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Ovitek: *Opuntia megacantha* Salm-Dyck 'Achefri' (foto: Mohamed Arba, 1–12)
Cover: Opuntia megacantha Salm-Dyck 'Achefri' (photo: Mohamed Arba, 1–12)

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doi:10.14720/aas.2021.117.2.1498 Original research article / izvirni znanstveni članek

Identification of proteins in sensitive and tolerant lines of sunflower *(Helianthus annus* L.*)* under water deficit

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Identification of proteins in sensitive and tolerant lines of sunflower (*Helianthus annus* L.) under water deficit

Abstract: The importance of examining environmental stresses and their role in predicting and evaluating the growth and yield of crops is very evident. Environmental stresses are the most important factor in reducing agricultural yields worldwide. In order to evaluate the environmental impact of water loss on the amount of proteins affected in sunflower, an experiment was conducted in randomized complete block design with three replications at Karaj Oil Seeds Research Institute. In order to study the response of susceptible line (BGK221) and tolerant line (RGK46) under drought stress conditions, extraction of protein by acetone deposition method was performed in 8 leaves and of 3 seedlings in each replication. The amount of extracted protein was determined by Bradford method using two-dimensional electrophoresis and existence of significant difference between the bands in sensitive and tolerant lines was investigated. A total of 467 repeatable bands were found in the tolerant line and 417 repeatable bands appeared in the sensitive line. Among these proteins, 6 bands in tolerant line (No 503, 1901, 904, 3301, 7011, 9005) and 6 bands in sensitive line (No 704, 811, 3205, 4108, 7307, and 9207) were significantly affected by drought stress. In both sensitive and tolerate lines the main consequence is increase in amount of protein. The results showed that the most important factor of tolerant line adaptive for environmental stress conditions is maintaining normal cell metabolism, keeping moisture in the cell, strengthening cellular structure and antioxidant defense. The study also showed that drought stress had the greatest effect on cytoplasmic and nucleus proteins, metabolism and energy of proteins.

Key words*:* sunflower; environmental stress; two-dimensional electrophoresis; proteomics.

Določanje beljakovin v občutljivih in toleratnih linijah sončnic (*Helianthus annus* L.) v razmerah pomanjkanja vode

Izvleček: Pomen preučevanja okoljskih stresov in njihove vloge v napovedovanju in vrednotenju rasti in pridelka gojenih rastlin je samoumevno. Okoljski stresi so v svetovnem merilu najpomembnejši dejavniki, ki zmanjšujejo pridelke kmetijskih rastlin. Z namenom ovrednotenja vpliva pomanjkanja vode na količino beljakovin v sončnicah je bil izveden popolni naključni bločni poskus s tremi ponovitvami na Inštitutu za preučevanje oljnih semen, Karaj, Iran. Za preučevanje odziva na sušni stres občutljive (BGK221) in tolerantne linije (RGK46) sončnic so bili narejeni izvlečki beljakovin iz osmih listov treh sejank v vsaki ponovitvi z acetonsko depozicijsko metodo. Količina beljakovin v vsakem izvlečku je bila določena z bradfordovo metodo z dvo dimenzionalno elektroforezo, kjer so bile preučevane značilne razlike med progami občutljivih in tolerantnih linij. Celokupno je bilo v tolerantnih linijah ugotovljenih 467 ponovljivih prog in 417 ponovljivih prog v občutljivih linijah. Med temi beljakovinami je bilo v tolerantni liniji 6 prog (No 503, 1901, 904, 3301, 7011, 9005) in v občutljivi liniji 6 prog (No 704, 811, 3205, 4108, 7307, 9207), na katere je značilno vplival sušni stres. V obeh linijah je bila značilna posledica sušnega stresa povečanje količine beljakovin. Rezultati so pokazali, da so pri tolerantni liniji najpomembnejši mehanizmi prilagoditve na okoljski stres vzdrževanje normalne celične presnove, ohranjanje vsebnosti vode v celicah, ojačevanje celičnih struktur in antioksidacijska obramba. Raziskava je pokazala, da ima sušni stres največji učinek na beljakovine citoplazme in jedra in na presnovo beljakovin.

Ključne besede: sončnica; okoljski stres; dvodimenzionalna elektroforeza; proteomika

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1 INTRODUCTION

Plants need the presence of nutrients in the soil, protection against pests and diseases, and the presence of appropriate environmental factors to grow well and maintain their health (Maghsoudi, 2006). The increasing world attention to the production of food to eliminate hunger and restore the food needs of the large consumer community has led to a massive effort across the world in the fields of establishment of wastelands, increasing the cultivating area, and increasing the efficiency of agricultural units, parallel with developing agricultural mechanization and soil fertility, evolving irrigation systems, combating pests and plant diseases, breeding and producing resistant seeds. Water is one of the most common and, at the same time, one of the most important compounds on the planet. The variation and abundance of plants in different parts of the earth depends on the amount of water available for the plants more than any other environmental factor. Drought tolerance is a complex feature that is affected by various plant and environmental variables. Identification of factors causing drought tolerance at the molecular level can be effective in the preparation of tolerant cultivars. Drought is one of the environmental stresses that causes harmful effects on most of the stages of plant growth, organ structure and their activities (Fulda et al., 2011, Passioura, 2007, Ishfaq et al., 2009). The response of plants to environmental stresses is different in morphological, anatomical, cellular and molecular levels (Dinakar et al., 2012). The ability of plants to adapt to environmental stresses depends on the type, intensity and duration of stress, plant species, and the stage of occurrence of stress (Passioura, 2007; Asl et al., 2003). Proteomics is referred as the analysis of a collection of proteins encoded by the genome (Karam et al., 2007). This technology provides quantitative and qualitative analysis of a large number of proteins; unlike the genome, which is a constant and unchanging nature, the proteome is fundamentally dynamic and variable, the proteome differs from organism to organism according to the type of tissue, cell, organ and environmental conditions (Goksoy et al., 2004). The study of the proteome of the intracellular organs may provide valuable information about the role of the protein. Several proteomics studies have been performed on proteome patterns of the organelles such as nucleus (Lawlor et al., 2002), chloroplasts (Ferro et al., 2003), mitochondria and plasma membranes (Adebayo et al., 2012). Sunflower is one of the important oily plants in the world and also Iran is in an arid and semiarid region, the preparation and identification of tolerant cultivars can help increase yield and develop its cultivation area.

2 MATERIALS AND METHODS

This research was carried out at the research farm of Seed and Plant Improvement and Production Institute located in the Mard Abad Road, Karaj. In this study, sensitive line (BGK221) and drought tolerant line (RGK46) were identified. For this purpose, sunflower inbred lines were cultivated in two separate experiments under normal irrigation and drought stress conditions in a randomized complete block design with three replications. The experimental plots had 3 x 3 meter Dimensions and included 3 planting lines, lenght of 2 meters (on agricultural land). The distance between the rows was 60 cm and the distance between the plants in each row was 25 cm. Normal irrigation was from 10 to 14 days and water stress was carried out through irrigation when irrigation was terminated in 2-8 leaf stages. Samples were randomly selected from 5 plants per plot and transferred were taken to a laboratory for protein identification. The quantity of extracted protein was determined by Bradford method (Bradford, 1976). Proteome pattern was obtained in two lines and in two aforementioned conditions using two dimensional electrophoresis. In the first dimension the proteins were separated by isoelectric focusing method based on isoelectric point and using IPG, and in the second dimension, the proteins were separated based on molecular weights using SDS-PAGE. After staining with Coomassie Brilliant Blue solution and scanning the gels, the difference between the appeared bands was investigated using PD Quest version 6 software-test.-The data of protein quantity and a significant difference between the bands in sensitive and tolerant lines was investigated. Significant differences between bandsof sensitive and tolerant lines were investigated by t-test.

3 RESULTS AND DISCUSSION

When plants are exposed to environmental stress, they respond to stress at the plant, cell, or molecule level. The pattern of production of many proteins changes in response to plant water depletion (Hajheidari et al., 2007). Plants to counteract or reduce the effects of drought stress may change the pattern of gene response or the amount of proteins within the tissue (Kanlaya et al., 2005).

3.1 CHANGE OF SENSITIVE LINE (BGK221) PROTEINS UNDER STRESS

Under stress conditions in sensitive line, 6 protein bands were investigated from 12 detected bands. Aforementioned bands have increased in expression (Fig. 1a,). Araus et al. (2002) in the effects of non-living stresses on the protein content of two wheat cultivars (sensitive and tolerant) in the seedling stage, has concluded that in drought stress, the root proteins in sensitive cultivar I increased significantly. These observations largely matched with the results of researchers who studied the changes in the protein content of wheat root and endosperm in the early stages of budding under dehydration stresses and reported that response to stress is specific to tissue conditions (Bakalova et al., 2008).

3.2 ENOLASE

Band No. 704 was identified as an enolase cytoplasmic form in the sensitive line, in which its expression was increased by 2.3 times in stress conditions. This increase reflects the ability of this line to produce this enzyme to supply energy and continue its vital processes. Increase of enolase due to drought stress in corn (Riccardi et al., 1998) and *Arabidopsis* (Wei et al., 2011) has been reported. Hajduch et al. (2007) has reported increase in the level of this enzyme in the sunflower oil line (Hajduch et al., 2007). Proteins such as enolase and 6 phosphoglycerate kinase play a vital role in controlling the key pathways of energy metabolism, such as glycolysis.

3.3 PHOSPHOGLYCERATE MUTASE INDEPENDENT OF 2-3 BISPHOSPHOGLYCIRATE

The cytoplasmic form of this protein (811) was identified in the sensitive line and its expression increased by 25.5 times under stress conditions. Gulcin (2012) has reported the increase in expression of this protein in soybeans under stress. This enzyme is the catalyzer of

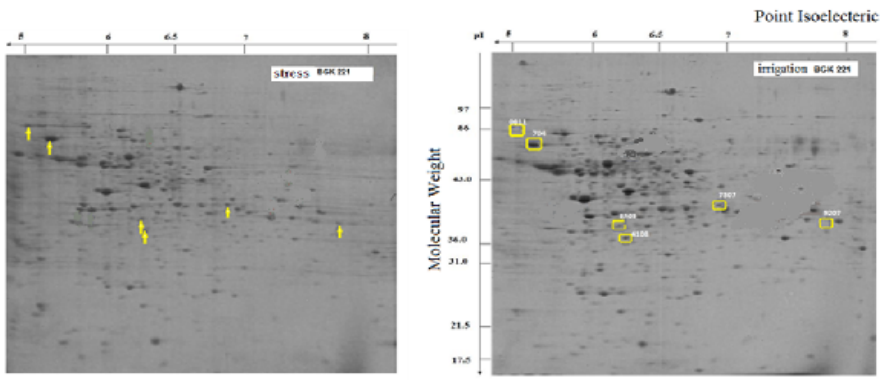

Figure 1a: Proteome pattern of sunflower sensitive line in irrigation and stress

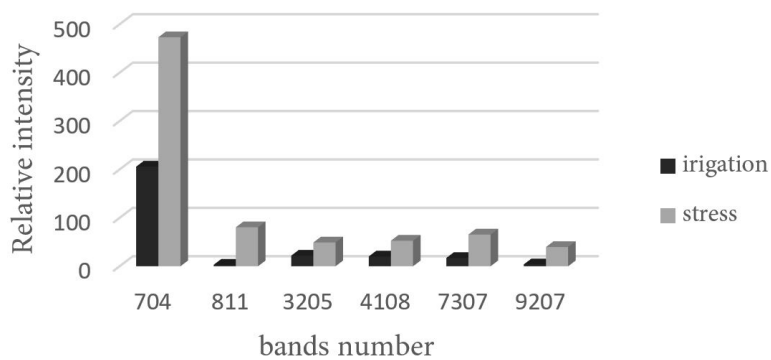

Figure 1b: Change of proteins on the sensitive line

the reversible catalytic conversion of 3-phosphoglycerate to 2-phosphoglycerate in the presence of magnesium ion, in process of glycolysis. According to Hajduch et al. (2007), this enzyme contributes to the balance between 3-phosphoglycerate and 2-phosphoglycerate (Hajduch et al., 2007). Increasing the level of this enzyme in sensitive line could be due to this regulatory role.

3.4 AUXIN CAUSED BY PROTEIN

The band number 4108 belongs to this enzyme, which its expression in cell nucleus has increased 2.6 times. The role of this protein is unknown.

3.5 MALATE DEHYDROGENASE

The cytoplasmic form of this protein was detected in the sensitive line (3205) and its expression increased by 2.2 times in stress conditions. Malate dehydrogenase is an important enzyme of cellular metabolism and catalyzes the two-way conversion of oxaloacetate, and malate (Musrati et al., 1998). This enzyme increases its activity under drought stress in plants to provide high energy requirements (Guicherd et al., 1997). Increased activity of this enzyme was observed in bread wheat (Wang et al., 2008) and *Arabidopsis* (Ndimba et al., 2005). Pereira et al. (2016) conducted a research on proteomic analysis of barley sensitive and tolerant genotype under drought stress. They have reported the increase of this enzyme in the barley tolerant genotype that shows the role of this enzyme in drought tolerance (Pereira et al., 2016), while the level of this enzyme declined in the sensitive line (Nezami et al., 2008).

3.6 CHALCONE SYNTHASE

The cytoplasmic form of this protein stain was detected in the sensitive line (7307), which was increased 3.8 times. Chalcone synthase (CHS) is a key enzyme in the pathway of biosynthesis of flavonoids, and its expression is one of the determining factors in the pathway of anthocyanins biosynthesis. CHS typically occurs in different plant species under various types of stress such as UV, injuries, and microbial pathogens and drought condition, resulting in the production of compounds with various activities such as antimicrobial activity (phytoalexins), insecticidal activity and antioxidant activity or suppressing direct or indirect UV light (Dao et al., 2011). CHS is a key enzyme in the flavonoid biosynthesis pathway. Increased chalcone synthase levels can play a role in antioxidant defense under stress.

3.7 GLYCERALDEHYDE TRIPHOSPHATE DEHYDROGENASE

The cytoplasmic form of glyceraldehyde triphosphate dehydrogenase (band 9207) was detected, which has 10.1 times higher expression. Considering the significant increase in the level of phosphoglycerate mutase enzyme, the increased activity of glycine aldehyde triphosphate dehydrogenase enzyme in this line was expected in line with the continuation of the glycolysis cycle. Increased glyceraldehyde 3-phosphate dehydrogenase can play role in plant tolerance under stress conditions by contribution in providing energy and antioxidative defense. (Table1).

3.8 CHANGE OF TOLERATING LINE (RGK46) PROTEINS UNDER STRESS

Under stress conditions, 6 protein bands were investigated from 18 detected bands. In tolerating line, the band No. 904 showed the highest percentage in increase expression (Fig2a).

3.9 SIMILAR TO DIHYDROFLAVONOL REDUCTASE

This protein was detected in the cytoplasm in the tolerant line with band No. 503. Its expression increased 2.3 times in stress conditions. Ghaffari et al (2013) in the study of the effect of drought stress in sunflower, reported a doubling of this protein in both sensitive and tolerant lines (Ghaffari et al 2013). This protein is involved in the pathway of biosynthesis of anthocyanin, which is part of the secondary metabolite biosynthesis (Dinakar et al 2012). Increasing the expression of this enzyme in this line, which is one of natural molecular antioxidants, is in line indicates its protective role and compatibility with drought stress. Anthocyanin has a protective role against various environmental stresses.

3.10 LINOLEATE 9S-LIPOXYGENASE

In the tolerant line, the cytoplasmic form of this protein band was identified with the number 1901 and its expression increased by 2.3 times in stress conditions. Ghaffari et al. (2013) reported the reduction of this protein in both sensitive and tolerant lines in the study of the effect of drought stress (Ghaffari et al., 2013). This protein is involved in the oxylipin biosynthesis pathway, which is part of the lipid metabolism. Lipoxygenase is involved in plant resistance to environmental stress. Its function involves oxidoreductase activity.

Table 1: Properties of expressed proteins in the sensitive line (BGK221)

SSP	Protein	Registration number	Bands	Percentage overlap	Irrigation	Stress	Protein ratio	Protein role	Protein location
704	Enolase	NP_001105896.1	149	17	205.9	473.2	2.30	Energy	Cytoplasm
811	Phosphoglycerate mutase,2,3-bisphosphoglycerate-independent	NP_187471.1	122	14	3.167	80.6	25.45	Energy	Cytoplasm
3205	Malate dehydrogenase, cytoplasmic	O48905.1	179	15	21.97	49.2	2.24	Metabolism	Cytoplasm
4108	Auxin-induced protein	AAB84222.1v	107	8	20.33	52.8	2.6	Unknown	nucleus
7307	Chalcone synthase	Q9ZU06	278	19	17.13	65.6	3.83	Metabolism	Cytoplasm
9207	Glyceraldehyde 3-phosphate dehydrogenase	AES72079.1	240	30	3.933	39.8	10.12	Energy	Cytoplasm

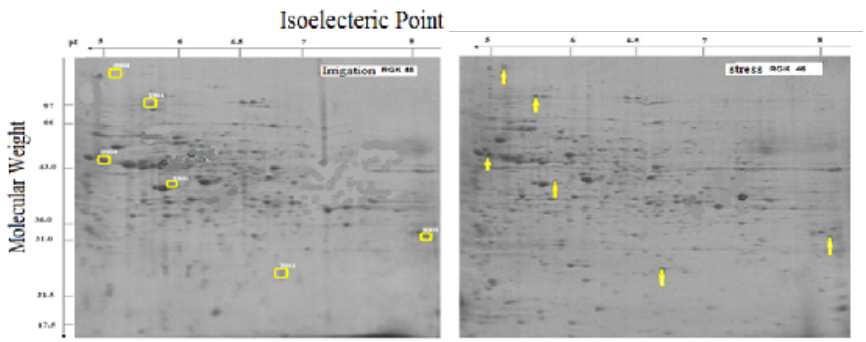

Figure 2a: Proteome pattern of sunflower tolerant line in irrigation and stress

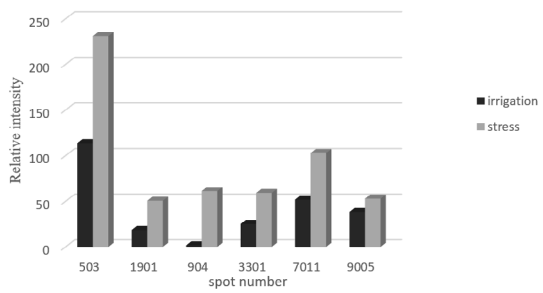

Figure 2: Change of proteins on the tolerance line

3.11 UBIQUITIN CARBOXYL- TERMINAL HYDROLYZE

In the tolerant line, the cytoplasmic form of this protein band was identified with the number 904 and its expression increased by 36.7 times in stress conditions. This enzyme has the activity of cysteine type peptidase (a catalyst for hydrolysis of peptide bands in the polypeptide chain by the mechanism in which the remained sulfhydryl group in the active center acts as a nucleophile) and hydrolase of ubiquitin (catalysis for hydrolysis of thiol depending on an ester, thioester, amide, peptide or iso-peptide chain formed by the C-terminus of glycine-ubiquitin). (Ubiquitin Proteasome System) is almost entirely involved in the regulation of all stages of growth in plants and is likely to play a major role in many hormonal pathways and cellular vital responses (Dreher and Callis, 2006).

3.12 POLYMYXIN BIFUNCTIONAL RESISTANCE PROTEIN

The aforementioned protein, was identified in cell cytoplasm with band number 3301 and its expression increased by 3.2 times in stress conditions. Ghaffari et al. (2013) reported the increase in expression of this protein in the tolerant line and its reduction in sensitive lines (Ghaffari et al., 2013) while investigating the effect of drought stress on sunflower. Its molecular function involves the coupling of coenzyme and oxidoreductase activity (catalysis of oxidation-reduction reactions in which a CH-OH group acts as a hydrogen or electron donor and reducer of NAD^+ and $NADP^+$) and transferase activity relating to hydroxymethyl- and hydroxyformyl. Increasing the level of this enzyme in the tolerant line could indicate its defensive role in removing free radicals and adapting to drought stress in the plant.

3.13 GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

In the tolerant line, the cytoplasmic form of glyceraldehyde 3-phosphate dehydrogenase (band No. 7011) was identified which shows its relation to glycolysis and energy. Increasing the level of this protein is a quick response to supply the needed energy in drought stress condition through glycolysis and oxidative defense. Ferro et al. (2003) reported the increase in levels of this enzyme in response to stress in Arabidopsis Thaliana (Ferro et al. 2003). Caruso et al. (2009) reported an increase in this protein in tolerant line of peanut under drought stress condition (Caruso et al., 2009). Balbuena et al. (2011) also reported the distinct expression of this protein in sunflower in conditions of cold stress (Balbuena et al., 2011).

3.14 BAND NO. 9005

Based on the standard protein comparison model, the type of the above band was not detected in tolerant line. Also, the location of the protein and its role are unclear, but increased by 1.38 times in stress conditions. (Table2)

3.15 RELATIVE CHANGES IN PROTEINS

According to the research (Ghaffari et al., 2013) and among the sensitive and tolerant sunflower lines, it can be identified BGK221 as a sensitive line and the line RGK46 as a tolerant line (Maghsoudi et al., 2020). A total of 467 repeatable bands were found on the tolerant line and 417 repeatable bands appeared on the sensitive line. Among these proteins, 6 bands in tolerant and sensitive lines were significantly affected by drought stress. Among these proteins, 6 bands in tolerant line were similar to dihydroflavonol reductase (503), seed linoleate 9S-lipoxygenase (1901), ubiquitin carboxyl--terminal hydrolas (904), bifunctional polymyxin resistance protein ArnA-like (3301), glyceraldehyde-3-phosphate dehydrogenase (7011), unknown (9005)} and 6 bands in sensitive line (enolase (704), phosphoglycerate mutase,2,3-bisphosphoglycerate-independent (811), malate dehydrogenase, cytoplasmic (3205), auxin-induced protein (4108), chalcone synthase (7307), glyceraldehyde 3-phosphate dehydrogenase (9207) were significantly affected by drought stress; in both sensitive and tolerate lines the main consequence is increase in amount of protein. (Fig 3, Fig 4)

Table 2: Properties of expressed proteins in the tolerance line (RGK46)

SSP	Protein	Registration number	Bands	Percentage overlap	Irrigation	Stress	Protein ratio	Protein role	Protein location
503	Similar to	AAK68820.1	103	11	113.7	230.9	2.03	metabolism	Cytoplasm
904	Ubiquitin carboxyl-terminal hydrolase	XP_002524120.1	112	5	1.67	61.17	36.63	metabolism	Cytoplasm
1901	Seed linoleate 9S-lipoxygenase	P24095.1	123	19	24.10	76.23	3.16	metabolism	Cytoplasm
3301	Bifunctional polymyxin resistance protein	XP_003538161.1	101	14	25.17	59.07	2.35	Disease Defense	Cytoplasm
7011	Glyceraldehyde-3-phosphate dehydrogenase	AEP71393.1	252	36	51.80	102.77	1.98	Energy	Cytoplasm
9005	Unknown	Unknown	-	-	38.40	52.87	1.38	Unknown	Unknown

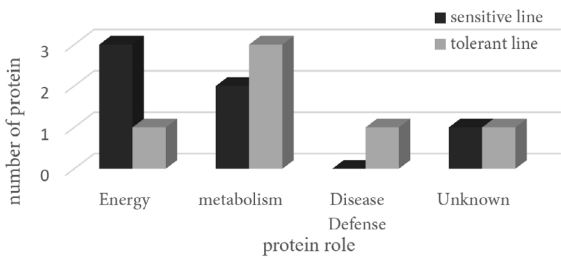

Figure 3: Protein classification by role

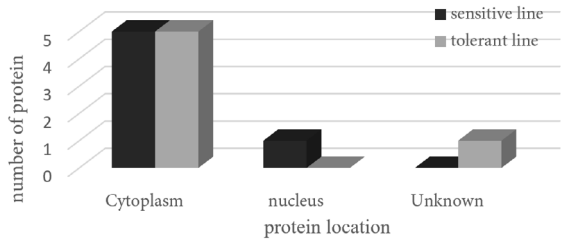

Figure 4: Protein classification by location

4 CONCLUSION

Given that water stresses have significant negative impacts; it is necessary to control these tensions to create a sustainable environment. Plants are very effective in protecting the environment. To prevent the extinction of plant species and the lack of water resources in the environment, it should be noted that the plant's characteristics and abilities are in the context of environmental stress. In this study, the classification of proteins based on the energy, metabolism, and defense function (Fig 3, Fig 4) showed that proteins had increased in sensitive and tolerant line. Research of protein in sensitive line and tolerant line, showed that the most important factors of tolerant line adaptive for environmental stress conditions are: maintaining normal cell metabolism, keeping moisture, strengthening cellular structure and antioxidant defense. The study also showed that water stress had the greatest effect on cytoplasmic/ nucleus proteins and metabolism/ energy proteins. The sensitive line tries to compensate for the damage caused by stress by increasing energetic proteins. But the key to success in the tolerant line is increased lipid synthesis in the cell membrane and increased hydrophilic proteins in the face of stress.

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Assessment of durum wheat (*Triticum durum* Desf.) genotypes based on their agro-physiological characteristics and stress tolerance indices

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Assessment of durum wheat (*Triticum durum* Desf.) genotypes based on their agro-physiological characteristics and stress tolerance indices

Abstract: The present study aimed to investigate the extent of variability and relationships between grain yield and morpho-physiological durum wheat traits. Sufficient variability was observed for most characters. Based on stress indices, either widely or specifically, adapted lines were identified. Path analysis pointed out to above ground biomass, harvest index, spike fertility and spike number as yield determinants, suggesting that these traits are of interest in the breeding program. The measured traits were classified within 6 principal components accounting for 79.45 % of the total variation. Breeding lines dispersed along first principal component exhibited substantial differences in performance and stress tolerance abilities. Cluster C3 lines were high yielding and stress tolerant. From this cluster, lines L24 and L14 were scored as the best for 7 and 5 traits out of 17 characters, respectively. Both lines are proposed for release and as parents in crosses to take advantage of their desirable characteristics. The results indicated that physiological traits were unrelated to each other and to morphological traits making difficult the concomitant selection for yield and stress tolerance driven by these traits. Complexes crosses, between parents carefully chosen for these specific characteristics, are necessary to enhance favorable genetic linkage and to generate new basic segregating populations with high genetic variability for these traits.

Key words: Durum wheat; grain yield; genotype x environment interaction; physiological traits; tolerance indices; path analysis; cluster

Ovrednotenje genotipov trde pšenice (*Triticum durum* Desf.) na osnovi agro-fizioloških lastnosti in indeksov tolerance na stres

Izvleček: Namen te raziskave je bil preučiti obseg spremenljivosti in razmerja med pridelkom zrnja in morfološko-fiziološkimi lastnostmi trde pšenice. Za večino lastnosti je bila ugotovljena zadostna variabilnost. Na osnovi indeksov stresa so bile določene širše in ožje prilagojene linije. Analiza povezanih znakov je pokazala, da so nadzemna biomasa, žetveni indeks, fertilnost klasov in njihovo število najpomembnejše lastnosti, ki določajo pridelek, kar kaže, da so te lastnosti zanimive za žlahtnjiteljske programe. Merjene lastnosti so bile razvrščene znotraj 6 glavnih komponent, kar je prispevalo kar 79,45 % celokupne variabilnosti. Linije križancev razvrščene vzdolž prve glavne komponenete so imele znantno raznolikost glede sposobnosti tolerance na stres. Linije v grozdu C3 so bile tolerantne na stres in imele velik pridelek. Iz te skupine sta bili liniji L24 in L14 prepoznani kot najboljši za 7 in 5 znakov izmed 17 lastnosti. Obe liniji sta predlagani prednostno za uporabo v križanjih kot starševski liniji zaradi njunih zaželjenih lastnosti. Izsledki so pokazali, da fiziološke lastnosti niso bile povezane med sabo niti z morfološkimi znaki, kar povzroča težave pri hkratni selekciji za pridelek in toleranco na stres na osnovi teh lastnosti. Za povečanje genetske povezave med preučevanimi znaki in vzgojo novih osnovnih raznolikih populacij so potrebna kompleksna križanja med pazljivo izbranimi starši, glede na te specifične lastnosti.

Ključne besede: trda pšenica; pridelek zrnja; interakcije genotipa in okolja; fiziološke lastnosti; indeksi tolerance; analiza povezanih znakov; klasterska analiza

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1 INTRODUCTION

Durum wheat (*Triticum durum* Desf.) is a major cereal crop grown in Algeria. Its production is based on adoption of modern varieties derived from CIMMYT (International Maize and Wheat Improvement Center) plant material and traditional cultivars issued from heritage landraces. Actually 1.5 million hectares are sown annually with a production varying from 0.42 (1986/87) to 3.2 million tons (2016/17), during the 1975-2017 period (CEIC, 2021). Because of differential ability to withstand drought and heat stresses, traditional cultivars are generally grown in poor yielding environments while recently released varieties are cultivated under relatively more favorable conditions. Sown in autumn, as rainfed crop, the vegetative growth phase occurs during cold and wet winter-early spring months while reproductive growth phase endures drought and terminal heat stresses. To meet the needs of a fast-growing population and to reduce the sharp rise in grain imports, larger production increases are sought. Since increasing sown area is not possible, future improved durum wheat varieties must then be capable of higher yields under lower management and uncertain climate scenarios. Because of climate changes, declining rainfall and increased temperatures are predicted for the Mediterranean basin which is likely to be a vulnerable hotspot (Lobell et al., 2007; Fraser et al., 2013).

Beside high yield potential, new cultivars should express stable performance and broad adaptation. Grain yield is a result of the combined effects of genotype (G), environment (E), and their interaction (GEI). Differences among genotypes, in their response to environmental changes, are caused by GEI (Annicchiarico et al., 2005; Haddad et al., 2016). A crossover GEI type reduces heritability and selection efficiency (Ceccarelli et al., 1991). Substantial efforts are therefore dedicated to reduce yield instability through plant breeding and crop management (Chamekh et al., 2015). A number of traits and indices have been proposed to be used along with grain yield to select more efficiently desirable genotype characterized by high yield potential, stress tolerance and acceptable stability (Li et al., 2012; Dorostkar et al., 2015). In this context, an understanding of the physiological mechanisms underlying abiotic stress tolerance, the identification of the trait-markers of these mechanisms and the analysis of their relationships with grain yield and yield attributes could be helpful to efficiently select for high and stable grain yield under variable environments. Plants have developed several mechanisms, which alleviate the harmful effects of stress (Ashraf, 2010). Among various environmental stresses, heat and drought are the main grain yield limiting factors. Yield reduction magnitude depends on plant growth stage subjected to stress and on stress severity (Nouri et al., 2011). Differences in stress tolerance among genotypes and species were reported in several studies (Marcinska et al., 2017; Grzesiak et al., 2012; 2017). Yield stability indices have been formulated and proposed among others by Di-Matteo et al. (2016) and Farshadfar et al. (2018).

The relation between grain yield obtained under stress and non stress conditions could be used as marker of stress tolerance. This relation is approached through stress tolerance indices, which identify genotypes with good performance under both non-stress and stress environments (Benmahammed et al., 2010; Grzesiak et al., 2012). Stress tolerance (STI), yield stability (YSI) and superiority genotypic (Pi) indices were, among several other indices, proposed as potential tools to identify high yielding, stable and/or stress tolerant genotypes (Lin and Binns, 1988, Farshadfar et al., 2018). Stress-induced cell membrane injury, relative water contain, rate of excised flag leaf water loss, canopy temperature, and leaf chlorophyll contain were rated as promising screening tools in the search of stress tolerance (Hura et al., 2007; Hasheminasab et al., 2014; Saed-Moucheshi et al., 2016). In this context, Awan et al. (2015) reported that grain yield was closely linked to relative water content, cell membrane stability and specific flag leaf area. The increased solute leakage, marker of decreased cell membrane thermostability, is used as a measure of heat-stress tolerance (Wahid et al., 2007; Khajuria et al., 2016). The present investigation aimed to assess the variability, stability and relationships of grain yield with physio-morphological traits in a set of durum wheat (*Triticum durum* Desf.) advanced breeding lines grown under south Mediterranean conditions.

2 MATERIAL AND METHODS

2.1. SITE, PLANT MATERIAL, AND EXPERIMENTAL DESIGN

Twenty four advanced durum wheat breeding lines and a check, cultivar Waha (Table 1), were evaluated in a field experiment during the 2016/17, 2017/18 and 2018/19 growing seasons at the Field Crop Institute, Agricultural Experimental Station of Setif (ITGC-AES, 36°12' N, 05°24'E, 1080 m above sea level, Setif, Algeria). The experiment was set-up in a randomized complete block design with four replications. Plot dimensions were 6 rows 5 m long with 0.20 m space between adjacent rows, and 0.30 m between adjacent plots. Recommended cultural practices for the area were followed to grow a good crop. Eighty kg ha^{-1} of mono-ammonium phosphate (52

% P_2O_5 + 12 % N) were applied just before sowing, and 80 kg ha^{-1} of urea (46 % N) were broadcasted at the tillering stage. Weeds were controlled chemically by application of 150 g ha^{-1} of Zoom (Dicamba 66 %, Triasulfuron 4 %) and 1.2 l ha^{-1} of Traxos (22.5 g l^{-1} of Pinoxaden, 22.5 g l^{-1} of Clodinafopropargyl and 6.5 g l^{-1} of Cloquintocet -mexyl) herbicides.

2.2. WEATHER CONDITIONS

Monthly rainfall and temperatures (maxi, mini, and average) recorded at the experimental site, during the three cropping seasons, are reported in Figure 1. Rainfall distribution, typical of Mediterranean climate, is quite variable within and between cropping seasons. The amount recorded, from September 1st to June 30th, reached 187.5, 442.1 and 346.6 mm, for 2016/17, 2017/18 and 2018/19 cropping seasons, respectively. Expressed relatively to the maximum value, these figures represent 42.4, 100.0 and 78.4 %, for the three cited cropping seasons, respectively. Rainfall amount available for the vegetative phase ranged from 73.2 % to 83.1 % of the cycle total, and from 16.9 to 26.8 % for the reproductive phase. Rainfall of the 2017/18 cropping season was evenly distributed along the crop cycle, while in 2016/17, a severe drought period occurred from the tillering stage onwards. June 2017 showers, coinciding with the grain filling period, avoided a complete crop failure. In fact, with less than 15 mm, during the March-April-May period, the 2016/17 cropping season was a stressful environment, while with 223.6 mm accumulated during the same period, the 2017/18 cropping season behaved as a favorable environment for wheat growth. Monthly mean maximum, mean minimum temperatures and their average exhibited, during the crop cycle, a bimodal evolution pattern, reaching their lowest values in January and February months and their highest values in June onwards (Figure 1). Under this climatic scenario, durum wheat growth is hampered by low temperature during the vegetative phase, when relatively appreciable soil water is available and by rising temperatures and water scarcity during the grain filling phase.

2.3. DATA COLLECTION

Relative water content (*RWC*) was determined as described in Pask et al. (2012). Fresh leaves were collected, at anthesis, weighted to record fresh mass (FM). The samples were placed in distilled water for 24 h and weighed to record turgid mass (TM). Samples were then subjected to oven drying at 72 °C for 24 h to record dry mass (DM). Relative water content was calculated as follow: $RWC = [(FM - DM)/(TM - DM)] \times 100$**.** Flag leaf chlorophyll content (CHL, CCI) was determined with a CCM - 200 chlorophyll meter (Opti-Sciences, Tyngsboro, MA, USA) at the anthesis growth stage. Chlorophyll measurements were taken from the middle of the flag leaf. Canopy temperature (CT) was measured, at heading, using a hand-held infrared thermometer (Fluke Corporation, Everett, WA, USA). Four measurements were taken per plot at approximately 0.5 m distance from plot edge. Readings were done between 11:00 to 14:00 hours on sunny days.

Table 1: Name and pedigree of the advanced durum wheat breeding lines tested during three successive cropping seasons (2016/17, 2017/18, 2018/19) at the ITGC- AES experimental site (Setif, Algeria).

N°	Name /Pedigree
1	Waha (check)
2	Jupare C
3	Sooty_9/Rascon_37//Storlom/5/Toska_26/Rasco
4	Ajaia_4/Canelo_3/4/Arment//Srn_3/Nigris_4/3/Ca
5	Canelo_9.1/Snitan//Plata_10/6/Mque/4/Usda573/
6	Bellaroi/4/Bicum//Lauretinia/3/Dukem_12/2*Raiscon $_{21}$
7	Islom_1/Dukem_2/Tarro_3/5/Crex//Boy/Yav_1/3
8	$P_{91.272.3.1}$/3*Mex_{175}//2*Jupare c
9	Guemgoum Rhem/4/Stj3//Bcr/Lks4/3/Ter_{-3}
10	Brak^2/Ajaia_2//Solga_3/3/Canelo_8//Sora
11	Sooty^9/Rascon_37//Stormlom/8/Rissa/Gan.
12	Silk_3/Dipper_6/3/ac089/Dukem_4/5*$Ac_{089.}$
13	Sooty_9/Rascon_37//Storlom/5/Toska_2
14	$Terbol_{975}$/$Geruftel_2$
15	Langdon/Kucuk
16	20048 Traikia/Mrb5//Stj_3
17	T.Polo/ZB//$lnrat_{69}$
18	Lcasyrl/3/Gen//Stj/Mrb_3
19	Ouasloukosl/5/Aznl/4Bezaizshf//SD19539/Waha/3/Gdr_2
20	Mohawk/9/$Usda_{595}$/3/D67.3/Rabi/Cra/4/Al05
21	Icasyrl/3/Bcr/sb1/5/Turaru/4/13376/Bcrchl//Ossll/Stj_5
22	Korifla/Aeg speltoïdes Syr/Amedakul
23	Amedakull/Triticum Dico Syr Col/Lukos
24	Terbol97-5/$Geruftel_2$
25	Ouasloukosl/5/Aznl/4/Bezaifshf//SD_{19539}/Waha/3/Gdr_2

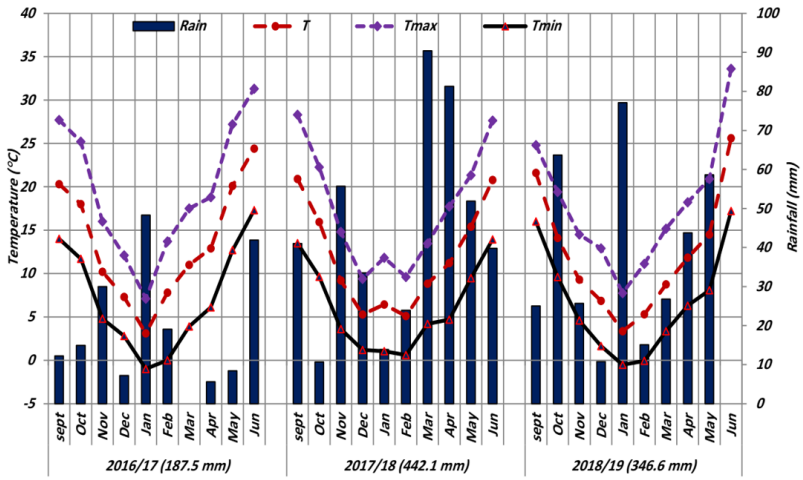

Figure 1: Monthly rainfall and mean temperatures recorded during the three cropping seasons test at the ITGC-AES, Setif, Algeria.

Membrane stability index (*MSI*) was estimated according to Ibrahim and Quick (2001). Two sets of leaf tissues, 10 leaf segments, 1 cm length each, were placed in test tubes containing 10 ml of double-distilled water. One set was kept at 40 °C for 30 min and its electrical conductivity recorded (C1) using a conductivity meter, type Eutech Instruments, Singapore, while the second set was kept in a boiling water bath (100 °C) for 30 min and its conductivity recorded (C2). MSI was calculated as follows: *MSI* = 100 * [1 – (C1/C2)]. To determine the rate of excised leaf water loss (ELWL), 10 flag leaves were randomly clipped per plot, and were immediately weighted to record the fresh mass (FM). The samples were wilted at 30 °C in an electrical oven for 4 hours, and weighted to obtain the wilted mass (WM). The samples were oven-dried at 70 °C for 72 h and weighed to record dry mass (DM). ELWL was worked out using the following formulae: ELWL (%) = 100 * (FM - WM) / DM (Dhanda and Sethi, 1998). Plants were scored for plant height (PHT, cm), measured just before harvest. Days to heading (DHE) were counted from January 1st to the date when 50 % of the spikes were half-way out of the flag leaf sheath. At maturity, a row segment, 1 m long, was harvested and used to determine above ground biomass (BIO, g m^{-2}), number of spikes m^{-2} (SN), and harvest index (HI, %). Grain yield (GY, g m^{-2}) and thousand-kernel mass (TKM, g) were determined from the combine harvested trial. The number of kernels per spike (NKS) was derived as the ratio of the number kernels m^{-2} divided by the number of spikes m^{-2}.

2.3. DATA ANALYSIS

Environment (E), genotype (G) and their interaction (GEI) effects of physiological, yield and yield-related traits were determined through a combined analysis of variance using balanced analysis of variance subroutine implemented in Cropstat software (Cropstat, 2007). Genotypic (σ^2_G) and error (σ^2e) components of variance were calculated to estimate the coefficient of experimental (CVe), and of genotypic variation (CV_G) and their ratio CV_G/CVe. This ratio is used to appreciate the magnitude of genotypic relatively to experimental variation as suggested by Cruz et al. (2012). A ratio value, greater than unity, suggests the presence of appreciable genotypic variability exploitable in selection. Pearson's correlation coefficients between grain yield and the measured traits were calculated per cropping season using Past software (Hammer et al., 2001). Path coefficients analysis was carried out on the across cropping seasons averaged values. For this purpose, mean values of the traits loaded in the full model were standardized, and subjected to a multiple regression analysis to determine standardized partial regression coefficients (Beta) or paths. The indirect effect of trait X_i via trait X_j, was obtained as the product of the path coefficient of the trait X_i and the correlation coefficient relating traits X_i and X_j, following the procedure described in Akintunde (2012). Genotypic superiority index (Pi) was derived according to Lin and Binns (1988) as: $P_i = \Sigma(X_{ij} - M_j)^2 / 2n$, where X_{ij} is the grain yield of the i^{th} breeding line in the j^{th} testing environment (cropping season), M_j is the grain yield of the best performing breeding line in the j^{th} environment, and n is the number of environments test. Grain yield stability index (YSI) was calculated using the following formulae: $YSI = Ys / Yp$; and stress tolerance index (STI) was determined as follow: $STI = (Yp \times Ys) / \bar{Y}p^2$, where Ys, Yp and $\bar{Y}p$ are grain yield means observed under stress, non stress and average of all assessed breeding lines under non stress envi-

ronments, respectively (Benmahammed et al., 2010). The ecovalence (W^2i) was calculated as described in Weedon and Finckh (2019) as follow: $W^2_i = \Sigma (X_{ij} - X_{i.} - X_{.j} + X_{..})^2$, where X_{ij} represents grain yield of the i^{th} genotype in the j^{th} environment, $X_{i.}$ is the genotype main effect, $X_{.j}$ is the environment main effect and $X_{..}$ is the grand mean. Principal components and cluster analyses, subroutine implemented in Past statistical software (Hammer et al., 2001), were carried out using standardized mean values of the measured traits averaged of across environments and the stress index values. Dendrogram, showing breeding lines classification, was generated adopting Ward's method based on squared Euclidean distance.

3 RESULTS AND DISCUSSION

3.1. MEAN PERFORMANCE AND VARIABILITY

The results of the combined analysis of variance for the measured traits are reported in Table 2. Season and genotype main effects as well as their interaction (GEI) were highly significant for all traits, except CHL season main effect and GEI which were not. Season main effect of GY, NKS, DHE, BIO, and PHT accounted for more than 70.0 % of the variation observed in the analyzed data. Season main effect of HI, SN and MTS explained between 30.0 to 60.0 % of the total variation expressed in these traits, while TKM, RWC and CHL season main effects accounted for less than 30 % of the total variation. Genotype main effect accounted for 15.0 % for GY, SN, NKS, DHE, BIO, HI and RWC. TKM and CHL genotype main effect accounted for 52.2 %, and 23.1 %, to the total variation, respectively. Contribution of GEI to the total variation was as low as 1.2 % and 2.7 % for PHT and DHE and increased to 38.5 % for HI (Table 2). These results suggested that the expression of the measured traits is affected essentially by changes in environment, while variation originated from differences between genotypes (G) and GEI effects is comparatively less marked. The large season main effect is attributed to differences in the amount and distribution of annual rainfall (Figure 1). The fact that GEI contributed more to the explanation of the variation observed than the contribution due to genotype main effect suggested that genotypes responded and ranked differently across the seasons test, as this is shown in Figure 2, which indicated the G + GEI contribution to the grain yield variation.

Breeding line L19 was the most reacting, exhibiting positive contribution during two seasons and a strong negative contribution during the third season. L9 appeared as a desirable entry as it exhibited positive contributions during the three cropping seasons. L6, L3 and to a lesser extent L4 were less yielding but have positive interactions during the three cropping seasons (Figure 2). The results of the present study show that selection and recommendation, based on grain yield of superior genotypes among those tested, is difficult because of the masking effect of the GEI.

The mean, maximum and minimum values, averaged over seasons, and the best scoring lines for the measured traits are reported in Table 2. GY ranged from 585.93 to 838.11 g m^{-2}, SN from 435.42 to 590.42, TKM from 33.08 to 41.17 g, NKS from 29.23 to 41.32 kernels per spike, DHE from 113.33 to 117.33 days, BIO from 1334.98 to 1976.38 g m^{-2}, HI from 34.45 to 47.53 %, PHT from 65.23 to 71.18 cm, RWC, from 66.90 to 83.01 %, MTS from 49.44 to 71.43 %, CHL from 33.78 to 46.36 cci, CT from 20.15 to 24.90 °C and ELWL from 23.58 to 55.32 %. The recorded values for the coefficient of experimental variation (CVe) were below 10 % for most of the measured traits, suggesting that an appreciable precision was achieved in the measurement of these traits. CVe values for HI, RWC, MTS, CHL and ELWL were higher than 10 % suggesting a lack of precision and an environment effect on the expression of these traits. Increasing replications for the measurement of these traits is justified to be able to detect significant genotypic differences, if any. The ratio CV_G/CVe is almost equal to 1 for CT and higher than unity for TKM and DHE, indicating sizeable genotypic variability. Values of this ratio were lower than unity for the remaining measured traits, suggesting relatively low genotypic variability.

3.2. GRAIN YIELD STABILITY AND STRESS TOLERANCE

Genotypic superiority index values ranged from 1.46 to 52.12 with an average of 33.41. The best breeding lines (≈10 % selection intensity) were L24 and L13 which exhibited the lowest Pi values of 1.5 and 8.4, respectively. YSI values ranged from 0.219 to 0.553 and an overall mean of 0.330. Since high YSI values are desirable, the best breeding lines for this trait were L25 (YSI = 0.180) and L19 (YSI = 0.553). STI values ranged from 0.184 to 0.439 and an overall mean of 0.324. High STI values are desirable in selection for drought tolerance. The best breeding lines for this trait were L1 (STI = 0.439) and L11 (STI = 0.423). The ecovalence values (W^2i) ranged from 3.03 to 120.0 with an average value of 25.34. Genotypes exhibiting low W^2i are desirable in selection. The best breeding lines for this characteristic were L15 (W^2i = 3.00) and L21 (W^2i = 7.1). Correlation coefficients analysis indicated that no significant relationship (r = 0.164, p > 0.05) existed between Yp (GY of 2017/18 season) and

Table 2: Combined analysis of variance mean squares, contribution to the total variation (%), mean values, best scoring lines, coefficient of experimental variation, ratio of genetic to experimental coefficients of variation and least significant difference at 5% probability level, for the measured traits.

Source	Season (E)	Blocks/E	Gen (G)	G x E	Pooled residual	Mean	Maxi	Min	CVe %	CV_G/CVe	Lsd5%
DF	2	9	24	48	216						
GY	11611800** 82.9%	14138.3	54003** 4.6%	52800** 9.0%	3853.6	674.04	838.11 L24, L14	585.93	9.21	0.16	50.7
SN	1069850** 56.7%	2920.9	19158** 12.2%	18253** 23.0%	1263.6	507.79	590.42 L24, L12	435.42	7.00	0.24	29.0
TKM	239.3** 13.1%	1.2	79.3** 52.1%	21.2** 27.9%	1.1	36.10	41.17 L23, L21	33.08	2.94	2.08	0.9
NKS	12985.3** 71.4%	4.4	155.2** 10.2%	119.1** 15.7%	4.2	35.50	41.32 L3, L15	29.23	5.78	0.84	1.7
DHE	5049.1** 93.6%	0.5	13.8** 3.1%	6.0** 2.7%	0.3	115.08	117.33	113.33 L24, L14	0.47	1.48	0.4
BIO	38690800** 72.3%	12655.7	406210** 9.1%	380943** 17.1%	7247.7	1641.45	1976.38 L14, L2	1334.98	5.19	0.54	69.5
HI	4053.4** 34.1%	37.7	111.1** 11.2%	189.6** 38.3%	16.3	40.15	47.53 L24, L17	34.45	10.06	0.00	3.3
PHT	38623.4** 91.9%	415.3	32.8** 0.9%	21.5** 1.2%	5.6	68.28	71.18 L24, L14	65.23	3.48	0.41	1.9
RWC	2146.2** 11.6%	188.5	229.9** 14.9%	138.6** 18.0%	87.5	77.86	83.01 L18, L2	66.90	12.01	0.29	7.6
MTS	29247.6** 49.5%	1568.7	301.3** 6.1%	221.7** 9.0%	128.7	62.29	71.43 L14, L21	49.44	18.21	0.23	9.3
CHL	506.5^{ns} 9.4%	183.5	103.3** 23.1%	22.8^{ns} 10.2%	20.9	39.44	46.36 L24, L12	33.78	11.58	0.57	3.7
CT[a]		11.63	4.80**		1.04	22.75	24.90	20.15 L1, L9	4.48	0.95	0.8
ELWL[a]		211.6	315.6**		116.1	37.13	55.32	23.58 L3, L6	29.05	0.66	8.8

GY = Grain yield (g m^{-2}),SN = Number of spikes m^{-2}, TKM = 1000-kernel mass (g), NKS = Number of kernels per spike, SM = Spikes mass (g m^{-2}), DHE = Number of days to heading, BIO = Above ground biomass (g m^{-2}), HI = Harvest index (%), STW = Straw yield (g m^{-2}), Yeco = economical yield (g m^{-2}), PHT = Plant height (cm), RWC = Flag leaf relative water content, MTS = Membrane thermo stability (%), CHL = Chlorophyll content(cci), CT = Canopy temperature (°C), ELWL = Excised leaf water loss (%). ns, * and ** = non significant and significant effects at 5 % and 1 % probability level, respectively, [a] = Data from one season only.

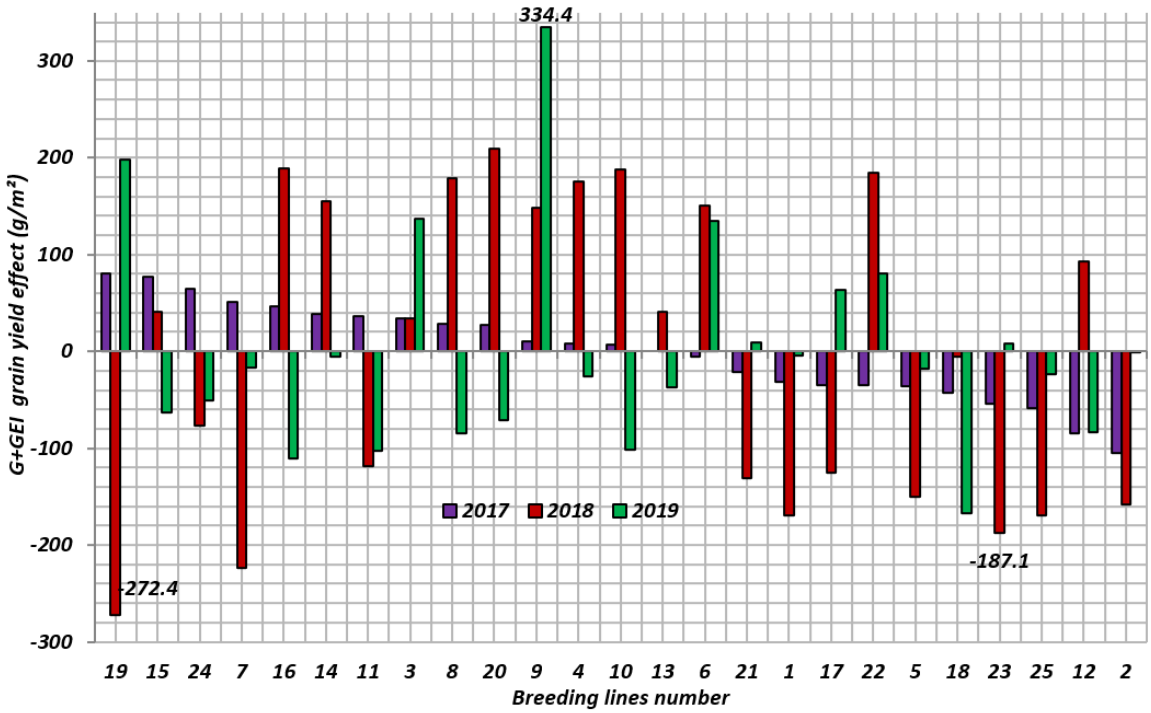

Figure 2: Genotype + Genotype x Environment interaction (G + GEI) contributions to grain yield variation of the advanced breeding lines assessed during three cropping seasons at the ITGC-AES of Setif (Algeria).

Ys (GY of 2016/17 season), suggesting that both environments ranked differently the assessed breeding lines. These results support findings reported by Nouri et al. (2011). In contrast, a positive and significant correlation, between Ys and Yp, was reported by Golabadi et al. (2006) and a negative one by Sio-Se-Mardeha et al. (2006). Different results may come out due to the nature of the GEI present (presence of crossover or not), stress intensity and to the degree of sensitivity of the plant materials tested. Pi index correlated negatively and significant with Yp ($r = -0.725$, $p < 0.01$) and with STI ($r = -0.629$, $p < 0.01$), but not with YSI ($r = 0.384$, $p > 0.05$), nor with W^2i ($r = -0.027$, $p > 0.05$).YSI index correlated negatively and significant with Yp ($r = -0.641$, $p < 0.01$) and positively and significantly with Ys ($r = 0.637$, $p < 0.01$) and with W^2i ($r = 0.470$, $p < 0.05$). Besides its negative and significant correlation with Pi, STI index correlated positively and significant with Ys ($r = 0.717$, $p < 0.01$) and Yp ($r = 0.800$, $p < 0.01$). Pi index measures the deviation of the performance of a given genotype from that of the best performing genotype in a given environment, and lower Pi values are desirable, because they identify high yielding and stable genotypes (Lin et Binns (1988).

The results of the present study corroborate those of Clarke et al. (1992) and Benmahammed et al. (2010) who reported significant and negative correlation between Pi and Yp. This correlation suggested that low Pi genotypes responded to improved growth conditions (fertility) more than they do under stress conditions which are less discriminating. Yield stability index (YSI) measures Ys as a fraction of Yp. Genotypes, with high YSI index, minimize yield decline under stressed conditions. The results of the present study corroborate those of Nouri et al. (2011) who reported a negative and significant correlation between YSI and Yp and a strong positive and significant correlation with Ys. Similar results were obtained by Sio Se-Mardeh et al. (2006). The positive and significant correlation between YSI and Wi, found in the present study, suggests that YSI identify stable genotypes too. Nouri et al. (2011) and Mohammadi et al. (2011) mentioned that STI was suitable for sorting out the best yielding and stable genotypes under Ys and Yp growth conditions. According to Lin et al. (1986), W^2i represents the genotypic contribution to the GEI, as such a W^2i value near or equal to zero is suggestive of dynamic stability. No significant correlations were found, in the present study, between W^2i and Ys, nor between W^2i and Yp. In this context Benmahammed et al. (2010) report a positive and significant correlation between W^2i and Yp, suggesting that best performing genotypes under favorable environment are generally instable. Non significant correlation between Yp and W^2i indicates the independ-

ence of this stability parameter from grain yield under non stress conditions and the potential trade-off between grain yield potential and stability as mentioned by Lopes et al. (2012). Comparison of Ys and Yp of the advanced breeding lines selected on the basis of the studied indices indicated that STI and Pi identify high yielding and stable genotypes which take advantage of the growth conditions available in the favorable environment (Figure 3).

Selection based on YSI identifies genotypes which minimize Ys decline at the expense of Yp which is drastically reduced. Selection, based on W^2i identifies stable genotypes which deviate little from the grand mean (Figure 3). These results corroborated those of Weedon and Finckh (2019) who reported that W^2i identifies stable genotypes which exhibit low GEI, achieving a yield response parallel to the mean yield response of all genotypes. These results suggest that selection based on W^2i and YSI parameters favors stable but below average yielding genotypes. In contrast, selection based on Pi and STI favors high-yield genotypes adapted to a wide range of conditions. Yield stability is either static or dynamic. Genotypes showing static stability tend to yield similarly across all environments, showing specific adaptation to stress environments. Genotypes showing dynamic stability exhibit a mean response parallel to the mean response of all genotypes in the test environments. They are adapted to a broader range of environments.

3.3. GRAIN YIELD AND MORPHO-PHYSIOLOGICAL TRAITS RELATIONSHIPS

Correlation coefficients analysis per environment indicated consistent positive and significant correlations across seasons ($r = 0.446$, $p < 0.05$; $r = 0.641$, $p < 0.01$ and $r = 0.615$, $p < 0.01$) between GY and SN, suggesting that SN exerted a strong influence on grain yield. The relationships between GY, on one hand, and NKS, BIO and HI, on the other hand, were inconsistent showing significance in two out of three cropping seasons. Relationships between GY and TKM, DHE and CT were inconsistent reaching significance in one out of three cropping seasons. These results suggested the dependence of these relationships on the environment for their expression. The correlation between GY and CT indicated that this relationship is likely expected under stress rather than under non stress environment. So, to be efficient, selection based on CT should be done under stress conditions which allow expression of CT controlling genes. No significant correlations were observed between GY and RWC, PHT, MTS, CHL and ELWL (Table 3). The results of the correlation coefficients analysis indicated that that GY is influenced consistently by SN, while the effects of BIO, NKS, TKM, DHE, HI, CT on GY were environment-driven. GY was insensitive to the expressed variation of the physiological traits (RWC, PHT, MTS, CHL and ELWL). This contrasts with what has been reported by Awan et al. (2015) who observed that GY was closely linked to RWC and MTS. Lopes et al., (2012) noted that RWC failed to correlate to GY, while Nouri et al. (2011) concluded that RWC is a valuable analytical selection tool to improve wheat GY under drought stress.

Phenotypic correlations depend on genetic and en-

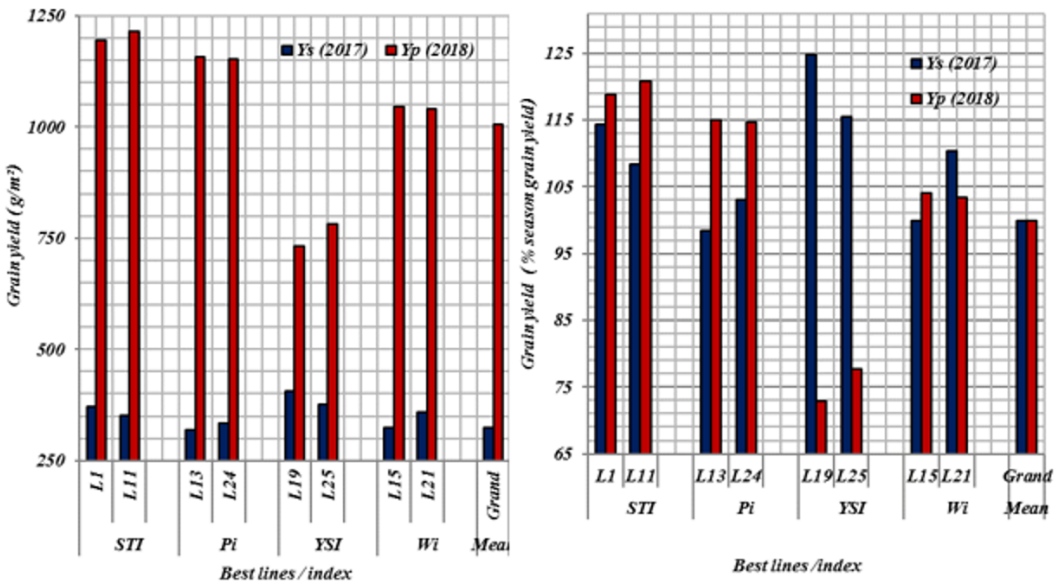

Figure 3: Grain yield performances (g m^{-2}, left figure and % of season grain yield, right figure) under stress (Ys) and non stress (Yp) growth conditions of the best breeding lines selected based on stress indices.

Table 3: Person's correlation coefficients between grain yield and morpho-physiological traits of the advanced durum wheat breeding lines tested during three successive cropping seasons (2016/17, 2017/18, 2018/19) at the ITGC- AES experimental site (Setif, Algeria).

	GY 2016/17	GY 2017/18	GY 2018/19
SN	0.446*	0.641**	0.615**
TKM	-0.099^{ns}	-0.392^{ns}	0.421*
NKS	0.700**	0.583**	0.232^{ns}
DHE	-0.096^{ns}	-0.158^{ns}	-0.503*
BIO	0.408*	0.693**	0.292^{ns}
HI	0.661**	0.211^{ns}	0.437*
PHT	0.032^{ns}	-0.274^{ns}	0.293^{ns}
RWC	0.054^{ns}	-0.178^{ns}	0.002^{ns}
MTS	-0.114^{ns}	-0.143^{ns}	-0.040^{ns}
CHL	0.124^{ns}	-0.109^{ns}	-0.246^{ns}
CT	-0.410*	-0.153^{ns}	-0.220^{ns}
ELWL	-0.163^{ns}	-0.069^{ns}	-0.103^{ns}
r 5%, 23 DF =		0.396	
r1%, 23 DF =		0.505	

GY = Grain yield (g m^{-2}), SN = Number of spikes m^{-2}, TKM = 1000-kernel mass (g), NKS = Number of kernels per spike, DHE= Number of days to heading, BIO = Above ground biomass (g m^{-2}), HI = Harvest index (%), PHT = Plant height (cm), RWC = Flag leaf relative water content, MTS = Membrane thermo stability (%), CHL = Chlorophyll content(cci), CT = Canopy temperature (°C), ELWL = Excised leaf water loss (%). ns, * and ** = non-significant and significant effects at 5 % and 1 % probability level, respectively, r 5% and r 1 %, = r table values at 5 and 1 % probability levels, respectively.

vironmental factors. Environmental factors may either enhance traits relationship or inhibit it, lessening the usefulness of such environment-driven traits in selection. The dependence of the relationship between traits on the environment was mentioned by Lopes et al., (2012) who found that TKM was positively associated with GY in some environments and negatively related in others environment, while CT was consistently associated with GY in all environments test. Furthermore, the absence of correlation between the independent and dependent variable could be due to the fact that the independent variable influences the dependent variable indirectly via other variables, rather than directly. These effects are not shown by the correlation coefficient. This inconvenient is avoided by using path analysis instead of the correlation coefficient analysis. In fact, path coefficient analysis subdivides the correlation coefficient into direct and indirect components, which allows determining which component influences substantially the dependent variable. Direct and indirect effects of the measured traits on grain yield are reported in table 4. Based on the categorization of the path coefficients suggested by Bhisma (2016) (absolute value < 0.100 is a negligible effect; < 0.300 > 0.100, small effect; > 0.300 < 0.500, medium effects, and > 0.500 is a large effect), the results of the present study indicated that NKS (0.738), SN (0.614), TKM (0.434), BIO (0.349) and HI (0.290), beside their appreciable direct effects, are a consistent route via which most of the measured traits influenced indirectly grain yield. This highlights their role as GY determinants. In fact, SN exhibited a large positive direct effect (0.614) on GY, and acted indirectly, on this trait, via NKS (-0.263) and BIO (0.218). The negative indirect effect via NKS comes out because of compensation effect between SN and NKS. This suggests that increased GY is amenable through selection for high SN. This trait, easily estimated visual, could be used to discriminate between assessed lines in the field. Khan et al. (2010) reported positive direct and indirect effects through PHT, NKS and TKM of SN on GY. TKM expressed moderate direct effect (0.434) on GY, associated with sizeable indirect and negative effects via NKS (-0.440). The indirect effects of this trait via the remaining measured traits were negligible. In contrast Khan et al. (2010) found a negative direct effect for TKM, associated to negative indirect effects via PHT and NKS. NKS influenced positively GY directly (0.738) and indirectly and negatively via SN (-0.219) and TKM (-0.259). No substantial indirect effects of NKS via the remaining measured traits were observed (Table 4). DHE showed negligible direct influence on GY (-0.095), since lateness affected negatively GY through SN (-0.229) and TKM (-0.295) decline, and through increase of spike fertility (indirect effect for NKS = 0.390). BIO acted directly (0.349) and indirectly via HI (-0.151) and via SN (0.383).

Table 4: Direct (diagonal) and indirect effects of the morpho-physiological traits on grain yield of the advanced durum wheat breeding lines tested during three successive cropping seasons (2016/17, 2017/18, 2018/19) at the ITGC-AES experimental site (Setif, Algeria): Data averaged across the three cropping seasons.

	SN	TKM	NKS	DHE	BIO	HI	PHT	RWC	MTS	CHL	CT	ELWL	rGY/yi	rGy/Yi -ß
SN	**0.614**	0.036	-0.263	0.035	0.218	-0.057	-0.024	0.002	0.001	0.004	0.007	0.001	0.574	-0.040
TKM	0.051	**0.434**	-0.440	0.065	-0.025	0.052	-0.008	-0.006	0.002	0.003	0.008	0.007	0.141	-0.293
NKS	-0.219	-0.259	**0.738**	-0.050	0.027	0.095	0.026	0.000	-0.002	-0.001	-0.025	-0.014	0.318	-0.420
DHE	-0.229	-0.295	0.390	**-0.095**	-0.028	-0.003	0.028	0.003	-0.002	-0.001	-0.004	-0.008	-0.242	-0.147
BIO	0.383	-0.032	0.057	0.008	**0.349**	-0.151	0.001	0.004	0.002	0.006	-0.006	-0.008	0.613	0.264
HI	-0.120	0.078	0.243	0.001	-0.182	**0.290**	0.006	-0.001	-0.001	-0.005	0.000	0.001	0.309	0.019
PHT	0.201	0.048	-0.264	0.036	-0.005	-0.024	**-0.074**	0.005	0.000	0.004	-0.003	0.000	-0.076	-0.002
RWC	-0.036	0.092	-0.010	0.009	-0.043	0.009	0.011	**-0.030**	0.001	0.003	-0.020	-0.003	-0.016	0.014
MTS	0.147	0.166	-0.240	0.029	0.101	-0.072	0.002	-0.007	**0.006**	0.002	-0.017	0.000	0.120	0.114
CHL	-0.089	-0.040	0.021	-0.002	-0.078	0.045	0.011	0.003	0.000	**-0.029**	0.018	0.002	-0.137	-0.108
CT	0.068	0.053	-0.282	0.006	-0.032	-0.001	0.003	0.009	-0.002	-0.008	**0.066**	0.008	-0.111	-0.177
ELWL	0.017	0.083	-0.294	0.021	-0.081	0.006	0.002	0.002	0.000	-0.002	0.015	**0.036**	-0.193	-0.229

GY = Grain yield (g m^{-2}), SN = Number of spikes m^{-2}, TKM = 1000-kernel mass (g), NKS = Number of kernels per spike, DHE = Number of days to heading, BIO = Above ground biomass (g m^{-2}), HI = Harvest index (%), PHT= Plant height (cm), RWC = Flag leaf relative water content, MTS = Membrane thermo stability (%), CHL = Chlorophyll content(cci), CT = Canopy temperature (°C), ELWL = Excised leaf water loss (%). Residual factor = 0.0264.

This trait showed negligible indirect effects via the other measured traits. Similarly, HI acted directly (0.290) and indirectly and negatively via BIO (-0.182), and via SN (-0.120) and positively via NKS (0.243). PHT showed sizeable indirect effects, positive via SN (0.201) and negative via NKS (-0.234). Khan et al. (2010) found positive direct effect of NKS on GY and positive indirect effects through PHT and TKM. The morphological traits measured (SN, TKM, NKS, DHE, BIO, HI and PHT) didn't express any sizeable indirect effect on GY via physiological traits. Similarly, physiological traits (RWC and CHL) didn't show any sizeable direct or indirect effects on GY, while MTS and CT and ELWL expressed moderate indirect effects. MTS affected indirectly and positively GY via SN (0.147) and via TKM (0.166) and negatively via NKS (-0.240). Similarly, CT (-0.282) and ELWL (-0.294) affected indirectly and negatively GY via NKS (Table 4). Globally these results suggested SN, NKS and BIO and HI should of interest to the breeding because of their role as GY determinants. These results corroborated those of Wolde et al. (2016) who found that HI and BIO impacted directly and indirectly GY, suggesting that these characters should be considered for selection either individually or combined under the form of an index.

3.4 TRAITS AND BREEDING LINES STRUCTURATION

Principal components analysis (PCA) grouped the recorded traits into 10 components, from which six exhibited an eigenvalue greater than unity, ranging from 4.03 for the first component to 1.30 for the sixth one. The retained principal components (PC) explained 23.7, 18.3, 10.9, 10.4, 8.5 and 7.6 %, respectively, with a cumulated variance reaching 79.45 %. The variability of the assessed breeding lines was interpreted based on the retained PC which indicated which of the measured traits were decisive in genotype differentiation. Based on their loading values, GY (0.474), STI (0.408), SN (0.345) and Pi (-0.438) were structured within the first component (PC1), while TKM (0.394), NKS (-0.516) and DHE (-0.418) determined the second component (PC2, Figure 4). BIO (0.418) and HI (-0.601) were well correlated with PC3.The physiological trait MTS (-0.423), and the stress indices YSI (0.416) and W^2i (0.522) were well represented on PC4. RWC (-0.417), CT (0.498) and ELWL (0.460) were more related to PC5, and PHT (-0.535) and CHL (0.420) were related to PC6. Based on traits assignation, PC1 could be used to target breeding lines with high GY, dynamic stability (high STI and low Pi indices value) and high SN. PC2 informs about breeding lines having high TKM, early to head but showing low NKS or lines having low TKM, late to head with high NKS (Figure 4). PC3 is a linear function of BIO production ability and partitioning (HI).

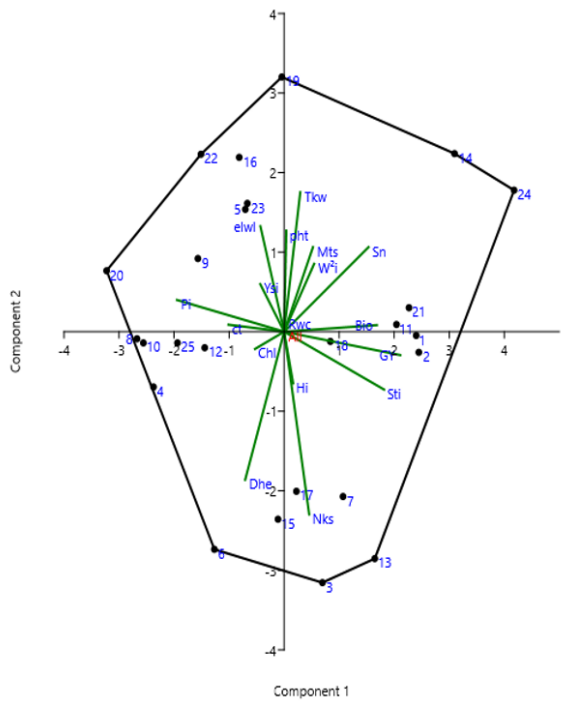

Figure 4: First two principal components (PC1, 23.7 % and PC2 = 18.3 %) biplot of the 25 durum wheat breeding lines based on standardized mean values of the morpho-physiological traits and stress indices.

Breeding lines expressing high heat tolerance (MTS) and yield stability could be found along PC4, lines characterized by high water status, low canopy temperature and low excised leaf water loss are present along PC5. Short stem breeding lines with high stay green ability could be looked for along PC6. Analysis of the relationships between the loaded variables and the differentiation of the breeding lines trait indicated that lines L1 (2.400), L2 (2.447), L11 (2.042), L14 (3.096), L21 (2.269) and L24 (4.178) with high positive scores on PC1 grouped separately from lines L4 (-2.375), L8 (-2.674), L10 (-2.558), L12 (-1.444) and L20 (-3.226) with negative scores along PC1 (Figure 4). Group of lines with positive scores gained, relatively to the mean value of the group of lines with negative scores, 21.40 % GY, 11.43 % SN, 27.98 % BIO, 73.07 % STI, -6.47 % CT, -17.93 % ELWL and -49.56 % Pi. Compared to the check cultivar Waha (L1), only L24 showed sizeable GY advantage (17.09 %), concomitant to a series of increases in TKM (19.94 %), HI (31.05 %), superiority genotypic (-95.61 %), stress sensitivity (-12.99 % reduction in STI), contribution to GEI (17.97 % increase in W^2i) and a slight reduction in BIO (-5.48 %). Based on its high GY ability L24 appears as a potential candidate for future release. From the group of lines well represented on PC2, L22 (2.231) had positive score and grouped separately from L3 (-3.155), L6 (-2.736), L7 (-7.072), L13 (-2.853), L15 (2.357) and L17 (-2.007) which had negative scores. These groups of lines diverge mainly for TKM and NKS. L22 appeared as potential genetic source to improve TKM (12.15 % increase over the check TKM mean), while L3, L6, L7, L13, L15 and L17 are good genetic sources of genes controlling spike fertility (12.78 to 17.77 % increase over check NKS mean), without any penalty on TKM (variation in TKM relative to check mean ranged from -1.41 to 4.94 %). Along PC3 were opposed line L5 (2.317) to line L18 (-2.198) which diverged essentially for BIO (15.10 % higher in L5 compared to L18) and HI (-21.80 % low in L5 compared to L18). Compared to the check cultivar, line L5 brought no sizeable change in BIO, nor in HI, but -14.40 % GY decline, while L18 exhibited no significant change in GY compared to the check but a decline in BIO (-16.20 %) and an increase in HI (+20.00 %). L18 appears as a good genetic source for future uses aiming to improve HI. Lines L16, L19 and L25 which were more related to PC4, as well as those related to PC5 (L9) and PC6 (L23) are less desirable as far as GY is concerned, in fact they yielded 6.54 to 14.66 % less than Waha, but L16 exhibited 10.02 % increase in MTS over Waha, associated with a decrease in YSI (-29.52 %), while L19 and L25 showed improvement in YSI over the check without sizeable change in MTS. L9 exhibited a decrease in CHL and L9 a reduction in ELWL. Based on these results, the assessed breeding lines were classified, at half way the maximum distance, into 5 clusters (Figure 5). The line L19 stemmed alone in a separate cluster, this line is a sister line to L25, whose cross pedigree is Ouasloukosl/5/Aznl/4Bezaizshf//SD19539/Waha/3/Gdr_2.

Cluster 2 included 9 lines (L25, L5, L22, L16, L20, L4, L8, L10 and L12). The cluster 3 contains 5 lines (L1, L2, L11, L14, and L24). L14 and L24 are sister lines with a cross pedigree $Terbol_{975}$/$Geruftel_2$ Cluster 4 included 3 lines (L21, L9 and L23), while cluster 5 included 7 lines (L17, L18, L7, L15, L6, L3 and L13). L3 and L23 are sister lines with a cross pedigree Sooty9/Rascon37//Storlom/5/Toska26/Rasco. The check cultivar Waha was included in cluster 3 (Figure 5). Deviation of the mean values of clusters C1, C2, C3, and C5, in %, from the mean values of the lowest grain yield cluster C4 are reported in Table 5. Compared to C4, breeding lines grouped in C3 showed appreciable increases in GY (13.06 %), BIO (17.72 %) and SN (20.15 %), associated with 10.76 % TKM decrease. They are stress tolerant and stable in the dynamic sense (23.94 % increase in STI, 39.6 % decrease in Pi), reacting to environment changes (210.72 % increase in W^2i). Among the lines included in this cluster the sister lines L24 and L14 were scored as the best lines for 7 (GY, SN, DHE, HI, PHT, CHL and Pi) and 5 traits (GY, BIO, DHE, PHT, and MTS) out 17 measured characters, respectively. Both lines are considered for released as cultivars, and may be used as parents in crosses to take advantage of the desirable characteristics they brought. L2 was scored as the best for its high RWC, and as such could be used as a genetic source for improving this characteristic in elite material. Similarly, L11, scored best for STI, could be crossed to transfer its stress tolerance and good yielding ability under stress and non stress conditions to advanced breeding plant material. L19 which stemmed alone as a cluster is sensitive to environmental changes (high W^2i) but was scored among the best for its ability to minimize GY reduction under stress conditions (high YSI value), and thus could be used in crosses as a source of germplasm to increase genetic variability of this characteristic in the segregating populations. Breeding lines included in cluster C4 are globally low grain yielding, but L9 from this cluster was scored as having the lowest CT, so it could be useful as a source for this desirable trait. Similarly, lines included in C5, are lower grain yield in general but some lines contain genes controlling desirable characteristics such as high RWC in L18 and low ELWL in lines L3 and L6.

Globally and as far as the objective of this study is concerned, the results indicated that if GY, BIO, SN, TKM, NKS and indices based on GY are more or less

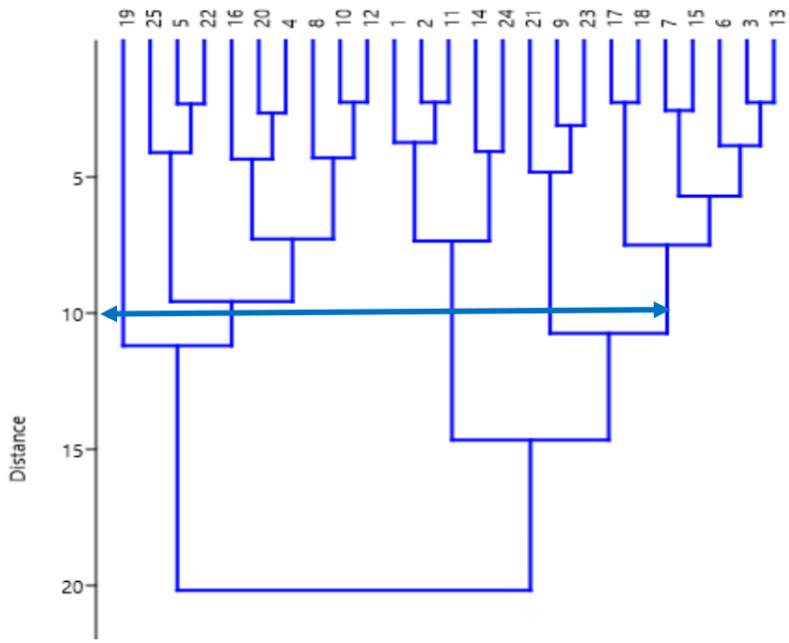

Figure 5: Clustering of the 25 durum wheat breeding lines based on standardized mean values of the morpho-physiological traits and stress indices.

Table 5: Deviation [(100 * (X_{Ci} - X_{C4}) / X_{C4})] of the mean values of clusters C1 (= L19), C2, C3 and C5 as % of the mean values of the lowest yielding cluster C4 for the measured traits and stress indices.

	C3	C5	L19	C2	Mean C4	Mean check (L1)
GY	13.05	5.44	1.4	-8.7	667.1	715.8
BIO	17.73	3.57	6.5	-3.5	469.2	568.3
SN	20.15	4.17	14.6	6.8	40.2	33.6
NKS	1.73	15.39	-9.4	-4.5	34.6	35.1
TKM	-10.76	-14.14	-4.3	-10.9	114.3	115.1
DHE	-0.07	1.32	0.2	0.9	1585.4	1883.1
HI	-4.62	2.09	-3.5	-4.1	41.0	36.3
PHT	2.22	-0.83	2.6	1.6	67.7	68.4
RWC	-5.97	-4.70	-6.1	-5.3	81.7	78.2
MTS	-2.86	-12.81	-13.7	-7.1	67.2	62.0
CHL	2.71	7.31	1.8	7.4	37.4	41.6
TCV	0.75	4.08	4.0	7.9	21.8	20.2
ELWL	1.95	-18.00	39.9	6.1	37.5	35.9
STI	23.94	13.34	-9.0	-21.7	31.8	33.2
Pi	-39.86	-16.25	31.3	45.1	0.4	0.3
YSI	-22.15	-17.11	48.7	-10.9	0.3	0.4
Wi	210.72	85.16	957.2	52.8	11.4	45.0

GY = Grain yield (g m^{-2}), BIO = Above ground biomass (g m^{-2}), SN = Number of spikes m^{-2}, NKS = Number of kernels per spike, TKM = 1000 - kernel mass (g), DHE = Number of days to heading, HI = Harvest index (%), PHT = Plant height (cm), RWC = Flag leaf relative water content, MTS = Membrane thermo stability (%), CHL = Chlorophyll content(cci), CT = Canopy temperature (°C), ELWL = Excised leaf water loss (%), STI = Stress tolerance index, Pi = genotypic superiority index, YSI = Grain yield stability index, W^2i = ecovalence.

correlated and easily accumulated in the plant materials, physiological traits on the contrary were unrelated to each other and to the first cited traits making difficult the concomitant selection for yield and stress tolerance driven by these traits. This is because selection is done under favorable conditions based mainly on GY. Under these conditions, physiological traits, markers of stress tolerance, are less expressed and not selected for. To bring altogether these traits and agronomic ones in the same genetic background, it is necessary to make complexes crosses between parents carefully chosen for these specific characteristics to enhance favorable genetic linkage and to generate new basic segregating populations with high genetic variability for these traits.

4 CONCLUSIONS

Even though the season main effect was the most preeminent source of variation, the results of the combined analysis of variance showed sufficient variability for most of the measured traits, justifying deeper analyses of the data. STI and Pi identify stress tolerant and high performing lines under Ys and Yp environments, showing wider adaptability. YSI is best suited to select drought tolerant lines which minimize grain yield reduction under stress but which are not responsive to fertility, showing biological stability. Wi^2 is best suited to select lines with reduced contribution to GEI. Both YSI and W^2i identify lines with specific adaptation. Based on correlation coefficients analysis GY showed a consistent correlation with SN, while its relationships with the remaining agro-morphological traits were inconsistent and environmentally-driven. The relationships with physiological traits were in most cases non significant. Path analysis results showed that BIO and HI, SN and to lesser extent NKS influenced directly GY, besides being a consistent route via which most of the measured traits influenced indirectly grain yield, suggesting that these traits should of interest to the breeding because. Physiological traits didn't show any sizeable direct effect on GY, their indirect effects were of varying sign and magnitude, via BIO and HI. Six PC explained altogether nearly 80.00 % of the total variation available in the data. PC1 was a function of GY, STI, SN and Pi. TKM, NKS and DHE were grouped within PC2. BIO and HI were well correlated with PC3. MTS, YSI and W^2i were represented on PC4 RWC, CT and ELWL were related to PC5, and PHT and CHL to PC6. Breeding lines dispersed along PC1 exhibited substantial differences in performances and stress tolerance abilities, among those lines L24 showed sizeable GY advantage which suggests it a potential candidate for future release. Potential genetic sources to improve morpho-physiological traits were identified on the retained PC's. The various breeding lines were grouped into 5 clusters. Breeding lines grouped in C3 were, in general, high yielding and stress tolerant, within this cluster L24 and L14 were scored as the best lines for 7 and 5 traits out of 17 measured characters, respectively. Both lines may be used as parents in crosses to take advantage of the desirable characteristics they brought. Globally and as far as the objective of this study is concerned, the results indicated that physiological traits were unrelated to each other and to morphological traits making difficult the concomitant selection for yield and stress tolerance driven by these traits. To bring altogether these traits and agronomic ones in the same genetic background, it is necessary to make complexes crosses between parents carefully chosen for these specific characteristics to enhance favorable genetic linkage and to generate new basic segregating populations with high genetic variability for these traits.

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Synthesis of a synthetic analogue for the Sitophilus weevil aggregation pheromone and study on its hygienic and toxicological indexes

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Synthesis of a synthetic analogue for the Sitophilus weevil aggregation pheromone and study on its hygienic and toxicological indexes

Abstract: The work was initiated to study hygienic and toxicological indices of a synthetic analogue for the *Sitophilus* weevil aggregation pheromone. The toxicity testing of 5-hydroxy-4-methyl-3-heptanone demonstrated its extremely low toxicity for the warm-blood animals, as compared to the one of the typical pesticides. The average lethal dose of the product per orally administered to the white mice was established to be 4375.0 mg kg^{-1} LD16 and LD84 being 2225.0 mg kg^{-1} and 6550.0 mg kg^{-1}, respectively. The average lethal dose for rabbits was 5900.0 mg kg^{-1} 5-hydroxy-4-methyl-3-heptanone proved to have a mild skin and conjunctival irritant action, and equally mild functional cumulation. As to chronic toxicity, the acceptable daily dose of 4.3 mg/person/d was calculated and scientifically substantiated. The odor threshold was determined at the dose ranging from 0.35 to 0.7 mg l^{-1} with the practical limit ranging from 0.35 to 1.5 mg l^{-1}, taste sensation threshold was found at the dose ranging from 1.0 to 3.0 mg l^{-1} with the practical limit ranging from 3.0 to 7.0 mg l^{-1}.

Key words: L-proline; aggregation pheromone; sitophilure; aldol condensation; pest insect, toxicological indexes

Sinteza sintetičnega analoga agregacijskega feromona in preučevanje higienskih in toksikoloških indeksov pri zatiranju žitnih žužkov (*Sitophilus* spp.)

Izvleček: Članek predstavlja raziskavo higienskih in toksikoloških indeksov pri sintezi agregacijskega feromona žitnih žužkov (*Sitophilus* spp.). Kot rezultat poskusov je bila izračunana dovoljena dnevna doza na osebo, in sicer 4,3 mg/osebo/dan; najmanjša izračunana doza za zaznavanje vonja je bila 0,42 mg l^{-1}, najmanjši vrednosti za okušanje in praktično uporabo sta bili določeni na ravni 1,44 in 3,3 mg l^{-1}. Največja dovoljena in priporočena doza agregacijskih feromonov za žitne žužke v vodnih zbiralnikih je bila 0,4 mg l^{-1}; pri čemer je bil vonj izbran kot omejujoč dejavnik za škodljivost; največja dovoljena koncentracija v zraku je bila določena kot 0,3 mg m^{-3} in največja dovoljena koncentracija v zraku v delovnem prostoru je bila določena kot 3,5 mg m^{-3}. Rezultati raziskave so pokazali, da je imel 5-hidroksi-4-metil-3-heptanon blag dražilni učinek na koži in na sluznicah ter šibke akumulacijske vplive na splošno počutje.

Ključne besede: optične izomere; L-prolin; agregacijski feromon; žitni žužki; aldolna kondenzacija; toksikološki indeksi

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1 INTRODUCTION

Production and reduction to practice of novel chemical, physical and biological products for pest management in agriculture are underway worldwide (Bohinc et al., 2020a). Search for novel highly efficient methods for pest control is driven by development of pesticide resistance in pest organisms caused by constant use of the products resulting in the uprise of the pesticide-resistant pest insect populations (Petrunya, 2011).

To prevent both quantitative and qualitative losses of agricultural products, the integrated pest management consisting of economically and environmentally feasible measures is needed. The system is based on the balanced combination of quarantine, preventive, physical-mechanical and biological measures, as well as of direct control to keep the proliferation of pest insects to reasonably safe level. The diversity of chemical products for crop protection suggests the increase of their toxic effects on living organisms making the search for environmental friendly protection products an urgent task.

Preventive and economic operations calling for high-level culture of storage and treatment of grain products at the granaries and grain mills comprise the backbone of the integrated test management. The fumigation or any other chemical treatment is supposed feasible in terms of grain infestation extent.

As natural bioregulators, the insect pheromones are thought to be promising in controlling the grain infestation extent at the granaries, maintaining the grain quality and avoiding the environmental pollution. The insect pheromones are known to exercise control over the pest insects resistant to existing insecticides or those requiring their multiple use (Trdan et al., 2019). This is especially true for covert living pests acquiring the pesticide resistance. The pheromone traps help bring to light the locations of pests with the lowest numbers when the visual inspection turns out inefficient. The synthetic analogues of the insect sex pheromones showed good results as tools for detection and monitoring of pest populations.

The synthetic chemicals, such as methyl bromide and aluminum phosphate were used in the largescale fumigations of granaries to control pests (Sousa et al., 2009). Furthermore, since larval and pupal instars develop within the rice and maize grains, any sustainable control strategy demands the timely and accurate monitoring of the prevalence of adult weevils (Ukeh et al., 2012). *Sitophilus* is a genus of weevils in the tribe *Litosomini*. Notable and the most menacing pest species include the rice weevil (*S. oryzae*, (Linnaeus, 1763), wheat weevil (*S. granaries*, Linnaeus, 1758), and maize weevil (*S. zeamais*, Motschulsky, 1858) (Ukeh et al., 2008), as well as the large mealworm, the cereal moth and other pests (Oehlschlager, 2016). The weevils of Sitophilus genus are the impactful pests of stored grains in tropical and sub-tropical regions, but they are common in Europe too (Bohinc et al., 2020b; Athanassiou & Buchelos, 2001). They attack various stored products including maize, wheat, oats, barley, rye, and dried cassava roots, as well as processed food such as macaroni, noodles, biscuits, and cakes. The post-harvest losses due to storage pests, such as weevils Sitophilus pose major problems to food security in the world (Orlov, 2006). The losses of stock products due to the insect pests like this have long been a serious farmer problem around the world. Any human efforts and finances committed in the crop production are wasted. Sitophilus infest ripening standing crops immediately prior to harvest and in storage, causing damage by boring into the grains and eating the inner part, which reduces maize mass and quality in terms of consumption and germination (Zakladnoi et al., 2003). The synthetic pheromones were successfully used to control the curculionid beetles by mass trapping, as the pheromones attract both males and females (Adda et al., 2002).

The pheromone-based monitoring helps determine the time and necessity of chemical treatment, avoid use of pesticides and save the stored products ecologically pure. The study on toxicological and hygienic characteristics is indispensable. Our work was initiated to study the toxicity of a synthetic analogue for the *Sitophilus* weevil aggregation pheromone.

2 MATERIAL AND METHODS

All chemicals and solvents were purified by standard techniques. For thin-layer chromatography (TLC), Silufol silica gel plates (Sigma-Aldrich, Germany) and ethyl ether/hexane (1:1) system of eluents was used; the compound was visualized by irradiation with iodine vapor. Flash chromatography was performed using TLC Silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) and ethyl ether/hexane (1:1) system of eluents. HPLC was performed using the Agilent 1100 Series HPLC System (USA) with a Daicel CHIRALPAK AS HPLC Analytical Column, 10 μm, ID 4.6 mm x L 250 mm - 20025, and Amylose tris [(S)--methylbenzylcarbamate] as a stationary phase coated on a 10μm silica support (Daicel Chemical Industries, Ltd. Japan); the mobile phase was acetonitrile (solvent A) and i-PrOH (solvent B) in the gradient mode: 0—10 min, 2—10 % B and 10—15 min 10 % B.

High-resolution mass spectra were recovered on 6420 Triple Quad LC/MS (Agilent Technologies, USA) with acetonitrile as an eluent in isocratic regime; the flow rate was 0.25 ml min^{-1}. The mass spectra were registered

with negative ionization. Conditions were as follows: the scanning range was 50-2200 m/z, dehydrating gas consumption – 3 min^{-1}, gas temperature 300 °C, gas pressure on needle sprayer 20 psi, evaporator temperature – 300 °C, tension on the capillary - 4000B.

2.1 GENERATION OF ALDOL PRODUCT

To generate the aldol product, propionaldehyde (3 mmol) was added to a heterogeneous mixture of anhydrous chloroform (10 ml), 3-pentanone (0.06 mol) and L-proline (25 %). The resulting mixture was stirred at the room temperature for 32 h. The reaction mixture was treated with the saturated NH_4Cl solution; the layers were separated, and the aqueous layers were extracted several times with ethyl acetate, dried with anhydrous Na_2SO_4 and evaporated. The pure aldol product was obtained by flash column chromatography with the yield of 74 %. HPLC was performed using ChiralPak AS - amylose tris [(S)-α-methylbenzylcarbamate] with 98 % MeCN and 2 % i-PrOH. Conditions were as follows: tR (major) = 7.998 VP, tR(minor) = 7.072 VV; S : R - 57.0722 : 28.0855, tR(major) = 8.007 VP, tR(minor) = 7.025 VV; S* : R*- 43.4765 : 30.0490. MS [C8H16O2], 143 [M – H+]-, 87 [M – C3H5O]-.

2.2 ACUTE TOXICITY

Acute toxicity assessment was performed on the laboratory white mice. The animals' behavior, general health condition, terms for manifestation of intoxication sign and eventually death were the key criteria for the product's acute toxicity following its single administration to the animals. 2.3 SKIN IRRITANT ACTION

To assess the skin irritant action of the compound, its native sample was applied on the skin of laboratory white rats and animals were carefully monitored for any skin irritation signs.

2.4. CONJUNCTIVAL IRRITATION

To assess the conjunctival irritant action of the compound, 2 drops of the product were put in the conjunctival sac of one rat's eye, while the second one served as the control. The effects were registered in 1-4 hours and in 1, 3 and 5 days.

2.5 CUMULATIVE PROPERTIES

To assess the cumulative properties of the compound, two groups of white 150-180g rats of both sexes were selected for the experiment. The first group of rats received the product at the dose of 1/10 LD_{50}, the second one served as the control.

2.6 CHRONIC TOXICITY

Chronic toxicity was expressed as effective and non-effective doses.

2.7 ASSESSMENT OF IMPACT IN THE ENVIRONMENTAL SAMPLES

To assess the impact of the compound in the environmental samples, its concentrations ranging from 0.17 to 8.0 g l^{-1} were used.

3 RESULTS AND DISCUSSION

The aggregation pheromone of *Sitophilus granarius* and *Sitophilus oryzae* is produced by the insects in the period good to the development of a population, that is, where there is enough food and possibilities for reproduction, as well as appropriate environmental humidity and temperature. The food attractants and sexual stimulants cannot supersede the aggregation pheromone produced by both females and males. The rise and corn weevils were found to produce the aggregation pheromone in the form of (4S, 5R) - hydroxy-methyl-3-heptanoneenantiomer (Fig.1) (Faustini et al., 1984).

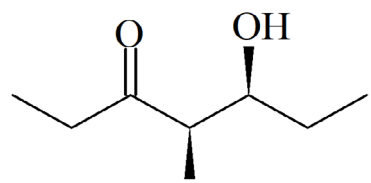

Figure 1: (4 S, 5 R) - hydroxy-methyl-3-hetanone

Diastereoisomers of 5-hydroxy-4-methyl-3-heptanone were identified as an aggregation pheromone produced by the male rice weevils (*Sitophilus oryzae*), and the maize weevils (S. *zeamais*). The compound has previously been reported as an aggregation pheromone produced by *Rhinostomus barbirostris* (Fabricius, 1775) (Coleoptera: Curculionidae, Fabricius, 1775) males. All three Sitophilus species belong to the subfamily Dryophthorinae, as *R. barbirostris* (Phillips et al., 1985; Ambrogi et

al., 2008; Ambrogi et al., 2009). This compound is known to cause a behavioral response in the adult weevils. According to Ismuxambetov (2015), and the findings from our trials, there is the weevils' response to various combinations of enantiomers. According to that formula, the compound can exist in the form of 4 steric enantiomers

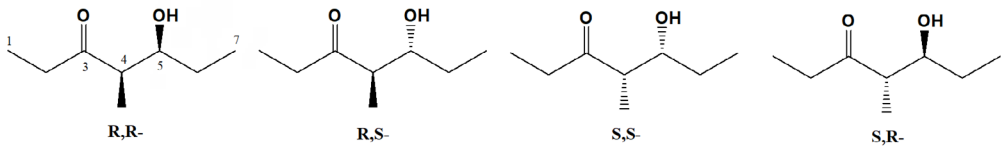

Figure 2: 4 steric enantiomers of (4, 5) - hydroxy- methyl-3-heptanone

(Fig. 2).

Unelius et al. resolved the four stereoisomers of 4-methyl-5-hydroxy-heptan-3-one by chiral gas chromatographic–mass spectrometric (GC-MS) analysis employing a CycloSil-B capillary column with the following elution order: (4R, 5S)-, (4S, 5R)-, (4R, 5R)-, (4S, 5S)-isomers (Unelius et al.,2012). Thus, to determine the absolute configuration of the *Rhinostomus barbirostris* pheromone, the mixture of all stereoisomers of synthetic sitophilure was also resolved in a CycloSil-B capillary column. The major component had the same retention time as the (4S, 5R)-isomer, and that of the minor pheromone component matched that of the (4S, 5S)-isomer (Unelius et al., 2012). The mixture of four stereoisomers of sitophilure has been synthesized previously by three different synthetic approaches. The first synthesis was reported even before the identification of this compound as a pheromone by coupling the lithium anion from 3-pentanone and propanal in 89 % yield (Smith & Levenberg, 1981).

Fauve et al. reported on the multigram synthesis as a more traditional approach with sodium hydroxide to generate the anion from 3-pentanone and subsequently to couple with propanal, giving hydroxyl-ketone in 46 % over-all yield (Fauve et al., 1984). The third method started by the alkylation of 3, 5-heptanedione following by the reduction of one carbonyl group in 76% yield for two steps (Kalaitzakis et al., 2006).

Unelius et al. identified the minor component of *Sitona discoideus* Gyllenhal 1834 (Coleoptera : Curculionidae, Gyllenhal, 1834) pheromone, as (4S, 5S)-5-hydroxy-4-methyl-3-heptanone by enantioselective GC analyses of the natural compounds on a modified β-cyclodextrine stationary phase in comparison with synthetic mixture of all four stereoisomers and derivatives obtained by enantiselective transesterification of the (4R, 5R)- and (4S, 5R)-isomers catalyzed by lipase from *Candida antarctica* (Goto, Sugiy. & Iizuka) Kurtzman, M.J. Smiley, C.J. Johnson & M.J. Hoffman (Unelius et al.,2012). We have synthetized diastereomers of 5-hydroxy-4-methyl-3-heptanone by means of different methods. The corresponding ketol has been obtained by aldol condensation between diethylketone and propylaldehyde; the asymmetric catalytic aldol reaction was carried out according to interaction of 3-pentanon and propylaldehyde by L-proline initiation, as well (Shakirzyanova et al., 2018). In the present work, in the conditions of aldol condensation between 3-pentanon and propyl aldehyde, we have obtained the corresponding ketol –

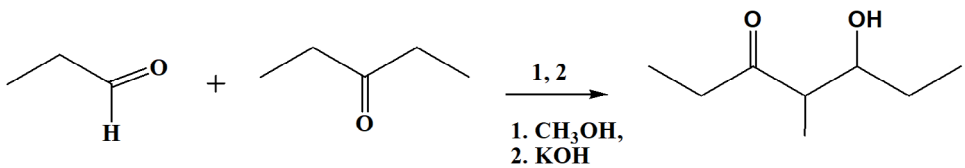

Figure 3: The synthesis of 5-hydroxy-4-methyl-3-heptanone

5-hydroxy-4-methyl-3-heptanone, an aggregation pheromone of granary and rice weevils (Fig. 3).

The ratio of R*S * and R*R* diastereomers was about 1:1. For aldol condensation we used 1.33 M 3-pentanon, 0.33 M propylaldehyde and 7 % KOH solution in methanol with reaction temperature 15°C and yield being 50 %. The findings from the preliminary studies taken into account, we performed the synthesis of the weevil aggregation pheromone adding aldol in the presence of L-proline as a catalyst.

Organocatalysis is a promising sphere in both organic and bioorganic chemistry. It is the way to get organic compounds from achiral predecessors in the presence of a small number of asymmetric metal-free organic molecules acting as the chiral inductors, but not getting involved into the process (Demyanovich et al., 2001).

Most enzymatic transformations have a synthetic counterpart. Often though, the mechanisms by which natural and synthetic catalysts operate differ markedly. The catalytic asymmetric aldol reaction as a fundamental C-C bond forming reaction in chemistry and biology is an interesting case in this respect. Chemically, this reaction is dominated by approaches that utilize preformed enolate equivalents in combination with a chiral catalyst (Denmark et al., 1998). Some of them, according to their activity and enantioselectivity, do not act as metal-complex catalysts. The aldol reaction is widely regarded to be one of the most important carbon-carbon bond-forming utilized in organic synthesis (List et al., 2010). The catalytic enantioselective carbon-carbon bond-forming is a significant problem in the synthesis of biologically active substances. The chiral amino acid catalysts have wide usage for most powerful enantioselective reactions (Hayashi, 2005). In nature, that reaction goes through the action of aldolases (types I and II). In the laboratory conditions, their effect could be simulated by natural amino acids, some chiral amines and small peptides. Amino acids use a fundamentally different strategy and catalyze the direct aldolization of two unmodified carbonyl compounds (Ramachandran et al., 1996). Many enantioselective aldol-krotonic reactions catalyzed by L-proline; and the reaction is performed via an enamine intermediate complex. The L-proline-catalyzed aldolyzation can be initiated with different aldehydes.

The low price and wide spread availability in two enantiometric forms are among the advantages of L-proline as a catalyst. Proline, as a small molecular compound, can be used for direct intramolecular aldol condensation (List et al., 2010), and many reactions with proline can be performed in industrial production. It is also important that the reaction be conducted in the room conditions without inert medium, requiring no transformations of the carbonyl substrate; that is, with neither deprotonation nor stimulation during reaction. Moreover, the catalyst is water soluble and can be easily extracted.

Here, we describe structure-activity relationships of proline catalysts of direct assymmetric aldol addition reactions, as well as the findings from study on the reaction mechanism. As the result of interaction of 3-pentanone, as a donor and propion aldehyde, as an acceptor into the presence of L-proline, the direct asymmetric aldolization has been performed. The L-proline-catalyzed asymmetric aldolization requires anhydrous solvents. The reaction was performed via an enamine intramolecular complex. As it is, the intramolecular complex of direct asymmetric aldol reaction is the enamine formation involving L-proline and appropriate 3-pentanone, a donor-substrate. Thereafter, the acceptor's carbonyl-group from propionaldehyde attacks the enamine intramolecular complex;

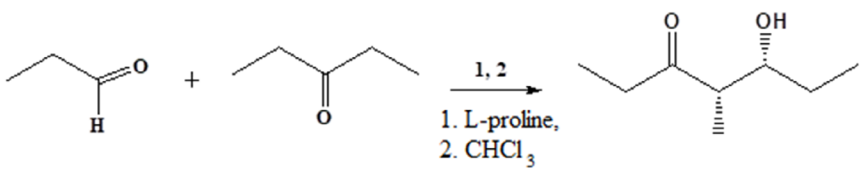

Figure 4: Synthesis of the L-proline-catalyzed aldol product

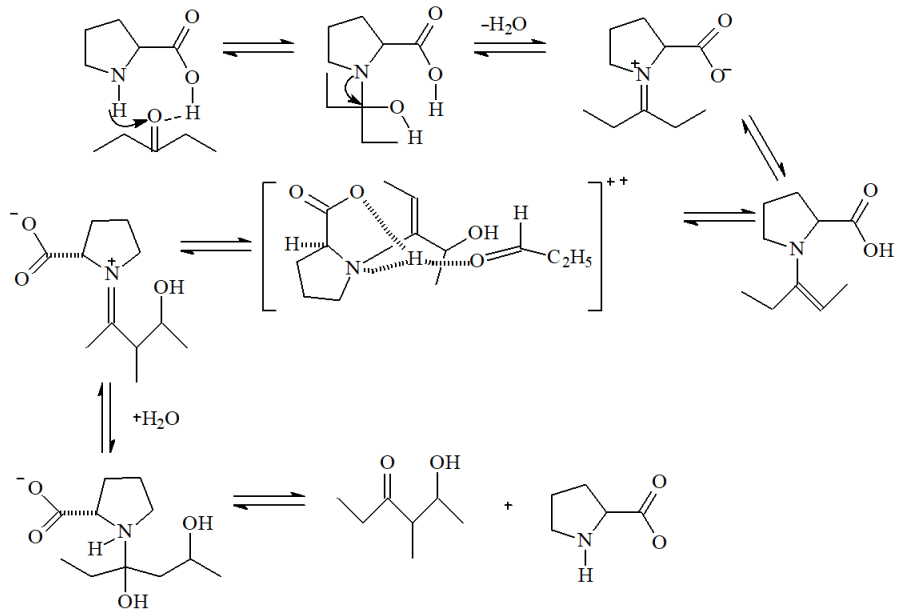

Figure 5: Proposed enamine mechanism of the proline-catalyzed asymmetric aldol reaction

the process is completed by formation of an aldol product (Fig. 4).

After separation, the reaction product was analyzed by HPLC (ChiralPak® AS – Amylose tris [(S)-α-methylbenzylcarbamate] with 98 % MeCN and 2 % i-PrOH.

In addition, we have analyzed the component consisting of (4S, 5S)-4-methyl-5-hydroxy-heptan-3-one by the electrospray ionization mass spectrometry (ESI-MS) with the registration of the mass spectra with negative ionization.

Thus, we have synthesized isomers of (5S, 4S)–-5-methyl-4-hydroxy-3-heptanone, an aggregation pheromone of rice, granary and maize weevils. The interaction of 3-pentanone and propionaldehyde in the aldol condensation reaction was realized in the presence of L-proline. Chloroform was more suitable solvent for the cross-coupling. The aldolization was performed via the enamine intramolecular complex. Changing terms of reaction couldn't influence the product's of enantio selectivity (Fig. 5).

Acute toxicity assessment revealed that animals' snorting and cheeping were among the typical clinical symptoms. They started rushing around the cages; the excitement was followed by the motor activity reduction; the breath was shallow. The animals died within the first experimental day. The average lethal dose of the product per orally administered to the white mice was established to be 4000.0 mg kg^{-1}; LD_{16} and LD_{84} being 2600.0 and 5300.0 mg kg^{-1}, respectively. The dose range from 1000.0 to 7000.0 mg kg^{-1} was used to be per orally administered to the white rats. Statistical processing of the data allowed establishing the average lethal dose to be 4375.0 mg kg^{-1}; LD_{16} and LD_{84} being 2225.0 mg kg^{-1} and 6550.0 mg kg^{-1}, respectively. According to Deichmann (1943), the average lethal dose for rabbits was 5900.0 mg kg^{-1}. In accordance with the Uzbekistan Sanitary Regulations and Standards for Classification of Pesticides by Toxicity and Hazard (Iskandarov et al., 2015), as a low-toxic compound 5-hydroxy-4-methyl-3-heptanone can be ranged in the IV class of hazard.

Following the 4-hour exposure, as well as in 1 and 16 hours after single exposure the dermal response was registered. At application the strong odor of the product made the animals sneeze and snort. The application removed and washed out, insignificant hyperemia of the chosen areas was observed to preserve after 1 hour and to be less marked one. In 24 hours after the experiment started, the residual hyperemic effect persisted; neither skin cracks nor peeling was registered. No signs of skin irritant action were found on the 2nd day of experiment. The findings could be the evidence for weak skin irritant action of the compound under study.

Conjunctival irritation results showed that following the administration, the rats cheeped and snorted trying to scratch the eye. In an hour, insignificant conjunctival hyperemia could be registered; the palpebral fissure was narrowed. In 3 hours, the signs of irritation subsided, while mild hyperemia and the palpebral fissure's narrowness persisted. In 24 hours, the palpebral fissure's size

was within normal limits; hyperemia was unregistered. During the whole period of assessment (up to 5 days), no signs of irritation could be seen to confirm the mild conjunctival irritation.

Evaluation of the cumulative properties showed that during the experiment no animals died; any calculations of the cumulation factor failed. But manifestation of some intoxication signs suggested the mild functional cumulation. Minimum effective and non-effective doses of the compound under study were established to be 18.0 and 3.6 mg kg^{-1}, respectively. The acceptable daily dose of 4.3 mg/person/d was calculated and scientifically substantiated.

According to environmental impact assessment data the sample was found to give a specific indescribable smell and bitter taste tang to water. The odor threshold (1 point) was determined at the dose ranging from 0.35 to 0.7 mg l^{-1} with the practical limit ranging from 0.35 to 1.5 mg l^{-1}, taste sensation threshold was found at the dose ranging from 1.0 to 3.0 mg l^{-1} with the practical limit ranging from 3.0 to 7.0 mg l^{-1}.

4 CONCLUSION

As it can be seen, negligible effective doses and the environmental safety are typical of pheromones used to assess the phytosanitary conditions, localization and eradication of pest centers and to reduce the numbers of quarantine pest insects. In contrast to pesticides (Abasov et al., 2015), the pheromones' effects are not toxic and environmentally safe. We have synthesized isomers of (5S, 4S)–-5-methyl-4-hydroxy-3-heptanone, an aggregation pheromone of rice, granary and maize weevils. Our toxicity testing of 5-hydroxy-4-methyl-3-heptanone demonstrated its extremely low toxicity for the warm-blood animals, as compared to the one of the typical pesticides.

The average lethal dose of the product per orally administered to the white mice was established to be 4375.0 mg kg^{-1}; LD_{16} and LD_{84} being 2225.0 mg kg^{-1} and 6550.0 mg kg^{-1}, respectively. According to D. LeBlanc, the average lethal dose for rabbits was 5900.0 mg kg^{-1}. 5-hydroxy-4-methyl-3-heptanone proved to have a mild skin and conjunctival irritant action, and equally mild functional cumulation. As to chronic toxicity, the acceptable daily dose of 4.3 mg/person/d was calculated and scientifically substantiated. The odor threshold was determined at the dose ranging from 0.35 to 0.7 mg l^{-1} with the practical limit ranging from 0.35 to 1.5 mg l^{-1}, taste sensation threshold was found at the dose ranging from 1.0 to 3.0 mg l^{-1} with the practical limit ranging from 3.0 to 7.0 mg l^{-1}.

As a part of the integrated pest management, the pheromone-based monitoring is successfully used to control the most menacing *Sitophilus* and *Rhinostomus barbirostris* species in Uzbekistan.

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Thermal and functional properties of starch extracted from tubers cultivated in the Ecuadorian Andean region

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Thermal and functional properties of starch extracted from tubers cultivated in the Ecuadorian Andean region

Abstract: Thermal and functional properties of starch extracted from American taro and Indian shot were determined to assess their use in food products. Starch was extracted by the wet-milling method. Physicochemical composition was determined following the Association of Official Agricultural Chemists (AOAC) protocols. Total fibre was measured by the Total Dietary Fiber Assay Kit. The morphology of starch granules was observed by scanning electronic microscopy (SEM). Gelatinization temperature and viscosity were measured by Differential Scanning Calorimetry (DSC) and with a rapid viscosity analyser (RVA), respectively. Swelling capacity, solubility index, and absorption index were measured at 15, 60, 70, 80, and 90 °C. The yield for Indian shot (72.5 %) was higher of that for taro (60.2 %). No significant differences ($p > 0.05$) were found for moisture, ashes, total fibres, and protein; significant differences were found for fat content, total carbohydrates, amylose, and amylopectin. Granules of Indian shot starch featured ovoid shapes (diameter, 30 μm), while granules of American taro starch presented round shapes (diameter, 15 μm. Gelatinization temperature for American taro (78.33 °C) was higher of that for Indian shot (65.28 °C). Maximum viscosity in Indian shot (3,535.5 cP) was higher of that in American taro (2,446.5 cP). Concerning functional properties, Indian shot starch yielded higher values. Moreover, at high temperature values, American taro starch presented better gelling results than those in Indian shot.

Key words*:* starch; functional properties; *Canna edulis* (Ker.) Gawl; *Xanthosoma sagittifolium* (L.) Shott ; thermal properties

Termične in funkcionalne lastnosti škroba pridobljenega iz gomoljnic, gojenih v ekvatorilnih območjih Andov

Izvleček: Termične in funkcionalne lastnosti škroba iz ameriškega tara (karibskega zelja) in užitne kane so bile določene za oceno njihove uporabnosti v prehrambenih izdelkih. Škrob je bil pridobljen z metodo mokrega mletja. Določitev njegove fizikalno-kemijske sestave je sledila protokolu Zveze kmetijskih kemikov (Association of Official Agricultural Chemists -AOAC). Vsebnost celokupne vlaknine je bila izmerjena s kitom za določanje celokupne prehranske vlaknine. Morfologija škrobnih zrn je bila pregledana z vrstično elektronsko mikroskopijo (SEM). Temperatura želiranja in viskoznost sta bili izmerjeni z metodo diferenčne dinamične kalorimetrije (DSC) in hitrim merilcem viskoznosti (RVA). Sposobnost nabrekanja, indeks topnosti in absorpcijski indeks so bili izmerjeni pri 15, 60, 70, 80, in 90 °C. Pridelek užitne kane (72,5 %) je bil večji kot pri ameriškem taru (60,2 %). Nobene značilne razlike ni bilo ($p > 0,05$) v vsebnosti vode, pepela, celukopne vlaknine in beljakovin; značilne razlike so bile ugotovljene v vsebnosti maščob, celokupnih ogljikovih hidratov, amiloze in amilopektina. Šrobna zrna užitne kane so bila jajčaste oblike (diameter, 30 μm), med tem, ko so bila pri ameriškem taru okrogla (diameter, 15 μm). Temperatura želiranja je bila pri ameriškem taru višja (78,33 °C) kot pri užitni kani (65,28 °C). Maksimalna viskoznost je bila pri užitni kani večja (3,535.5 cP) kot pri ameriškem taru (2,446.5 cP). Škrob iz užitne kane je imel boljše funkcionalne lastnosti, medtem ko je imel škrob iz ameriškega tara pri višjih temperaturah boljše želirne lastnosti.

Ključne besede: škrob; funkcionalne lastnosti; *Canna edulis* (Ker.) Gawl.; *Xanthosoma sagittifolium* (L.) Shott.; termične lastnosti

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1 INTRODUCTION

American taro (*Xanthosoma sagittifolium* (L.) Shott) and Indian shot (*Canna edulis* (Ker.) Gawl.) are among the rhizomatous plants cultivated in the Ecuadorian Andean region and used as food sources. Corms of both rhizomes are counted as botanical sources of relevant nutritional value (López et al., 1995). A significant amount of starch, representing circa 68.50 %, dry basis, occurs in taro tubers (Palomino et al., 2010), thus representing a practical raw material for a number of uses in food systems and other industrial applications (Torres, et al., 2013). Taro starch is considered as a polymer of high nutritional value, as substantial amounts of dietary fibre occur in its chemical composition (Palomino et al., 2010). Recommendations towards taro starch consumption in human diets have been previously acquainted (Huang et al., 2007). Indian shot rhizomes bestow relevant nutritional values in terms of carbohydrate, protein, and fibre contents, respectively (Barrera et al., 2004). Starch percentage in Indian shot ranges from 75 to 80 % (Gallant et al., 1982). Gelatinization temperatures of starch granules vary accordingly to intrinsic factors, e.g., granule size (bigger granules undergo through swelling processes and retain water), and botanic source of the starch, (García, 2015), amylose/amylopectin ratio (Paredez-López, 1994); extrinsic factors affecting gelatinization temperatures include heating speed, moisture content, mechanical damage over granules, and conditions of starch extraction (Parker y Ring, 2001). Gelatinization effects over a water/starch solution and the influence degree of heating speed over transition processes can be measured by Differential Sweeping Calorimetry (DSC) (Pineda-Gómez et al., 2010). Starch viscosity analysis aids to determine starch uses such as a food component or other applications. Maximum viscosity temperature is higher to that of gelatinization. Maximum viscosity denotes the degree of swelling in starch granules before the breakdown process at certain concentrations (Cortés, 2015). In addition, size and shape variation of starch granules affect gelatinization processes. Size and shape may vary depending on the botanical source from where the starch in question was extracted (Amaya et al., 2011). Due to gelatinization processes, starch granules start to swell, hence affecting the starch solubility. Swelling and releasing of soluble material starts from the hilum to the periphery of the granule (Singh et al., 2003). Scanning Electron Microscopy (SEM) is a technique widely used to observe the size and the shape occurred in starch granules. Concerning starch availability, American taro and Indian shot may replace conventional raw materials commonly used in the food industry such as corn, yam, cassava, potato, etc. (Vázquez, 2013). The aim of the present research work was to determine thermal and functional properties in starch extracted from American taro (*Xanthosoma sagittifolium*) and Indian shot (*Canna edulis*), crops cultivated in the Ecuadorian Andean region, in order to seek for potential uses in processed food products.

2 MATERIAL AND METHODS

2.1. RAW MATERIAL

For the purposes of the present research work, taro tubers were purchased in Santo Domingo de los Tsáchilas, Ecuador; conditions for growth were the following: rainy season, 625 meters above sea level (MASL), average temperature 21 °C, and 87 % of relative humidity. Indian shot rhizomes were purchased in Loja, Ecuador; conditions for growth were the following: rainy season, 2065 MASL, average temperature 16 °C, and 74 % of relative humidity.

2.2. STARCH EXTRACTION

Starch was extracted by the wet-milling method (Bello-Pérez et al, 2010). Weighed, peeled, and chopped rhizomes of both taro and Indian shot were separately embedded in a 5 % citric acid/water solution. Each concoctions were left stand for 40 minutes and afterwards was blended with water (1 : 5 ratio, concoction : water) at 2000 rpm in a blender (Fleetwood by Skymsem LAR-15/25L, Skyfood Equipment LLC., North Miami, FL, United States). The resulted solutions were sieved consecutively through meshes of 0.841, 0.149, and 0.047 mm. Further water washings were carried out over the retained material in the meshes upon any turbid washing liquid was obtained. The filtered liquids were allowed to stand for 4 hours. Sediments containing starch were separated from the liquid phase and were dried at 55 °C for 24 hours in a laboratory oven (INB 500, Memmert GmbH + Co. KG, Schwabach, Germany).

2.3. STARCH PHYSICOCHEMICAL ANALYSIS

Percentages of moisture (925.10), ashes (923.03), protein (920.87), and crude fat (920.85), were determined after AOAC methods (AOAC, 2005). Total carbohydrates were determined by subtraction from the total sum of the other physicochemical parameters. Total fibre was determined by the Total Dietary Fiber Assay Kit (Megazyme Ltd., Bray, Ireland).

2.4. STARCH GRANULES SIZE AND SHAPE

Size and shape of starch granules were identified by Scanning Electronic Microscopy (SEM). To improve conductivity and thus to enhance imagining resolution, starch samples were coated with gold. Samples were observed and photographed with a scanning electron microscopy JSM 6300 SEM (SEMTech Solutions, Inc. North Billerica, United States) at X500, X1000, and X1500 zoom values, respectively.

2.5. STARCH GRANULES SWELLING CAPACITY, SOLUBILITY RATES, AND WATER ABSORPTION

1 g of each starch sample was weighed in an analytical balance AES 200 (Kern & Sohn GmbH, Balingen, Germany) and then transferred to centrifuge tubes of 50 ml volume capacity. 10 ml of deionized water were added to each tube; tubes were agitated in a vortex (WISD VM-10) for 30 seconds at room temperature. Afterwards, four centrifuge tubes per each starch sample were heated in a water bath at 60, 70, 80, and 90 °C, respectively, for 30 minutes. Then, tubes were cooled in a water bath at 10 °C and were allowed to stand until reaching room temperature. Cooled tubes were spun at 1200 rpm for 30 minutes in a universal centrifuge Z 326 (Hermle Labortechnik GmbH, Wehingen, Baden-Württemberg, Germany). The resulted supernatant from the centrifugation process on each tube was decanted and weighed in previously-tared-and-dried aluminium trays. The resulting gel, wet and dry supernatant were weighed (soluble material mass) in order to determine:

Water absorption index (WAI) = Gel mass/Sample mass

*Starch solubility index (SSI) = (Soluble material mass/ Sample mass)*100*

Swelling capacity (SC) = Gel mass/(Sample mass-Soluble material mass)

2.6. GELATINIZATION TEMPERATURE

2 mg (dry basis) of each starch sample were weighed onto previously weighed aluminium sample pans. To prepare a solution containing 20 % of solids, water was added with a micropipette over samples. Calorimetry measurements were determined over sealed sample pans in a DSC Q2000 (TA Instrument, New Castle, United States) at a heating rate of 5 °C min^{-1} until 100 °C. An empty sample pan was used as the blank reference to develop the baseline. Analyses were carried out threefold. Initial Temperature (Ti), Peak Temperature (Tp), Final temperature (Tf), and Gelatinization Enthalpy (ΔH) of each sample identified from the generated charts (thermograms).

2.7. VISCOSITY ANALYSIS

Viscosity profiles of both starch samples were determined by a Rapid Viscosity Analyzer RVA 4500 (PerkinElmer, Waltham, United States) at the following conditions: profile temperatures from 50 °C to 92 °C, temperature increment of 5 °C min^{-1}, stirring rate of 160 rpm. Samples were analysed threefold. According to the equipment specifications, 3 g of starch were weighed (dry basis) and then 25 ml of water were added. Parameters obtained from RVA graphs were: peak viscosity (PV, maximum viscosity reached by the sample during starch gelatinization), peak time (tP, time required by samples to reach maximum viscosity), peak temperature (TP, time temperature required by samples to reach maximum viscosity), hot paste viscosity (HPV, minimum viscosity to reach 92 °C), cold paste viscosity (CPV, viscosity when the paste cooled down to 50 °C), breakdown (BD, stability of granules after gelatinization), calculated as the difference between PV and HPV, and setback (SB), obtained as the difference between CPV and HPV.

2.8. STATISTICAL ANALYSIS

Significant differences ($p \leq 0.05$) were determined by a multiple comparison test of treatment means (Tukey Post-Hoc test) performed by the statistical package SPSS, version 21 (SPSS Institute Inc., Cary, United States). Graphical representation of data was generated by Origin 50 (Originlab, Northampton, United States).

3 RESULTS AND DISCUSSION

3.1. STARCH PHYSICOCHEMICAL COMPOSITION AND YIELD

The chemical composition for taro and Indian shot showed similar values to those reported by Palomino et al. (2010) and Quicaña-Avilés (2014), respectively. The chemical composition occurred in starch samples of taro and Indian shot is detailed in Table 1 showed below. Yields obtained were 60.2 % in taro and 72.5 % in Indian shot, respectively on fresh mass basis.

Table 1: Chemical composition of taro (TS) and Indian shot starches (IS)

Composition (%)	TS	IS
Moisture	$13.18^{a} \pm 0.17$	$16.57^{b} \pm 0.65$
Ashes	$0.20^{a} \pm 0.01$	$0.15^{b} \pm 0.13$
Protein	$2.05^{a} \pm 0.01$	$0.33^{b} \pm 0.07$
Fat	$0.13^{a} \pm 0.012$	$0.11^{a} \pm 0.02$
Carbohydrates	$84.45^{a} \pm 0.02$	$82.85^{b} \pm 0.83$
Total fibre	$4.11^{a} \pm 0.03$	$4.54^{a} \pm 0.11$
Purity	$93.52^{a} \pm 0.45$	$94.88^{b} \pm 0.37$
Amylose	$35.62^{a} \pm 1.72$	$28.59^{b} \pm 1.05$
Amylopectin	$64.38^{a} \pm 2.72$	$71.41^{b} \pm 1.78$

Average of 3 repetitions (n = 3) ± standard deviation
* Different letters in each row show significant differences ($p < 0.05$)

3.2. STARCH GRANULES SIZE AND SHAPE

Differences regarding size and shape between taro and Indian shot were found, this was attributed to the different biological and chemical nature on each botanical source. Indian shot starch granules featured homogeneous oval-shaped, smooth surface, and 30 μm of size, approximately. Taro starch granules were of circular shape, rough surface, and a size of 15 μm on average. Fig. 1 and Fig 2 feature size and shape of granules in both starch samples.

3.3. GELATINIZATION TEMPERATURE

Indian shot starch presented a lower peak gelatinization temperature (65.28 °C) than that in taro starch (78.33 °C). Starch granules with larger diameters gelled in shorter times and lower temperature (García, 2015); howbeit the required enthalpy value for such process was higher in Indian shot starch than that in taro starch: 14.14 and 12.16 (J g^{-1}), respectively. Another salient factor influencing over gelatinization temperature in starch is amylose concentration, i.e., when comparing the gelatinization temperatures between two types of starch of different botanical origin, the one bestowing higher amylose concentration requires higher temperatures in order to reach a gelatinization peak (Paredez-López, 1994). Table 2 depicts gelatinization temperature values in taro starch and Indian shot starch. Fig. 3 shows the gelatinization behaviour of taro starch and Indian shot starch, respectively.

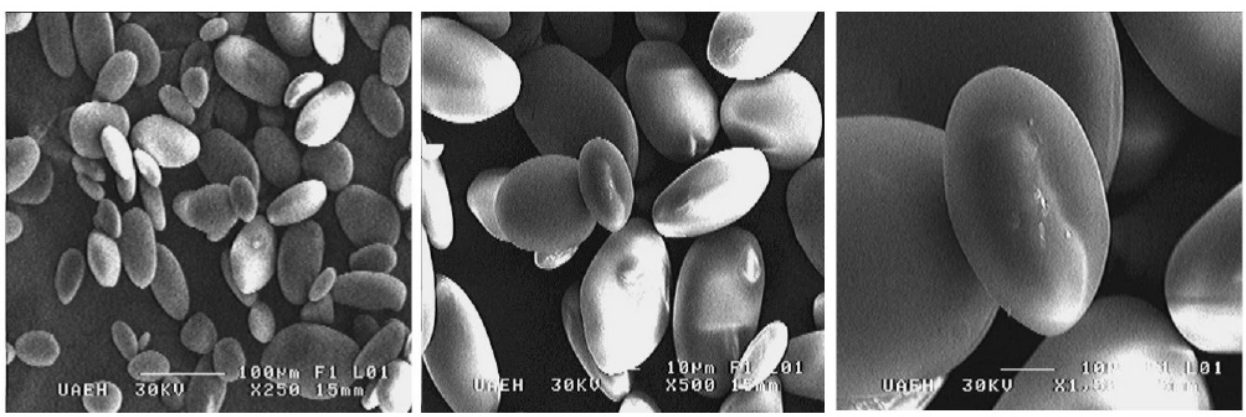

Figure 1: Indian shot starch granules at x 250, x 500, and x 1500, respectively

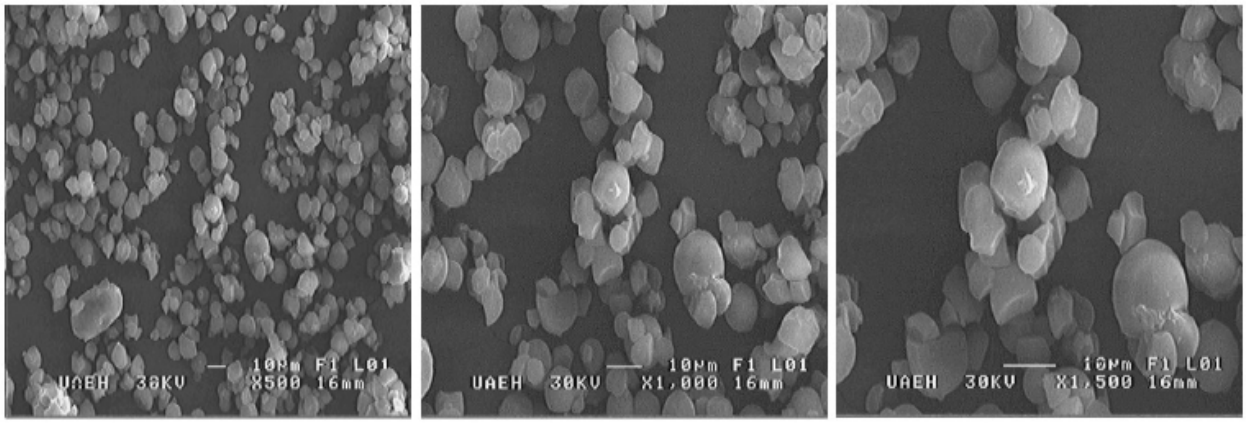

Figure 2: Taro starch granules at x 250, x 500, and x 1500, respectively

Table 2: Gelatinization temperature in taro starch and Indian shot starch

Sample	Ti (°C)	PGT (°C)	Tf (°C)	ΔH (J/g)
TS	76.09[a] ± 0.21	78.33[a] ± 0.12	84.72[a] ± 0,03	12.16[a] ± 0,31
IS	62.48[b] ± 0.05	65.28[b] ± 0.07	73.12[b] ± 0.18	14.14 [b] ± 0,04

Ti: initial temperature, PGT: peak gelatinization temperature, Tf: final temperature, ΔH: gelatinization enthalpy.
Average of 3 repetitions (n = 3) ± standard deviation
* Different letters in each row show significant differences ($p < 0.05$)

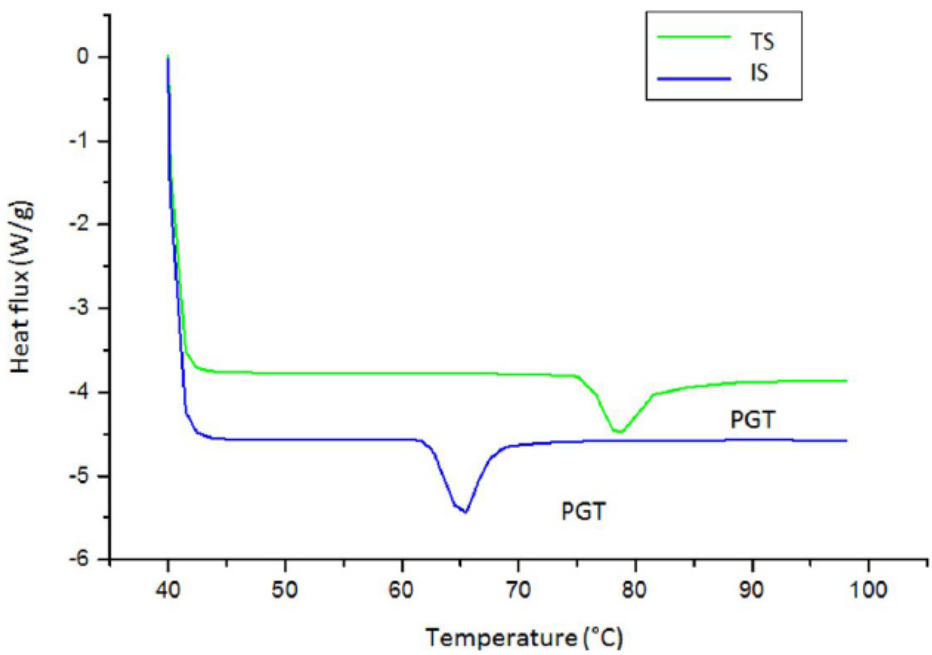

Figure 3: Gelatinization behaviour of taro and Indian shot starches

Hernández-Medina et al. (2008), reported gelatinization temperatures for starch extracted from Makal (*Xanthosoma yucatanensis* Engl.) and Sago (*Cycas circinalis* L.) of 78.4 °C and 74.9 °C, respectively. Gelatinization temperatures found in taro starch were consistent to the previously reported. Indian shot starch showed a gelatinization temperature values similar to those reported for starch extracted from sweet potato, cassava, oat, and barley (61.3, 65.2, 66.5, and 67.3 °C, respectively) (Cantellano-Jarrillo et al., 2016). Starch featuring high gelatinization temperatures may be used in food products requiring high processing temperatures, e.g., canned. Starch featuring lower gelatinization temperatures may be incorporated into formulations of products such as sweets, puddings, and soups (Hernández-Medina et al., 2008).

3.4. RAPID VISCOSITY ANALYSIS (RVA)

As depicted in Fig. 4, starch extracted from Indian shot showed a higher peak viscosity in shorter time and a lower heating temperature (3535.5 cP; 3.4 min at 76.45 °C) compared to starch extracted from taro (2446.5 cP; 4.56 min at 89.4 °C). This difference is related to the size of starch granules. Starch extracted from Indian shot presented granules with a larger average size than those occurred in starch extracted from taro. Accordingly, the former absorbs larger quantities of water than the latter. Fig. 4 shows the viscosity amylograms found for starch extracted from taro and Indian shot, respectively.

Gel generated from taro starch showed a lesser degree of instability, i.e., breakdown, than that of the gel obtained from Indian shot starch. The evaluation of the breakdown provides information regarding gel stability and resistance when starch is subjected to shear in further food processing. The lower the value, the more stable the starches are opposite to mechanical fragmentation (Lucas, et al., 2013). Table 3 presents the results for RVA over starch extracted from taro and Indian shot, respectively.

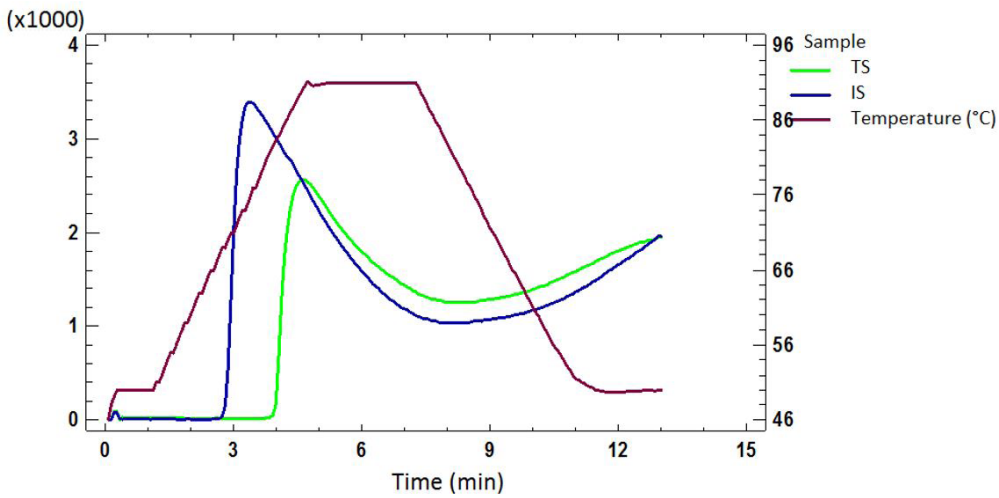

Figure 4: Viscosity amylograms for taro and Indian shot starches

Table 3: RVA results for starch extracted from taro (TS) and Indian shot (IS)

Parameters	TS	IS
Peak viscosity (cP)	2,446.50[a] ± 113.34	3,535.50[b] ± 197.28
Viscosity peak time (min)	4.56[a] ± 0.12	3.40[b] ± 0.02
Viscosity peak temperature (°C)	89.40[a] ± 0.56	76.45[b] ± 0.07
Hot paste viscosity (cP)	1,315.00[a] ± 69.29	1,176.00[b] ± 80.61
Cold paste viscosity (cP)	1,845[a] ± 104	2,021.00[b] ± 97.58
Breadkdown (cP)	1,131.50[a] ± 32.63	2,359.50[b] ± 86.67
Setback (cP)	530.00[a] ± 29.30	845.00[b] ± 58.19

Starch extracted from Indian shot showed a greater capacity for setback and retrogradation than the starch extracted from taro. The setback in soluble polymers of starch and insoluble granular fragments during the cooling phase is associated with retrogradation (Álzate et al., 2013). Starch samples studied in this research presented higher maximum viscosity values than those in starch samples extracted from other tubers, such as cassava (1,116 cP), and two malanga varieties (*Colocasia esculenta* (L.) Schott; 1,170 cP and 975 cP) (Escobar et al., 2009 and Torres et al., 2013).

3.5. STARCH GRANULES SWELLING CAPACITY, SOLUBILITY RATES, AND WATER ABSORPTION

As the temperature of a starch solution rose above 30 °C, rates of solubility, water absorption, and swelling capacity increased, this due to the interaction between water hydrogen bonds and starch glycosidic bonds, therefore resulting in gelatinization of starch. Due to higher amylopectin contents, indices were higher for starch extracted from Indian shot than those for starch extracted from taro. Amylopectin enhances the acquainted properties; in the other hand, amylose is an inhibitor of those (Paredez-López, 1994). Figs, 5, 6, and 7 depict swelling capacity, solubility index, and water absorption index for starch extracted from taro and Indian shot, respectively.

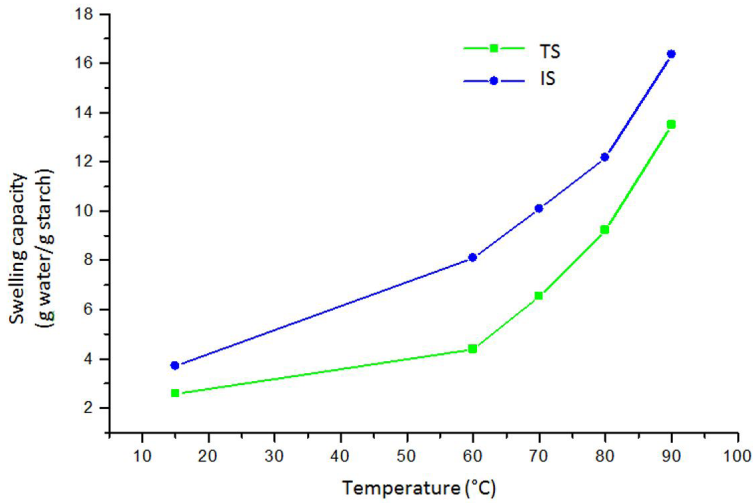

Figure 5: Swelling capacity for taro and Indian shot starches

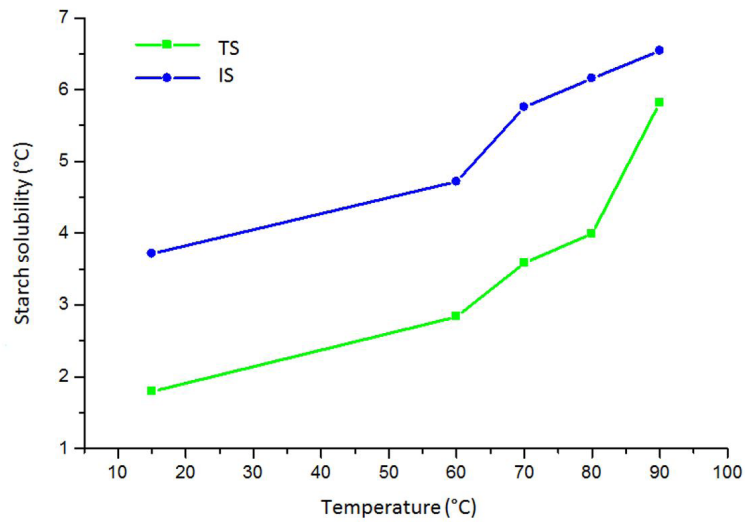

Figure 6: Solubility index for taro and Indian shot starches

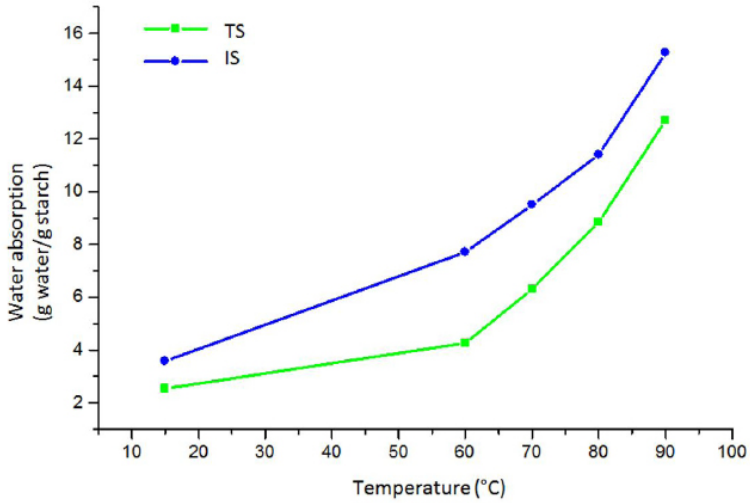

Figure 7: Water absorption index for taro and Indian shot starches

A positive inflection in the slope obtained for these indices can be observed from 70 °C in starch extracted from taro; in starch extracted from Indian shot, the inflection is less pronounced; this is related to the viscosity peak temperature. At 90 °C, the swelling capacity was 13.49 and 16.53 g water/g starch for taro and Indian shot, respectively. Regarding water absorption, values found were 12.70 and 15.27 g water/g starch for Indian shot and taro, respectively. The values of the solubility index values for the studied starches were 3.99 % for taro and 4.54 % for Indian shot. These results were similar to those previously reported, as well as for swelling capacity. Alvira-Maníos et al. (2015) and Fonseca et al. (2016) declared 13.69 and 15.23 g water/g starch for taro and Indian shot, respectively. Low values for swelling capacity are related to low peak viscosity in starch and a lower degree in the rupture in granules (García, 2015); this was observed in taro starch. In the other hand, starch granules with higher swelling capacity usually have higher peak viscosity values, behaviour observed for Indian shot starch, as reported by Ktenioudaki et al. (2013).

4 CONCLUSIONS

American taro and Indian shot rhizomes denote high yields for starch extraction and may be considered as a sound alternative to obtain this biopolymer. Starch granules with larger diameters and higher water absorption capacity reached maximum viscosity values in shorter periods and at lower temperatures, phenomenon observed in Indian shot starch. Taro starch featured higher amylose contents than that in Indian shot starch, higher peak gelatinization temperature was observed in the former. However, due to a smaller average granule size, lower values for gelatinization enthalpy, swelling capacity, absorption, and solubility were found. According to the results found for thermal and functional properties in starch samples extracted from Indian shot and American taro, promising results may occur when adding these types of starch as an alternative matrix or input in food processing, especially when the process demands of high temperatures and where starch has been used as an essential ingredient, such as some types of canned foods (e.g. ready-made soups), ready-made food for infants, and acting as a wall material in the use of encapsulated active principles dedicated to release flavours and colours in food products, to name a few.

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An efficient protocol for in vitro regeneration from the nodal explants of *Withania coagulans* (Stocks) Dunal: a valuable medicinal herb

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An efficient protocol for in vitro regeneration from the nodal explants of *Withania coagulans* (Stocks) Dunal: a valuable medicinal herb

Abstract: In order to develop a protocol for the effective micropropagation of the important medicinal plant *Withania coagulans* (Stocks) Dunal, the effects of different concentrations and combinations of growth regulators on the nodal explants in two independent experiments were investigated. For shooting, a MS medium fortified with different concentrations and combinations of IBA (0.01, 0.1 and 0.5 mg l^{-1}), BA (0.5, 1 and 2 mg l^{-1}), Kin (0.5 and 1 mg l^{-1}), PG (0.5 mg l^{-1}) and GA (0.5 mg l^{-1}) was used and the highest shooting response, shoot number and shoot length were obtained in the MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1}) + GA (0.5 mg l^{-1}) treatment. In the second experiment, the effect of MS supplemented with different combinations and concentrations of IBA (0.1, 0.5, 1 and 2 mg l^{-1}), NAA (0.1 and 1 mg l^{-1}) and PG (1 mg l^{-1}) on rooting of the nodal explants was investigated, which showed that the highest rooting response (%) was observed in the MS fortified with NAA (0.1 mg l^{-1}), NAA (1 mg l^{-1}), NAA (0.1 mg l^{-1}) + PG (1 mg l^{-1}), and NAA (1 mg l^{-1}) + PG (1 mg l^{-1}) treatments, as well as the highest number of roots at NAA (0.1 mg l^{-1}) and the highest root length at IBA (1 mg l^{-1}). Our findings highlight a complete micropropagation method for *W. coagulans* from the nodal explant that can make a significant contribution to the development of *W. coagulans* material for medical applications.

Key words*: Withania coagulans*; micropropagation; *in vitro*; nodal explant; phloroglucinol

Učinkovit protocol za in vitro regeneracijo nodijskih izsečkov vrste *Withania coagulans* (Stocks) Dunal, cenjene zdravilne rastline

Izvleček: Z namenom izboljšanja protokola za učinkovito mikropropagacijo pomembne zdravilne rastline (*Withania coagulans* (Stocks) Dunal) so bili preučevani učinki različnih koncentracij in kombinacij rastnih regulatorjev na izsečkih kolenc v dveh neodvisnih poskusih. Za razvoj poganjkov je bilo uporabljeno MS gojišče, obogateno z različnimi koncentracijami in kombinacijami IBA (0,01; 0,1 in 0,5 mg l^{-1}), BA (0,5; 1 in 2 mg l^{-1}), Kin (0,5 in 1 mg l^{-1}), PG (0,5 mg l^{-1}) in GA (0,5 mg l^{-1}). Največji odziv v rasti poganjkov, v njihovem številu in dolžini je bil dosežen pri obravnavanju MS + IBA (0,01 mg l^{-1}) + BA (0,5 mg l^{-1}) + PG (0,5 mg l^{-1}) + GA (0,5 mg l^{-1}). V drugem poskusu je bil preučevan učinek MS z dodatkom različnih koncentracij in kombinacij IBA (0,1; 0,5; 1 in 2 mg l^{-1}), NAA (0,1 in 1 mg l^{-1}) in PG (1 mg l^{-1}) na zakoreninjenje nodijskih izsečkov, pri čemer je bil dosežen največji odziv zakoreninjenja (%) pri obravnavanju MS obogatenim z NAA (0,1 mg l^{-1}), NAA (1 mg l^{-1}), NAA (0,1 mg l^{-1}) + PG (1 mg l^{-1}), in NAA (1 mg l^{-1}) + PG (1 mg l^{-1}). Največje število korenin je bilo pri obravnavanju s NAA (0,1 mg l^{-1}), največja dolžina korenin pa pri obravnavanju z IBA (1 mg l^{-1}). Izsledki raziskave pojasnjujejo celotno metodo mikropropagacije vrste *W. coagulans* iz izsečkov kolenc, kar je pomemben prispek k vzgoji sadilnega materiala te vrste za uporabo v zdravstvu.

Ključne besede: *Withania coagulans*; mikropropagacija; *in vitro*; nodijski izsečki; floroglucinol

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1 INTRODUCTION

Withania coagulans Dunal is one of the most important species in the Solanaceae family, growing mainly in the eastern Mediterranean to South Asia, including Iran, Afghanistan, Pakistan and India. *W. coagulans* is widely used due to its numerous medicinal properties such as hypo-lipidemic, cardiovascular, hepato-protective, anti-hyperglycemic, anti-diabetic and anti-tumor (Haq et al., 2013; Maurya & Akanksha, 2010). *W. coagulans* fruits are used in cheese production due to their ability to coagulate the milk. The numerous medicinal properties of *W. coagulans* are mainly due to the compounds of withanolides that are naturally synthesized by the plant (Haq et al., 2013; Chen et al., 2011). Due to the accumulation of the medicinal compound withanolide A in the above-ground parts of *W. coagulans* in comparison with the root of *W. somnifera* (L.) Dunal, indicates the economical and easy harvesting of withanolides (Rathore et al., 2016). Due to the lack of proper cultivation practices, *W. coagulans* plants are harvested from wild, which represents a threat to the natural diversity of its germplasm. Reducing the chance of seed setting due to self-incompatibility and polygamous-dioecious nature of flowers reduces the rate of natural regeneration that cannot meet the rate of exploitation (Rathore et al., 2012; Gilani et al., 2009).

Various factors, such as reproductive failure, habitat disturbances, hostile environmental factors and overexploitation, pose a serious threat to valuable medicinal plants, which may expose them to complete extinction (Gerami et al., 2018; Ghorbani et al. 2018). Therefore, collecting plants from nature is not a viable way to meet commercial requirements, and it is important to establish the appropriate strategies to meet the needs (Ghorbani et al. 2019). *In vitro* culture is one of the biotechnology powerful tools that can be effective in the propagation of genetically uniform plants from the elite lines in large numbers, which can eliminate the need to collect medicinal plants from wild (Ghasemi-Omran et al. 2021). Hence the propagation of endangered or rare plants using *in vitro* culture can help maintain germplasms and prevent extinction (Rathore et al., 2016). Furthermore, due to the propagation of genetically uniform plants by *in vitro* culture, it allows the accurate study of stress tolerance and the regulation of secondary metabolites between different treatments, which could have potential application in elite breeding lines. A simple and efficient method for *in vitro* propagation of *W. coagulans* is a necessity for its sustainable use in order to meet pharmaceutical requirements. It can also provide the primary needs for genetic improvement through genetic transformation, genetic restoration programs through true-to-type propagation, and phyto-pharming improvement. Therefore, in the current study, the aim was to investigate the potential of the nodal explants in order to develop an effective protocol for the *in vitro* propagation of *Withania coagulans* Dunal.

2 MATERIAL AND METHODS

Young, non-lignified stems of *W. coagulans* were collected from Iran (Saravan region, Sistan and Baluchestan Province). The stems were soaked in liquid detergent (10 % (vv^{-1}) Teepol) for 5 min after rinsing with running tap water for 30 min. After rinsing with running tap water (10 min), the stems were cut into 2 cm segments (explants), each segment containing a node. Then, after disinfection with an $HgCl_2$ (0.1 %) for 5 min and rinsing with sterile distilled water (five times), the nodal segments were used for culture.

The explants were implanted vertically on MS medium (Murashige & Skoog, 1962) containing agar (C, 0.8 %) and sucrose (mg l^{-1}, 3 %) at pH 5.8. In order to investigate the effect of different hormonal combinations on W. *coagulans* shooting and rooting, the culture media were supplemented with different concentrations and combinations of growth regulators. The treatments applied for shooting and rooting are represented in Table 1. The plant material was kept at 25/18 °C with 14 h photoperiod and 60-80 μmol m^{-2} s^{-1} light intensity. After 6 weeks, the number of differentiated shoots per node, shoot length, shoot multiplication percentage, number roots per shoot, root length and rooting percentage were recorded.

For acclimatization, the rooted plants were transferred to plastic pots containing an autoclaved mixture of cocopeat, soil and sand (1:1:1) after rinsing with tap water and removing agar. To retain humidity, the pots were covered with clear plastics and kept in the tissue culture laboratory. After 10 days, the plastic cover was removed from the pots and the pots were kept at 25 °C with 16 h photoperiod. After 3 weeks, the plantlets were transferred to normal field conditions.

All experiments were repeated three times and the means value were calculated based on four independent replicates (Each replication contained 5 explants). Statistical analysis of the results was calculated using SAS v. 9.1.3 software and the mean comparison was carried out with a least significant difference (LSD) test (at the 5 % level).

Table 1: Treatments applied in two experiments of the induction of shooting and rooting from the nodal explants of *W. coagulans*

	Treatments applied for shooting	Treatments applied for rooting
T1	MS (Control)	MS (Control)
T2	MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1})	MS + IBA (0.1 mg l^{-1})
T3	MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1})	MS + IBA (0.5 mg l^{-1})
T4	MS + IBA (0.5 mg l^{-1}) + BA (2 mg l^{-1})	MS + IBA (1 mg l^{-1})
T5	MS + Kin (0.5 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1})	MS + IBA (2 mg l^{-1})
T6	MS + PG (0.5 mg l^{-1}) + BA (1 mg l^{-1})	MS + NAA (0.1 mg l^{-1})
T7	MS + Kin (1 mg l^{-1}) + IBA (0.5 mg l^{-1}) + BA (1 mg l^{-1})	MS + NAA (1 mg l^{-1})
T8	MS + IBA (0.1 mg l^{-1}) + BA (2 mg l^{-1})	MS + IBA (0.1 mg l^{-1}) + PG (1 mg l^{-1})
T9	MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1}) + GA (0.5 mg l^{-1})	MS + IBA (1 mg l^{-1}) + PG (1 mg l^{-1})
T10	MS + Kin (1 mg l^{-1}) + BA (1 mg l^{-1}) + PG (0.5 mg l^{-1})	MS + NAA (0.1 mg l^{-1}) + PG (1 mg l^{-1})
T11	----------	MS + NAA (1 mg l^{-1}) + PG (1 mg l^{-1})

IBA: Indole-3-butyric acid, BA: 6-Benzyladenin, PG: Phloroglucinol, Kin: Kinetin, GA: Gibberellic acid, NAA: 1-Naphthaleneacetic acid

3 RESULTS

In the present study, the effect of different concentrations and combinations of plant hormones (auxin, cytokinin, and gibberellin) and phenolic composition (phloroglucinol) on induction of branching in nodal explants of *W. coagulans* under *in vitro* conditions were investigated. The results showed that supplement of MS medium with different concentrations and combinations of IBA (0.01, 0.1 and 0.5 mgl^{-1}), BA (0.5, 1 and 2 mgl^{-1}), Kin (0.5 and 1 mgl^{-1}), GA (0.5 mgl^{-1}) and PG (0.5 mgl^{-1}) increased shooting compared to control treatment (Fig. 1). As shown in Table 2, the highest increase in the number of shoots per explant was observed in T9 treatment by 170 % compared to T1 (MS medium). Furthermore, a high number of shoots per explant was also observed in T7, T4 and T3 treatments, respectively (Table 2). The initiation of bud break and the emergence of buds from explants were induced within 8 to 10 days in all treatments, except for T1 and T10 treatments, which began within 15 to 20 days. The highest response to nodal segments of *W. coagulans* in terms of shoot multiplication (%) was obtained in T9 treatments. Following T9 treatment, the highest shoot multiplication was observed in T7, T2 and T3 treatments, respectively (Table 2). The results showed that adding different concentrations and combinations of plant hormones and phenol compound to the MS medium caused a significant increase in shoot length, so that the highest shoot length in T9 and T5 treatments was observed by 163 % and 92 %, respectively, compared to the MS treatment alone (Table 2).

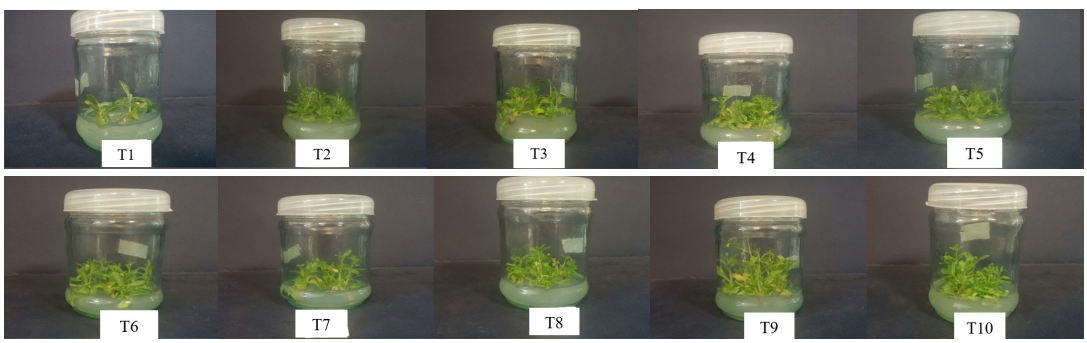

Fig. 1: The effect of different treatments on shooting from the nodal explants of *W. coagulans* in *in vitro* conditions.

Table 2: The effect of different concentrations and combinations of growth regulators on shooting from the nodal explants of *W. coagulans* in *in vitro* conditions.

Treatments	Number of shoots/nodes	Shoot multiplication (%)	Shoot length
T1 (MS (Control))	2.50 ± 0.58 cd	37.75 ± 3.31 f	1.075 ± 0.17 e
T2 (MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1})	4.25 ± 0.50 b	72.75 ± 4.57 c	1.810 ± 0.27 bc
T3 (MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1})	4.50 ± 0.58 b	72.51 ± 3.70 cd	1.883 ± 0.27 bc
T4 (MS + IBA (0.5 mg l^{-1}) + BA (2 mg l^{-1})	4.51 ± 0.48 b	66.50 ± 4.43 d	1.695 ± 0.23 cd
T5 (MS + Kin (0.5 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1})	4.25 ± 0.57 b	66.50 ± 4.65 d	2.063 ± 0.25 b
T6 (MS + PG (0.5 mg l^{-1}) + BA (1 mg l^{-1})	4.23 ± 0.51 b	67.51 ± 5.51 cd	1.890 ± 0.21 bc
T7 (MS + Kin (1 mg l^{-1}) + IBA (0.5 mg l^{-1}) + BA (1 mg l^{-1})	4.51 ± 0.56 b	79.75 ± 4.27 b	1.975 ± 0.17 bc
T8 (MS + IBA (0.1 mg l^{-1}) + BA (2 mg l^{-1})	3.25 ± 0.51 c	51.00 ± 4.32 e	1.490 ± 0.22 d
T9 (MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1}) + GA (0.5 mg l^{-1})	6.75 ± 0.96 a	96.00 ± 3.37 a	2.830 ± 0.17 a
T10 (MS + Kin (1 mg l^{-1}) + BA (1 mg l^{-1}) + PG (0.5 mg l^{-1})	2.25 ± 0.94 d	52.00 ± 3.37 e	1.680 ± 0.15 cd

Values marked with same letters are not significantly different (LSD, $p < 0.05$). All the values are means of four replicates ± SD.

IBA: Indole-3-butyric acid, BA: 6-Benzyladenin, PG: Phloroglucinol, Kin: Kinetin, GA: Gibberellic acid, NAA: 1-Naphthaleneacetic acid

The results of the present study showed that the MS medium fortified with IBA (0.1 mg l^{-1}), NAA (0.1 and 1 mg l^{-1}) and combinations of IBA (0.1 mg l^{-1}) + PG (1 mg l^{-1}), NAA (0.1 mg l^{-1}) + PG (1 mg l^{-1}) and NAA (1 mg l^{-1}) + PG (1 mg l^{-1}) increased the rooting response of the nodal explants relative to the MS medium alone, however, adding IBA (0.5, 1 and 2 mg l^{-1}) and combination of IBA (1 mg l^{-1}) + PG (1 mg l^{-1}) to the MS medium reduced the rooting response compared to the MS medium alone. The highest percentage of rooting was observed in T6, T7, T10 and T11 treatments (Table 3). The results showed that different treatments applied, except for T9 treatment, increased the number of roots per explant. The highest and lowest number of roots per shoot were recorded in T6 (20.5 ± 3.4 per shoot) and T9 (10.5 ± 2.4 per shoot) treatments, respectively (Table 3). The results also showed that T3, T4, T5, T6, T8, T9 and T10 treatments significantly increased the root length and T7 and T11 treatments reduced the root length compared to the MS medium alone, while there was no significant difference between control treatment and T2 treatment. The highest and lowest root lengths were observed in T4 (4.95 cm) and T7 (0.475 cm) treatments, respectively (Table 3).

Table 3: The effect of different concentrations and combinations of growth regulators on rooting from the nodal explants of *W. coagulans* in *in vitro* conditions

Treatments	Rooting response (%)	Number of roots	Root length
T1 (MS (Control)0	70 ± 8 c	13.0 ± 1.2 de	1.925 ± 0.22 d
T2 (MS + IBA (0.1 mg l^{-1}))	80 ± 8 b	14.0 ± 0.8 d	1.925 ± 0.22 d
T3 (MS + IBA (0.5 mg l^{-1}))	50 ± 7 d	13.8 ± 1.7 d	3.150 ± 0.29 b
T4 (MS + IBA (1 mg l^{-1}))	65 ± 6 c	15.0 ± 2.2 bcd	4.950 ± 0.21 a
T5 (MS + IBA (2 mg l^{-1}))	65 ± 9 c	13.3 ± 1.7 de	2.350 ± 0.13 c
T6 (MS + NAA (0.1 mg l^{-1}))	100 ± 0 a	20.5 ± 3.4 a	3.075 ± 0.10 b
T7 (MS + NAA (1 mg l^{-1}))	100 ± 0 a	17.8 ± 3.1 ab	0.475 ± 0.10 f
T8 (MS + IBA (0.1 mg l^{-1}) + PG (1 mg l^{-1}))	85 ± 9 b	14.3 ± 2.5 cd	2.975 ± 0.17 b
T9 (MS + IBA (1 mg l^{-1}) + PG (1 mg l^{-1}))	50 ± 7 d	10.5 ± 2.4 e	4.900 ± 0.18 a
T10 (MS + NAA (0.1 mg l^{-1}) + PG (1 mg l^{-1}))	100 ± 0 a	17.3 ± 2.6 bc	3.050 ± 0.13 b
T11 (MS + NAA (1 mg l^{-1}) + PG (1 mg l^{-1}))	100 ± 0 a	15.5 ± 1.7 bcd	1.050 ± 0.13 e

Values marked with same letters are not significantly different (LSD, $p < 0.05$). All the values are means of four replicates ± SD.

IBA: Indole-3-butyric acid, BA: 6-Benzyladenin, PG: Phloroglucinol, Kin: Kinetin, GA: Gibberellic acid, NAA: 1-Naphthaleneacetic acid

For acclimatization, the plantlets from the rooting experiment were transferred to pots. The results showed that the highest acclimatization was observed in T6, T7 and T10 treatments by 86, 75 and 72 %, respectively. The lowest acclimatization was recorded in T9 and T3 treatments by 36 % and 53 %, respectively (Fig. 2).

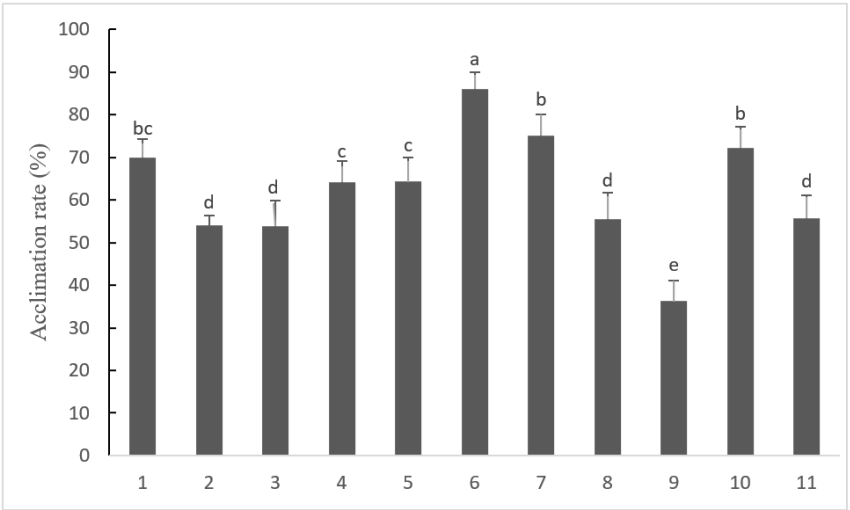

Fig. 2: The rate of hardened plants from well-rooted plantlets obtained from the second experiment (rooting experiment). Values marked with same letters are not significantly different (LSD, $p < 0.05$).

4 DISCUSSION

The populations of *W. coagulans* are in their natural habitat in Iran in danger of extinction due to the weak seed setting and germination created by its poor reproductive system. Irregular and uncontrolled collection of *W. coagulans* for medicinal purposes is another reason for the extinction of *W. coagulans* in Iran. In order to prevent the disappearance of the local *W. coagulans* populations, it is therefore necessary to take timely measures for their conservation (Gilani et al., 2009). Since there are many limitations (low seed viability and self-incompatibility) to conventional propagation of *W. coagulans* plants, *in vitro* culture can be effective in the propagation of genetically uniform plants in large numbers (Valizadeh & Valizadeh, 2011). Induction of rooting, elongation of micro-shoots, differentiation and development of stem buds in *W. coagulans* plants under different concentrations and combinations of plant growth regulators are different. The results of the present study showed that the highest induction of shooting and shoot length was obtained in the MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1}) + GA (0.5 mg l^{-1}) treatment compared to other treatments. Saritha & Naidu (2007) indicated that MS media fortified with 0.1 mg l^{-1} α-naphthalene acetic acid (1.5 mg l^{-1}) and 2 mg l^{-1} BA (1.5 mg l^{-1}) was the best treatment to induce shooting of *W. somnifera* from axillary buds. In another report, Jain et al. (2011) showed that the multiple adventitious shoots of *W. coagulans* plant were differentiated in the MS medium containing BA (5 mg l^{-1}) and kinetin (0.5 mg l^{-1}) from leaf explants. Rathore et al. (2016) studied the effect of different concentrations of plant hormones (6-Benzylaminopurine (BAP), Kin and TDZ) on shoot regeneration from leaf explant of *W. coagulans* and showed that the highest shoot regeneration (74 %) was observed under 1 mg l^{-1}BAP treatment. The effect of different concentrations and combinations of growth regulators on the callus induction from the nodal explants of *W. coagulans* was performed by Valizadeh & Valizadeh (2009), who indicated that the highest growth of callus was observed on the MS medium supplemented with BA (0.25 mg l^{-1}) and 2, 4-D (4 mg l^{-1}). PG is an important phenolic compound that effectively induces the growth of root and shoot in stem culture. The results of the present study showed that PG had a positive effect on the shooting and shoot length, and the similar results have been reported on the effect of PG on shoot multiplication on *Minuartia valentina*

(Pau) Sennen by Ibanez & Amo-Marc (1998). Our findings confirmed that the highest shoot multiplication and shooting were achieved on the MS medium containing MS + IBA (0.01 mg l^{-1}) + BA (0.5 mg l^{-1}) + PG (0.5 mg l^{-1}) + GA (0.5 mg l^{-1}), which can be considered in the propagation of *W. coagulans* medicinal plant.

Auxin has been reported to induce lateral rooting and improve primordium growth (Rathore et al., 2016). The results of the present study showed that NAA was more effective in rooting and number of roots than IBA. Valizadeh & Valizadeh (2011) investigated the effect of different concentrations of IBA, auxin and Kin on rooting of *W. coagulans* and showed that the highest percentage of rooting and number of roots were obtained under the IBA (2 mg l^{-1}) treatment. NAA-induced rooting has also been reported in other medicinal plants (Ahmed et al., 2007b; Sivansean & Murugesan, 2008). In another report, Ahmed et al. (2007a) indicated that auxin and NAA treatments induced the highest rate of rooting in stevia plant in *in vitro* condition. In general, various studies have shown that various auxin hormones are effective in inducing rooting in *in vitro* conditions. Different effects by some compounds can be due to differences in plant species, genotype, age and physiological status of the mother plants. The results also showed that adding PG to the MS medium containing IBA or NAA reduced number of roots compared to IBA and NAA treatments alone, indicating a negative effect of PG on rooting induction.

5 CONCLUSION

In summary, the results of the present study showed that the nodal explants of *W. coagulans* have a high organogenic potential for rooting and shooting response, however, the concentration and combination of growth regulators have a significant effect on rooting and shooting rate. Our findings highlight a complete propagation method for *W. coagulans* plants from the nodal explant that can also be used in genetic transformation studies to improve the plant and protect plant from extinction.

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Analysis of energy balance and global warming potential in tangerine (*Citrus tangerina* Tanaka) orchards versus soybean (*Glycine max* (L.) Merr.) production system

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Analysis of energy balance and global warming potential in tangerine (*Citrus tangerina* Tanaka) orchards versus soybean (*Glycine max* (L.) Merr.) production system

Abstract: With the aim of evaluation and comparison of the greenhouse gas emissions from soybean and tangerine production in Golestan province, Iran, a pilot experiment was carried out. In this experiment, 43 fields of soybeans and 43 orchard tangerines were selected by various management in the province using questionnaires. The greenhouse gas emissions were examined using the Global Warming Potential (GWP). The results of this study showed that fossil fuel was the highest energy consumption in the production of soybeans (6906.5 MJ ha^{-1}) and tangerines (17205.1 MJ ha^{-1}). The lowest amount of energy consumption among inputs was related to micro fertilizers, that was 9 MJ ha^{-1} for soybeans and 17.6 MJ ha^{-1} for tangerine. In both of production system, the most energy consumed was shown for the harvesting sector. Irrigation and planting were the highest contributors to greenhouse gas emissions in soybean field by 387.7 and 109.4 kg CO_2 ha^{-1}, respectively; while in the tangerine production, the most greenhouse gas emissions were related to irrigation and harvesting process by 5828.4 and 394.7 kg CO_2 ha^{-1}. In general, input energy in soybean and tangerine were 17512.8 and 33879.8 MJ ha^{-1}, total output energy was calculated 48310.5 and 105463 MJ ha^{-1}. Finally, the energy use efficiency was computed for soybean and tangerine 2.9 and 3.3, respectively.

Key words: tangerine; soybean; energy use efficiency; greenhouse gas emissions

Primerjalna analiza energetske bilance in potenciala globalnega segrevanja med pridelovalnima sistemoma sadovnjakov mandarin (*Citrus tangerine* Tanaka) in gojenjem soje (*Glycine max* (L.) Merr.)

Izvleček: Z namenom ovrednotenja in primerjave emisij toplogrednih plinov med pridelavo soje in mandarin je bil v provinci Golestan , Iran, izveden pilotni poskus. Za poskus je bilo z vprašalnikom izbranih 43 polj soje in 43 sadovnjakov mandarin z različnimi načini upravljanja. Emisije toplogrednih plinov so bile preučene z uporabo protokola potencialnega globalnega segrevanja (GWP). Rezultati so pokazali, da je predstavljala uporaba fosilnih goriv največjo porabo energije, tako pri soji (6906,5 MJ ha^{-1}) kot pri mandarinah (17205,1 MJ ha^{-1}). Najmanjši energetki vložek pri gojenju obeh kultur so predstavljala mikrohranila, 9 MJ ha^{-1} pri soji in 17,6 MJ ha^{-1} pri mandarinah. V obeh sistemih pridelave je bilo največ energije porabljene za spravilo pridelka. Pri pridelavi soje sta prispevala največji delež emisije toplogrednih plinov namakanje in setev, 387,7 in 109,4 kg CO_2 ha^{-1}, med tem, ko sta pri pridelavi mandarin k temu prispevala največ namakanje in spravilo pridelka, 5828,4 in 394,7 kg CO_2 ha^{-1}. Na splošno je bil energetski vložek pri pridelavi soje 17512,8 in pri pridelavi mandarin 33879,8 MJ ha^{-1}. Celokupen izplen energije je bil pri soji 48310,5 in pri mandarinah 105463 MJ ha^{-1}. Izračunana učinkovitost izrabe energije je bila za sojo 2,9 in za mandarine 3,3.

Ključne besede: mandarine; soja; učinkovitost izrabe energije; emisije toplogrednih plinov

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1 INTRODUCTION

In the agricultural sector, energy consumption has increased over the last several decades due to population growth, decrease in arable land and improvement of living standards. To feed a growing population, intensive use of chemical fertilizers, pesticides, agricultural machines, electricity and natural resources is needed (Barut et al., 2010). Fossil resources are limited and, proper and high-efficiency use is necessary to preserve these resources for future generations of humans. On the other hand, the increased intensive use of energy sources causes environmental problems (Alluvione et al., 2011). Unfortunately, most of the time farmers use more energy to increase crop production, but they do not have enough knowledge on how to increase the efficiency of energy consumption (Ozkan et al., 2004). Therefore, it is not possible to analyze the input and output energies in production systems to design agronomic and policy-making models in agricultural sales sector without examining the efficiency of energy consumption. Energy relationships in agricultural production are correlated with production techniques, inputs, yield levels, and environmental factors. Given that energy consumption and environmental problems resulting from agriculture are increasing year by year, efficient energy consumption is therefore an important issue in sustainable agriculture (Singh et al., 1997). On the other hand, calculating energy inputs in agriculture is more difficult than in industrial sectors because many controllable and uncontrollable factors affect production.

In these days, the agricultural methods are toward developing systems that can generate more energy with lower input consumption (Dalgaard, 2000; Tzilivakis et al., 2005). Increasing energy efficiency and reducing greenhouse gas emissions in crop production are among the fundamental issues in achieving sustainable production (Dyer and Desjardins, 2003). Aydin (2019) stated that the largest share of input energy in the production of tangerines in Turkey with 36 % is associated with the use of chemical fertilizers and the total input energy was estimated at 42.3 GJ ha^{-1}.

In the year 2017, out of about 2.85 million hectares of the orchards in Iran, about 818 thousand hectares, or 28.7 %, were allocated to subtropical fruits. Among the 7.7 million tons of tropical fruit production in the country, about 10 % was related to tangerine production. Golestan province is one of the northern provinces of Iran. The area under cultivation of crops in this province is 724697 hectares, out of which 694618 hectares are for agricultural crops and the rest for orchards. Golestan province is one of the most important areas for citrus production in the country. According to the Agricultural Jihad Statistics of 2017, citrus cultivation area in Golestan province has been reported at 6500 hectares, which has been allocated to cultivation of tangerine, orange and bitter orange. The total area under cultivation of tangerine in Iran was 43525 hectares with production of about 757000 tons in 2017. The area under cultivation in Golestan province was 1549 hectares and produced 20217 tons (Ministry of Agriculture, 2018).

According to the Statistics and Information Office of the Ministry of Agriculture Jihad, Iran, the total area under soybean cultivation in Iran in the year 2017 was about 40000 hectares that, 91000 tons of soybean have been produced from this area. The area under soybean cultivation in Golestan province has been 21000 hectares where 41000 tons of grain has been produced. Golestan province, with 45 % of total grain production, is in the first place of soybean production in the country (Ministry of Agriculture, 2018).

Considering the fact that more than 45 % of the country's soybean is cultivated in Golestan province also, due to the abundance of citrus orchards in the province, study of the energy and greenhouse gas emissions of the two soybean and tangerine products and comparing them together to identify the factors and methods with the maximum energy use in producing these crops seem necessary. This will help agricultural policy makers in the pattern of cultivation in different regions to guide farmers to cultivate tangerine or to grow soybean. These results will also help farmers decide whether to establish tangerine orchards or soybean farms.nally, a risk assessment for consumers was conducted.

2 MATERIAL AND METHODS

2.1. DATA COLLECTION

For this research, 43 soybean fields and 43 tangerine orchards were selected in villages of Golestan province in Gorgan, Bandar Turkmen, Kordkouy, Fazelabad and Aliabad. The farms and orchards were selected in a way to cover all soybean and tangerine production management in the area.

For this purpose, a questionnaire was designed and data were collected through personal (face-to-face) interviews with farmers and taking notes of various field operations. In these farms and orchards, information on duration of field operations, amount of fuel consumed for each operation, machines used, number of conduction of each operation, cultivar and amount of seed consumed, type and amount of fertilizer consumed, name and amount of herbicides and insecticides, irrigation duration, irrigation water supply location, energy used

for pumping water (diesel or electricity) and orchard and field yields were recorded. In order to collect information on the type and amount of inputs and all input and output energies, the number of samples was obtained from the following (Eq.1) (Newbold, 1994):

$$n = N \times S^2 / (N-1)S^2 x + S^2 \qquad \text{(Eq. 1)}$$

where n is the required sample size, N is population size, S is standard deviation, S_X is standard deviation of sample mean ($S_X = d/z$) , d is the allowable error in the sample size was defined to be 5 % of the mean for a 95 % confidence interval and z is the reliability coefficient (1.96 which represents the 95 % reliability).

2.2. ENERGY CALCULATION

Energy flows in farms and orchards can be divided into two types of input energy and output energy. To calculate the input energy in the farms and orchards, the energy inputs were first calculated. Finally, the amount of energy output was calculated by measuring the yield of seed and fruit produced. In order to calculate the amount of input energy for each of the soybean and tangerine sectors, apart from the energy used by the machines and the energy resulted by the electricity consumption, the consumption rate of each input (human labor in hour per hectare, fertilizer in kilograms of nutrients consumed per hectare per kilogram of effective ingredient per hectare) is multiplied by their energy equivalent. The energy equivalent for each of the inputs used and the soybean and tangerine seeds produced have been presented in Table 1.

2.3. CALCULATION OF ENERGY INDICES

The following equations were used to calculate each of the energy indices (Eq. 2-5) (Ghorbani et al., 2011):

$$\textit{Energy use efficiency} = \textit{Energy output}\,(\text{MJ ha}^{-1}) / \textit{Energy input}\,(\text{MJ ha}^{-1}) \qquad \text{(Eq. 2)}$$

$$\textit{Energy productivity}\,(\text{t GJ}^{-1}) = \textit{Crop output}\,(kg\ ha^{-1}) / \textit{Energy input}\,(\text{MJ ha}^{-1}) \qquad \text{(Eq. 3)}$$

$$\textit{Specific energy}\,(\text{GJ t}^{-1}) = \textit{Energy input}\,(\text{MJ ha}^{-1}) / \textit{Crop output}\,(t\ ha^{-1}) \qquad \text{(Eq. 4)}$$

$$\textit{Net energy}\,(\text{MJ ha}^{-1}) = \textit{Energy output}\,(\text{MJ ha}^{-1}) - \textit{Energy input}\,(\text{MJ ha}^{-1}) \qquad \text{(Eq. 5)}$$

2.4. ESTIMATES OF GREENHOUSE GAS EMISSIONS

Global Warming Potential (GWP) Index was used to evaluate the greenhouse gas emissions. This index is the sum of the greenhouse gases produced, expressed as carbon dioxide equivalent (IPCC, 1997). In order to calculate GWP emissions, three greenhouse gases of carbon dioxide, nitrous oxide and methane resulting from energy consumption were considered to produce agricultural inputs and perform various agricultural and horticultural operations.

The GWP was calculated during the following steps:

1. The energy equivalent for the production and use of each of the inputs as well as the energy equivalent for each crop production for soybean and tangerine were calculated according to the method described in the energy section.

2. The share of different energy sources used, including electricity, natural gas, diesel, oil and petroleum, was estimated for the production of each of the inputs (Green, 1987; Tzilivakis et al., 2005). The amount of electricity, natural gas, diesel, oil and petroleum determined by the questionnaires was multiplied at the equivalent energy and its energy equivalent was calculated.

3. After determining the contribution of each energy source to the production of different inputs, the emission rate of each of greenhouse gases of carbon dioxide, methane and nitrous oxide resulting from the use of different energy sources by using greenhouse gas emission factors for each joule of energy consumed was calculated separately for each energy source.

4. Given the different potential of greenhouse effect for the three carbon dioxide, methane and nitrous oxide gases (each kg of nitrous oxide and methane have 310 and 21 kg carbon dioxide of greenhouse effect, respectively), total greenhouse gas emissions was calculated as carbon dioxide equivalent, which is the GWP index.

5. After calculating the total GWP, GWP values per unit area (kg CO_2 eq per hectare) per yield unit produced (kg CO_2 eq per ton of soybeans) were calculated per unit of energy input (kg CO_2 eq in Giga-Joule) and in units of output energy (kg CO_2 eq in Giga-Joule).

2.5. STATISTICAL ANALYSIS

In this study, SAS (Ver. 9.2) and Excel software were employed for data analysis and drawing the graphs.

Table 1: Energy equivalents of inputs and outputs in tangerine orchard and soybean field

Particulars	Unit	Energy equivalent (MJ unit^{-1})	Reference
Inputs			
Human labor	h	1.96	Ozkan et al. (2004)
Machinery	h	142.7	Kaltsas et al. (2007)
Diesel fuel	l	38.00	Ministry of oil (2007)
Nitrogen	kg	60.60	Akcaoz et al. (2009)
Phosphate (P_2O_5)	kg	11.10	Akcaoz et al. (2009)
Potassium (K_2O)	kg	6.70	Akcaoz et al. (2009)
Insecticide	kg or	237	Tzilivakis et al. (2005)
Herbicides	kg or l	278	Tzilivakis et al. (2005)
Manure	kg	0.30	Taylor et al. (1993)
Electricity	kW h	3.60	Pimental and Pimental (1996)
Irrigation Water	m^3	0.63	Taylor et al. (1993)
Seed	kg	30.50	Pimental and Pimental (1996)
Outputs			
Grain yield	kg	15.05	Pimental and Pimental (1996)
Fruit	kg	1.9	Kitani (1999)

3 RESULTS AND DISCUSSION

3.1. FUEL CONSUMPTION RATE DURING OPERATIONS

Among the various soybean operations, fertilization had the lowest amount of diesel consumption, averaging 1.15 liters per hectare (Table 2). The highest fuel consumption in soybean production was related to irrigation operations that consumed, on average, 81.1 liters per hectare (diesel) for diesel wells and 972 kWh per hectare of electricity energy for electric wells (Table 2). After irrigation, the highest fuel consumption in soybean production was related to harvesting operations, which averaged 32.4 liters per hectare (Table 2). One thing to note about fuel consumption and operation is that the way of the operations also affects fuel consumption. For example, land preparation with combine harvester consumes less fuel than land preparation with a plow and a deep farm disk (Table 2).

Regarding the production of tangerines, the highest fuel consumption was for harvesting operations, which consumed an average of 157.8 l ha^{-1}. After harvesting, the highest fuel consumption in the tangerine production process was related to the irrigation operation, which consumed an average of 126.1 l ha^{-1} (Table 2). The lowest amount of fuel consumed in the tangerine production operation was associated with weed control, pest control, which consumed an average of 9.8 and 15.5 l ha^{-1}, respectively. It should be noted, however, that the chemical fertilization operation had no fuel consumption because it was fully manned (Table 2). Weed control also had high fuel consumption in relation to the production of tangerines such as animal manure, in a way that animal manure consumed an average of 38.1 liters of fuel per hectare and weed control consumed an average of 42.1 l ha^{-1} (Table 2). Yilmaz and Aydin (2019) stated that the highest share of input energy in the production of tangerines in Turkey with 36 % is associated with the use of chemical fertilizers and the total input energy was estimated at 42.3 GJ ha^{-1}.

3.2. ENERGY CONSUMPTION IN THE INPUTS SECTOR

Based on the results of Table 3, the energy consumption rate per seed input varied from 948.1 to 752.5 MJ ha^{-1}, so that the average soybean fields studied in this experiment consumed 840.7 MJ ha^{-1} per seed (Table 3). Among the fertilizers used for soybean production, nitrogen fertilizer had the highest energy consumption, with the highest energy consumption for nitrogen fertilizer at 6363 MJ ha^{-1}. As some of the fields studied did not use nitrogen fertilizer, the lowest energy consumption for

Table 2: Amount of used gasoline (l ha^{-1}) and electricity (kWh ha^{-1}) for each operation in tangerine orchard and soybean field

Operation	Tangerine	Soybean
Land Preparation	30.7 ± 5.2	15.5 ± 2.5
Sowing	27.3 ± 3.2	7.4 ± 1.1
Fertilization and manure	38.1 ± 3.3	15.1 ± 2.0
Pest control	15.5 ± 1.2	19.4 ± 3.6
Weed control	9.8 ± 1.2	3.8 ± 0.3
Irrigation	126.1 ± 9.2	81.1 ± 6.6
Harvesting	157.8 ± 6.9	32.4 ± 3.2
Land operation	14.7 ± 2.8	0
Total	19.8 ± 420.0	12.3 ± 174.7
Electricity for irrigation	1113 ± 114.5	972 ± 59.6

Table 3: Consumed energy (MJ ha^{-1}) for different input of tangerine orchard and soybean field

Input	Tangerine	Soybean
Labor	2312.5 ± 116.7	535.5 ± 88.5
Seed / Seedling	2533.0 ± 145.3	840.7 ± 2.2
Machinery	11910.6 ± 791.8	3754.9 ± 881.6
Fuel	17205.1 ± 1050.8	6906.5 ± 391.2
Electricity	1156.1 ± 548.1	269.2 ± 86.4
Nitrogen	1087.8 ± 102.3	3878.4 ± 115.9
Phosphorus	1063.7 ± 81.2	1041 ± 102.2
Potassium	391.9 ± 59.3	251.2 ± 39.2
Micro nutrient	17.6 ± 1.2	9 ± 1.1
Herbicide	204.6 ± 10.7	88.9 ± 10.2
Pesticide	206.9 ± 9.0	36.3 ± 2.1
Winter pest control	56 ± 8.6	-
Manure	187 ± 19.8	-
Total	38332.8	17611.6

fertilizers was zero, so the average energy consumption for nitrogen fertilizer was 3878.4 MJ ha^{-1} (Table 3). In the case of phosphorus and potash fertilizer, because some farms did not use these two types of fertilizer, the lowest energy consumption for potassium and phosphorus fertilizer was zero (Table 3). The average consumption of phosphorus and potassium fertilizers for soybean production was 1041 and 252.2 MJ ha^{-1}, respectively (Table 3). In the studied farms, the amount of fertilizer consumed depends on the management of the farms and the finances of the farmers. Generally, in the Gorgan area, the farms who consider the crop rotation and have planted legumes in the fall and also in the case of returning crop residues to the soil, the farmers do not use nitrogen and potash fertilizers without affecting crop yields. Also, farmers who did not have high financial resources refused to use fertilizer to reduce production costs, the opposite was also true, farmers who had relatively good financial status used micro-fertilizers in addition to common fertilizers.

Regarding tangerines, the average energy consumption for nitrogen, phosphorus and potassium fertilizer inputs for tangerine production were estimated at 1087.7, 1063.7 and 391.9 MJ ha^{-1}, respectively (Table 3). Maximum energy consumption for nitrogen, phosphorus and potassium fertilizer inputs was 1087.8, 1063.72775 and 391.9 MJ ha^{-1}, respectively (Table 3). An average energy of 187 MJ ha^{-1} has been consumed in the studied orchards in relation to livestock manure (Table 3). Among the herbicide, pesticide and winter spraying inputs, the

highest amount was related to pesticide 206.9 MJ ha^{-1}, after the pesticide input, the herbicide input with the highest energy consumption of 204.6 MJ ha^{-1} had the highest energy consumption for tangerine production.

Dehshiri and Aghaalikhani (2012) reported the average energy use in soybean seed production in Golestan and Mazandaran was 1836 MJ ha^{-1}. Rajabi et al. (2012) reported an average energy consumption of 5964 MJ ha^{-1} for wheat production. In their study, the researchers calculated the highest and lowest energy consumption of this input, respectively at 1248 to 12366 MJ ha^{-1}. Mousavi Aval et al. (2011) estimated the amount of energy consumed by nitrogen, phosphorus, potassium, and sulfur fertilizers for soybean production in Gorgan, at 6281, 627, 102, and 4 MJ ha^{-1}, respectively. The difference in the energy consumption rate in the fertilizer sector in this study was in line with other studies in the Golestan province due to the recent increase in fertilizer prices that farmers preferred to use less fertilizer to reduce production costs. In soybean production studies, the energy consumption of herbicides for soybean production in the Golestan region has been estimated at 631 MJ ha^{-1}.

In the production of tangerines, the average energy consumed for human labor was 2312.5 MJ ha^{-1} (Table 3). Like soybeans, the way in which various operations are performed has a huge impact on the amount of energy consumed in the tangerine sector, for example, the irrigation method can affect the energy consumption rate of the work force sector. Drip irrigation did not have any human labor, while flood irrigation of the orchards required a great deal of work force and consumed a great deal of energy in the work force sector. Yilmaz and Aydin (2019) reported that the highest energy consumption in the orchards of tangerine and lemon production was associated with the use of chemical fertilizers and fuel.

3.3. CONSUMPTION ENERGY FOR VARIOUS OPERATIONS IN SOYBEAN AND TANGERINE PRODUCTION

The results of Table 4 showed that among the various operations for soybean production, the highest average energy consumption was related to harvesting with average value of 2955 MJ ha^{-1} (Table 4). The lowest average energy consumption in the soybean production process was related to manual weeding, cultivator and hoeing, which consumed 62.3 and 107.0 MJ ha^{-1} on average (Table 4). The average energy consumption in land preparation, irrigation, sowing and fertilization in soybean production was estimated at 556.4, 543.3, 1115.2 and 1755.6 MJ ha^{-1}, respectively (Table 4).

In the process of tangerine production, the highest average energy consumption is related to the harvesting process (picking the fruit and transporting it to the desired location) which is significantly different with other operations in energy consumption, so that, on average, 13817.6 MJ ha^{-1} energy is consumed for the tangerine harvesting operations (Table 4). In fact, the tangerine harvesting process consumes a lot of energy because of the human labor. On the other hand, because of the high fruit yield in the tangerine orchards, the energy for transport is also very high. These are two factors could raise energy consumption for the tangerine harvesting operation. It should be noted, however, that the energy consumption rate in tangerine harvesting operations largely depends on the proximity or location of the storage or sale of tangerines, which also increases with increasing destination distance.

After harvesting, the highest energy consumption was related to irrigation operations with an average value of 5763.9 MJ ha^{-1} in tangerine production (Table 4). Fertilization also had less tangerine energy consumption of on average 1098.99 MJ ha^{-1} than the other operations (Table 4). In terms of comparing soybean and tangerine energy consumption, it should be stated that energy consumption in tangerine production is very different from soybean, for example, the energy consumption rate in soybean irrigation is on average 543.3 MJ ha^{-1}, while this amount for tangerine was 5763.9 MJ ha^{-1} (Table 4). In general, it can be stated that the energy consumption of similar operations in soybean production is much lower than that of tangerine production, which could also be due to the presence of tangerines throughout the year.

Filipović et al. (2006) estimated fuel consumption for three types of tillage systems including conventional tillage, low tillage and direct tillage at 71, 35 and 7.5 liters per hectare, respectively. Mari and Changying (2007) compared conventional tillage, low tillage and direct tillage systems in terms of fuel and energy consumption and showed the highest energy consumption (7969 MJ ha^{-1}) in fuel consumption for the conventional system and the lowest energy consumption (4099 MJ ha^{-1}) for direct cropping system. Farmers and orchardist can reduce production costs by reducing fuel consumption (Tabatabaefar et al., 2009).

3.4. ENERGY USE INDICATORS

Table 5 presents the energy inputs, outputs, as well as energy indicators of soybean and tangerine production. Total input energy in soybean production was on average 17512.8 MJ ha^{-1} (Table 5). Soybean yield was on average 3210 kg ha^{-1}, the lowest and highest grain yield

Table 4: Consumed energy (MJ ha^{-1}) in different operations of tangerine orchard and soybean field

Operation	Tangerine	Soybean
Sowing	2173.6 ± 99.6	1115.2 ± 58.6
Land preparation	2533.0 ± 95.2	556.4 ± 41.6
Fertilization	1098.9 ± 45.2	1719.2 ± 115.4
Pest control	3139.5 ± 148.6	216.6 ± 11.6
Weeding	906.4 ± 74.5	62.3 ± 8.4
Irrigation	5763.9 ± 188.2	543.3 ± 18.1
Harvest	13718.6 ± 521.2	2955 ± 156.5
Cultivator and hoeing	2180.7 ± 113.2	107.0 ± 15.2
Total	31513.8	7275.0

in the studied soybean fields were 2700 and 3800 kg ha^{-1}, respectively. The total energy output for soybean fields was on average 48310.5 MJ ha^{-1} (Table 5).

The net energy produced in the studied soybean fields was 30797.7 MJ ha^{-1} on average (Table 5). The energy efficiency index in the studied fields was 2.9 on average. The energy efficiency index is obtained by dividing the total output energy by the total input energy in soybean fields. This index indicates that the output energy in the farms is several times the input energy, in the soybean fields the energy efficiency index ranged from 1.8 to 5.5. The energy productivity index in soybean fields was at 5.5 t GJ^{-1} on average, the energy efficiency index is obtained by dividing the grain yield by the input energy values of the soybean fields. This indicator shows the ratio of grain yield to input energy in the field. In our investigation it varied between 8.1 to 2.7 t GJ^{-1}. The higher energy efficiency index causes the higher yield for less input energy.

In the soybean fields, the specific energy index was at 0.19 GJ t^{-1} on average. The specific energy index is the opposite of the energy efficiency index and is obtained by dividing the input energy by soybean grain yield. Net energy performance index calculated from subtraction of output energy and input energy is in fact the same amount of net energy which was 30.8 GJ ha^{-1} on average for the soybean field (Table 5).

But in the tangerine orchards studied, the average energy input for all orchards was 33879.8 MJ ha^{-1} (Table 5). The average tangerine yield in orchards was 56033.3 kg ha^{-1}, with the highest and lowest tangerine yields of 80000 and 37000 kg ha^{-1}, respectively (Table 5). The average total energy output in the orchards under study was 105463.3 MJ ha^{-1}. The net energy in the studied orchards varied between 129213.1 MJ ha^{-1} to 29629.4 MJ ha^{-1}, with an average net energy value of 72583.5 MJ ha^{-1} in the studied orchards (Table 5).

Energy efficiency index of tangerine orchards was 3.3 on average. The highest energy use efficiency was 6.7 and the lowest energy efficiency index was 1.6. Average energy use efficiency in tangerine production was higher than soybean production, this indicates that tangerine orchards produce more net energy than soybean fields, but a very important point is that the soybean production process in Gorgan is about half the length of the growing season, while tangerine orchards have been occupied by tangerine trees throughout the growing season, it is therefore reasonable to expect less net energy in soybean fields than in tangerine orchards (Table 5).

Energy efficiency index in tangerine orchards was on average 0.63 GJ t^{-1} (Table 5). The average net energy yield index in the tangerine orchards under study was 72.5 GJ ha^{-1} (Table 5). Yilmaz and Aydin (2019) by examining the energy balance in citrus orchards reported the energy efficiency in tangerine orchards at 2.03, the energy yield at 0.85 kg MJ^{-1}, the net energy at 1.18 MJ, and the net energy at 43.6 GJ ha^{-1}, respectively. In the overall comparison between tangerine and soybean, total input energy in soybean production was much lower than in tangerine production. Also, the total energy output and net energy in soybean were also lower than those of soybean. The net energy output for soybean production was 30797.7 MJ ha^{-1}, but it was estimated at 72583.5 MJ ha^{-1} for tangerine, this indicates a much higher output of tangerine than soybean. Comparison results between energy indices in soybean and tangerine production showed that energy efficiency index in soybean (2.9) was lower than tangerine (3.3), but energy productivity in soybean (5.5) was higher than tangerine (1.3). In addition, net yield energy index in soybean was 30.8 GJ ha^{-1}, which was much lower than net energy index in tangerine (72.5 GJ ha^{-1}) (Table 5).

Ozalp et al. (2018) showed that the input energy of pomegranate in Turkey was 50.5 GJ ha^{-1} and the output energy was 76.3 GJ. The energy use efficiency was also 1.51. Aydin et al. (2018) stated that in the apple produc-

Table 5: Input, output and energy indexes of tangerine orchard and soybean field

Index	Unit	Tangerine	Soybean
Total Input	MJ ha^{-1}	33879.8 ± 688	17512.8 ± 596
Fruit/Seed Yield	kg ha^{-1}	56033.3 ± 791	3210 ± 256
Total output	MJ ha^{-1}	105463.3 ± 822	48310.5 ± 671
Net energy	MJ ha^{-1}	72583.5 ± 511	30797.7 ± 822
Energy use efficiency	-	3.3 ± 0.9	2.9 ± 0.3
Energy productivity	ton GJ^{-1}	1.3 ± 0.2	5.5 ± 0.6
Specific energy	GJ t^{-1}	0.63 ± 0.07	0.19 ± 0.03
Net yield energy	GJ ha^{-1}	72.5 ± 5.3	30.8 ± 3.6

tion system with good agricultural method, energy efficiency, energy yield and specific energy were 1.36, 0.56 and 1.77, respectively. In a study, Ghorbani et al. (2011) compared energy consumption in dry land wheat (low input) and irrigated wheat (high input). They stated that the total energy consumption in dryland wheat cultivation was 9354.2 MJ/ha, but the total energy input in irrigated wheat was 45367.6 MJ ha^{-1}. Sahabi et al. (2013) stated that the input energy for wheat and barley production was 514040 and 44866 MJ/ha, respectively. They stated that profit-to-cost ratio in wheat was higher than in barley, which was 1.59 versus 1.35. Generally speaking, lower consumption of inputs can reduce energy consumption in the production of horticultural products but it should be noted that the decrease in inputs does not have a negative effect on the profit-to-cost ratio.

3.5. GREENHOUSE GAS EMISSIONS

3.5.1. Emissions of greenhouse gases from consumption of inputs

The results of the study of greenhouse gas emissions in the fields and orchards under study for different inputs have been presented in Fig. 1. The results of soybean field surveys showed that the highest greenhouse gas emission rate was related to fuel and electricity inputs with the average values of respectively 501.6 and 163.7 kg of CO_2 per hectare. The lowest values for greenhouse gas emission rate in the soybean fields were in human labor and pesticide, respectively, which produced, on average, 0.22 and 0.78 kg CO_2 per hectare, respectively (Fig. 1). The average greenhouse gas emission rate was higher for herbicides than for pesticides, because most of the farms used herbicides, this led to a higher energy consumption for herbicides than for pesticides.

In the production of tangerines like soybeans, the highest greenhouse gas emission rate was related to fuel input, which produced an average 1249.6 kg of CO_2 per hectare (Fig. 1). The average greenhouse gas production rate for tangerine input was lower than for pesticides, this is because fruit trees are more susceptible to pests and diseases than weeds, which is why most of the orchards studied used pesticides, and another reason is that some of the orchards under study used cultivators instead of herbicides to combat weeds (Fig. 1). Ozalp et al. (2018) showed that 1.73 tons of CO_2 was emitted in the production of one hectare of pomegranate. An 88.1 kg of CO_2 is also emitted to produce 1000 kg of fruit.

The average greenhouse gas emissions induced from human labor for tangerines were 11.79 kg CO_2 on average per hectare, which was much higher than for soybeans. This seems to be due to the large amount of human labor being used in harvesting in tangerines and so the greenhouse gas emissions in the tangerine human labor sector are also increasing. In the production of tangerine manure fertilizer also caused a significant greenhouse gas emissions, averaging 78.5 kg CO_2 ha^{-1} (Fig. 1). In general, it can be said that greenhouse gas emissions are much higher in the production process of tangerines than soybeans, so, in similarly used inputs between soybean production and tangerine, greenhouse gas emissions were higher in tangerine (Fig. 1).

3.5.2. Released greenhouse gases resulting from agricultural and horticultural operations

Different amounts of greenhouse gases are produced in different operations to produce soybeans and tangerines, which have been summarized in Fig. 2.

According to the results of Figure 2 in the soybean production process, the highest amount of greenhouse gas emissions was produced in irrigation operations, which was on average at 387.7 kg CO_2 ha^{-1} (Fig. 2). Harvesting and irrigation were the operations that cause

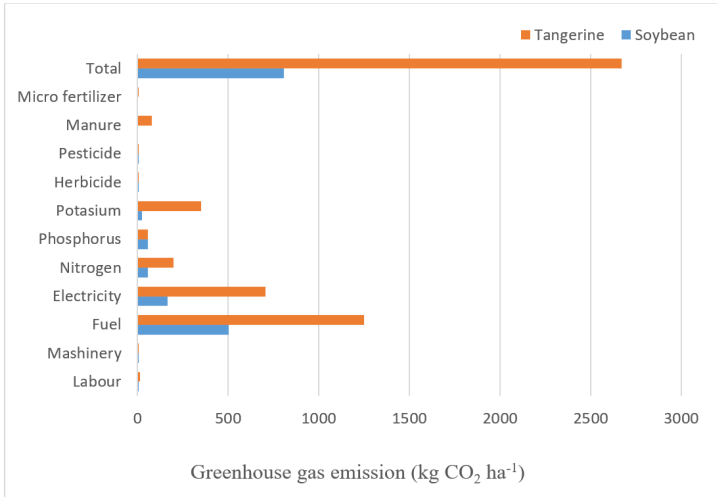

Figure 1: Amount of greenhouse gas emission (kg CO_2 per hectare) for different inputs in tangerine and soybean production systems

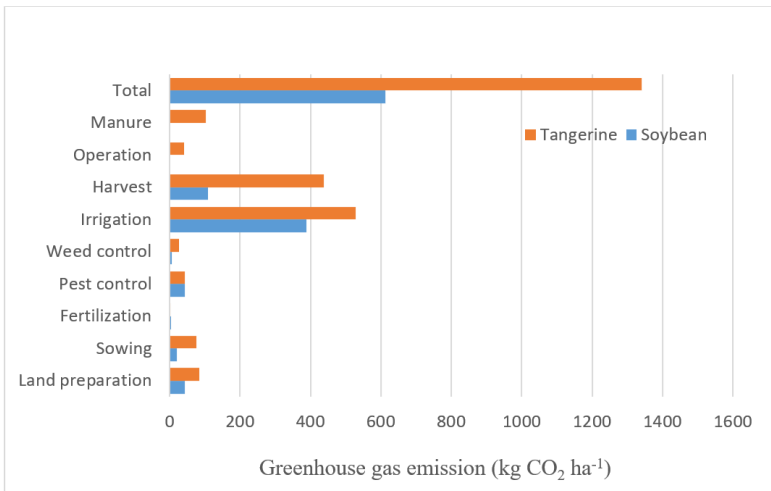

Figure 2: Amount of greenhouse gas emission (kg CO_2 ha^{-1}) for different operations in tangerine and soybean production systems

most important greenhouse gas emissions to produce tangerines in the orchards under study. These two irrigation and harvesting operations have, on average, released 528.4 and 439.7 kg CO_2 ha^{-1} of greenhouse gas, respectively. Greenhouse gas emissions in different tangerine production operations showed that these operations produced more greenhouse gases than similar operations in soybean production, in fact, it should be stated that the amount of greenhouse gas emission derived from fuel consumption, human labor and etc. in tangerine production was higher than soybean. This ultimately produces more greenhouse gases in the tangerine production process (Fig. 2).

The average carbon emissions for soybean production for conventional tillage, low-tillage and no-tillage systems were reported at 168, 146, and 137 kg CO_2 ha^{-1}, respectively (West and Marland, 2002).

4 CONCLUSION

In general, the results showed that the total energy consumed per hectare of tangerine orchard far exceeds the energy required for soybean production. This difference is mainly due to the high fruit production rate per hectare and therefore the high energy needed to harvest and transport it. In tangerine production system irrigation also consumed more energy than soybeans. In soybean production, the highest energy consumption was associated with irrigation and harvesting operations. The greenhouse gas emission rate in citrus production was

higher comparing to soybean production because of fuel consumption and human labor, which in the end has led to the production of more greenhouse gases in the tangerine production process. Considering the fact that in the management of the orchards, farmer has to carry out different operations throughout the year because this plant is a perennial plant compared to a soybean plant which is annual and farming operations are conducted in only a part of the year. In addition, because of the much higher crop production in tangerine orchards, so energy consumption and carbon dioxide emissions are much higher in tangerine orchards. Thus farmers can use the findings of this study to decide whether to establish a tangerine or soybean farm in terms of energy balance and carbon dioxide production. However, economic, social, climatic, and edaphic comparisons are also needed to make more precise decisions.

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Chemical components of volatile oil and fatty acids of wild *Bunium persicum* (Boiss.) B. Fedtsch. and cultivated *Cuminum cyminum* L. populations

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Chemical components of volatile oil and fatty acids of wild *Bunium persicum* (Boiss.) B. Fedtsch. and cultivated *Cuminum cyminum* L. populations

Abstract: Volatile oil and fatty acids components of six various populations of wild *Bunium persicum* Boiss. (Bam and Zirkuh/Iran) and cultivated *Cuminum cyminum* L. (Rayen/Iran; Cukurcak, Taskopru and Asagialicomak/Turkey) species were investigated. The volatile oil content of Bam and Zirkuh populations were 3.9 and 4.7 %, respectively. The analysis of volatile oils by the GC/FID-MSD showed that γ-terpinene (33.62-39.62 %), cuminal (17.9-19.3 %), o-cymene (5.3-11.1 %), benzenemethanol, α-methyl- (7.4-9.5 %), 1-phenyl-1-butanol (6.4-8.4 %) and limonene (6.4-8.6 %) were the major components of *B. persicum* populations. Rayen, Cukurcak, Taskopru and Asagialicomak populations of *C. cyminum* had 2.6, 2.2, 2.0 and 2.5 % of volatile oil, respectively. Cuminal (22.8-37.6 %), benzenemethanol, α-methyl- (5.3-22.6 %), γ-terpinene (16.7-19.4 %), β-pinene (11.2-11.9 %) and 1-phenyl-1-butanol (5.4-12.5 %) were identified as the main components of *C. cyminum*. Fatty acids were detected by the GC/FID. In total, 15 fatty acids were characterised in *B. persicum* populations from Iran. Petroselinic acid (26.3-52.6 %), lauric acid (16.2-37.0 %) and linoleic acid (18.3-33.0 %) were the predominant fatty acids identified in Iranian populations. *C. cyminum* populations were rich in the same fatty acids but, the order was: petroselinic acid (47.5-55.5 %), linoleic acid (22.5-25.4 %) and lauric acid (13.4-24.2 %). Monounsaturated fatty acids (27.4-56.2 %) were the major subgroup. Overall, *B. persicum* populations from Iran and *C. cyminum* from Turkey were almost similar in fatty acids profile although they had wide diversity in the volatile oils compositional profile.

Key words*: Bunium persicum; Cuminum cyminum;* essential oil; fatty acid; GC

Kemijska sestava hlapnih olj in maščobnih kislin samoniklih populacij črne gomoljaste kumine (*Bunium persicum* (Boiss.) B. Fedtsch.) in gojenih populacij rimske kumine (*Cuminum cyminum* L.)

Izvleček: Preučena je bila sestava hlapnih olj in maščobnih kislin dveh samoniklih populacij črne gomoljaste kumine (*Bunium persicum* Boiss.) (Bam and Zirkuh/Iran) in štirih populacij gojene rimske kumine (*Cuminum cyminum L.*); (Rayen/Iran; Cukurcak, Taskopru and Asagialicomak/Turčija). Vsebnost hlapnih olj je v populacijah Bam in Zirkuh znašala 3,9 in 4,7 %. Analiza hlapnih olj z GC/FID-MSD je pokazala, da so bile v populacijah črne gomoljaste kumine njihove glavne sestavine γ-terpinen (33,62-39,62 %), kuminal (17,9-19,3 %), o-cimen (5,3-11,1 %), benzenmetanol, α-metil- (7,4-9,5 %), 1-fenil-1-butanol (6,4-8,4 %) in limonen (6,4-8,6 %). Populacije rimske kumine iz rastišč Rayen, Cukurcak, Taskopru in Asagialicomak so vsebovale 2,6; 2,2; 2,0 in 2,5 % hlapnih olj. Kuminal (22,8-37,6 %), benzenmetanol, α-methyl- (5,3-22,6 %), γ-terpinen (16,7-19,4 %), β-pinen (11,2-11,9 %) in 1-fenil-1-butanol (5,4-12,5 %) so bile glavne sestavine hlapnih olj v rimski kumini. Maščobne kisline so bile analizirane z GC/FID. Celokupno je bilo v populacijah črne gomoljaste kumine iz Irana določenih 15 maščobnih kislin, pri čemer so imele največji delež petršilova (26,3-52,6 %), lovorjeva (16,2-37,0 %) in linolenska kislina (18,3-33,0 %). Populacije rimske kumine so vsebovale enake maščobne kisline, a njihov delež je bil sledeč: petršilova (47,5-55,5 %), linolenska (22,5-25,4 %) in lovorjeva kislina (13,4-24,2 %). Enkrat nenasičene maščobne kisline so bile glavna podskupina (27,4-56,2 %). Nasplošno so imele populacije črne gomoljaste kumine iz Irana in rimske kumine iz Turčije podobno sestavo maščobnih kislin a veliko različnost v sestavi hlapnih olj

Ključne besede: *Bunium persicum; Cuminum cyminum*; eterična polja; maščobne kisline; plinska kromatografija (GC)

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1 INTRODUCTION

Bunium persicum (Boiss.) B. Fedtsch. [syn: *Elwendia persica* (Boiss.) Pimenov & Kljuykov] as a member of the Apiaceae family is a perennial and herbaceous plant that grows in a limited area of Central to West Asia. The fruits (seeds) of *B. persicum* have been widely used as medicinal, aromatic and spice plants in food and cosmetic industries (Azizi et al., 2009; Omidbeigi, 2013). The active ingredients of this plant are volatile oils extracted from the ripe fruits (*Kan et al., 2007*). Based on numerous studies, *B. persicum* has biological and pharmacological properties including antimicrobial (Rustaie et al., 2016), antioxidant (Sharafati Chaleshtori, 2018), antifungal (Takayuki et al., 2007; Khaledi and Hassani, 2018), antibacterial (Demirci et al., 2008; Oroojalian et al., 2010), hypoglycaemic and anti-inflammatory activities (Hajhashemi, 2011). The *B. persicum* fruit samples from different locations of Kerman province/Iran contained 3.5, 4, 7 and 8.5 % of volatile oil (Omidbaigi and Arvin, 2009). ρ-cuminaldehyde (23.50 %), α-methylbenzenemethanol (14.59 %), γ-terpinene (13.10 %) and β-cymene (8.48 %), sabinene (5.82 %) and α-pinene (4.03 %) were reported as major constituents of *B. persicum* essential oil (Sanei Dehkordi et al., 2016).

Cuminum cyminum L. is a valuable medicinal and aromatic plant that originated from Egypt, Central Asia and Eastern Mediterranean regions (Omidbeigi, 2013). The fruits of *C. cyminum* are applied as a popular spice in the kitchen and food industries (Hajlaoui et al., 2010). This plant possesses anti-inflammatory, diuretic, carminative and antispasmodic characteristics. It is also used to treat toothache, epilepsy, dyspepsia, jaundice, diarrhea, flatulence, and indigestion (Evanse et al., 1996; Dhandapani et al., 2002; Rebey et al., 2012). Also, *C. cyminum* has high antioxidant and antibacterial activities (Guo et al., 2018). The volatile oil content of this plant has been reported from 1 to 5 % (Lee, 2005; Ladan Moghadam, 2016). The main components of the volatile oil are monoterpenes and sesquiterpene derivatives such as cuminal (36.31 %), cuminic alcohol (16.92 %), γ-terpinene (11.14 %), safranal (10.87 %), p-cymene (9.85 %) and β-pinene (7.75 %) (Li and Jiang, 2004).

Another valuable by-product of the Apiaceae family is their fatty oil which is widely used in various industries (Kooti et al., 2015). Petroselinic acid is the major component of *C. cyminum* fatty oil (Dubey et al., 2018). Also, petroselinic acid, oleic acid, linoleic acid, lauric acid and palmitic acid were introduced as the main components of *B. persicum* fatty oil (Khalid et al., 2009). Although comprehensive information on cumin oil is available in the literatures, there are a few available scientific records about *B. persicum*. The production and accumulation of secondary metabolites and their qualities are affected by various biotic and abiotic factors such as genetic characteristics, climatic conditions (light, temperature, rainfall, irrigation, soil, height, location, etc.), environment organisms, applied agro-techniques and post-production processing (Soltanbeigi & Sakartepe, 2020).

The aim of this investigation was the comparison of volatile oil content and the volatile and fatty oils chemical component and its diversity in various populations of *B. persicum* and *C. cyminum* from different locations of Iran and Turkey.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS

The fruits of two populations of wild *Bunium persicum* were collected from the mountains of Bam (Kerman Province/Iran) and Zirkuh (Khorasan Province/Iran). Also, four cultivated *Cuminum cyminum* fruits samples were obtained from Rayen county (Kerman Province/Iran), Cukurcak (Çukurcak/Sultandağı), Taskopru (Taşköprü/Sultandağı) and Asagialicomak (Aşağıaliçomak/Emirdağ) villages (Afyonkarahisar/Turkey). The geographical and climatic conditions of the sampling regions are outlined in Table 1. The plants were taxonomically identified by a senior expert from the Agricultural Research, Education and Extension Organization of West Azerbaijan Province of Iran and Afyonkarahisar Directorate of Provincial Agriculture and Forestry, Republic of Turkey.

2.2 ISOLATION OF VOLATILE OILS

50 g of powdered dried fruits of plant samples were subjected to hydro-distillation by using a Clevenger type apparatus for 3 hours and volatile oil content of the samples was calculated as:

Oil content (v/M) = observed volume of oil (ml)/ mass of sample (g) × 100

The volatile oil samples were dried over anhydrous sodium sulfate and were stored at 4 °C in ambered vials till GC-MS analysis.

2.3 DETERMINATION OF VOLATILE OIL COMPONENTS

A gas chromatography (GC) system (Agilent Technologies, 7890B) equipped with a flame ionization detector (FID) and coupled to a mass spectrom-

Table 1: The geographical and some climatic data of the plants sampling locations

	Bam[1]	Zirkuh[1]	Rayen[1]	Cukurcak[2]	Taskopru[2]	Asagialicomak[2]
Coordinates	29°04′N 58°21′E	33°36′N 59°59′E	29°35′N 57°26′E	38°42′N 31°22′E	38°34′N 31°18′E	38°58′N 31°42′E
Elevation (m)	1050	1330	2201	1309	953	980
Climate Type	Hot and dry climate	Normal tropical climate	Moderate mountainous/ dry	Continental climate	Continental climate	Continental climate
Rainfall (mm/ year)	68	150	93.8	501	501	421

1: Iran Meteorological Organization, 2: Turkish State Meteorological Service

etry detector (MSD) (Agilent Technologies, 5977A) was used. An HP-Innowax column (Agilent 19091N-116: 60 m × 0.320 mm internal diameter and 0.25 µm film thickness) was used for the separation of the components. Samples were analyzed with the column held initially at 70 °C with 5 min hold time. Then, the temperature increased to 160 °C with 3 °C min-1 heating ramp. Finally, the temperature was raised to 250 °C with 6 °C min-1 heating ramp with 5 min hold time. Helium (99.999 % purity) was the carrier gas at 1.3 ml min-1 flow rate. The injection volume was 1µl (20 µl of volatile oil was dissolved in 1 ml of n-hexane). The solvent delay time was 8.20 min. The injection was in split mode (40 : 1). Detector, injector and ion source temperatures were 270 °C, 250 °C and 230 °C, respectively. MS scan range was (m z-1): 50-550 atomic mass units (AMU) under electron impact (EI) ionization of 70 eV.

Retention indices were determined by the co-injection of C7-C30 n-alkanes (Sigma-Aldrich) to (GC/FID) system (Agilent Technologies, 7890B) under the same conditions mentioned above. The volatile oils constituents were identified by the comparison of their retention indices and mass spectra by the computer library search database of US National Institute of Standards and Technology (NIST), Wiley libraries, other published mass spectra data (Adams, 2007) and the available data from our database.

2.4 LIPID EXTRACTION

50 g of grinded fruit samples were dissolved in 150 ml n-hexane at laboratory temperature for 12 h. n-hexane was removed with a rotary evaporator (40 °C) and the residue was stored at -10 °C until the fraction of the fatty acids could be determined. During the lipid extraction, evaporation and storage steps, the samples were kept away from light (Özgul Yücel, 2005).

2.5 ESTERIFICATION OF FATTY ACIDS

Methyl esters of samples were prepared by a cold transmethylation using 2 ml KOH in methanol and n-hexane with minor modifications (IUPAC, 1987). The extracted oil (0.5 g) was dissolved in 10 ml n-hexane followed by the addition of 1 ml of 2 ml methanolic KOH. The tubes were vortexed for 2 min. Finally, 1 ml of n-hexane layer was taken for GC analysis.

2.6 DETERMINATION OF FATTY ACIDS

Gas Chromatography analyses were carried out on a GC-2025 (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector (FID). A capillary column DB-23 (60 m, 0.25 mm ID and 0.25 µm film thickness, J & W Scientific, Folsom, USA) was used. The oven temperature was scheduled as follows: 180 °C for 5 min, increased to 200 °C with 10 °C min^{-1} heating ramp with 18 min hold time. Further, raised to 240 °C at the rate of 10 °C min-1 for 20 min. Helium (99.999 %) was used as the carrier gas at 40 ml min-1 flow rate. The injection was performed in split mode (100 : 1). Detector and injector temperatures were 250 °C. Fatty acids standards had linear calibration curves (R^2 = 0.99). The GC method used was validated for fatty acids determination of cumin seed oil samples with 95 % confidence limits. Mean analytical recoveries from the individual fatty acids in the oil samples were changed from 99.7 % to 100 %. The results were calculated as percentage peak area. The identification of FAMEs of samples was performed using a standard FAMEs mixture (Sigma-Aldrich Chemicals 189-

19). In addition, some parameters including the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) as well as petroselinic acid : linoleic acid ratio (PS : L), linoleic acid : linolenic acid (L : LN) ratio, iodine values (IV), oxidative susceptibility (OS) and theoretical oxidative stability (TOSI) were determined. Iodine values were calculated from the fatty acid percentages by using the formula given by Maestri et al. (1998):

IV = palmitoleic % × 1.001) + (oleic % × 0.899) + (linoleic % × 1.814) + (linolenic % × 2.73)

Oxidative susceptibility (OS) was estimated from fatty acid values by using the formula given by Cert et al. (1996):

Oxidative Susceptibility (OS) = MUFA + (45 × linoleic) + (100 × linolenic)

Theoretical oxidative stability (TOSI) was calculated from fatty acids data by using the formula given by Chu and Kung, (1998):

TOSI(h) = 7.5125 + palmitic % × (0.2733) + stearic % × (0.0797) + petroselinic % × (0.0159) + linoleic % × (-0.1141) + linolenic % × (-0.3962)

3 RESULTS AND DISCUSSION

3.1 VOLATILE OIL COMPONENTS

The volatile oils from wild *B. persicum* and *C. cyminum* were pale yellow to brown and pale yellow, respectively. As shown in Tables 1 and 2, location and climate significantly influenced the quantity and quality of volatile oils. The volatile oil content of Bam and Zirkuh *B. persicum* populations were 3.9 and 4.73 %, respectively. Volatile oil content for cultivated Rayen, Cukurcak, Taskopru and Asagialicomak populations of *C. cyminum* were 2.65, 2.2, 2 and 2.5 % (Table 2). According to the results, the volatile oil content of *B. persicum* populations was higher than that of *C. cyminum* populations. Various studies have reported similar results. Mazidi et al. (2012) reported that the volatile oil content of *B. persicum* was about 4.18 % and yellow. Another study on wild *B. persicum*, reported that the volatile oil content of plants collected from seven locations of Khorasan Province/Iran were about 3.1, 6.4, 6.7, 7.1, 7.5, 7.7 and 7.9 % (Talebi et al., 2018). Overall, the chemical differences between species are inevitable, and the yield and chemical constituents of medicinal and aromatic plants (like other types of plants) are responsive to the genetic, geographical, climatic and seasonal conditions, agronomic practices, and harvest time (Yanive and Palevitch, 1982; Omidbaigi and Arvin, 2009). Elevation and temperature are the most important of these factors (Talebi et al., 2018). The essential oil is made gradually from the beginning of the fruit, but its amount is low. The highest amount of essential oil is at a stage when the fruits are not yet fully mature. At the full maturity stage, a small amount of essential oil is reduced (Hornok, 1978).

Monoterpenes were found as the main chemical group components in both species (Table 3). The levels of monoterpene hydrocarbons in *B. persicum* (57.17-64.53 %) were significantly higher than *C. cyminum* (39.37-39.5 %). Maximum monoterpene hydrocarbons in *B. persicum* and *C. cyminum* were recorded in Bam and Cukurcak populations, respectively. The amounts of oxygenated monoterpenes in *C. cyminum* (36.11-46.16 %) were richer than *B. persicum* (27.24-31.81 %). Sesquiterpenes levels were lower than monoterpenes. Biosynthesis of sesquiterpene hydrocarbon components in *C. cyminum* species was more than twice that of *B. persicum*. Even oxygenated sesquiterpenes components were not observed in *B. persicum* populations. Except for Cukurcak population in *C. cyminum*, other populations of this species had considerably high content of alcohol components than *B. persicum*. Esters and ethers were obtained in very minor amounts (Table 3). The results of chemical analysis (GC-MS/FID) of populations showed that *C. cyminum* species had a relatively higher compositional diversity. Bam and Zirkuh populations of *B. persicum* contained 31 and 30 components, respectively. Rayen and Cukurcak populations of *C. cyminum* had 38 constituents. Taskopru (43) and Asagialicomak (44) contained a higher number of components (Table 2). From the oil constituents, 21 components were exclusive of *C. cyminum* populations. In contrast, nine components were specific to *B. persicum* populations.

γ-terpinene (39.62 %), cuminal (17.95 %), o-cymene (11.12 %), benzenemethanol, α-methyl- (7.49 %), 1-phenyl-1-butanol (6.41 %), limonene (6.41 %), β-pinene (2.29 %), α-pinene (1.90 %), isopulegone (1.09 %) and sabinene (1.01 %) were identified as the major constituents of Bam population. The major components of Zirkuh were γ-terpinene (33.62 %), cuminal (19.34 %), benzenemethanol, α-methyl- (9.52 %), limonene (8.66 %), 1-phenyl-1-butanol (8.41 %), o-cymene (5.37 %), α-pinene (2.12 %), terpinolene (1.38 %), isopulegone (1.27 %) and cuminol (1.05 %) (Table 2). Previous studies on *B. persicum*, support our findings on the major constituents of volatile oil (Foroumadi et al., 2002; Ehsani et al., 2016; Rustaie et al., 2016; Sanei Dehkordi et al., 2016; Khaledi and Hassani, 2018). Talebi et al. (2018), in their study with seven populations of wild *B. persicum* Boiss. from Northeast of Iran noted that γ-terpinene (29.2-40.1 %), cumin alcohol (16.4-28.4 %), cumin aldehyde (9-18.9 %), ρ-cymene (9.4-15.6 %), safranal (3.4-7.9%), limonene (3.7-6.4 %), β-pinene (0.8-2.3 %), α-pinene (0.3-1.7 %),

and sabinene (0.8-1.2 %) were the main constituents of the volatile oils; very similar to the findings of our study.

The results demonstrated that cuminal (22.80 %), benzenemethanol, α-methyl- (22.65 %), γ-terpinene (19.41 %), β-pinene (11.22 %), 1-phenyl-1-butanol (10.32 %), o-cymene (4.90 %) and isopulegone (1.49 %) were the dominant constituents in Rayen population of *C. cyminum*. The main constituents of essential oils from Cukurcak were cuminal (37.64 %), γ-terpinene (16.79 %), o-cymene (12.67 %), β-pinene (11.92 %), 1-phenyl-1-butanol (5.45 %), benzenemethanol, α-methyl- (5.3 %), isopulegone (1.36 %), α-phellandrene (1.02 %) and α-pinene (1.01 %). The predominant components of Taskopru sample volatile oil were cuminal (26.75 %), γ-terpinene (16.76 %), benzenemethanol, α-methyl- (15.25 %), 1-phenyl-1-butanol (12.59 %), β-pinene (11.64 %), o-cymene (6.5 %) and isopulegone (1.54 %). Furthermore, in Asagialicomak population essential oil cuminal (24.18 %), γ-terpinene (18.26 %), benzenemethanol, α-methyl- (17.48 %), β-pinene (11.35 %), 1-phenyl-1-butanol (9.76 %), o-cymene (6.41 %), α-phellandrene (2.36 %), isopulegone (1.23 %) and α-pinene (1.02 %) were found as the main components (Table 2). Moghaddama and Ghasemi Pirbalouti, (2017), compared 20 *C. cyminum* accessions and determined γ-terpinene (26.53-37.81 %), ρ-cymene (12.84-21.22 %), cumin aldehyde (9.45-20.66 %), cumin alcohol (1.63-15.22 %), β-pinene (8.32-13.84 %) and safranal (2.3-6.37 %) as the major constituents. in another study, propanal (26.19 %), benzenemethanol (25.4 %), 1-phenyl-1-butanol (16.49 %), γ-terpinene (13.04 %), β-pinene (7.28 %), cymene (4.24 %) and pulegone (2.58 %) were identified as the main components of this plant (Haghiroalsadat et al., 2011). In another study, the oil constituents of fruit samples from Emirdag (Turkey) were cumin aldehyde (19.25-24.80 %), p-mentha-l,3-dien-7-al (7.54-9.30 %), p-mentha-l,4-dien-7-al (36.51-44.91 %), γ-terpinene (8.61-9.72 %), ρ-cymene (5.94-6.45 %) and β-pinene (4.99-5.60 %) (Baser et al., 1992). The results of our study are in line with the findings of various studies on the chemical components of cumin (Rihawy et al., 2014; Esmaeili, 2015; Moghaddama et al., 2015; Tahir et al., 2016). Among the major components identified in all populations, γ-terpinene (39.62 %) was the highest in Zirkuh and cuminal (37.64 %) in Cukurcak.

3.2 FATTY ACID COMPONENTS

In total, 15 fatty acids were identified from the fatty oil of *B. persicum* populations of Iranian origin (Table 4). Based on the results, palmitic acid, petroselinic acid, linoleic acid, linolenic acid and behenic acid were the major components of Bam and Zirkuh populations of *B. persicum*. Lauric acid (37.08 %) and linoleic acid (33.60 %) were determined as the major fatty oil components of Bam and Zirkuh populations of *B. persicum*, respectively. Besides, capric acid and gadoleic acid from Bam and stearic acid from Zirkuh were the other major constituents.

Except for lignoceric acid, which was not present in *C. cyminum* population oils, the other components were common in both species. Petroselinic acid (47.53-55.51 %), linoleic acid (22.58-26.32 %), lauric acid (13.46 %) and palmitic acid (2.68-3.03 %) were identified as the major components of *C. cyminum* populations. Petroselinic acid content in *C. cyminum* was significantly higher than in *B. persicum*. Comparisons between *B. persicum* populations from two different countries showed no dominant differences in terms of fatty acid components.

The monounsaturated fatty acids especially petroselinic acid have great importance because of their high nutritional value and the contribution to the oxidative stability of oils (Bettaib et al., 2012; Rebey et al., 2012; Rebey et al., 2013). The oils from *C. cyminum* populations fruits were characterized by the presence of a high proportion of monounsaturated and polyunsaturated fatty acids. Our findings are similar to the previous studies (Bettaib et al., 2012; Rebey et al., 2012; Rebey et al., 2013; Keskin and Baydar, 2016; Milica et al., 2016; Hajib et al., 2018). Oil samples from two species were rich in petroselenic acid (29-55 %). This fatty acid is the iconic characteristic of the seeds oil from Apiaceae species. These oils have potential industrial significance, especially in the paint industry (Bettaib et al., 2012; Rebey et al., 2013).

Linoleic acid as a predominant polyunsaturated fatty acid was also present in both species at appreciable levels. Considering linoleic acid and other polyunsaturated fatty acids profiles, our finding is similar to Milica et al. (2016) and Hajib et al. (2018). Nevertheless, some other studies reported lower values than our research (Bettaib et al., 2012; Rebey et al., 2012; Rebey et al., 2013). The saturated fatty acids (lauric and palmitic acids acids) exhibited a vast variability (15.13-37.08 %).

The minimum recommended value for PUFA : SFA ratio is 0.5 g (HMSO, 1994), which is significantly lower than our findings (0.79-1.41) except for Bam population of *B. persicum* (0.39). There is no scientific information for the PUFA : SFA ratio in previous studies on *C. cyminum* and *B. persicum* (Table 4).

The changes for petroselinic acid : linoleic acid ratio, which is important for the estimation of oxidative stability, were 0.78-1.59 for *B. persicum* populations and 2-2.18 for *C. cyminum* samples. Overall, it can be pointed out that the oxidative stability of *C. cyminum* populations appears to be relatively higher than *B. persicum* populations. The present results are in agreement with Bettaieb et al. (2013). The above-mentioned components have in-

Table 2: Volatile oil components of *B. persicum* and *C. cyminum* populations from Iran and Turkey

RT[a]	RI[b]	Components (%)	*B. persicum*		*C. cyminum*				ID
			Bam	Zirkuh	Rayen	Cukurcak	Taskopru	Asagialicomak	
8.813	1032	α-pinene	1.903	2.12	0.855	1.01	0.962	1.029	1
9.838	1079	camphene	-	0.338	-	-	-	-	1
10.936	1120	β-pinene	2.292	2.61	11.225	11.928	11.649	11.352	1
11.256	1131	sabinene	1.015	0.981	0.688	0.695	0.684	0.724	1
12.103	1158	δ-3-carene	0.074	-	0.04	0.043	0.043	0.04	1
12.418	1168	β-myrcene	0.789	0.759	0.704	0.617	0.691	0.746	1
12.635	1175	α-phellandrene	-	-	0.603	1.024	0.985	2.362	1
13.121	1190	α-terpinene	0.318	0.429	0.172	0.118	0.153	0.166	1
13.791	1210	limonene	6.415	8.665	0.333	0.39	0.318	0.39	1
14.146	1219	1,8-cineole	-	0.343	-	-	-	-	1
14.163	1220	β-phellandrene	0.462	0.355	0.396	0.466	0.458	0.569	1
14.919	1240	*cis*-ocimene	-	0.18	-	-	-	-	1
15.547	1256	γ-terpinene	39.627	33.62	19.418	16.795	16.762	18.264	1
16.503	1281	o-cymene	11.122	5.377	4.904	12.676	6.505	6.413	1
16.932	1293	terpinolene	0.357	1.386	0.044	0.055	0.05	0.056	1
24.125	1469	*trans*-sabinene hydrate	0.046	-	0.036	0.041	-	0.046	1
25.418	1501	α-copaene	-	-	0.252	0.318	0.326	0.334	2
27.186	1546	β-gurjunene	-	-	0.046	0.039	0.07	0.08	1
27.369	1550	linalool	-	-	0.03	-	0.03	-	1
27.506	1554	*cis*-sabinene hydrate	-	-	0.092	-	0.067	0.058	1
28.119	1569	*trans*-2-menthenol	-	-	-	0.084	0.047	0.081	1
28.582	1581	isopulegone	1.096	1.272	1.49	1.363	1.547	1.233	1
28.948	1590	bornyl acetate	0.053	0.554	0.035	0.041	0.035	0.071	1
29.046	1592	*trans*-α-bergamotene	-	-	0.063	0.089	0.09	0.106	1
29.67	1608	caryophyllene	-	0.261	0.135	0.334	0.255	0.433	1
29.721	1610	terpinene-4-ol	0.471	0.528	-	-	-	-	1
30.625	1633	*cis*-2-menthenol	-	-	-	0.061	0.036	0.058	1
31.781	1664	*trans* -pinocarveol	-	-	0.055	0.059	0.073	0.067	1
32.05	1671	*trans*-β-farnesene	-	-	0.264	0.285	0.358	0.395	1
32.181	1674	(-)-isoledene	-	-	-	-	0.043	0.048	3
32.433	1681	α-humulene	-	-	-	-	0.033	0.07	1
32.908	1693	β-farnesene	0.046	-	0.034	-	-	-	1
33.177	1701	(-)-β-acoradiene	-	-	0.156	0.325	0.185	0.335	1
33.251	1702	γ-muurolene	0.114	0.146	0.188	-	-	-	1
33.303	1704	γ-curcumene	-	-	-	-	0.231	0.239	1
33.898	1720	germacrene D	0.15	0.172	-	-	-	-	1
34.167	1727	zingiberene	0.137	-	-	-	0.028	-	1
34.396	1733	phellandral	0.18	0.215	-	0.182	0.207	0.167	1
34.745	1742	β-selinene	-	-	0.042	0.071	0.07	0.073	1
34.831	1745	(-)-carvone	0.215	0.201	0.138	-	-	-	1

RT[a]	RI[b]	Components (%)	*B. persicum*		*C. cyminum*				ID
			Bam	Zirkuh	Rayen	Cukurcak	Taskopru	Asagialicomak	
35.025	1750	*cis*-piperitol	-	-	-	0.045	-	0.052	1
36.038	1777	β-sesquiphellandrene	0.085	-	-	-	-	0.145	1
36.974	1802	cuminal (cumin aldehyde)	17.95	19.345	22.809	37.642	26.758	24.181	1
37.125	1805	1-phenyl-1-butanol	6.417	8.412	10.32	5.45	12.59	9.765	1
37.480	1813	benzenemethano, α-methyl-	7.493	9.525	22.654	5.3	15.25	17.487	1
38.447	1835	anethole	-	-	-	0.134	0.04	0.073	1
40.289	1877	isoterpinolene	0.157	0.355	0.119	0.05	0.118	0.121	3
44.072	1977	cuminyl acetate	0.045	0.185	-	-	-	-	1
45.138	2008	caryophyllene oxide	-	-	0.058	0.133	0.097	0.081	1
45.743	2029	carotol	-	-	0.282	0.949	0.702	0.758	1
46.435	2054	α,α'-dihydroxy-m-diisopropylbenzene	-	-	0.032	0.082	0.07	0.074	3
46.982	2073	p-mentha-1,4-dien-7-ol	0.172	0.37	0.403	0.279	0.365	0.368	1
47.551	2093	viridiflorol	-	-	-	-	0.103	0.05	1
47.922	2107	cuminol (cumin alcohol)	0.644	1.051	0.686	0.638	0.832	0.75	1
49.874	2190	thymol	0.054	0.077	-	-	-	-	1
50.52	2220	carvacrol	-	-	0.051	0.188	0.084	0.087	1
53.644	2384	dill apiole	0.101	0.092	-	-	-	-	1
Volatile oil content (%)			3.9	4.73	2.65	2.2	2.0	2.5	

RT: Retention time; RI: Retention indices calculated against n-alkanes (C7-C30) on HP-Innowax column; ID: Identification method 1: RI-MS; 2: RI-Rf; 3: MS

Table 3: Grouped chemical components of *B. persicum* and *C. cyminum* populations essential oil from Iran and Turkey

Grouped chemical components(%)	*B. persicum*		*C. cyminum*			
	Bam	Zirkuh	Rayen	Cukurcak	Taskopru	Asagialicomak
Monoterpene hydrocarbons	64.531	57.175	39.501	45.867	39.378	42.232
Oxygenated monoterpenes	27.245	31.814	36.11	46.166	42.676	36.986
Sesquiterpene hydrocarbons	0.532	0.579	1.18	1.461	1.689	2.258
Oxygenated sesquiterpenes	-	-	0.34	1.082	0.902	0.889
Alcohols	7.493	9.525	22.654	5.3	15.25	17.487
Esters	0.098	0.739	0.035	0.041	0.035	0.071
Ethers	0.101	0.092	-	-	-	-
Others	-	-	0.032	0.082	0.07	0.074
Total (%)	100	99.924	99.852	99.999	100	99.997

dustrial applications especially in the manufacturing of oil based paints (Bettaib et al., 2012; Rebey et al., 2013).

The ratio of linoleic acid : linolenic acid is a prominent indicator for comparing the relative nutritional value of oils from different plant sources (Rebey et al., 2013). This value varied widely among the populations of both species tested. *C. cyminum* had significantly higher values than *B. persicum* (Table 4).

The iodine values were calculated according to the fatty acid components. The saturated fatty acids, mono-

unsaturated fatty acids and polyunsaturated fatty acids levels of samples influenced the iodine values (Maestri et al., 1998). Oxidative susceptibility was estimated based on the fatty acids profile. According to Table 4, no significant variation was observed in the oxidative susceptibility of *C. cyminum* populations. However, this value for Zirkuh population was twice more than that of *B. persicum* (Bam population).

The variations for Iodine values, oxidative susceptibility and theoretical oxidative stability index for all the samples were almost similar due to the homogenous unsaturated fatty acids profiles. All these values represent the theoretical stability of the oil (Chu and Kung, 1998).

Generally, the oxidative susceptibility and theoretical oxidative stability index values of samples were correspondingly increased with high linoleic acid content more than monounsaturated fatty acids (especially petroselinic acid). Linoleic acid is much more susceptible to oxidation than monounsaturated fatty acids (Chu and Kung, 1998). However, there is no available data on iodine values, oxidative susceptibility and theoretical oxidative stability index on *C. cyminum* and *B. persicum*.

The differences in fatty acids profiles of *C. cyminum* and *B. persicum* populations from various localities of Iran and Turkey are seemingly dependent on the genetic factors (Bettaib et al., 2012; Rebey et al., 2013), environmental and edaphic characteristics and agricultural practices (Bettaib et al., 2012; Rebey et al., 2013; Keskin and Baydar, 2016) as well as they depend upon the large geographical variations (Keskin and Baydar, 2016; Hajib et al., 2018).

Table 4: Fatty acid components and some parameters related with fatty oil quality of *B. persicum* and *C. cyminum* populations from Iran and Turkey

Fatty Acid (%)	*B. persicum*		*C. cyminum*			
	Bam	Zirkuh	Rayen	Asagialicomak	Cukurcak	Taskopru
Capric acid C 10 : 0	3.09	0.71	0.24	0.82	0.65	0.68
Lauric acid C 12 : 0	37.08	22.92	16.21	15.13	24.22	13.46
Myristic acid C 14 : 0	0.86	0.34	0.1	0.23	0.75	0.21
Palmitic acid C 16 : 0	4.53	6.24	3.03	2.89	2.68	2.99
Palmitoleic acid C 16 : 1	0.43	0.25	0.35	0.39	0.34	0.35
Margaric acid C 17 : 0	0.21	0.51	0.03	0.02	0.03	0
Margoleic acid C 17 : 1	0.59	0.26	0.04	0.03	0.06	0.07
Stearic acid C 18 : 0	0.97	1.99	0.60	0.67	0.56	0.72
Petroselinic acid C 18 : 1 (n-6)	29.12	26.36	52.64	53.51	47.53	55.51
Linoleic acid C 18 : 2 (n-6)	18.34	33.60	26.32	25.76	22.58	25.49
Linolenic acid C 18 : 3 (n-3)	1.01	4.82	0.24	0.27	0.30	0.17
Arachidic acid C 20 : 0	0.12	0.14	0.05	0.04	0.06	0.04
Gadoleic acid C 20 : 1	1.18	0.55	0.13	0.20	0.14	0.27
Behenic acid C 22 : 0	2.05	1.04	0.03	0.61	0.10	0.06
Lignoceric acid C 24 : 0	0.33	0.28	ND	ND	ND	ND
Saturated fatty acids (SFA %)	49.24	34.17	20.05	20.41	29.05	18.16
Monounsaturated fatty acids (MUFA %)	31.32	27.42	53.16	54.13	48.07	56.20
Polyunsaturated fatty acids (PUFA %)	19.35	38.42	26.56	26.03	22.88	25.66
PUFA : SFA	0.39	1.12	1.32	1.28	0.79	1.41
MUFA : PUFA	1.62	0.71	2	2.08	2.1	2.19
Petroselinic acid : Linoleic acid	1.59	0.78	2	2.08	2.1	2.18
Linoleic acid : Linolenic acid	18.16	6.97	109.67	95.41	75.15	149.94
Iodine value	62.64	98.09	96.08	95.96	84.85	96.96
Oxidative susceptibility	957.62	2021.42	1261.56	1240.33	1094.17	1220.25
Theoretical oxidative stability index (TOSI) hours	6.79	4.04	6.12	6.15	6.34	6.29

4 CONCLUSIONS

Our findings showed significant differences in the volatile oil content, volatile oil and fatty oil constituents' profile of *B. persicum* and *C. cyminum* populations from Iran and Turkey. It can be inferred that genetic characteristics, location (region) and climatic conditions have sensible effects on the oil contents and its ingredients. As known, the production of primary and secondary metabolites in plants is directly and continuously associated with multiple biotic and abiotic factors. The identification and characterization of secondary metabolites profile in medicinal plants and especially in native plants are crucial to assign a specific characteristic for those precious species and, to make them new candidates for the multidisciplinary use with several industries. Besides, the identification of compositional profile of native neglected plant species labels the plants a recognizable criterion for the commercial cultivation and exploitation. Moreover, by the characterized secondary metabolites profile; natural habitats will be safer and intact since, the agricultural systems try to concentrate on a defined species production and, the miss and over-harvesting of the related species will be limited in favor of natural habitats diversity.

Inline, identifying the major components and the possible chemotypes and the characterization of bioactive substances with the potential pharmaceutical application from medicinal and aromatic plants provide a broaden way and horizon in front of the producers, entrepreneurs and policy makers for the efficient utilization of natural habitats and cultivated plants.

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Euphorbia bupleuroides Desf. latex as biopesticide against the red flour beetle (*Tribolium castaneum* [Herbst, 1797]) and khapra beetle (*Trogoderma granarium* Everts, 1898)

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***Euphorbia bupleuroides* Desf. latex as biopesticide against the red flour beetle (*Tribolium castaneum* [Herbst, 1797]) and khapra beetle (*Trogoderma granarium* Everts, 1898)**

Abstract: Laboratory evaluation of *Euphorbia bupleuroides* latex as biopesticide against the red flour beetle (*Tribolium castaneum*) and khapra beetle (*Trogoderma granarium*) were evaluated at ambient temperature. The insecticidal activity of latex was determined by direct contact application. Different concentrations were prepared by dilution of 2.5, 5.0, 7.0 and 10.0 µl of latex into 0.1 ml acetone. 1 µl was pumped regularly in the thorax of different insects. The latex of *E. bupleuroides* showed insecticidal activity against *T. granarium* and *T. castaneum*. High levels of mortality were associated with the increase in the concentration and time of exposure as well. *T. granarium* adults are generally more prone to latex insecticidal effects than *T. castaneum* adults. After 6 days of exposure to *E. bupleuroides* latex, the LC_{50} recorded was 14.12 µl for *T. granarium* adults, and 14.7 µl for *T. castaneum*. LC_{90} numbers, on the other hand, were 38.8 µl for the former, and 51.44 µl for the latter.

Key words: *Euphorbia bupleuroides*; biopesticide; latex; *Tribolium castaneum*; *Trogoderma granarium*; mortality

Mleček prerastolikega mlečka (*Euphorbia bupleuroides* Desf.) kot bioinsekticid za zatiranje riževega mokarja (*Tribolium castaneum* [Herbst, 1797]) in indijskega žitnika (*Trogoderma granarium* Everts, 1898)

Izvleček: Laboratorijsko vrednotenje mlečka iz prerastolikega mlečka (*Euphorbia bupleuroides* Desf.) kot bioinsekticida za zatiranje riževega mokarja (*Tribolium castaneum* (Herbst, 1797)) in indijskega žitnika (*Trogoderma granarium* Everts,1898) je bilo izvedeno pri sobni temperaturi. Insekticidna aktivnost mlečka je bila določena z neposrednim nanosom. Različne koncentracije so bile pripravljene z razredčenjem 2,5; 5,0; 7,0 in 10,0 µl mlečka v 0,1 ml acetona. 1 µl raztopine je bil previdno vbrizgan v oprsje hroščev. Mleček prerastolikega mlečka je izkazal insekticidno delovanje na oba preučevana hrošča. Velika smrtnost hroščev je bila povezana s povečanimi koncentracijami in daljšim časom izpostavitve. Odrasli osebki indijskega žitnika so bili na splošno bolj dovzetni za strupeni učinek mlečka kot odrasli osebki riževega mokarja. Po šestih dnevih izpostavitve mlečku je bila LC_{50} za indijski žitnik 14,12 µl in 14,7 µl za riževega mokarja. LC_{90} vrednost je bila za prvega 38,8 µl in 51,44 µl za drugega.

Ključne besede: *Euphorbia bupleuroides*; bioinsekticid; mleček; *Tribolium castaneum*; *Trogoderma granarium*; smrtnost

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1 INTRODUCTION

Pest insects can potentially be carriers of pathogens, and are a substantial contributing source of allergens. That is because of their large cosmopolitan population and high numbers at homes and other buildings (Chang and Anh, 2002). Interest in developing safer alternatives to potentially replace toxic chemicals in pest control is increasingly growing. Bioactive substances and plant insecticides serve different functions in pest control: act as repellents, impact oviposition or feeding, disrupt development, or serve as pest insecticides (Isman, 2017, as cited in Bohinc et al. (2020).

The use of plant extracts is one of the most desirable pest control methods (Salvadores et al., 2007). Plants secondary metabolites are recognized for their crucial role in pest control due to the selective, biodegradable, non-toxic nature of their products, as well as possessing fewer harmful side-effects on non-targeted organisms and the environment (Wink, 1993). Approximately 250,000 species of plants on earth have been labelled as possessing compounds with insecticidal properties (Rafael, 2001).

Euphorbiaceae family is one of the largest and most diverse family in the plant kingdom; comprising of 7800 species in 300 genera (Webster, 1994). Diterpenoids and triterpenoids secondary metabolites are substantially present in *Euphorbia* species (Giner et al., 2000). They are endowed with striking biological anti-cancer qualities; for instance, they can serve antitumor purposes (Tanaka et al., 2000), anti-proliferative (Cateni et al., 2010), anti-oxidant and cytotoxic (Aslanturk et al., 2013), and modulators of multidrug resistance (Vasas et al., 2012).

Euphorbia bupleuroides Desf. is labelled as an herbaceous plant, characterized by plain and simple leaves. It is commonly found in mountain rock areas (Quezel and Santa, 1963), and is utilized in Algeria as an endemic medicinal plant in traditional medicine with varied uses ranging from the extirpation of thorns to the treatment of warts, as well as the use of the decoction of roots for anti-inflammatory purposes. Furthermore, two of the major chemical compounds of *E. bupleuroides* are diterpenoids and triterpenoids (Aichour et al., 2014). They are the most relevant in the insecticidal nature of *E. bupleuroides* and similar plants (Singh, 2012; Vimal and Das, 2014).

This study is conducted to assess the toxicity of *Euphorbia bupleuroides* latex against grain pests, red flour beetle (*Tribolium castaneum* [Herbst]) and khapra beetle (*Trogoderma granarium* Everts).

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

In April 2018, samples of *Euphorbia bupleuroides* were collected from their natural habitat of mountain rock areas around Tazoult, east of the city of Batna in the North East of Algeria (35° 28' 54" N, 6° 15' 39" E).

2.2 LATEX PREPARATION

The stems of the collected samples of *Euphorbia bupleuroides* were cut using a knife, allowing the latex to come out into a container. Latex was then collected in beakers, which were then corked tightly to prevent both evaporation and solidification. They were, after that, labeled and preserved in a refrigerator to maintain freshness. The whole procedure took approximately three days overall.

2.3 INSECTS

Cultures of the red flour beetle (*Tribolium castaneum*) and khapra beetle (*Trogoderma granarium*) were maintained at 27 °C and 65 % relative humidity (RH) on a wheat flour of a growth culture room in the dark. Adult insects, 1–7 days old, were used for bioassay.

2.4 BIOASSAY METHODS

2.4.1 Insecticidal Activity

The insecticidal activity of latex was determined by direct contact application. Different concentrations were prepared by dilution of 2.5, 5.0, 7.0, and 10.0 µl of latex into 0.1 ml acetone. For each preparation, 1 µl was pumped regularly in the thorax of different insects; 10 adult insects were enclosed in a Petri dish. Controls were treated similarly but exposed only to acetone. Each concentration and control were replicated four times. Mortality percentage was determined at 2, 4 and 6-days following treatment. Upon the observation of no leg or antennal movements were, insects were considered dead. The percentage of insect mortality was calculated using Abbott's correction formula for natural mortality in untreated controls (Abdel-Sattar et al.,2010).

2.5 STATISTICAL ANALYSIS

Probit analysis of concentration-mortality data was conducted to estimate the LC_{50}, LC_{90} values, their 95 % confidence intervals and related parameters (Finney, 1971). Probit analysis was fitted using the ''LC_probit" function in the ''ecotox" package in R (Robertson et al., 2007).

3 RESULTS AND DISCUSSION

The insecticidal activity of *E. bupleuroides* latex was tested against *T. granarium* and *T. castaneum* (Table 1). Data on the accumulative mortality of the two stored products insect species along 6 days of exposure to latex revealed that *E. bupleuroides* latex was significantly toxic for both species, though at different concentrations, all slope regressions were qualified significant ($p < 0.05$) (Table 1).

The mortality rates of *T. castaneum* are summarized in Figure 1. At a concentration of 5 µl, mortality rates remained negligible from day 2 up to day 6. However, after 6 days, mortality rates increased from 5 to 90 %. The higher the concentration, the less time is required to achieve high levels of toxicity. At 7.5 µl and 10 µl, 10 and 42.5 % mortality rates are observable after 2 days, 7.5 and 20 % after 4 days, and 57.5 to 90 % after 6 days. The highest concentration (10 µl) caused 42.5 % a mortality rate just after 2 days of exposure (Figure 1).

The results on Figure 2 demonstrate that the mortality rate of *T. granarium* adults varied with concentrations and time. In fact, after 6 days, the recorded mortality rate was 15, 22.5, and 40 % using 5.0, 7.5, and 10.0 µl concentrations respectively.

Lethal concentrations of latex were calculated after 2, 4, and 6 days of exposure for both species. Results showed that *T. granarium* adults are generally more sensitive to latex insecticidal effects than *T. castaneum* adults (Table 1). Evidently, after 6 days of exposure to *E. bupleuroides* latex, the LC_{50} recorded was 14.12 µl for *T. granarium* adults, and 14.7 µl for *T. castaneum*. LC_{90} numbers, on the other hand, were 38.8 µl for the former, and 51.44 µl for the latter (Table 1).

Mortality rates increased with rising concentration levels of *E. bupleuroides* latex. Additionally, mortality rates of *T. castaneum* and *T. granarium* differed in accordance with the different concentration levels of *E. bupleuroides* latex.

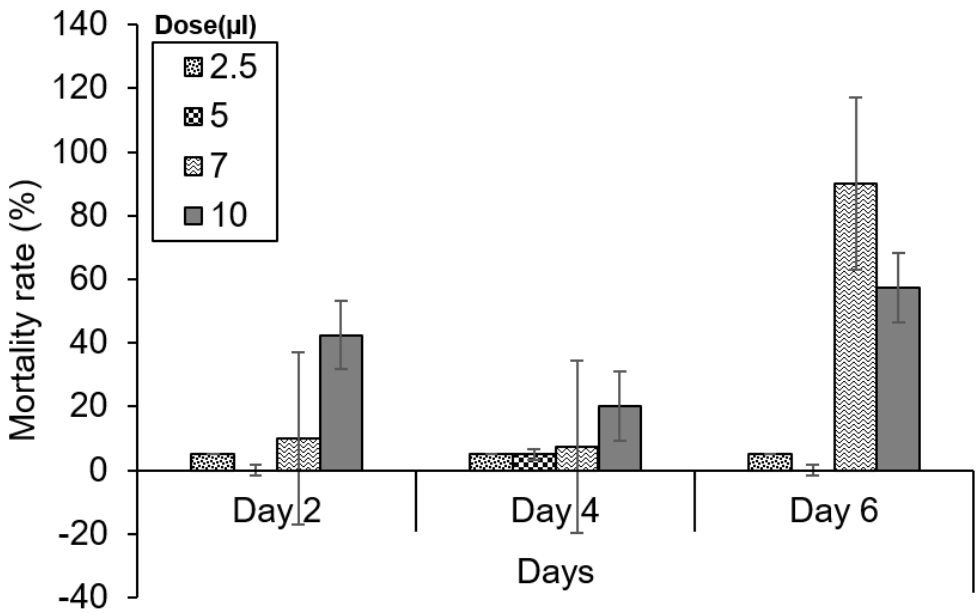

Figure 1: Mortality rates in T. castaneum treated with different concentrations of latex.

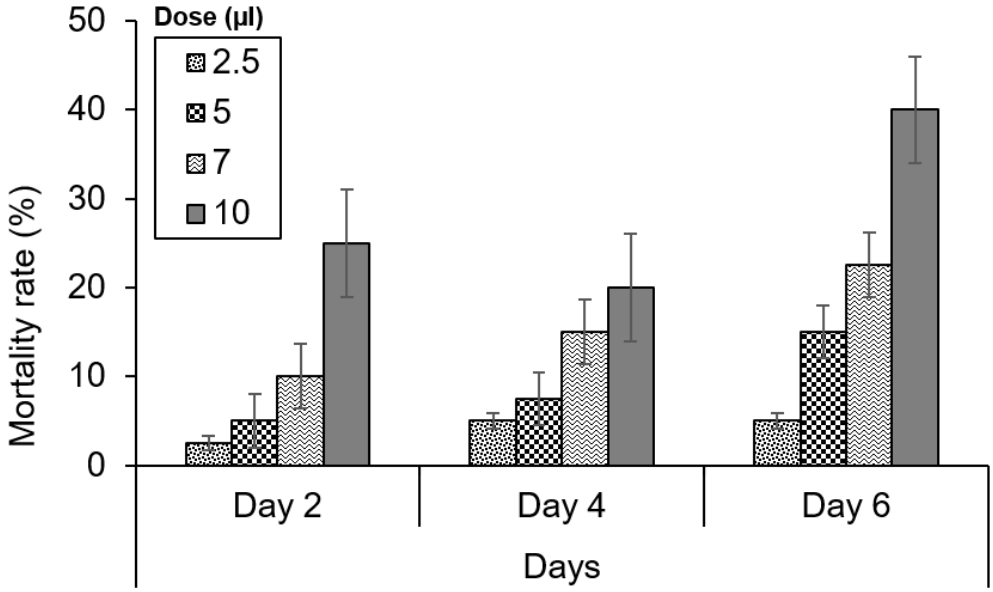

Figure 2: Mortality rates in *T. granarium* treated with different concentrations of latex.

Table 1: Latex toxicity on T. castaneum and T. granarium adults (LC_{50} and LC_{90}) after 2, 4, and 6 days.

Species	Assay time (days)	LC_{50} (µl)	95% LCL – UCL	LC_{90} (µl)	95% LCL – UCL	Slope ±SE	P
Tribolium castaneum	2	14.7	9.76 – 9.81E+3	38.8	17.02 – 1.93E+8	3.04 ± 0.860	0.0004
	4	56.03	17.98 – 5.23E-42	482.05	53.42 –3.77E-84	1.37 ± 0.715	0.049
	6	14.7	9.76 – 9.81E+3	38.8	17.02 – 1.93E+8	3.04 ± 0.860	0.0004
Trogoderma granarium	2	22.12	13.26 – 323.8	78.12	28.54 – 21222.5	2.33 ± 0.835	0.005
	4	40.74	16.10 – 3.613E+09	320.08	48.72 – 2.040E+19	1.43 ± 0.663	0.031
	6	14.12	9.44 – 105.5	51.44	21.14 – 9780.8	2.28 ± 0.625	0.0002

4 DISCUSSION

Different plants belonging to the Euphorbiaceae family have been studied all over the world for their toxic constituents. For instance, according to Govindarajan et al. (2008), Leaf extract of *Acalypha indica* L. exhibits larvicidal and ovicidal activities against malaria vector - *Anopheles stephensi* Liston, 1901. *Acalypha alnifolia* Klein ex Willd. extracted leaves demonstrated, in similar fashion to *Acalypha indica* L., larvicidal properties, but differed in having pupicidal effects—rather than ovicidal—against the same species (Murugan et al., 2011). De Silva et al. (2008) studied the insecticidal properties of *Euphorbia antiquorum* L. latex againt rice insect pests, whilst *Euphorbia fischeriana* Steud. had anti-feeding effects on stored-product insects according to Geng *et al.* (2011).

Euphorbia bupleuroides latex was tested several times against common species and pest insects that are widespread in houses, restaurants, and food stockages (Saito and Hama, 2000). For example, It was proven effective and toxtic against *Blattella germanica* Linnaeus, 1767 adults and larvae (Azoui et al.,2016) .

Vimal and Das (2014) confirmed that mortality rates increased with the increase in concentration of *Euphorbia antiquorum* L. latex extract. It was also found that latex was a strong pesticide against *Aedes aegypti* (Linnaeus and Hasselquist,1762) larvae, where LC_{50} val-

ue was 14.34 ml dl^{-1} after 24 hours, 10.70 ml dl^{-1} after 48 hours, 6.62 ml dl^{-1} after 72 hours of exposure.

The insecticidal effects of *Sebastiania corniculata* Müll. against *Laodelphax striatellus* (Fallén,1826), *Nilaparvata lugens* (Stål, 1854) and *Sogatella furcifera* (Horvath 1899), were evaluated by Lee et al. (2010). Results indicated that the chloroform fraction of *S. corniculata* possessed the highest potential for insecticidal activity against *L. striatellus* (DL50 = 1.09 µg/female), *N. lugens* (DL50 = .46 µg/female), and *S. furcifera* (DL50 = 2.32 µg/female). (LD stands for "Lethal Dose". LD_{50} is the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test insects).

Mwine et al. (2010) examined larvicidal properties of *Euphorbia tirucalli* L. latex against larvae of *Anopheles* mosquitoes. Results showed that the latex made total mortality at the highest dilution used of 1:250 in 5 days. Plant latex comprises of a substantial mixture of proteins and specialized products that include alkaloids, terpenoids, cardenolides, and many other components, most of which are toxic against insects and pathogens (Agrawal and Konno, 2009; Hua et al., 2015; Huber et al., 2015; Konno, 2011; Konno et al., 2006).

Numerous ingredients have been isolated from the extracts of *Euphorbia* species (Jain et al., 2008). Al-Younis and Abdullah (2009) identified flavonoids and phenolic acids from several species of *Euphorbia* genus including *Euphorbia granulata* Forssk. and *Euphorbia heliscopia* L. Different triterpenoids and diterpenoids were isolated from various *Euphorbia* species (Sutthivaiyakit et al., 2000; Sun et al., 2011; Aichour et al., 2014). According to Lima et al (2006), terpenoids are the most significant element in the insecticidal property of several plant species.

5 CONCLUSION

This study confirms the success of latex as a biopesticide against certain stored products insect species; namely, *T. castaneum* and *T. granarium*. Hence, latex could serve as an alternative to synthetic insecticides for the protection of stored grain. Further studies and investigations are necessary to isolate the active insecticidal compounds of the latex and study the insecticidal effects of these compounds.

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Impact of irrigation during flowering and fruit growth on fruit yield and quality of the cactus *Opuntia* spp.

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Impact of irrigation during flowering and fruit growth on fruit yield and quality of the cactus *Opuntia* spp.

Abstract: Most plantations of cactus pear are not irrigated in Morocco and fruits are tiny. The impacts of irrigation during flowering (FLO) and fruit growth (FRG) on fruit yield and quality were monitored along two years on three varieties of cactus pear. In 2011, irrigation treatments were: T1 (0 mm), T2 (30 mm during FLO and 30 mm during FRG) and T3 (30 mm during FRG only). In 2012, irrigation treatments increased to 60 mm during FLO and FRG. The irrigation treatments were applied in 8 (T3) or 16 (T2) watering, once every three days. Interactions between varieties and treatments were significant for fruit yields and for yield components. In 2011, T2 and T3 irrigations had a negative effect on 'Aissa' and 'Moussa' (-2.8 kg/plant) and T2 had a positive effect on 'Achefri' (+2.7 kg/plant). Fruit quality was not affected by irrigation. In 2012, all the varieties responded positively to irrigation: 'Achefri' and 'Aissa' yielded very significantly more with T3 (plus 63 % and 30 % resp.) and 'Moussa' with T2 (+30 %). All irrigations increased fruit number and size. Irrigation had no significant effect on the fruit quality or slightly decreased the content of total sugars and titratable acidity.

Key words: cactus pear; fruit yield; fruit quality; irrigation; irrigation scheduling.

Vpliv namakanja kaktusov iz rodu *Opuntia* med cvetenjem in rastjo plodov na njihov pridelek in kakovost

Izvleček: Večina nasadov opuncije v Maroku ni namakanih zaradi česar so njihovi plodovi drobni. Vpliv namakanja med cvetenjem (FLO) in rastjo plodov (FRG) na njihov pridelek in kakovost je bil spremljan v dveh letih na treh sortah. V letu 2011so bila namakanja naslednja: T1 (0 mm), T2 (30 med cvetenjem in 30 mm med rastjo plodov) in T3 (30 mm samo med rastjo plodov). V letu 2012 je bilo namakanje povečano na 60 mm med cvetenjem in med rastjo plodov. Namakanja so bila izvedena v 8 (T3) ali 16 (T2) zalivanjih, enkrat vsake tri dni. Medsebojni vpliv obravnavanj in sort je bil značilen za pridelek plodov in njegove komponente. V letu 2011sta imeli obravnavanji T2 in T3 negativni učinek na sorti 'Aissa' and 'Moussa' (-2,8 kg/plant), obravnavanje T2 pa je imelo pozitiven učinek na sorto 'Achefri' (+2,7 kg/plant). Namakanje ni vplivalo na kakovost plodov. V letu 2012 so se vse tri sorte odzvale pozitivno na namakanje, pri čemer sta imeli sorti 'Achefri' in 'Aissa' značilno večji pridelek pri obravnavanju T3 (več kot 63 % in 30 %), sorta 'Moussa' pa pri obravnavanju T2 (+30 %). Vsa namakanja so povečala število in velikost plodov. Namakanja niso značilno vplivala na kakovost plodov, le neznatno sta ste zmanšali vsebnosti celokupnih sladkorjev in titrabilnih kislin.

Ključne besede: plodovi opuncije; pridelek plodov; kakovost plodov; namakanje; plan namakanja.

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1 INTRODUCTION

The prickly pear cactus *Opuntia* plays an important role in the system of agriculture of arid and semi-arid zones, due to its numerous non-food (forage, pharmaceuticals, cosmetic oil from the seeds and for anti-erosion), and food uses (cladodes as vegetables, fruit, and fruit juice) (Nefzaoui & Ben Salem, 2000; Le Houérou, 2002; Felker & Inglese, 2003; Arba, 2009; Inglese, 2010). Cultivated historically in Morocco, the cactus *Opuntia* is a source of food and feed and of incomes for the rural populations in the arid and semi-arid regions. Cactus pear fruits are appreciated by the consumer for their flavour, taste and dietetic properties.

It occupies an important area of about 150 000 ha, but plantations are often poorly managed and usually not irrigated. Recently, high density plantations of cactus pear were established with drip irrigation in South Morocco, notably in the areas of Haouz, Tiznit and Guelmim (Arba et al., 2018). The plant has the reputation of low water demand (Nobel, 2002; Nobel & Bobich, 2002) and of rarely requiring irrigation in the Mediterranean region (Nobel, 2002; Inglese, 2010). As a crassulacen acid metabolism plant, cactus pear is grown under rainfed conditions in the arid and semi-arid areas where low and erratic rainfall is the main limiting factor for its productivity (Potgiter & D'Aquino, 2017). In the dry areas of Morocco, the hot summer season can extend over 7 to 8 months without rain (from March-April to October-November). Even if the cactus *Opuntia* is resistant to drought and its water requirements are low, it is difficult for it to overcome the hot and dry periods in the south part of the Mediterranean region. The cladodes and fruits become dehydrated and the pads are marked by desiccated spots. Fruit yield is low and fruit size is poor. However, irrigation may be required in hot and dry zones in the summer, especially for intensive commercial production as in Mexico, USA, Chile, Italy, South Africa, Morocco, Tunisia (Inglese, 2010). In these areas, an addition of limited amounts of water can improve yields when rainfall fails to be sufficient for the plant growth (Oweis & Hachum, 2012). Several authors also reported that the productivity of cactus pear in the arid and semi-arid areas can be increased by supplemental irrigation which is a common practice in many countries, such as in Italy (Gugliuzza et al., 2002), Jordan and Morocco (Potgieter & D'Aquino, 2017) and Mexico (Zegbe & Serna-Perez, 2018, Zegbe et al., 2019; Zegbe & Sevin-Palestina, 2020). The amount of rain received during the fruit development period (FDP) of the cactus *Opuntia*, which extends from the beginning of the formation of floral buds until fruit ripening, influences the mean fresh mass of fruit (Zegbe Dominguez et al., 2015). Felker et al. (2002) also suggested that high rainfall during the last two months of fruit maturation led to an increase in fruit size (calibre) and in the content of pulp. The amount of rain received during the FDP also affects the total soluble solids (TSS) of the fruits (Zegbe Dominguez et al., 2015).

This study aims to evaluate the impacts of complemented irrigation at critical phenological stages, particularly during the flowering and fruit-growth stages, on fruit yield and fruit quality, mainly in terms of calibre and composition.

2 MATERIAL AND METHODS

The trials were conducted in the orchard of the experimental station of the Hassan II Institute of Agronomy and Veterinary Medicine, Horticultural Complex of Agadir, Morocco (30°36' N, 9°36' E, and 32 m elevation) (Figure 1). The soil has medium depth and good water retention. It has a sandy silty texture with 19–23 % coarse sand, 30–35 % fine sand, 19–22 % coarse silt, 20–21 % fine silt and 5–7 % clay. The content in organic matter is low to passable, porosity is 45 %, and moisture at field capacity is 30 %.

The experiments were on three varieties originated from the Sidi Ifni area, south of Agadir: two spineless varieties 'Aïssa' and 'Moussa' of *O. ficus-indica* L. and one thorny variety 'Achefri' of *O. megacantha* Salm-Dyck (Figure 2). The plants were planted in 1997 in plots as described hereafter. At the start of the experiment, plantations were fourteen years old, plant size was 2 to 2.5 m high and each plant had four to five principal branches.

Climatic data relating to rainfall and evapo-transpiration (Figure 3) were obtained from the Saouda Station located 10 km from the experimental site.

In order to manage correctly the amounts and timing of irrigation, drip irrigation has been installed including flexible perforated pipes at the plant feet and valves, allowing water to pass through for a pre-determined time and water distribution adapted to every single small plot. Details on the scheduled irrigation treatments are presented in Table 1.

The experiment has been set up in an area of 1404 m^2. The experimental design was a split-plot with four replicates (4 blocks of 350 m^2) (Figure 4). The variety factor was the main plots (3 rows at 3 m inter-rows x 8 plants at 1 m distance plus 2 m paths), area 90 m^2, and the irrigation factor, the subplot (one row 8 m of plantation x 3 m inter-row).

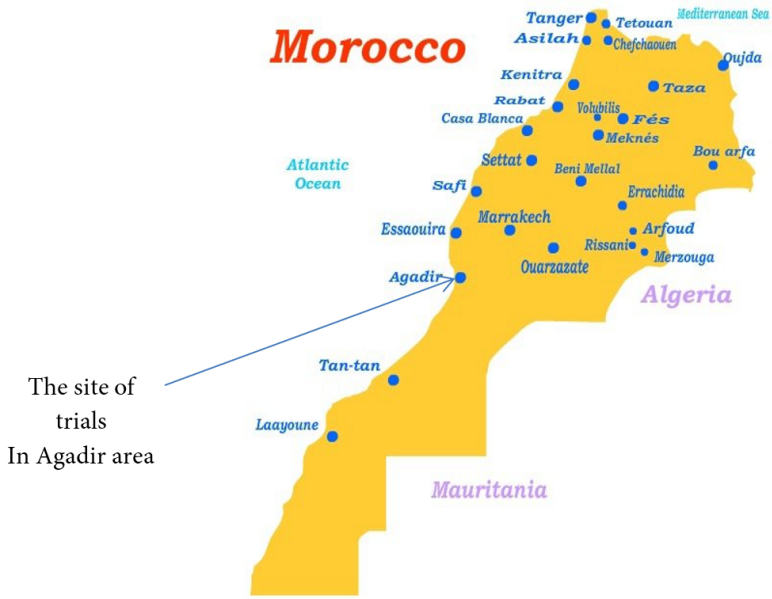

Figure 1: Trials site location in Agadir area within the map of Morocco.

O. ficus-indica 'Aissa'.

O. ficus-indica 'Moussa'.

O. megacantha 'Achefri'.

Figure 2:
Opuntia spp. varieties used in the study. Photos were taken in the orchard of the experimental station of the Hassan II Institute of Agronomy and Veterinary Medicine, Horticultural Complex of Agadir, during the ripening phase in July 2011.

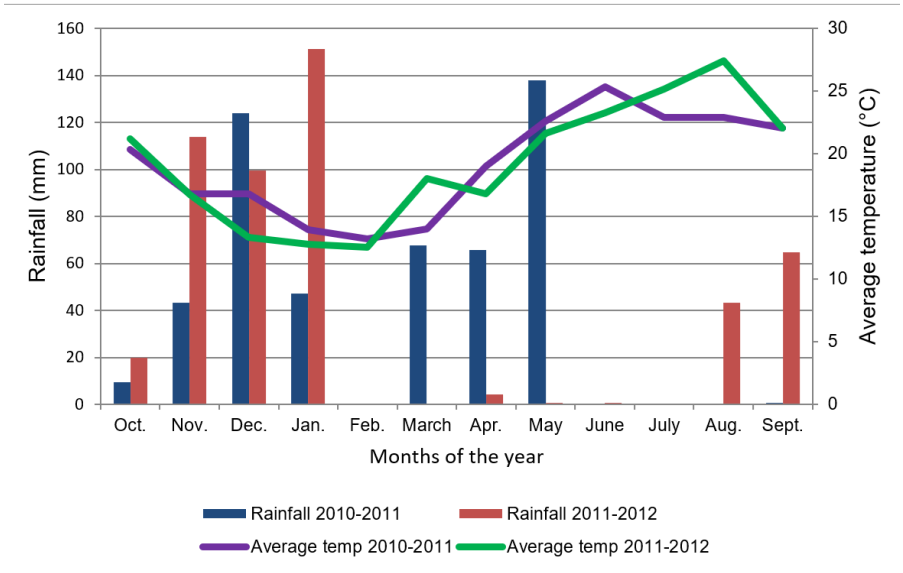

Figure 3: Average monthly rainfall and air temperature for the study site Agadir, Morocco during the two seasons of study (2010-2011 and 2011-2012).

Table1: Treatments of irrigation applied during the two years of experiments (2011 and 2012), number of irrigations per treatment and amounts of water per irrigation.

	Treatments of irrigation, irrigation frequency, number of irrigations and amount of water per irrigation			
	T1 Control without irrigation	T2 30 mm during flowering (mid-April) (60* at end February) + 30 mm during fruit growth (mid-June) (60* during may)		T3 30 mm only during fruit growth (mid-June) (60* during May)
		Irrigation frequency: every third day		
		Number of irrigations	Amount of water per irrigation (mm water)	Number of irrigations	Amount of water per irrigation(mm water)
First year experiments		7 + 7 1 + 1	4 2	7 1	4 2
Second year experiments		7 + 7 1 + 1	8 4	7 1	8 4

*Total amount of water in the second year experiments

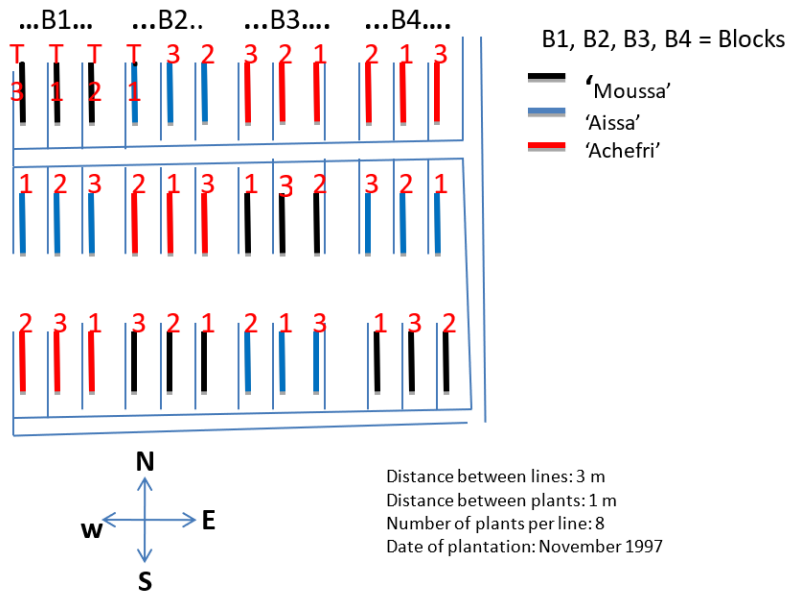

Figure 4: Experimental design in split-plot used in the trials.

Fruit yields expressed as fruit yield per plant were determined by harvesting fruits on two central plants per treatment of irrigation, per variety and per replication. The single fruit average masses have been measured on samples of 20 fruits per plot. The third yield component-fruit number per plant- has been determined by two ways; an 'observed' fruit number on the harvested plants and a "computed" number resulting from the ratio yield/ average fruit mass.

The_following fruit quality_parameters were evaluated: mass, length and diameter, pulp proportion, juice content, as well as fruit organoleptic characteristics, namely amount of juice, titratable acidity, pH, degree Brix, total sugars and juice dry matter. Measurements of the physical characters of fruits were performed on samples of 20 fruits for each treatment and variety. Chessa & Nieddu (1997) established five size categories for harvested fruits: very small calibre (< 80 g), small (81 to 120 g), medium (121 to 150 g), large (151 to 200 g) and extra-large (> 200 g). Organoleptic properties were determined by chemical analysis of samples of five to six combined fruits per variety in each block (large plots) and per irrigation treatment in a variety (experimental units) (Figure 4). Statistical analyses included ANOVA and Tukey's tests for comparing treatments means.

In order to illustrate the relationship between crop yield and water status, crop water requirements during the two years of the study are presented. Rainfall RAIN and potential evapotranspiration ET_0 data displayed by decades were available by the Saouda meteorological station. To estimate the real evapotranspiration ETR (= Kc * ET_0), a Kc value for a cactus pear crop has been searched. Attempts to find reliable values of Kc for *Opuntia* in the region were not successful, despite consulting numerous sources, e.g., Allen et al. (1998), Lazzara & Rana (2010), Consoli et al. (2013). On the basis of various indications for perennial fruit ligneous plants in the Mediterranean-type environments, a value of 0.7 for Kc has been adopted. Furthermore, it had been assumed that the annual cycle of O*puntia* begins immediately at the end of fruit maturation, and thus, in this case, covered the period from 1 October to 30 September (i.e. 36 periods of ten days). In order to simplify, the initial water status (or residual hydrous state at the end of the previous cycle) was not taken into account and rainfall and irrigations during the annual cycle were fully taken into consideration (assuming no losses by run-off or by drainage).

3 RESULTS AND DISCUSSION

3.1. RESULTS

3.1.1. Effects of irrigation treatments on fruit yield and physical features in the first year experiments

Yields of the first year experiments (Table 2) revealed a significant interaction between varieties and irrigations; they indicate the positive effect of irrigation doses on fruit yields for 'Achefri' and no or negative effects on the yields of 'Aissa' and 'Moussa'.

Table 2: Fruit yields of the varieties Achefri, Aissa and Moussa under the treatments of irrigation T1: not irrigated, T2: 30 mm during flowering and 30 mm during fruit growth and T3: 30 mm only during fruit growth in the 1st year experiments (2011).

Variety	Fruit yield (kg plant^{-1})		
	T1 Not irrigated	T2 30 mm + 30 mm	T3 0 mm + 30 mm
Achefri	14.6±0.4	17.3±0.2**	15.6±0.2
Aissa	13.4±0.3	11.3±0.2*	10.7±0.4**
Moussa	12.4±0.4	9.6±0.3**	10.8±0.3*

interaction: significant * at $p \leq 0.05$; effect variety: ** ($p \leq 0.01$); effect irrigation: not significant.

Table 3: Fruit mass and sizes of Achefri, Aissa and Moussa under the treatments of irrigation T1: not irrigated, T2: 30 mm during flowering and 30 mm during fruit growth and T3: 30 mm only during fruit growth in the first year's experiment.

(a) Fruit mass and pulp mass

	Fruit mass (g)				Pulp mass (g)				Pulp/fruit (%)		
	T1	T2	T3		T1	T2	T3		T1	T2	T3
Achefri	112 ± 4a	122 ± 3a	124 ± 3.5a	ns	59.3 ± 2.3a	66.9 ± 3a	67.4 ± 2.3a	ns	53	55	54
Aissa	135 ± 2b	130 ± 2b	129 ± 2.3b	ns	72.6 ± 1.5a	68.3 ± 1.5a	69.6 ± 1.2a	ns	54	52	54
Moussa	122 ± 3a	131 ± 1.8a	130 ± 2.3a	ns	67.2 ± 1.3a	67.8 ± 1.9a	71.7 ± 1.5a	ns	55	52	55

(b) Fruit length and diameter

	Fruit length (cm)				Fruit diameter (cm)			
	T1	T2	T3		T1	T2	T3	
Achefri	7.17 ± 0.1a	7.40 ± 0.1b	7.53 ± 0.1bc	*	4.85 ± 0.06a	4.99 ± 0.06ab	5.08 ± 0.05b	*
Aissa	7.24 ± 0.1a	7.09 ± 0.04b	7.12 ± 0.05b	*	5.27 ± 0.04a	5.35 ± 0.04ab	5.23 ± 0.05a	*
Moussa	7.14 ± 0.1a	7.12 ± 0.01a	7.17 ± 0.02b	*	5.02 ± 0.1a	5.25 ± 0.07b	5.28 ± 0.07b	*

*: significant difference at $p \leq 0.05$; ns: no significant difference; interaction: a: not significant
For each variety and each variable, different letters a,b,c indicate significant differences

Table 4: Effect of irrigation treatments T1: not irrigated, T2: 30 mm during flowering and 30 mm during fruit growth and T3: 30 mm only during fruit growth on the number of fruits produced per plant and by 10 cladodes in 2011.

	(a) Number of fruits /plant (calculated on the basis of the ratio yield/mean fruit mass)		
	T1 (0 + 0)	T2 (30 + 30)	T3 (0 + 30)
Achefri	130.7±4.5	141.9±4.5	125.6±4.3
Aissa	99.3±4	86.9±4.3	82.6±4.3
Moussa	101.5±7	73.3±6	83.2±7.5
	(b) Number of fruits observed on 10 cladodes		
	T1 (0 + 0)	T2 (30 + 30)	T3 (0 + 30)
Achefri	75.4±11.5	117.0±12	94.9±12
Aissa	67.4±2.2	61.7±2	61.9±2
Moussa	59.7±3.5	70.7±3	65.3±2

Table 5: Effect of irrigation dose T1: not irrigated, T2: 60 mm during flowering and 60 mm during fruit growth and T3: 60 mm only during fruit growth on the fruit yield of the varieties Achefri, Aissa and Moussa in 2012.

Variety	Fruit yield (kg plant^{-1})			
	T1 Not irrigated	T2 60 mm + 60 mm	T3 0 mm + 60 mm	
Achefri	20.4 ± 0.8a	26.4 ± 0.7b	33.2 ± 0.7c	**
Aissa	17.7 ± 0.5a	23.5 ± 0.5b	27.4 ± 0.6c	**
Moussa	18.3 ± 0.4a	24.6 ± 0.3c	21.5 ± 0.3b	**
	**	**	**	

**: significant difference at $p \leq 0.01$
For a variety, different letters *a,b,c* indicate significant differences

Fruit and pulp mass and the content of fruit in pulp were not significantly different according to treatments of irrigation as well as fruit length and diameter (Table 3). All these features only differed according to variety, except for the pulp/fruit ratio, which was remarkably constant between 52-55%.

The numbers of fruits calculated (a) or observed (b) (Table 4) are highly correlated between them ($R^2 = 0.792$) and are also significantly correlated with the yields (resp. R^2(a) = 0.976 and R^2(b) = 0.836)

3.1.2. Effects of irrigation treatments on fruit yield and fruit quality in the second year's experiments

Statistical analysis of yields revealed a significant interaction between the factors variety and treatments of irrigation. Results (Table 5) must be considered separately for each variety. For 'Achefri', the application of both T2 and T3 irrigations caused a very significant increase in the yields by 6 and 13 kg plant^{-1} (or 30% and 65%), respectively. For the variety 'Aïssa', irrigations T2 and T3 increased the yields by nearly 6 and 10 kg plant^{-1} (or 30 % and 55 %) respectively. For 'Moussa', irrigations significantly increased the yield by 6.3 and 3.2 kg plant^{-1}.

On 'Aissa' and 'Achefri', irrigation schedule T3 with 60 mm applied at the start of the ripening stage has given higher yields than T2 with 120 mm (60 mm during flowering and 60 mm during fruit growth), indicating that_irrigation during flowering and fruit growth had less positive effect than irrigation at the start of ripening stage_ Conversely, for 'Moussa' irrigating during flowering and fruit growth had positive effect on fruit growth. Fruit and pulp mass (Table 6) very significantly increased in the irrigated treatments (with the exception of the 'Moussa' pulp mass). The irrigations also increased the fruit length and diameter and reduced the 'pulp/fruit' ratio for 'Achefri' (55 vs. 58 %) and for 'Moussa' (52 vs. 59 %). Yields and mean fruit mass were positively correlated ($R^2 = 0.802$).

Table 6: Effect of irrigation treatments (T1: not irrigated; T2: 60 mm during flowering and 60 mm during fruit growth; T3: 60 mm only during fruit growth) on fruit and pulp mass of the varieties 'Achefri', 'Aissa' and 'Moussa' in 2012.

Variety	Fruit mass (g)				Pulp mass (g)				Ratio Pulp/ Fruit (%)			Fruit length(cm)				Fruit diameter (cm)			
	T1	T2	T3		T1	T2	T3		T1	T2	T3	T1	T2	T3		T1	T2	T3	
Achefri	115	143	143	**	66	76	79	**	58	53	55	7.4	8.0	7.9	*	4.8	5.2	5.3	**
Aissa	122	141	142	**	67	72	79	**	55	51	56	7.3	7.7	7.8	*	5.0	5.3	5.2	*
Moussa	117	136	135	**	69	70	71	ns	59	52	52	7.5	7.8	7.7	*	5.0	5.1	5.2	ns

*: significant difference ($p \leq 0.05$); **: very significant difference ($p \leq 0.01$); ns: no significant difference.

Table 7: Effect of irrigation treatments T1: not irrigated, T2: 60 mm during flowering and 60 mm during fruit growth and T3: 60 mm only during fruit growth on the number of fruits produced per plant and by 10 cladodes in 2012.

Variety	(a) Number of fruits /plant (calculated on the basis of the ratio yield/mean fruit mass)		
	T1 0 mm + 0 mm	T2 60 mm + 60 mm	T3 0 mm + 60 mm
Achefri	178±12	184±16	232±18
Aissa	145±10	167±11	193±14
Moussa	157±3	180±8	159±6
Variety	(b) Number of fruits observed on 10 cladodes		
	T1 0 + 0	T2 60 + 60	T3 0 + 60
Achefri	76.0±6.0	82.0±8.0	99.5±6.0
Aissa	75.3±5.0	92.5±6.5	95.0±6.0
Moussa	75.0±5.0	87.5±4.0	91.8±4.5

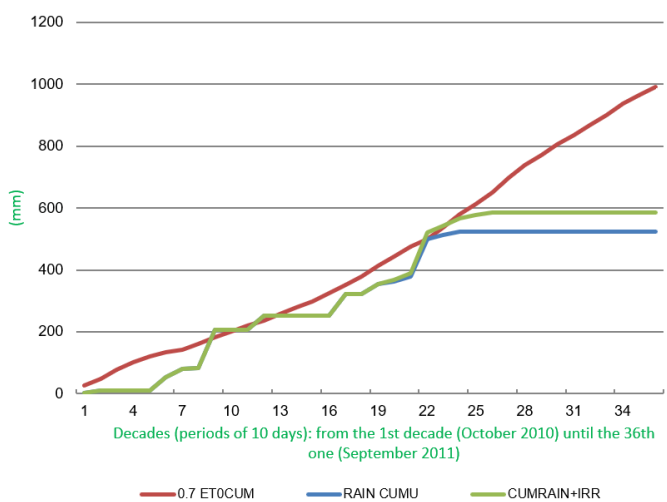

Figure 5: Hydric values (in mm) on the basis of Kc= 0.7 and taking into account rainfall and irrigation. Agadir area, 2010–2011 season. (RAIN CUMU = cumulative rainfall; CUMRAIN+IRR = cumulative rainfall + irrigation).

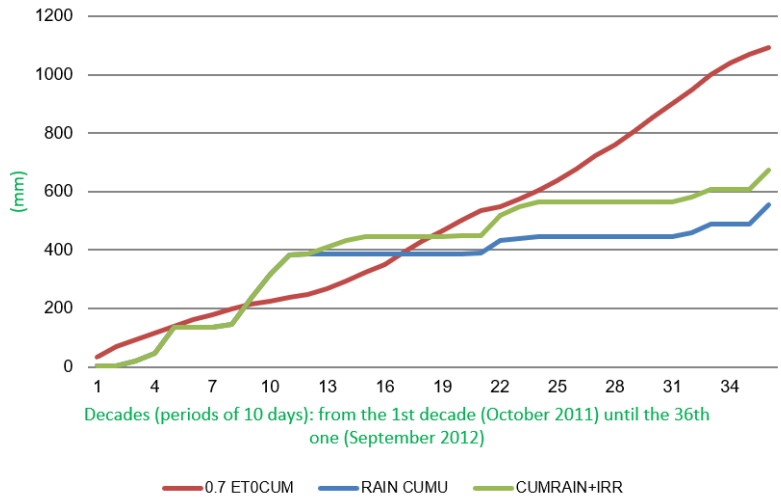

Figure 6: Hydric values (in mm) on the basis of Kc= 0.7 and taking in account rainfall and irrigation. Agadir area, 2011–2012 season. (RAIN CUMU = cumulative rainfall; CUMRAIN + IRR = cumulative rainfall + irrigation).

Table 8: Effect of irrigation treatments (T1: not irrigated; T2: 30 or 60 mm during flowering and 30 or 60 mm during fruit growth; T3: 30 or 60 mm only during fruit growth) on fruit organoleptic characteristics of the varieties 'Achefri', 'Aissa' and 'Moussa' for 2011 and 2012.

Organoleptic Compounds		'Achefri'			'Moussa'			'Aissa'		
		T1	T2	T3	T1	T2	T3	T1	T2	T3
The rate of juice (%)	2011	71.23	69.06	72.18	71.14	69.61	70.35	70.44	71.44	71.45
	2012	63.85	72.16	67.04	63.85	70.84	67.20	-	-	-
Total sugars (%)	2011	48.49	49.26	54.55	67.05	56.78	55.86	52.47	51.87	56.56
	2012	63.68	44.06	57.99	62.33	61.70	59.46	-	-	-
Titrable acidity (g^{-1})	2011	0.51	0.63	0.41	0.54	0.45	0.57	0.61	0.68	0.71
	2012	0.69	0.45	0.50	0.85	0.78	0.68	-	-	-
Dry matter of the juice (%)	2011	4.36	4.38	4.49	4.35	4.41	4.12	4.35	4.42	-
	2012	4.46	4.43	4.40	4.38	4.36	4.37	-	-	-
°Brix	2011	13.90	13.36	14.04	13.98	13.40	14.06	13.40	13.90	14.26
	2012	14.43	12.40	12.85	14.20	13.06	14.43	-	-	-
pH	2011	6.21	5.96	6.19	-	6.12	-	6.10	5.89	6.19
	2012	6.03	5.99	6.10	6.13	5.67	5.99	-	-	-

Although the number of fruits produced by 10 cladodes (observed) and per plant (computed) (Table 7) were not significantly correlated (R^2 = 0.409), the means indicate that the T3 treatment increased the number of fruits more than the T2 treatment at 'Aissa' and 'Achefri' varieties. Therefore, for 'Moussa' variety the number of fruits produced per plant was higher in T2 than in T3.Yield variations are well explained by the number of fruits per plant (coefficient of determination 89 %) and poorly by the numbers of fruits observed on ten cladodes (coefficient of determination 38 %), illustrating the difficulties of constituting representative samples.

3.1.3. Relations between the two seasons yield and climatic conditions

Referring to the climatic data (Figure 3), the results indicate a link between fruit yield and climatic conditions during the growing season, as well as with the supplemental irrigation timing. Figures 5 and 6 display the cumulated ETR (= 0.7 * ET_0), the cumulated rainfall and the cumulated rainfall + irrigation for the first year and second year experiments.

3.1.4. Effects of irrigation treatments on the fruit quality in the 1st year and 2nd year's experiments

In 2011, irrigation treatments did not significantly modify the qualitative components of the fruit and the juice. In the second year experiments, the amount of juice in fruit was higher in irrigated treatments; the content of total sugars and the titratable acidity of fruit were lower. Brix value and pH of fruit juice were not affected by the irrigation regime (Table 8).

3.2. DISCUSSION

Our results are globally consistent with those obtained by Van Der Merwe et al. (1997) in South Africa, and by Gugliuzza et al. (2002) and Mulas & D'hallewin (1997) in Italy. But they clearly illustrate the negative effects of irrigations applied when the rainfall is sufficient in relation to the variety's water demand. Zegbe and Sevin-Palestina (2020) who studied the effect of supplemental irrigation (SI) (application of irrigation to reach field capacity every time soil water content was close to or around permanent wilting point) and full irrigation (FI) (application of irrigation weekly to reach field capacity) on fruit yield and quality of four cactus pear varieties in Mexico, also reported that fruit yield and fruit size in terms of fruit diameter and fruit mass were higher in SI and FI irrigated plants than in not irrigated plants under rainfed conditions (NI). Fruit yield was similar in irrigated SI and FI plants during the first two growing seasons, but in the third growing season fruit yield was higher in SI plants than in FI plants. Supplemental irrigation has also enhanced fruit yield and quality of fruit trees, such as peaches and olives (Oweis & Hachum, 2012; Razouk et al., 2013).

Considering that the aim was not an extensive study of the hydrous relationships, and accepting the approximations, it can be observed that on these bases, the periods of significant risk of water stress without irrigation were as follows: (i) in the season 2010/2011, the period starting from the third decade of May (decade 23) to the end of the cycle; (ii) in the season 2011/2012, the period starting from the first decade of April (decade 17), whereas the period between the end of December and the end of March showed a positive water supply for the cactus. This clearly explains the differences between the irrigation efficiencies in the first season and in the second season of trials. As water for irrigation is scare in arid and semi-arid areas, it's important to explore the saving water strategies and supplemental irrigation during critical crop stages, such as the period of flowering and fruit growth, could be one such strategy (Oweis & Hachum, 2012; Zegbe & Sevin-Palestina, 2020). Supplemental irrigation is a feasible irrigation for cactus pear in areas where water availability is limited for agricultural activities. It saved water by 51-52% and reduced crop water use by 38-42%, in comparison to full irrigation (Zegbe & Sevin-Palestina, 2020).

The results regarding fruit quality were similar to those reported in Italy where an application of a micro spray irrigation of 60 mm in three applications (the first one week before flowering, the second two weeks after flowering and the third six weeks after flowering) on a 10-year old plantation of *O. ficus-indica* (L.) Mill. had a positive effect on fruit size and no significant effect on the organoleptic properties (°Brix, pH, malic acid) (Gugliuzza et al., 2002). In the same country, during three consecutive years, a drip irrigation of 566 m^3 ha^{-1} per year applied from May to September to adult plantations of four varieties of *Opuntia* increased yield by 40% to 50% for the first two years, and 130% in the third year (Mulas & D'hallewin, 1997). In South Africa, a drip irrigation of 12.5 mm per week applied during the dry period (from 15 July to 15 February) on five varieties of *O. ficus-indica* increased fruit yield from 9.2 to 10.7 kg $plant^{-1}$ (+16%) and fruit and pulp mass by 6% and 3%, respectively (Van Der Merwe et al., 1997). Zegbe and Sevin-Palestina (2020) also reported that supplemental irrigation plants maintained fruit yield, fruit mass and marketable fruit size at the level of full irrigation plants. The later authors indicated that supplemental irrigation could be a better irrigation strategy than full irrigation in areas where a drought period occurs during the fruit development period or during the whole growing season.

For the all varieties, fruits obtained in irrigated treatments have a medium calibre (121-150 g) (Chessa & Nieddu, 1997) and meet the South African commercial criteria for their pulp/fruit ratio which is more than 50% (De Wit et al., 2010). Despite no statistical significance, our results also illustrate that irrigation could possibly slightly affect the content of juice in the fruit, the content of sugar and the acidity of the juice.

4 CONCLUSIONS

From the results, it is obvious that the optimization of the irrigation in relation with yield and fruit physical properties (fruit mass and shape) must be considered for each individual variety and growth stage, and must take into account the climatic conditions notably the rainfall_ or better the evapotranspiration_cumulated from the start of a productive cycle. The varieties Aissa and Moussa yielded less when irrigated in the first season, when obviously the climatic water supply was sufficient. They have responded very positively to irrigation in the second and dryer season. The variety Achefri responded

positively in the first season and very positively in the second season, much better with one single irrigation in the fruit growing period than with two irrigations. This illustrates the limits of irrigation, and the disadvantage of over-irrigating. A simplified water status allows both delivering water as soon as needed and avoiding water excesses resulting in harmful effects to the crop. Considering the fruit chemical quality, all parameters measured did not vary in the function of the irrigation: irrigation did not decrease the quality. Another beneficial effect of irrigation is the intense increase of the shoot number emitted per cladode in the three varieties as reported in Arba et al. (2018).

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CAP post 2022 scenarios and income impacts – a case analysis for selected typical farms in Slovenia

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CAP post 2022 scenarios and income impacts – a case analysis for selected typical farms in Slovenia

Abstract: assessment based on representative farms is an established approach in the modern assessment of the effects of changes in agricultural policy. In line with previous CAP reforms, we can expect income redistribution impacts also with the implementation of the legislative and financial framework of the CAP for the next period. This paper discusses a scenario analysis using the farm model. The model is based on linear programming, which enables to address various technological challenges at farm level. We formed the scenarios for the analysis following the example of the scenarios contained in the impact assessment that the European Commission prepared for the CAP after 2020. The analysis involves selected farm types from selected sectors. The results suggest that the expected reduction in the envelope will generally lead to lower farm-level revenues from CAP direct payments. Consequently, economic performance will deteriorate, what is likely to be amplified in some sectors by the abolition of historical payments. The range of consequences at farm level will likely be considerable, especially for sectors and production types with a high share of CAP payments in the structure of total farm income. In certain sectors, however, there is even an improvement regarding the current situation.

Keywords: direct payments; CAP strategic plan; farm model; impact assessment

SKP po letu 2022 scenariji in vpliv na dohodek – analiza primerov izbranih tipičnih kmetijskih gospodarstev v Sloveniji

Izvleček: Ocena vpliva na podlagi reprezentativnih kmetijskih gospodarstev je uveljavljen pristop v sodobnih ocenah učinkov sprememb kmetijske politike. Glede na prejšnje reforme SKP, lahko pričakujemo vplive prerazporeditve dohodka tudi z izvajanjem zakonodajnega in finančnega okvira SKP za naslednje obdobje. V prispevku obravnavamo analizo vpliva z uporabo modela kmetijskih gospodarstev. Model temelji na konceptu linearnega programiranja, ki omogoča reševanje različnih tehnoloških izzivov na ravni kmetijskih gospodarstev. Scenarije za analizo smo oblikovali po zgledu scenarijev iz ocene učinka, ki jo je Evropska komisija pripravila za SKP po letu 2020. Analiza vključuje izbrana kmetijska gospodarstva različnih proizvodnih usmeritev. Rezultati kažejo, da bo pričakovano zmanjšanje sredstev na ravni kmetij na splošno povzročilo manjše prihodke iz naslova neposrednih plačil SKP. Posledično se bo ekonomska slika poslabšala, kar se bo v nekaterih sektorjih dodatno odrazilo zaradi ukinitve zgodovinskih plačil. Verjeten razpon posledic na ravni kmetijskih gospodarstev bo sicer precejšen, zlasti pri sektorjih in proizvodnjah z velikim deležem plačil SKP v strukturi celotnega dohodka kmetij. Pri določenih sektorjih pa se nakazuje celo izboljšanje trenutne situacije.

Ključne besede: neposredna plačila; SKP strateški načrt; model kmetijskih gospodarstev; analiza vpliva

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1 INTRODUCTION

A new round of changes to the Common Agricultural Policy (CAP) began in 2017 and will culminate in the changing of the fundamental regulations of the CAP. The direction and tone of these changes were outlined by the Communication from the European Commission, and set out more concretely in its legislative proposals (European Commission, 2018a). The most obvious changes are the shift toward a more result-oriented agricultural policy and a clear commitment to policy that is based on facts and an established intervention logic (Lovec et al., 2020), which is to be specified by the Member States' Strategic Plans. These plans will for the first time encompass all of the CAP's measures; each Member State will choose its agricultural policy priorities and, in accordance with common principles, also determine the type, allocated funds and scope of individual measures. It is evident that there is a targeted expansion of policy toward societal goals related to food production, natural resources and the countryside. The substantive areas and the framework of CAP measures remain largely unchanged, except for a clear intention to strengthen environmental measures (Šumrada et al., 2020).

EU Member States have different discourses on agricultural policy (Coleman, 1998; Daugbjerg & Swinbank, 2016; Alons, 2019; Erjavec & Erjavec, 2020), which can be classified into three basic groups, each with its own emphases and accompanying policy priorities and preferred measures: a production-oriented (mercantilist) discourse, a neoliberal discourse and an environmental discourse (multifunctionality). Using the same logic, it is possible to distinguish three fundamental approaches to agricultural policy and formulate different agricultural policy scenarios.

Impact assessment of different scenarios should be part of an evidence-based approach of agricultural policy and should help decision-makers in planning policies, including the EU's Common Agricultural Policy (Lee et al., 2006). Impact assessment needs to be linked to the various indicators that are relevant to the assessment and monitoring of agricultural policy. In line with the CAP concept, indicators pertain to all three areas of sustainability by assessing economic, environmental and social aspects (European Commission, 2020).

Among the economic aspects of the CAP, the issue of farm income retains its dominant role (Hill 2018). The amended agricultural policy measures can have a significant impact on incomes in agriculture at the aggregate level and at the level of individual farms. A particularly politically sensitive issue is the redistribution of payments between farms (Sinabell et al., 2013; Severini & Tantari, 2015), which can also significantly determine the choice of the direct payment scheme in each Member State. Analytical insight into the effects of different scenarios on the economics of farms is therefore an important issue for planning future agricultural policy measures, as well as for directing the further development of the entire industry.

In support of planning, farm models have been developing for almost two decades, gradually complementing the previously prevailing sectoral models based on partial and general equilibria (Van Tongeren et al., 2001; Langrell et al., 2013). Farm level modelling requires comprehensive data sources at farm level. This becomes a central issue for both EU policy makers and researchers. Langrell et al. (2013) mention (i) data availability and (ii) quality as the key challenges in using farm models for policy analysis at EU level. The effects of changed income conditions are then habitually monitored at the level of average farms that use accountancy (e.g. in the EU FADN system). However, the evaluation of results at the level of typical farms is increasingly used as well (Reidsma et al., 2018). These are generally either real or hypothetical farms that best represent the situation in a certain segment of an individual sector (representative farms) and allow for generalisation at the aggregate level.

The aim of this work is to examine the income issue and redistributive effects of different potential scenarios of the future CAP at the level of typical farms in Slovenia. Unlike the analytical tools developed in Slovenia so far, which primarily examined the effects of the CAP on the income position of Slovenian agriculture at the level of individual agricultural markets (Kavčič & Erjavec, 2003; Salomon et al., 2018) or the distributive effects of the changed direct payments policy on Slovenian agriculture (Rednak et al., 2005), this paper presents analyses based at the level of typical farms taken from (Rednak et al., 2009).

To this end, we upgraded the farm model, which allows to simulate economic indicators at farm level by means of Model-calculations[3] prepared by Agricultural institute of Slovenia (AIS, 2019). This approach builds on our previous work (Volk et al., 2017) and we aspired to test it and determine its applicability to scenario assessment of changes in agricultural policy.

In this paper, we thus try to evaluate gross margin changes in individual selected types of farms. Our premise was that the changed method of implementing CAP measures also affects the production decisions of farms, and thus indirectly also the economic indicators of their operations.

3 Static simulation models that enables simulating incomes and costs at the level of individual production activities.

The aim of our research was to explore:
- The possibilities of using model calculations as a basic data source for modelling with a farm model.
- The applicability of developed farm model to assess the effects of future CAP reforms.
- The income effects of the basic scenarios of future agricultural policy that emphasize the environmental, production or social aspects of agricultural policy.
- The redistributive effects of scenario changes for individual farm types.

In the continuation, we briefly present the approach used, starting with a short description of developed farm model and followed by presentation of the farms considered within the framework of this survey. We then briefly present the key assumptions of the reform scenarios, followed by presentation of the results and key findings.

2 MATERIAL AND METHODS

2.1 FARM MODEL

For the purpose of the research, we used the farm model that is based on Model calculations (MC), developed at the Agricultural Institute of Slovenia (AIS 2019). The farm model is based on a modular approach; independent modules have been developed for individual phases of analysis, which enable a comprehensive analysis of the farm production plan. The farm model is organised in the form of spreadsheets in MS Excel, with the majority of operations being automated using Visual Basic (VBA) macros, which enables relatively simple microeconomic analyses and adjustment of MC to the production activities of analysed farms.

The basic purpose of Model calculations is to monitor the costs and income situation in the production of individual agricultural product (farm activity). MCs are independent simulation models that, based on the defined (selected) initial technological parameters, enable the estimation of input consumption and thus of production costs for an individual agricultural product. The consumption of inputs depends on production technology, intensity (yield), the size of the plot or herd, the slope and in some places also some other technological parameters. MCs for an individual crop include all costs associated with production, which also allows for a direct comparison of total costs with total revenue and the calculation of various economic indicators.

The farm model is a complex tool that enables the adaptation of model calculations for each individual production activity at different levels to the analysed farm. It is based on mathematical programming with constrained optimisation. This enables the use of different operation research techniques in the automated preparation of production plans, which is also the starting point for impact assessment of the CAP reform at farm level.

In the version of the farm model that was used for the purpose of this research, the model is based on classical deterministic linear programming (LP). This is a single-criterion approach that assumes that, in light of changed circumstances, the decision-maker (i.e., farm manager) makes decisions mainly based on one main goal when defining a production plan, while taking into consideration all the production and technical constraints at the farm level. The matrix of production possibilities thus represents an example of finding (optimal) production plan, in which we focus on maximizing the objective function. In our case, this was expected gross margin, which was maximised while taking into consideration a set of constraints, operating under the assumption that it is, in addition to the farm's features, one of the more important goals influencing decision-making. Both the production constraints and the production activities that can enter optimal production plan, stem from the original farm model and are described in more detail in the report by Volk et al. (2017).

The key purpose of utilising farm model, however, is not to optimise the entire production plan, but mainly to reconstruct baseline production plan and balance it based on key information that we had for each farm. Among production activities these includes also nutrition balances and other flows of intermediate consumption at the farm level.

However, due to "normative" nature of LP solutions from an economic standpoint and to partially bypassed it and to get more "positive" solution (production plan) we used a partial optimization approach (Žgajnar & Kavčič, 2016), which is also based on LP. Namely, the characteristic of LP is that the solution can change quickly during optimization due to its relatively sensitive system of equations and basically normative approach, which can be problematic for an analysis such as this one. In the analysis of CAP reform measures, we are mainly interested in short-term changes, which do not entail a complete restructuring and reorientation of farms. We thus used this approach that enables the reconstruction of the (baseline) farm production plan in order to assess the current state of the farm and calculate various economic and physical indicators, assuming that the production plan is also technologically appropriate (e.g. nutrition balance, stock balance). Mathematically, this means a complex system of additional equations, which enables finding the values of variables that are unknown in such a

way that the farm production plan is complete and technologically consistent.

When we define all the given activities or at least the lower and upper limits, the described problems of reconstruction can be solved in a relatively simple way with partial optimization (equations 1 to 4). Partial optimization refers to the fact that we fix a certain part of the activities (x_f) and demand that the solver also includes them into the optimal solution (b_f).

$$maxEGM = \sum_{j=1}^{n} c_j x_j + \sum_{f=1}^{n} c_f x_f \quad \ldots (1)$$

so that

$$\sum_{j=1;f=1}^{n;r} a_{ij} x_j + a_{if} x_f \leq b_i \quad \text{for all } i = 1 \text{ to } m \quad \ldots (2)$$

$$x_f = b_f \quad \text{for all } f = 1 \text{ to } r \quad \ldots (3)$$

$$x_j \geq 0 \quad \text{for all } j \quad \ldots (4)$$

The basic idea is to estimate or calculate the missing data – variables (x_j) using a linear program while maximizing the expected gross margin (*maxEGM*). Whereas all activities (x_f) whose values (e.g. number of dairy cows, feeders, market crops, etc.) we know, we fix with additional restrictions (b_f). In our example objective function coefficients (c_j and c_f) represent expected gross margin (*EGM*) per each production activity in the model. It is calculated as the three-year average for variable costs and revenues for each variable, where probability for each year is the same (1/3).

2.2 TYPICAL FARMS

We conducted the research on income effects of various agricultural policy options beyond 2022 using a simulation of changes in expected revenues and expected variable costs (EGM) on 11 selected types of farms (typical farms), which were defined for Slovenia within the research conducted by Rednak et al. (2009). Typical farms differ in size, production orientation and production intensity, and are located in different areas (flatlands, different kinds of less favoured area – LFA) (Table 1). Individual types characterise interesting production types of farms for agricultural policy, but they are not totally representative for Slovenian farm structure as such. They are more or less specialized in individual production activities, but above all they are such that they can be visualised in Slovenian conditions.

In terms of price-cost ratios, the base year is 2017. However, since 2017 was extreme in weather and price conditions, we took into account the three-year average of prices for both production factors and products in or-

Table 1: Basic characteristics of typical farms (adapted from Rednak et al., 2009)

Production orientation	Description
(1) Arable farming	Smaller specialised crop farm (38 ha), good conditions, grains and potato in crop rotation; flat area.
(2) Viticulture	Specialized viticultural farm (5.2 ha), white grapes, own processing, sale of wine at an average price. Integrated production.
(3) Fruit farming	Apple production on a larger specialized farm (13 ha), large portion of plantation at peak productivity, intensive yield. Integrated production; LFA: hilly.
(4) Milk production (intensive)	Specialised farm (66 ha), Holstein cattle (63), highly intensive production, sale of calves and heifers, no fattening, significant proportion of grass in ration; flat area.
(5) Milk production (medium intensity)	Specialised farm (17 ha), Holstein cattle (22), medium intensive production, sale of calves and heifers, no fattening, significant proportion of grass in ration; flat area.
(6) Cattle farming (combined breeding)	Full-time farm (15.5 ha), Simmental cattle (16), intensive production, fattening offspring (10), fodder predominantly from grassland; LFA: hilly.
(7) Cattle farming (suckler cows)	Part-time farm (42 ha), Simmental cattle (38), extensive production, fattening offspring (28), fodder predominantly from grassland, additional purchase of a few calves; LFA: hilly.
(8) Cattle farming (fattening bulls)	Specialised (mixed) farm (29 ha), fattening bulls (70), ration based on corn silage, hay and purchased concentrated feed, purchase of 200-250 kg calves; flat area.
(9) Sheep farming (lamb breeding)	Extensive sheep farming on lower quality land (147 ha), Istrian pramenka sheep (235 breeding sheep); LFA: karst; gene bank.
(10) Pig farming (breeding sows + fattening)	Larger specialised pig breeding and crop farm (50 ha), breeding of piglets (45 breeding sows) and fattening (878 fattening pigs) at the same farm, ration based on home feed, with purchased protein components. An important part of grain sold, flat area.
(11) Pig farming (fattening)	Larger pig feedlot on the farm (1660), purchase of piglets, ration based on home feed (46 ha), surplus of grains sold, flat area.

der to avoid these ratios blurring a more realistic picture of an individual typical farm.

2.3 KEY ASSUMPTIONS OF SCENARIO ANALYSIS

We formed the scenarios for the analysis following the example of the scenarios contained in the impact assessment that the European Commission prepared for the requirements of the preparation of its legislative proposals for the CAP after 2020 (European Commission, 2018b). The individual scenarios define simplification, environmental orientation, and production and societal aspects in line with the possibilities offered by the European Commission's 2018 proposal for future measures and financial framework (European Commission, 2018a) and the 2018 European Commission's original proposal for the CAP budget (Matthews, 2020). The key assumptions of individual scenarios are presented in more detail in Table 2.

Scenarios were created by determining the choice of individual measures and the amount of funds for each measure, which also enabled the calculation of the value of payments per unit of area or animal. In the simulation analysis, we mainly included measures that have a direct income effect:

- Production-decoupled support of the first CAP pillar (various forms of basic payments, green component, support for areas with natural constraints, eco-scheme, redistribution payment, support for young farmers). It is important to point out that all scenarios anticipate the abolition of existing historical payments, which are part of the basic payment in the period 2014-2020.
- Production-coupled support for various purposes (grains, vegetable growing, beef feeders, milk in mountain areas, suckler cows, protein crops).
- LFA payments under the second pillar.

Other agricultural policy measures have not been modelled directly, but the envelope for the above-mentioned payments was reduced because of them. Thus, we have not modelled agri-environmental and climate measures, which can play an important role on an individual farm and may have substantial income impacts. The reason for this is that the implementation of environmental and climate payments for the period after 2022 has not yet been defined in Slovenia at the time of the analysis. We also did not model the effects of the introduction of risk management measures, nor any measures for young farmers, as the attribution of these measures on typical farms would be arbitrary.

All scenarios are based on the assumption of a reduced budget in real terms for direct payments (- 4 %) and reduced resources for rural development policy measures (Matthews, 2020).

3 RESULTS AND DISCUSSION

Results of scenario analysis for selected typical farms (Table 3) are presented individually. The effects of different scenarios on typical farms' economic results are illustrated by gross margins (GM), estimated total revenues (R) and budgetary payments (BP). For the base year (2017) and the currently valid CAP scheme, we present values in EUR and percentage changes for the other scenarios.

Arable farm (1) considered in the scenario analysis could be described as being of medium size in terms of socio-economic status (full-time farm) and production orientation; the market value of production (around EUR 68000) is improved by the production of potatoes with relatively higher revenues per unit of area. The analysed scenarios significantly change the current level of CAP income payments (around EUR 14000). A broadly set eco-scheme with a weaker environmental ambition (Scenario C_b) would result in an 11 % increase in the policy-related revenues, while a single payment per hectare or an eco-scheme with stricter environmental requirements would reduce policy-related revenues by a quarter. Redistributive approaches to the design of basic payments (scenarios D and E) would lead to a one-third reduction in budgetary payments on this farm. The share of budgetary support in total revenues thus fluctuates between around 10 and 20 %, which places the farm among those that are relatively sensitive to potential changes in the implementation of direct CAP payments (changes in GM fluctuate between an increase of 6 % (C_b) and a decrease by almost a fifth).

Budgetary support on the analysed viticultural farm (2) is relatively low (EUR 2200) in the baseline scenario, especially compared to the market revenues from wine sales. All scenarios reduce budgetary support, some by as much as two-thirds, but this does not have a large impact on the farm's revenues themselves (drop in revenues of 1-2 %). Changes in the form of agricultural support practically do not affect this farm. This farm's key challenges are related to the level and fluctuation of prices and to fluctuations in yields due to weather and climate conditions.

We see a similar picture in the case of the fruit-growing farm (3), where the base level of CAP income is around EUR 7000. The environmentally oriented scenario (C_b) leads to an increase in support here as well, while

Table 2: Presentation of scenarios for assessing the effects of agricultural policy changes beyond 2022

Scenario label	Short title	Explanation (choice of measures and shares for individual measures in the national envelope for direct support)
A - Baseline 2017	Baseline scenario under the conditions of 2017	Production-coupled support: 13 %; of this 5 % support for grains; 1.4 % for vegetable growing; 3 % for beef; 3.5 % for dairy farming in mountain areas; 0.1 % support for protein crops; Production-decoupled support: 53 % of envelope is basic payment with substantial historical payments for farms that had substantial production-coupled payments before 2006, mainly dairy, beef and arable crops; 30 % of envelope is green component; 2 % of envelope for young farmers; 2 % Areas facing natural or other specific constraints (ANCs); LFA under second pillar
B (single payment)	Simplified scheme with equal payments	No Production-coupled support; Production-decoupled support: 88 % is a basic payment scheme; 2 % of the envelope for young farmers; 0 % eco-schemes; LFA under second pillar and reduced due to given smaller size of envelope.
C_a (dark green)	Ultra-green model	No production- coupled support; Production-uncoupled support: 28 % is basic payment; 2 % of envelope for young farmers; 60 % eco-schemes; LFA under second pillar reduced given smaller size of envelope and raised by 2 %, as was baseline scenario A.
C_b (light green)	Green model medium Eco-scheme	Production-coupled support in the amount of 10 %; Of this 4 % for grains, 3 % for suckler cows, 0 % for beef, and 3 % for dairy in mountain areas. Production-decoupled support: 38 % is basic payment scheme (35 % in baseline); 2 % of envelope for young farmers; 25 % eco-schemes; Redistributive payment 15 % (for all surfaces up to 20 ha); LFA under second pillar and reduced due to given smaller size of envelope.
D (production-oriented)	Production model with 100 % difference given intensity	Production-coupled support in the amount of 5 %, of which 2 % for grains, 2 % for suckler cows, 0 % for beef and 1 % for support for dairy in mountain areas; Production-uncoupled support: 83 % is a basic payment scheme (amount of payment depends on intensity and is corrected by a coefficient): extensive grassland (<0.2 LU/ha) and medium-intensity grassland (0.2-0.5 LU/ha), meadow orchard, olive grove – baseline payment; Intensive grassland (>0.5 LU/ha) – 2x baseline payment; Fields – 2x baseline payment; permanent crops, hop fields, greenhouses and gardens – 3x baseline payment); 2 % of envelope for young farmers. LFA under second pillar reduced given smaller size of envelope.
E (socially oriented)	Social model with 25 % difference depending on amount of land in different categories (farm size) and LFA in second pillar	Production-coupled support in the amount of 10 %, of which 3 % for grains, 4 % for suckler cows, 0 % for beef and 3 % for dairy in mountain areas; Production-uncoupled support: 78 % a basic payment scheme (30 % derived from earlier funds intended for LFA), (amount of payment depends on size of arable lands and is corrected by a coefficient (factor in each category is 25 % of baseline payment); 0-5 ha – 4x baseline payment; 5-10 ha – 3x baseline payment; 10-20 ha – 2x; 20-50 ha – 1x baseline payment; Surfaces larger than 50 ha basic payment; 2 % of envelope for young farmers. LFA under second pillar and reduced due to given smaller size of envelope.

*LU = Livestock units

Table 3: Economic indicators for selected typical farms under the envisaged CAP reform scenarios

		SCENARIOS (Baseline = 100)					
		A (Baseline) 2017	B (single payment)	C_a (dark green)	C_b (light green)	D (production-oriented/land types)	E (socially oriented/ farm size)
Type of farming		EUR	% of baseline				
(1) Arable farm	BP	14014	74	74	111	82	66
	R	88571	96	96	102	86	86
	GM	24977	86	86	106	90	81
(2) Viticulture	BP	2236	97	99	80	38	38
	R	81452	100	100	99	98	98
	GM	54115	100	100	99	97	97
(3) Fruit (apple) growing	BP	6681	84	85	115	35	35
	R	217467	99	100	100	98	98
	GM	116214	99	99	101	96	96
(4) Milk production (intensive)	BP	29916	51	51	45	60	55
	R	208748	93	93	92	87	89
	GM	127666	88	88	88	91	90
(5) Milk production (medium intensity)	BP	9072	51	51	60	61	68
	R	69600	94	94	95	88	90
	GM	44124	90	90	92	92	94
(6) Cattle breeding (combined breeding)	BP	7852	55	55	60	64	70
	R	55530	94	94	94	88	90
	GM	32112	89	89	91	92	93
(7) Cattle breeding (suckler cows)	BP	18003	80	82	62	91	77
	R	85045	96	96	92	85	86
	GM	33918	89	90	80	95	88
(8) Cattle breeding (bull fattening)	BP	15797	49	49	74	54	45
	R	112100	92	92	96	87	87
	GM	24699	68	68	83	71	65
(9) Sheep farming (lamb breeding)	BP	35971	152	156	201	34	95
	R	58888	132	134	162	60	60
	GM	30289	147	151	205	23	94
(10) Pig farming (breeding sows + fattening)	BP	18767	70	70	110	79	63
	R	185400	97	97	101	91	91
	GM	38931	86	86	105	90	82
(11) Pig farming (fattening)	BP	16005	77	77	47	84	65
	R	284983	99	99	97	95	95
	GM	58969	94	94	86	96	90

Legend: BP – budgetary payments, R – revenue, GM – gross margin

single payments or a more environmentally demanding eco-scheme would lead to a reduction of around 15 %. Scenarios D and E importantly reduce support, which can, in extreme cases, even fall down to a third of current funds. The impact of revenue changes in agricultural policy measures is low (a 2 % reduction at most). For this type of farms, the focus of agricultural policy should also be shifted from direct payments to establishing a more effective system of risk management measures.

On the type of farm engaged in intensive milk production (4), CAP income payments would currently amount to EUR 30000 and are strongly based on historical rights. This represents about 15 % of revenues and a quarter of GM. A farm with GM of EUR 127000 seems an attractive option, and if the farm is not over-indebted, it enables good management. Notwithstanding the reliance of this farm's performance from the assumptions about its indebtedness, it allows for a discussion on the fundamental issues of intensive dairy cow breeding. Budgetary payments for this farm will be significantly reduced after 2022 – by at least 40 to 55 % regardless of the scenario, mainly as a result of the projected loss of historical rights. Scenarios that assign higher values to arable land (scenario D) turn out somewhat better, and those that favour smaller farms (scenario E) turn out worse, but these differences are not very large. Regardless of these losses in budgetary payments (around EUR 15000), such changes alone may not pose a direct threat to the existence of such farms (in all scenarios, they would still reach around 90 % of baseline GM). The share of budgetary payments is relatively low, so the success of such breeding is not solely due to budgetary support, although the reduction of payments and the consequent reduction of the GM by more than 10 % will present a significant challenge. However, we believe that the key challenge of such farms is in the optimization of technology, prudent investment management, as well as economies of scale.

The second type of specialised milk farm (5) illustrates the potential effects for smaller, medium-intensity dairy farms. In the farm's revenue, which is estimated at around EUR 70000, the CAP payments in question contribute around EUR 9000. The gross margin is around EUR 44000, so the economic situation of such a farm is highly dependent on cost management and the purchase price of milk. The conclusions of the scenario analysis are similar to the previous case (larger specialized dairy farm). This means a reduction in direct payments by approximately half due to the loss of historical payments; there are no major differences between the scenarios (the 'socially oriented' scenario E, which favours smaller farms, would be the most favourable for this farm). The decrease in GM ranges up to 10 % in all scenarios.

The scenario analysis also reveals similar effects on the economic performance for the farm that combines dairy production and beef (6). The estimated baseline revenue of the farm is about EUR 56000, of which about EUR 8000 is from CAP payments. GM is estimated at EUR 32000 per year. Budgetary support represents 14 % of revenue and 24 % of gross margin. All scenarios significantly reduce budgetary support by 30 to 45 %. Here, too, this is mainly the result of the loss of historical support. On this farm, changes in budgetary payments lead to approximately the same changes as on other cattle farms with milk production, which is again mainly the result of the loss of historical part of the payments. The farm has long-term problems with accumulating funds for investment, and the value of labour inputs relative to gross margin depends substantially on the level of purchase prices.

On farms with a more extensive production orientation, such as the farm focused on rearing suckler cows and fattening offspring (7), the share of budgetary payments in the structure of total revenues (around EUR 85000) is higher, in this case around one fifth, or more than half of the created margin. All of the envisaged scenarios reduce the amount of support due to a general reduction in payments and the loss of historical bonuses. These losses are largest in the 'social' scenario E. On the other hand, the production-oriented scenario (D), with higher differences between extensive and intensive land use, approaches the current payment levels the most. The results of the scenario analysis predict a deterioration in the economic situation of the farm up to a 15 % reduction in created gross margin. Dependence on support is significant, so this type of farm should think strategically about future production or market orientations that would lead to a significant increase of revenue (e.g. entering quality schemes, as well as increasing farm size and/or production volume).

Among the analysed types of farms, the largest expected changes in the model of implementing CAP direct payments are found on the farm that is specialised in fattening (mainly purchased) bulls (8). Estimated revenue is about EUR 112000, of which 14 % is budgetary payments. Due to high variable costs of such production laying on purchase of calves, gross margin is low (EUR 25000) and is greatly determined by CAP payments (64 %). These are largely the result of historical support, which the reform is abolishing. This is also reflected in the dramatic reduction in budgetary support for this type of farm by up to 65 % (scenario E). This type of farm and its variants are therefore quite endangered. The question of gradually abolishing historical rights is more than appropriate. Strategic consideration is necessary not only at the level of the individual farm, but also at the level of the entire production orientation.

There are several alternatives in the proposal for the future CAP payment regime that allow high payments for production orientations aimed at extensive animal husbandry on grassland. A typical example of such an orientation is the analysed sheep farm (9), whose estimated revenues in the initial situation amount to EUR 59000, 36000 of which (61 %) stem from budgetary payments. In the market area, it even operates at a loss, as direct support exceeds gross margin by 18 %. This is the type of farm where the biggest differences between scenarios occur. The simplified scheme (B) increases revenues by 50 % and the environmentally oriented eco-scheme of scenario C_b increases them by as much as 100 %. However, a different level of payments for different land uses, which allocates smaller amounts to extensive grassland, and the socially oriented scenario E, which gives more to smaller farms, can dramatically reduce payments, by as much as 80 %. Due to the high level of dependence on subsidies, the gross margin and thus the viability of this farming model is highly dependent on the choice of policy model.

We also included two pig farms in the scenario analysis. In the first case (10) we are dealing with a crop-growing farm that supplements its income with breeding of home-bred piglets at a small scale. The revenues of this type of farm are estimated at EUR 185000, of which EUR 19000 are area-based direct payments (10 %). The farm would reach EUR 39000 of GM, with direct payments amounting to almost half of that value. As in the case of larger arable farms, policy change is accompanied by the same effect of the predicted scenario change. Most support is decreasing, which is especially evident in the socially-oriented scenario that supports smaller farms (E), while including a farm in an eco-scheme with a weaker environmental ambition (C_b) would lead to a 10 % increase in revenues. Budgetary support is an important, but not a key factor in maintaining and developing such farms. The key lies in the level and stability of purchase prices, as well as in greater technological efficiency, but especially in economies of scale and improved breeding productivity parameters.

The second type of pig-rearing farm (11) is marked by high market sales and consequently has a high yearly revenue of EUR 285000. Budgetary payments contribute 6 % to this, and represent a quarter in the structure of GM. Of course, this is a type of farm where the economic situation can change dramatically very quickly with changes in the prices of piglets and fatteners. All the scenarios in question bring this farm a reduction in support; from 16 % in the scenario rewarding intensive land use (D), to a reduction of more than 50 % (due to the size of the farm) in scenario E. Despite the not-insignificant revenue consequences of CAP income payments, we allow ourselves the conclusion that the long-term resilience of this type of farm depends primarily on price ratios in pig farming, as well as an uninterrupted supply of weaners.

4 CONCLUSIONS

We conducted the research on the income effects of the various agricultural policy options beyond 2022 by simulating changes in revenues and variable costs (gross margin) on selected 11 typical farms. These are types of farms that are above-average in size, volume of production, and most also in production intensity. They are located in different types of areas (plains, various less favoured areas). Following the example of the Impact Assessment that was carried out for the same purpose for the European Commission (European Commission, 2018b), we developed a set of scenarios that define simplification, environmental orientation, production and social aspects in line with the possibilities offered by the European Commission's proposal for future measures (European Commission, 2018a) and the 2018 CAP budget (Matthews, 2020).

4.1 ANALYSIS OF INCOME EFFECTS BY TYPES OF (SPECIALISED) FARMS

Future changes will lead to a reduction in budgetary support in most of the farm types and scenarios considered. Farms with predominant extensive animal husbandry on grassland are an exception if a sufficiently funded income based eco-scheme is introduced. The reductions are mainly the result of a reduction in the 2018 envisaged total amount of available funds, in certain types also of the abolition of historical payments. Cattle farms are a story into themselves, where a larger drop in CAP direct payments is expected due to the loss of historical support, making this decrease an important, perhaps even key change for cattle-breeding types of farms. Namely, the current (2015-2020) programming period was marked by an only gradual adjustment of direct payments and therefore an important part of the historical element was still present in 2017. Scenarios for changes in agricultural policy assume an immediate transition to equal area-based payments, depending on the type of measure, but they no longer include top-ups for individual farms.

The magnitude of the effects of agricultural policy on farms' gross margins is most visible in those sectors and types of Slovenian agriculture where budgetary transfers represent the largest revenue item. This definitely includes beef fattening as well as small ruminant

production. The situation in these two cases is different, as the abolition of historical rights affects cattle fattening the most. In other types there are reductions, but changes in gross margins can still be managed with appropriate strategic changes on holdings. Changes in agricultural policy must therefore be viewed mainly in light of changes in GM, and not so much in terms of what share of funds an industry or farm type may lose (in absolute or relative terms). Fruit growing, viticulture, pig farming, and to a large extent also milk production, can overcome changes more easily than cattle fattening, for example.

An important message of the results of the scenario analysis is that, in the discourse on strategic planning of agricultural policy for the future, more attention needs to be paid than before to those farm resiliency strengthening measures that bring qualitative progress in terms of higher and more stable market revenues. Improvements in technology are related to this, as are improvements in farm management and marketing, and not only in primary agricultural production, but along entire value chains. Improvements are also needed in risk management. The proposal for the future CAP predicts the possibility of allocating part of the funds of the first pillar (up to 10 %) for the upgrade of the risk management system. Sectors that are particularly exposed to production risks (e.g. viticulture, fruit growing, crop-growing) would benefit the most from a more ambitious agricultural policy approach in setting up a risk management system. However, here we must take into account that the introduction of risk management support will bring an additional redistribution of money between sectors or farm types, which would not significantly impact the economic situation of most of the types of farms considered.

4.2 SCENARIO ANALYSIS OF INCOME EFFECTS

The scenario with a simplified payment scheme (B) provides virtually all of the funds of the first pillar in the form of a single payment. The results on the economics of production are neither particularly bad nor good. Because the sum is relatively large, it supports extensive producers the most, but it also brings some stability for intensive producers, regardless of orientation. In terms of agricultural policy, it could be understood as some sort of support for all producers, who then have to decide what they will produce. The model of agricultural policy support would certainly be simplified.

Both 'green' scenarios (C_a and C_b) are based on different types of eco-schemes, depending on how many resources and for which areas they would be allocated. The conditions tied to these measures were not explicitly modelled, as all conditioning is also associated with certain costs, which we could not predict at this stage of drafting the new policy. Therefore, these particular results need to be taken with a grain of salt. In some types of farms (crop-growing and pig farms, to a lesser extent also milk production) eco-schemes have proved to be a possible way of compensating for the loss of budgetary support. This is logical, because we can direct additional funds to certain types of holdings with them, but of course they must also yield certain environmental results. We must therefore be careful in interpreting the results of this analysis, which has, however, certainly indicated that the combination of the basic payment and the appropriate choice of eco-scheme can give sound economic results.

The production scenario (D) is based on different levels of payments for different types of land use (extensive uses would receive less, whereas arable land and permanent crops, for example, would receive more). Based on the model results, we are not convinced by this scenario. Extensive grassland was used in the breeding of small ruminants, and there the effect was seen (correction of "high" public support for this category of use). For others, it has brought slightly smaller losses in budgetary support, but these are not very pronounced. For some types of farms, the effect is offset because they have diversified land-use; otherwise the effect depends on the degree of differentiation of payment for more extensive grassland. However, the administrative complexity of introducing and implementing such a payment system should not be underestimated.

The social scenario (E) is based on different levels of payments for different sizes of holdings, as well as on production-coupled payments, thus supporting smaller holdings. In this scenario, too, the seekers of solutions to all issues within the proposed agricultural policy model find themselves in a trap. Small farms cannot be helped merely with per-hectare payments. No matter how much we try to increase these amounts, the total yield is too poor for this way of farming to be maintained in the long run. Smaller farms could only be helped by an income payment, which is a payment per person working on such farm. This would lead to a radical change, which the CAP does not envisage at the moment, and the funds for such plans would also run out very quickly.

4.3 MODEL AND APPROACH

Our scenario analysis also served as a test of the method and applied approach. It turns out to be useful, however applied approach needs to be strengthened in the future by choosing a selection of farms that is wider and more adapted to realistic conditions, which will

show the full diversity of conditions and variety of potential effects. The farm model and its basis also need to be adapted. However, model calculations that are based on the detailed breakdown of the production function did turn out to be a good substitute for bookkeeping data. Due to the weakness of the FADN database in Slovenia, which stems from the facts that it does not record detailed sectoral costs, is practically not used and does not have sufficiently developed control assessments that would raise quality, the chosen approach is also the only feasible one. Such an approach can also be an alternative for other countries with poorer quality of bookkeeping data and ought to be tested, but establishing such a system does require a great deal of effort and investments.

Regardless of the narrow selection of typical farms and the limitations of the methodological tool, we estimate that the presented approach enables the implementation of agri-policy relevant analyses and evaluations. The scenario analysis of future changes in agricultural policy provides a broader insight into the extent of changes and the state of the economic situation of individual types and production orientations of farms and thus of agriculture as a whole. The assessment opens up new insights and provides an opportunity for in-depth discussion, which, considering the new, more strategic and goal-oriented manner of defining measures, could be important for its future development. The biggest advantage of this approach is that we assess the redistributive effects of different direct payment schemes in light of the total revenues and gross margins, where it is clear that not all sectors and types within them are equally economically dependent on support.

A specific limitation of the proposed approach is that the microeconomic analysis, by not including fixed costs, does not take into account the overall financial and operating situation of a farm, which can have a significant impact on its economic situation. If a farm is indebted, its position is significantly more fragile and budgetary support is an important part of its liquidity and solvency. The inability to cover at least the bulk of amortisation most often occurs with larger and more technologically demanding types of farms and can be a significant obstacle to a farm's operation. In the future, it will be necessary to take this into account and also provide for an analysis of total costs, taking into account the different levels of indebtedness of farms.

We did not include agri-environmental and climate measures and measures to support organic farming in the analysis. For some of the types of farms considered, this is an important source of income, sometimes even more than direct payments. We did not consider them because future environmental measures are not defined, but if we simply continued with the same ones as before, this could yield a wrong picture, while leaving their actual impact un-analysed.

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Antioxidant defense and secondary metabolites concentration in hyssop (*Hyssopus officinalis* L.) plants as affected by salt stress

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Antioxidant defense and secondary metabolites concentration in hyssop (*Hyssopus officinalis* L.) plants as affected by salt stress

Abstract: Salt stress is one of the major limiting factors for plant production, and the quality of medicinal plants is also affected by soil salinity. Hyssop (*Hyssopus officinalis* L.) plants were cultivated for four weeks in perlite: sand and irrigated with Hoagland nutrient solution containing 0, 50, 100, 150, and 200 mM NaCl. Plants growth was decreased by salt stress while the leaf relative water content was not affected, and the chlorophyll content decreased only by the highest salt concentration (200 mM). Sodium was accumulated at small amounts, indicating a high ability of this species to exclude salt. Soluble sugars and proline were accumulated up to 1.6 and 4.5 fold, respectively. The antioxidant enzymes activity (peroxidase, catalase, ascorbate peroxidase) were increased by the salt treatments, particularly in the leaves. The levels of secondary metabolites (saponins, phenolics, flavonoids, anthocyanins, and iridoids) were all increased under salt stress, and the total antioxidant capacity of alcoholic extract of the leaves and roots was significantly higher in the salt-treated compared with control plants. Our results showed that hyssop is a salt-tolerant species, and the quality of this medicinal plant is improved when grown under saline conditions.

Key words: salinity; hyssop; *Hyssopus officinalis*; secondary metabolites; antioxidant enzymes

Antioksidativna obramba in vsebnost sekundarnih metabolitov v navadnem ožepku (*Hyssopus officinalis* L.) v razmerah solnega stresa

Izvleček: Solni stres je eden izmed dejavnikov, ki najbolj omejuje rast rastlin, v razmerah zasoljenih tal je prizadeta tudi kakovost zdravilnih rastlin. Navadni ožepek (*Hyssopus officinalis* L.) je bil gojen v mešanici perlita in peska in zalivan s Hoaglandovo hranilno raztopino, ki je vsebovala 0, 50, 100, 150, and 200 mM NaCl. Rast rastlin se je s solnim stresom zmanjšala, a relativna vsebnost vode v listih ni bila prizadeta in vsebnost klorofila se je zmanjšala le pri največji koncentraciji (200 mM NaCl). Natrij se je v rastlinah kopičil v majhnih količinah, kar nakazuje sposobnost te vrste, da izloča sol. Vsebnost topnih sladkorjev in prolina se je povečala za 1,6 ,oziroma 4,5 krat. Aktivnost antioksidacijskih encimov (peroksidaze, katalaze, askorbat peroksidaze) se je povečala po obravnavanjih s soljo, še posebej v listih. V razmerah solnega stresa se je povečala raven sekundarnih metabolitov (saponinov, fenolov, flavonoidov, antocianinov in iridoidov), celokupna antioksidacijska sposobnost alkoholnega ekstrakta listov in korenin je bila značilno večja pri rastlinah izpostavljenih soli kot pri kontroli. Rezultati so pokazali, da je navadni ožepek na sol strpna rastlina in, da se kakovost te zdravilne rastline izboljša, če jo gojimo v razmerah slanosti.

Ključne besede: slanost; navadni ožepek; *Hyssopus officinalis*; sekundarni metaboliti; antioksidacijski encimi

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1 INTRODUCTION

Soil salinity is one of the main abiotic stress factors threatening agricultural production worldwide. Salt in the soil is also considered the main factor limiting the dispersal of plants in their natural habitats (Acosta-Motos et al., 2017; Mushtaq et al., 2020). Salinity causes both osmotic and ionic stresses and affects all the plant major processes such as germination, photosynthesis, growth, water and nutrients balances and yield (Parida & Das, 2005; Parihar et al., 2015).

Salt stress affects the plant's primary metabolism and alters primary metabolites' concentrations, including soluble sugars and amino acids (Gupta and Huang, 2014). Among amino acids, proline plays a pivotal role in the plant's adaptation to salt stress by protecting cells from damages caused by excess accumulation of ions and salt-induced dehydration (Verbruggen and Hermans, 2008). As the second important group of compatible solutes, soluble sugars protect from dehydration and help sustain the structural integrity of plant cells under salt stress (Rosa et al., 2009).

Under environmental stress conditions such as salinity, the generation of higher reactive oxygen species (ROS) causes oxidative stress. It results in membrane damage characterized by elevated levels of malondialdehyde (MDA). Plants employ defensive systems for scavenging ROS and protecting from damaging oxidative reactions through different antioxidant enzymes such as peroxidases (POD), catalase (CAT), and ascorbate peroxidase (APX) (Foyer et al., 1994; Gupta and Huang, 2014; Akyol et al., 2020). Proline protects plant cells from ionic and osmotic stresses and contributes to the scavenging ROS such as hydroxyl radicals (Verbruggen and Hermans, 2008).

In addition to primary metabolism, secondary plant metabolism is also influenced by salt stress (Ahmad and Sharma, 2008). The concentration of secondary metabolites highly depends on plants' growth stage, especially environmental conditions, including light intensity and stress factors such as salt (Ahl and Omer, 2011). Salt stress has a positive or negative impact on secondary metabolites' biosynthesis depending on plant species or the severity of stress (Verma and Shukla, 2015). Salt stress led to about 8–35 % increase in total phenolics and about 35 % increase in total flavonoid content in *Portulaca oleracea* L. (Alam et al., 2015). An enhancement of antioxidant activity and flavonoid and phenolics contents has also been observed in *Cichorium spinosum* L. under salt stress (Petropoulos et al., 2017).

In the members of Lamiaceae, salinity may cause substantial changes in the compositions and yield of secondary metabolites (Taarit et al., 2009). Salt stress led to about 20-40 % increase in the total phenolics and flavonoids content in *Thymus* species (Zrig et al., 2016). Salt stress significantly induced the biosynthesis of some crucial essential oil and phenolic compounds in *Salvia mirzayanii* Rech.f. & Esfand. (Valifard et al., 2014). Considering the medicinal application of most plant species from Lamiaceae, it is important to know the effect of salt stress on the quality and quantity of secondary metabolites. The content of secondary metabolites is also a determining factor for the total antioxidant activity of plant extract defined either by FRAP (Ferric Reducing Ability of Plasma) or through DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay (Fukumoto and Mazza, 2000; Sethi et al., 2020).

Hyssop (*Hyssopus officinalis* L.) is a perennial subshrub belonging to the Lamiaceae family and distributes in the eastern Mediterranean to central Asia (Fathiazad and Hamedeyazdan, 2011). Hyssop is a popular medicinal herb with carminative, tonic, antiseptic, and expectorant properties and is used to remedy congestion, lung complaints, and cardiovascular disorders (Hristova et al., 2015). Extracted essential oils from the shoots of hyssop plants possess a unique aroma and are widely used in the food, pharmaceutical, and cosmetics industries (Kazazi et al., 2007). Different polyphenolic compounds identified in this species contain different glycones and aglycones such as flavonoids, quercetin, apigenin, diosmin, luteolin, and other phenolic compounds such as chlorogenic, ferulic, protocatechuic, syringic, caffeic, and p-hydroxybenzoic acids (Fathiazad and Hamedeyazdan, 2011).

Hyssop is a xerophyte species and is well adapted to drought conditions (Khazaie e al., 2008). Effect of salt stress on the activity of antioxidant enzymes has been investigated in this species (Jahantigh et al., 2016). There is no report, however, on the secondary metabolites levels under salt stress in hyssop plants. This work aimed to investigate the effect of salinity on growth, ROS scavenging activity, the content of various secondary metabolites, and the antioxidant capacity of the leaf and root extract in this species.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND TREATMENTS

Seeds of hyssop (*H. officinalis*) were purchased from

Pakan-Bazr Company (Isfahan, Iran). The seeds were surface-sterilized using 1 % sodium hypochlorite then were sown in the 2 L pots (15 seeds in each pot) containing sterilized perlite: sand (1:3) mixture and placed at 4 °C for stratification. After 4 days, the pots were transferred to the greenhouse conditions at 25/22 °C day/night temperature regimes, at a 16/8 h day/night cycle, and relative humidity of 60 %. After germination, plants were irrigated with 20 % Hoagland solution once a week and with 100 ml distilled water twice a week throughout the experiment.

Salt stress was imposed at a four-leaf stage with four NaCl concentrations (50, 100, 150, and 200 mM) applied with irrigation water to the pots gradually within one week. Plants were grown for four weeks after starting salt stress and then were harvested. At harvest, fresh mass (FM) of shoot and roots were determined, and subsamples were taken and immediately frozen in liquid nitrogen and then stored at –80 °C until analysis. Another group of samples was oven-dried and, after determination of dry mass (DM), were used for the analysis of secondary metabolites and elemental concentrations.

2.2 BIOCHEMICAL MEASUREMENTS

Soluble carbohydrate content was determined according to the phenol-sulfuric acid method (Dubois et al., 1956). Proline was quantified according to the methods of Bates et al. (1973), and the content of soluble proteins was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard.

To determine the leaf content of chlorophyll (Chl) and carotenoids, 0.2 g of fresh leaf samples were homogenized with 2 ml of 80 % acetone and centrifuged at 4000 *g* for 10 min. After that, the samples' absorbance was recorded by a spectrophotometer (Bausch & Lomb 70) at 663, 645, and 480 nm, and the contents of pigments were calculated according to the following equations where A corresponds to the absorbance (Flores-de-Santiago et al., 2016):

$$\text{Chl a} = 12.21A_{663} - 2.81\ A_{646}$$
$$\text{Chl b} = 20.13A_{646} - 5.03A_{663}$$
$$\text{Carotenoids} = [1000A_{470} - 3.27\ (\text{Chl a}) - 104\ (\text{Chl } b)] / 229$$

The leaf relative water content (RWC, %) was measured according to the following equation:

$$RWC\ (\%) = [(FM - DM) / (TM - DM)] \times 100$$

For determination of turgid mass (TM), leaf disks (5 mm diameter) were submerged for 5 h in distilled water, thereafter, they were blotted dry gently on a paper towel and weighed.

2.3 OXIDATIVE STRESS MARKERS AND ACTIVITIES OF ANTIOXIDANT ENZYMES

Sergiev et al. (1997) method with slight modification was used for quantification of H_2O_2 content. 100 mg of samples were ground in liquid nitrogen, extracted with trichloroacetic acid (TCA) in an ice bath, centrifuged at 13,000 *g* for 15 min. The 500 µl of supernatant was added to potassium phosphate buffer (pH 7.0), and the H_2O_2 content was determined based on supernatant absorbance at 390 nm. Malondialdehyde (MDA) content was evaluated by the method of Heath and Packer (1968). 100 mg of samples were homogenized in 1 ml 0.1 % (v/v) TCA and centrifuged at 12,000 *g* for 10 min. The extracted supernatant (0.1 ml) was mixed with 20 % TCA containing 0.5 % (w/v) thiobarbituric acid (TBA). The mixture was incubated at 100 °C for 10 min then centrifuged at 10,000 *g* for 15 min. TBA reactive substances' content was calculated based on the difference in absorbance at 532 and 600 nm using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

For assay of catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), 100 mg of the samples was extracted in 5 ml of 100 mM phosphate buffer. The homogenate was then centrifuged for 10 min and used for enzyme assays. CAT activity was analyzed by Beers and Sizer (1952) method. The 2 ml of reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0) was mixed with 400 ml of 6 % H_2O_2 and 100 µl of enzyme extract. CAT activity was calculated to reduce the H_2O_2 absorption at a wavelength of 240 nm using an extinction coefficient of 0.036 $mM^{-1}\ cm^{-1}$. POD activity was measured by the method of Lin and Kao (1999). The reaction mixture (2 ml) contained potassium phosphate buffer (50 mM, pH 7), guaiacol solution (9 mM), H_2O_2 (19 mM) and root or leaf extract (100 µl). POD activity calculated the absorbance changes at 470 nm using the extinction coefficient of 26.6 $mM^{-1}\ cm^{-1}$. For assay of APX, the reaction mixture contained 250 mM phosphate buffer (pH 7), 1.2 mM H_2O_2, 0.5 mM ascorbic acid, and 0.1 mM EDTA. The reaction was started by adding H_2O_2 to the mixture. Total APX activity was calculated to reduce the absorbance at 290 nm for 2 min using the extinction coefficient of ascorbic acid (2.8 $mM^{-1}\ cm^{-1}$) (Dazy et al., 2008).

2.4 SECONDARY METABOLITES QUANTIFICATION

For analyzing secondary metabolites, 100 mg of leaf or root dried samples were dissolved in 80 % ethanol (5 ml) and sonicated for 20 min at room temperature. The resulted mixture was centrifuged at 3,000 *g* for 15 min.

The extraction was repeated three times, and the supernatants were pooled and stored until analysis.

A colorimetric method was used to determine the total amount of saponins (Hiai et al., 1975). In a test tube, 0.5 ml of plant ethanol extract was mixed with 0.5 ml of vanillin and 5 ml of 72 % sulfuric acid. The mixture was shaken and heated for 10 min at 60 °C in a water bath. After cooling in the water at room temperature, the extract's absorbance was determined spectrophotometrically at 545 nm. To determine the concentration of the total phenolics, 2.5 ml of Folin-Ciocalteu-Deniz indicator and 2.5 ml of 2 % sodium carbonate solution were added to 0.5 ml plant ethanol extract. The resulting mixture was homogenized and incubated in the dark for 30 min. The absorbance of the solution was measured spectrophotometrically at 750 nm (Seevers et al., 1971). The total flavonoids concentration was determined using the aluminum chloride colorimetric method (Zhishen et al., 1999). The extract's 0.5 ml was mixed with 4.5 ml of distilled water and 0.5 ml of 5 % sodium nitrite solution. After 5 min, 0.5 ml of 10 % aluminum chloride was added, and the mixture was incubated for 6 min. After that, 4 ml of 1 M NaOH was added, and after 15 min, the absorbance of the mixture was read at 510 nm by a spectrophotometer. Quercetin was used for the creation of a standard curve. For analyzing the total anthocyanins concentration, 0.2 ml of alcohol extract was diluted separately with 4.8 ml potassium chloride (pH 1) and sodium acetate buffer (pH 4.5). The solutions were incubated in the dark for 15 min. The absorbance of both groups of samples was determined at 510 and 700 nm, and the concentration of anthocyanins (A) was calculated according to the following equation and reported as cyanidin-3-O-glucoside equivalent using an extinction coefficient of 26.9 mM^{-1} cm^{-1} (Giusti and Wrolstad, 2001):

$$A = [(A_{510}\ nm - A_{700}\ nm)]_{pH\ 1.0} - [(A_{510}\ nm - A_{700}\ nm)]_{pH\ 4.5}$$

A colorimetric method based on the color reaction of acobin with glycine was used to determine the total iridoid concentration (Narayanan and Akamanchi, 2003). One ml of ethanol extract was mixed with 2 ml distilled water, 1 ml of 10 % glycine, and 1 ml sulfuric acid (0.1 M). The mixture was shaken and heated in a water bath (95 °C) for 1 h. After cooling at room temperature, the absorbance was measured at 554 nm.

2.5 DETERMINATION OF TOTAL ANTIOXIDANT ACTIVITY

Different plant extract concentrations in methanol (10, 25, 50, 100, 250, and 500 μg ml^{-1}) were prepared and incubated with freshly-prepared 80 μg ml^{-1} DPPH (2, 2-diphenyl-1-picrylhydrazyl). The mixtures were shaken and placed in the dark for 30 min, then the absorbance of the samples in parallel with a solution without plant extract (as blank) was read at 517 nm. The inhibition (%) of DPPH radical formation was calculated according to the following equation:

$$\text{Inhibition (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Plant extract's antioxidant activity was reported as the sample concentration providing 50 % inhibition (IC_{50}) calculated by plotting inhibition percentages against the samples' concentration (Sarkar et al., 2006).

2.6 DETERMINATION OF ELEMENTS CONCENTRATIONS

To determine potassium (K) and sodium (Na) concentrations, 100 mg milled oven-dried samples were digested in concentrated nitric acid overnight, then heated at 80 °C for one hour and dissolved in 1 % HCl. The concentrations of K and Na were determined by flame photometry (Kalra, 1997).

2.7 STATISTICAL ANALYSIS

The experiment was undertaken as a completely randomized block design with three pots as independent replicates for each treatment. Data were presented as means ± standard deviation (SD). Comparison of means was performed by Tukey test ($p < 0.05$) using SPSS (version 23, for Windows; SPSS Inc., Chicago, IL, USA).

3 RESULTS AND DISCUSSION

Plants' growth was expectedly decreased under salt stress conditions (Fig. 1). A significant effect of salt stress on the shoot and root fresh weight was observed at 100 mM salt and higher. Dry biomass of plants was depressed up to 75 % at a salt concentration of 200 mM. Shoot height and length of the taproot significantly decreased by 50 mM salt and higher (Fig. 2).

The leaf content of Chl a and Chl b were not affected by salt concentration up to 150 and 100 mM NaCl, respectively. Leaf carotenoid content and RWC, were not significantly influenced by applied salt levels (Table 1).

Activities of all three analyzed antioxidant enzymes were higher in salt-stressed plants both in the leaves and roots (Fig. 3). At 200 mM salt concentration, the leaf activities of POD, CAT and APX increased up to 2.3, 6.3 and 6.9 fold, respectively, compared to the control treat-

Figure 1: Hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions.

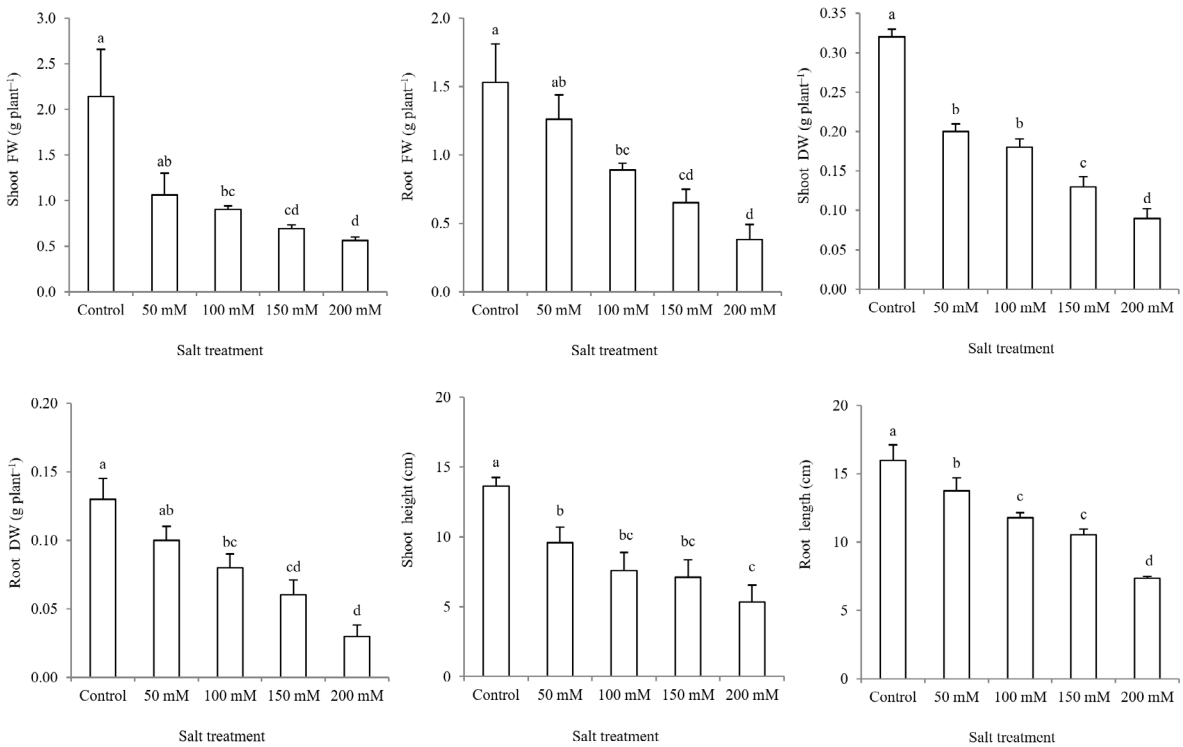

Figure 2: Fresh and dry biomass (g plant^{-1}), shoot height and root length (cm) of hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions. Bars indicated by the same letter are not significantly different ($p < 0.05$).

Table 1: Content of chlorophyll (Chl) *a*, *b* and carotenoids (mg g^{-1} FM) and relative water content (RWC, %) in the leaves of hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions. Data of each column indicated by the same letter are not significantly different ($p < 0.05$).

NaCl concentration	Chl *a*	Chl *b*	Carotenoids	RWC
Control	3.01 ± 0.27 [a]	1.27 ± 0.18 [a]	4.95 ± 0.53 [a]	0.58 ± 0.05 [a]
50 mM	2.95 ± 0.21 [a]	1.07 ± 0.06 [a]	5.40 ± 3.74 [a]	0.50 ± 0.11 [a]
100 mM	2.18 ± 0.04 [ab]	1.09 ± 0.01 [a]	3.74 ± 0.13 [a]	0.55 ± 0.12 [a]
150 mM	2.48 ± 0.19 [a]	0.74 ± 0.07 [b]	4.19 ± 0.35 [a]	0.46 ± 0.04 [a]
200 mM	1.36 ± 0.79 [b]	0.76 ± 0.09 [b]	2.51 ± 0.25 [a]	0.49 ± 0.03 [a]

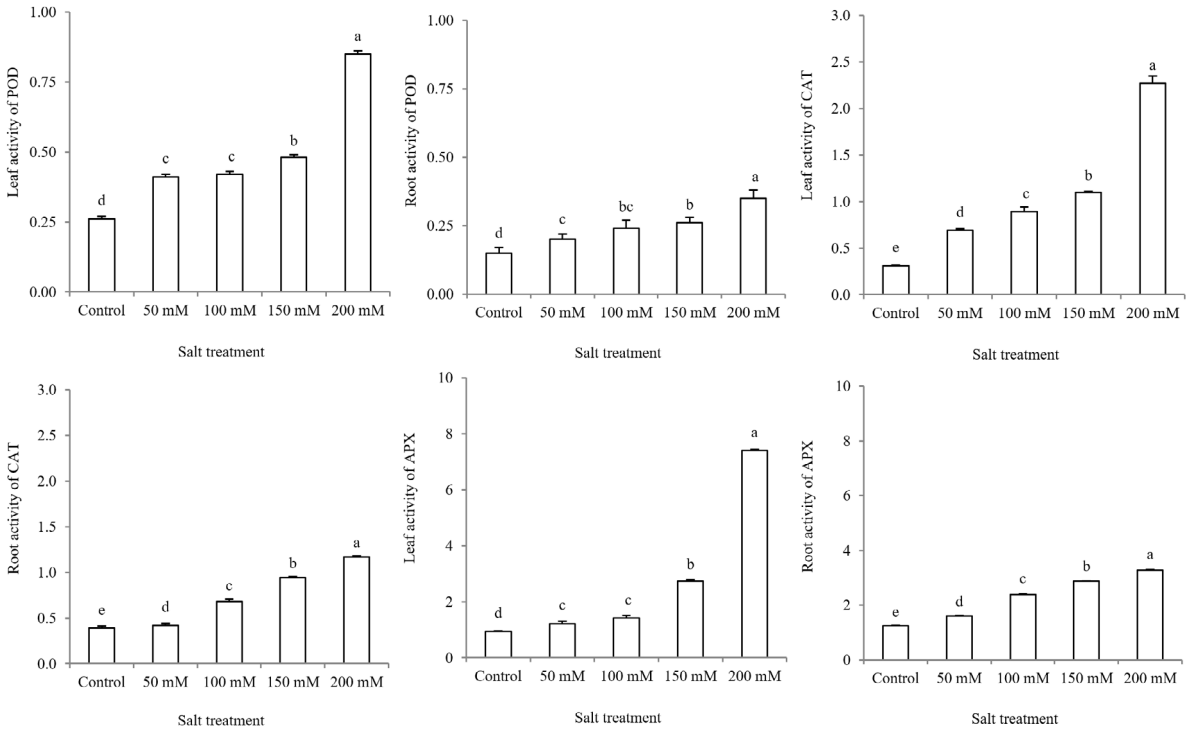

Figure 3: Activity of peroxidase (μmol mg^{-1} protein min^{-1}), catalase (μmol mg^{-1} protein min^{-1}) and ascorbate peroxidase (μmol mg^{-1} protein min^{-1}) in the leaves and roots of hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions. Bars indicated by the same letter are not significantly different ($p < 0.05$).

ment. In the roots, the activities of POD, CAT and APX were 1.3, 2.0 and 1.6 fold higher than the control plants (Fig. 3).

The soluble sugar content increased gradually in response to increasing salt levels in the medium, both in the leaves and roots. The extent of the increase was higher for the leaves (60 % at 200 mM salt) than the roots (20 % at 200 mM salt). The content of soluble proteins decreased by salt stress both in the leaves and roots. However, different salt levels did not differ in their effect on the root protein content, while in the leaves, it was continuously decreased by increasing salt levels (Table 2). Proline was accumulated both in the leaves and roots upon exposure to salt stress. In the leaves, proline content responded to low salt level (50 mM) and accumulated up to 4.5 fold in the presence of 200 mM salt. In comparison, in the roots, salt's significant effect was not observed at a low level (50 mM) and accumulated to much less extent, i,e, 1.8 fold under 200 mM salt. The concentration of K was steadily decreased under salt treatment while that of Na increased both in the leaves and roots (Table 2).

Leaf content of MDA was increased by salt stress both in the leaves and roots. The treatment effect was more prominent in the roots with up to 2.3 fold MDA accumulation at the salt treatment of 200 mM, while the corresponding value for the leaves was only 1.2 fold. The

Table 2: Contents of soluble sugars (mg g^{-1} FM), soluble proteins (mg g^{-1} FM), proline (µmol g^{-1} M), potassium and sodium (mg g^{-1} DM) in the leaves and roots of hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions. Data of each column indicated by the same letter are not significantly different ($p < 0.05$).

NaCl concentration	Soluble sugars	Soluble proteins	Proline	Potassium	Sodium
			Leaves		
Control	21.43 ± 0.84 ^{d}	12.3 ± 0.02 ^{a}	5.03 ± 0.01 ^{e}	4.72 ± 0.15 ^{a}	2.39 ± 0.09 ^{d}
50 mM	23.05 ± 1.27 ^{cd}	10.7 ± 0.10 ^{b}	5.88 ± 0.01 ^{d}	4.39 ± 0.06 ^{b}	3.63 ± 0.24 ^{c}
100 mM	26.23 ± 1.67 ^{bc}	9.61 ± 0.23 ^{c}	6.53 ± 0.05 ^{c}	4.26 ± 0.01 ^{b}	4.13 ± 0.13 ^{c}
150 mM	28.27 ± 0.32 ^{b}	9.04 ± 0.36 ^{d}	12.2 ± 0.10 ^{b}	3.66 ± 0.01 ^{c}	4.84 ± 0.26 ^{b}
200 mM	34.05 ± 1.90 ^{a}	7.01 ± 0.07 ^{e}	22.5 ± 0.03 ^{a}	3.29 ± 0.15 ^{d}	5.58 ± 0.40 ^{a}
			Roots		
Control	31.27 ± 0.16 ^{b}	10.8 ± 1.64 ^{a}	2.70 ± 0.27 ^{d}	7.16 ± 0.10 ^{a}	0.35 ± 0.01 ^{e}
50 mM	31.41 ± 0.07 ^{b}	8.44 ± 0.02 ^{b}	2.94 ± 0.20 ^{cd}	6.56 ± 0.10 ^{b}	0.59 ± 0.01 ^{d}
100 mM	33.51 ± 0.12 ^{b}	7.51 ± 0.02 ^{b}	3.57 ± 0.13 ^{bc}	6.26 ± 0.10 ^{c}	0.75 ± 0.07 ^{c}
150 mM	36.63 ± 1.81 ^{a}	7.14 ± 0.05 ^{b}	4.05 ± 0.14 ^{b}	5.46 ± 0.10 ^{d}	1.05 ± 0.02 ^{b}
200 mM	37.51 ± 0.75 ^{a}	6.76 ± 0.20 ^{b}	4.79 ± 0.37 ^{a}	4.36 ± 0.10 ^{e}	1.42 ± 0.07 ^{a}

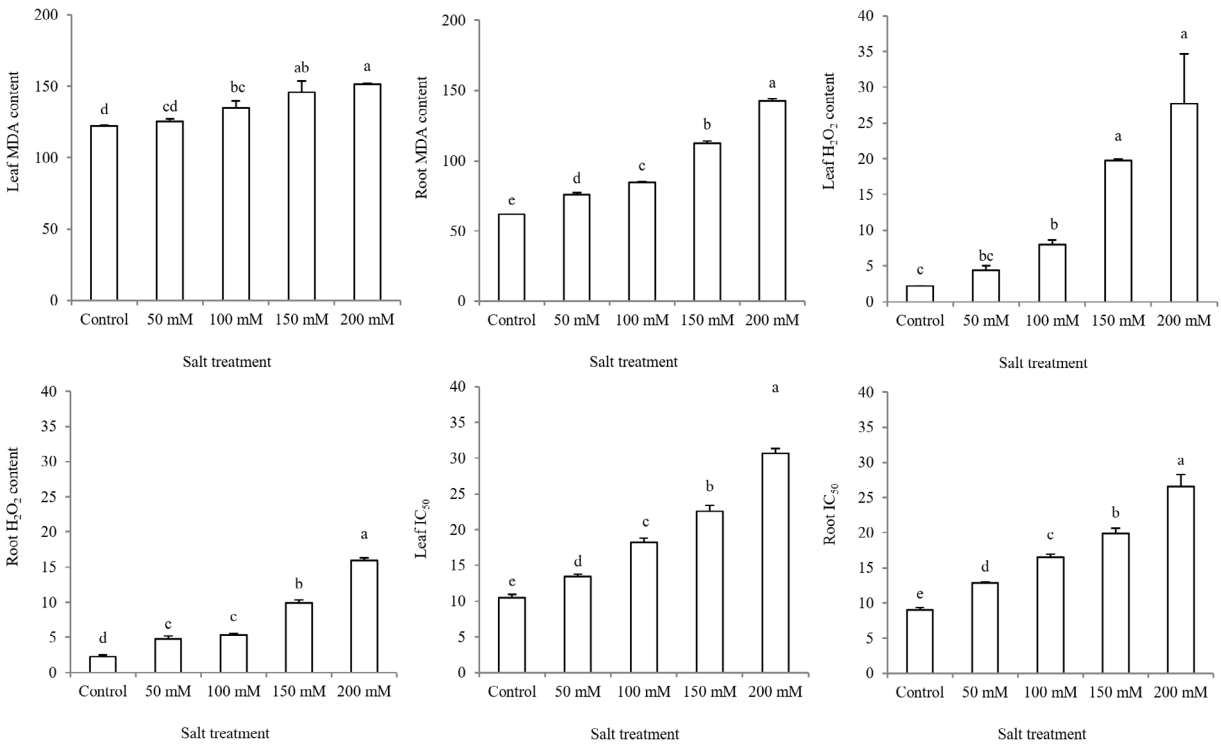

Figure 4: Contents of malondialdehyde (MDA, µmol g^{-1} FM), H_2O_2 (µmol g^{-1} FM) and total antioxidant activity (IC_{50}) in the leaves and roots of hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions. Bars indicated by the same letter are not significantly different ($p < 0.05$).

Table 3: Concentrations of saponins (mg g^{-1} DM), total phenolics (mg g^{-1} DM), total flavonoids (µg g^{-1} DM), anthocyanins (µg g^{-1} DM) and iridoids (mg g^{-1} DM) in the leaves and roots of hyssop (*Hyssopus officinalis* L.) plants grown for 4 weeks under control or different salt concentrations (50, 100, 150 and 200 mM NaCl) under greenhouse conditions. Data of each column indicated by the same letter are not significantly different ($p < 0.05$).

NaCl concentration	Saponins	Phenolics	Flavonoids	Anthocyanins	Iridoids
			Leaves		
Control	330 ± 2.8 ^{c}	19.3 ± 0.10 ^{d}	75.4 ± 3.96 ^{d}	10.15 ± 0.57 ^{d}	50.7 ± 1.59 ^{d}
50 mM	371 ± 24.7 ^{c}	22.1 ± 0.98 ^{c}	87.6 ± 3.22 ^{c}	18.70 ± 0.62 ^{c}	55.0 ± 2.57 ^{cd}
100 mM	475 ± 83.6 ^{c}	24.4 ± 0.33 ^{b}	93.9 ± 1.83 ^{c}	20.55 ± 0.78 ^{c}	73.7 ± 3.13 ^{bc}
150 mM	644 ± 54.6 ^{b}	25.7 ± 0.90 ^{ab}	109 ± 4.58 ^{b}	26.25 ± 0.83 ^{b}	92.2 ± 5.78 ^{b}
200 mM	801 ± 68.9 ^{a}	27.4 ± 0.48 ^{a}	118 ± 2.37 ^{a}	50.09 ± 1.98 ^{a}	129.1 ± 15.2 ^{a}
			Roots		
Control	390 ± 23.7 ^{c}	45.31 ± 1.22 ^{d}	199 ± 18.31^{c}	0.90 ± 0.10 ^{d}	70.5 ± 0.33 ^{a}
50 mM	446 ± 10.1 ^{c}	48.79 ± 0.82 ^{cd}	224 ± 1.64 ^{bc}	1.50 ± 0.10 ^{d}	75.3 ± 0.98 ^{a}
100 mM	610 ± 109 ^{b}	52.75 ± 0.38 ^{bc}	239 ± 8.38 ^{b}	2.52 ± 0.50 ^{c}	76.8 ± 0.18 ^{a}
150 mM	755 ± 18.3 ^{a}	55.03 ± 0.43 ^{b}	249 ± 1.77 ^{ab}	4.00 ± 0.56 ^{b}	78.5 ± 0.64 ^{a}
200 mM	859 ± 5.81 ^{a}	59.39 ± 3.10 ^{a}	267 ± 9.39 ^{a}	6.98 ± 0.07 ^{a}	75.9 ± 6.79 ^{a}

content of H_2O_2 was consistently increased by increasing salt level. The H_2O_2 accumulation in response to higher salt levels (150 and 200 mM) was more prominent in the leaves than in the roots. The total antioxidant activity (IC_{50}) was increased up to 2.9 fold in the salt-stressed plants (Fig. 4).

The leaf and root concentrations of all analyzed secondary metabolites were increased by exposure to salt stress in the leaves and roots except iridoids in the roots that remained unchanged (Table 3). Lower salt level (50 mM) was effective in the increasing phenolics, flavonoids, and anthocyanins in the leaves, while in the roots, a significant effect was observed by higher salt level (100 mM). The extent of salt-induced increase in the concentration of analyzed secondary metabolites was in the range of 1.5-2.5 fold except for anthocyanins. This metabolite showed up to 4.9 and 7.8 fold increase upon exposure to 200 mM salt in the leaves and roots, respectively (Table 3).

4 DISCUSSION

4.1 EFFECT OF SALT STRESS ON GROWTH, NA CONCENTRATION AND LEAF CHL CONTENT

Hyssop is a drought-tolerant species (Khazaie e al., 2008); however, its salt tolerance has not been studied so far. Our data demonstrated that hyssop is also tolerant to salinity stress as the plants in our study survived after 4 weeks of salt treatment of 200 mM. Such high salt tolerance has been rarely reported in the members of Lamiaceae. In the studies on the salt tolerance in other Lamiaceae species such as *Thymus, Perilla,* and *Salvia*, much higher growth inhibition by salt has been reported, and plants were killed by salt concentrations higher than 100 mM (Paiva et al., 2018; Bistgani et al., 2019; Salachna et al., 2019).

The Na concentration data showed that this species is a Na-excluder salt-tolerant plant and can avoid root Na uptake. The low Na accumulation in the roots and leaves was accompanied by stable amounts of RWC showing that this species maintains tissue water content despite exposure to low water potentials in the rooting medium. On the other hand, a constitutively lower RWC (0.49-0.58 %) shows that these species cope with low water potentials through passive water content reduction. A similar mechanism for salt tolerance has been observed in *Thellungiella,* a halophyte close relative of *Arabidopsis* (Lugan et al., 2010).

In agreement with the conclusion mentioned above on high salt tolerance in hyssop plants, leaf Chl content remained unaffected by salt treatment up to 150 mM suggesting that leaf photosynthetic capacity remained mainly unaffected under these conditions. The maintenance of photosynthesis and carbon metabolism may help plants retain an ability to synthesize organic osmolytes, including soluble sugars and proline (Chaves et al., 2009). These two organic osmolytes were accumulated in the leaves up to 1.6 and 4.5 fold, respectively, which may contribute significantly to plants' osmotic homeostasis under salt stress conditions. In addition to osmotic functions,

these osmolytes contribute to protecting cell structures, ROS scavenging, and nitrogen and carbon sources under stress conditions (Verbruggen and Hermans, 2008; Mattioli et al., 2009; Rosa et al., 2009).

4.2 EFFECT OF SALT STRESS ON THE ACTIVITY OF ROS ACCUMULATION, SCAVENGING AND MEMBRANE INTEGRITY

The activities of ROS scavenging enzymes were expectedly increased by salt treatment both in the leaves and roots. The salt-induced activity of all three analyzed enzymes was higher in the leaves compared with the roots that may contribute to high protection of leaves against salt-induced damage. Better protection of leaves than roots was confirmed by the maintenance of a high Chl content under high salinity treatments and much less increase of MDA content under salt stress (24 % at 200 mM salt) compared with the roots (130 % at 200 mM salt).

Nevertheless, the accumulation of H_2O_2 was higher in the leaf than in the roots indicating that higher enzyme activities were not sufficient for inhibition of H_2O_2 accumulation in the leaves. Although H_2O_2 belongs to ROS, it is known to be much less damaging in comparison to superoxide and hydroxyl radicals (Cheng et al., 2006). The prevailing effect of H_2O_2 is a signaling role. It has been observed that H_2O_2 is an important signal that is raised under salt stress and is responsible for the activation of various defense pathways in salt-stressed plants (Shu-Hsien et al., 2005). We suggest that the higher capability of leaves for H_2O_2 accumulation, and activation of defense pathways may be partly responsible for higher protection of leaves against salt stress than the roots.

4.3 EFFECT OF SALT STRESS ON THE CONCENTRATION OF SECONDARY METABOLITES

The concentration of all analyzed secondary metabolites was higher in the salt-stressed hyssop plants in our study. The effect of salt treatment on the levels of phenolics, flavonoids, and anthocyanins has been reported in other Lamiaceae species (Kotagiri et al., 2017; Bistgani et al., 2019; Salachna et al., 2019; Becerra-Gudiño et al., 2019). However, in hyssop, the quantity of bioactive compounds as affected by salinity has not been investigated so far. Our study is also the first report on the effect of salt stress on the saponins and iridoids. Iridoids are a type of monoterpenoids found in plants, mainly as glycosides (Wang et al., 2020). The iridoids produced by plants act as a defense against herbivores or microorganisms (Fuchs et al., 2004). From a medicinal point of view, these compounds have wound-healing and anti-inflammatory effects with therapeutic potential for Alzheimer's and Parkinson's diseases (Dinda et al., 2019; Hussain et al., 2019). Saponins with one or more hydrophilic glycoside moieties combined with a lipophilic triterpene molecule (El Aziz et al., 2019) exhibit medicinal properties such as hemolytic factor, anti-inflammatory, antibacterial, antifungal, antiviral, anticancer, and cholesterol-lowering action in animals and human (Sparg et al., 2004). Besides, saponins formed the backbone of modern medicine or drugs and were considered a starting precursor for the semi-synthesis of steroidal drugs in the pharmaceutical industry (Netala et al., 2015).

It is noteworthy that higher concentrations of the secondary metabolites accompanied by reduction of biomass suggests a 'concentration-effect' in our hyssop plants. Nonetheless, it indicates that salt treatment did not inhibit the secondary metabolism in this species.

4.4 EFFECT OF SALT STRESS ON THE ANTIOXIDANT ACTIVITY OF LEAF AND ROOT EXTRACT

The DPPH scavenging activity is defined as the antioxidant activity of food and medicinal plants (Fukumoto and Mazza 2000; Sethi et al., 2020), has been reported for hyssop plants (Fathiazad et al., 2011; Pirbalouti et al., 2019; Rezaei Savadkouhi et al., 2020). However, the effect of salt on this parameter has not been studied so far. Here in our work, the DPPH free radical scavenging activity was increased by salt treatment for the leaf and root extracts. Electron donation is an important mechanism in which plants bioactive compounds convert free radicals to nonradical forms and thus, end the radical chain reactions (San Miguel-Chávez, 2017; Shahidi and Ambigaipalan, 2015). By analyzing various plant species, it has been observed that the main component of DPPH scavenging activity is phenolics, flavonoids, and anthocyanins (Fukumoto and Mazza 2000; Kim et al., 2007). Phenolic compounds act as a reducing agent and a hydrogen donator and show antioxidant effects (Oke et al., 2009).

5 CONCLUSION

Our data demonstrated that, hyssop plants are a salt-tolerant species, and secondary metabolites are increased upon growth under salinity. Regarding the fact

that, the plants dry matter production was reduced under higher salt levels. i.e., 200 mM equivalent with 14.5 dS m^{-1}, cultivation of this species is recommended in the soils with electrical conductivity up to 10 dS m^{-1}. Thus, the cultivation of this species on salinized soils that are unsuitable for most crop species is an alternative for low-income farmers.

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Evaluation of Ethiopian chickpea (*Cicer arietinum* L.) genotypes for frost tolerance

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Evaluation of Ethiopian chickpea (*Cicer arietinum* L.) genotypes for frost tolerance

Abstract: Frost stress is one of the most significant abiotic factors affecting chickpea (*Cicer arietinum* L.) production in the Ethiopian highlands. To investigate the frost tolerance of chickpea, 673 genotypes were characterized using an augmented design at Bakelo, Debre Berhan, Ethiopia for two years. A significant ($p < 0.01$) variability amongst genotypes was recorded for all agronomic traits considered. A considerable number of accessions better performing over the frost susceptible genotypes were identified for agronomic traits. Stem/leaf pigmented genotypes showed a better reaction for frost stress than non-pigmented genotypes. The majority of black seeded chickpea adapted well under frost stress when compared to with brown and white seeded genotypes. According to the freezing tolerance rate (FTR) and plant survival rate (SR), 83 (12.3 %) and 85 (12.6 %) genotypes were identified as frost tolerant. There was a strong correlation ($p < 0.01$) in grain yield with FTR, SR, seed shriveling score, stem/leaf pigmentation and seed color. Based on our findings, Ethiopian chickpea landraces has a good genetic potential for frost resistance traits for use in breeding programs.

Key words: chickpea; Ethiopian landraces; frost survival rate; frost tolerance; germplasm characterization

Ovrednotenje etiopskih genotipov čičerke (*Cicer arietinum* L.) za toleranco na mraz

Izvleček: Mrazni stres je eden izmed najznačilnejših abiotskih dejavnikov, ki vpliva na pridelavo čičerke (*Cicer arietinum* L.) v etiopskem višavju. Za preučevanje tolerance na mraz je bilo v izboljšanem poskusu analiziranih 673 genotipov čičerke v Debre Birhan, Etiopija, v obdobju dveh let. Med genotipi je bila ugotovljena značilna variabilnost ($p < 0,01$) za vse preučevane agronomske lastnosti. Prepoznano je bilo znatno število akcesij, ki so se izkazale boljše v preučevanih agronomskih lastnostih kot tiste občutljive na mraz. Genotipi z obarvanimi stebli ali listi so se boljše odzvali na mrazni stres kot neobarvani. Večina genotipov čičerke s črnimi semeni je bila bolje prilagojena na mrazni stres v primerjavi s tistimi z rjavimi ali belimi semeni. Glede na toleranco na mraz (FTR) in preživetje rastlin (SR), je bilo 83 (12,3 %) in 85 (12,6 %) genotipov na mraz toleratnih. Ugotovljena je bila močna povezava ($p < 0,01$) med pridelkom semena in FTR, SR, nagubanostjo semena, obarvanostjo stebla in listov ter barvo semena. Na osnovi teh ugotovitev imajo etiopske tradicionalne sorte čičerke dober genetski potencial za odpornost na mraz in so lahko uporabne v žlahtniteljskih programih.

Ključne besede: čičerka; tradicionalne etiopske sorte; lastnosti tolerance na mraz; ovrednotenje genotipov

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1 INTRODUCTION

Chickpea (*Cicer arietinum* L.) cultivation and utilization are profoundly notable within Ethiopian culture and produced by smallholder farmers under rainfed condition (Ferede et al., 2018). The cultivation is so profound that chickpea production in Ethiopia is one of the most widespread legume in terms of both area and volume. Across Ethiopia chickpea cultivation occupies ~1,620,497.30 hectares of land annually with an estimated production of 30,113,480570 kg (CSA, 2019). Both the land dedicated to chickpea production and the volume of production itself has been increasing over the last decade in Ethiopia (Fikre & Bekele, 2020; Fikre et al., 2018). Ethiopia is thus the largest producer, consumer, and exporter of chickpea in Africa, and is among the top ten most vital chickpea producers in the world (FAOSTAT, 2020). Chickpea production is suited to areas having vertisol-dominated soil with an altitudinal range of 1400 to 2300 meters above see level (Bejiga et al., 1996). Nevertheless, it is cultivated across a wide selection of zone (Fikre et al., 2018). Moreover, Ethiopia is considered to be the second greatest diversity hotspot of chickpea amongst major chickpea growing countries (Van der Maesen, 1987). Taking into consideration both immense variability among the chickpea germplasm and many agroecological zones as well as the increased demand for animal feed and processed foods (Fikre et al., 2020; Muoni et al., 2019; Shiferaw &Hailemariam, 2007), Ethiopia features great potential to expand chickpea production within the highland areas if the chickpea varieties are resistance to frost stress.

Chickpea is important for Ethiopian highland cultivation and is preferably sown in early- to mid-September. Previously, mid-August was considered the appropriate sowing date, but due to the "*belg*" rainy season, chickpea cultivation was heavily impacted by root rot. Root rot issues can be avoided by planting in mid-September, leading to higher yields. However, the later sowing date presents a new issue, due to the elevation of highlands, which is frost stress. The frost stress takes place late in the podding and flowering stages. Frost stress during these stages causes issues such as flower abortion, poor pod set, and impaired pod filling, leading to a drastic reduction in yield and quality (Croser et al., 2003). These stressors can be classified as chilling (0 °C to 12 °C) or freezing/frost (< 0 °C) temperatures (Gogoi et al., 2018; Toker et al., 2007). Moreover, temperatures lower than 10 °C at flowering can reduce grain yield by 15–20 % (Chaturvedi et al., 2009). Therefore, the need for improving frost-tolerance in chickpea has become evident which requires characterization of chickpea germplasm for frost tolerance.

Determining the nature of genetic diversity and variability existing among chickpea genotypes for frost resistance is mandatory to identify promising genotypes that are productive in Ethiopian highlands with late sowing dates. However, few studies have been conducted so far in this regard. Hence, research is needed to further understand the optimal utilization of landraces as sources of novel traits for frost resistant chickpea variety development. Therefore, the aim of this research is to identify chickpea genotypes that are both highly productive and frost resistant through use of field screening of genotypes for frost-tolerance. The long-term goal is to establish highly productive and frost tolerant chickpea varieties supporting Ethiopian highland farmers by enhancing food security and improving rural livelihoods.

2 MATERIAL AND METHODS

2.1 EXPERIMENT SITE

The experiment was conducted at Bakelo, Debre Berhan Agricultural Research Center experimental site (Debre Berhan, Ethiopia) for two consecutive growing seasons (2018/19 and 2019/20). The experimental site is located 147 km away from Addis Ababa at a N 09°41'42" latitude and E 39°37'20" longitude. Its altitude is 2,837 meter above sea level and receives an annual mean precipitation of 965.25 mm. The temperature ranges from 6.5 °C to 20.1 °C with mean annual temperature of 13.3 °C. The dominant soil type of Bakelo is black vertisol. The daily minimum and maximum temperature values are indicated in Fig 1.

2.2 PLANT MATERIALS

A total of 673 genotypes (559 Ethiopian genotypes from the Ethiopian Biodiversity Institute (EBI), 83 elite frost resistant genotypes from the International Center for Agricultural Research in the Dry Areas (ICARDA), three susceptible local checks and 28 improved chickpea varieties released from Ethiopian Agricultural Research Centers were screened for their tolerance against frost stress under field condition at Bakelo, Debre Brehan, Ethiopia, which is a frost prone area (see Supplementary Table S1 for further details) using freezing tolerance rate, plant survival rate and other frost resistant-related agronomic traits. The geographical origin of the Ethiopian chickpea germplasm used in the study is indicated in Fig. 2.

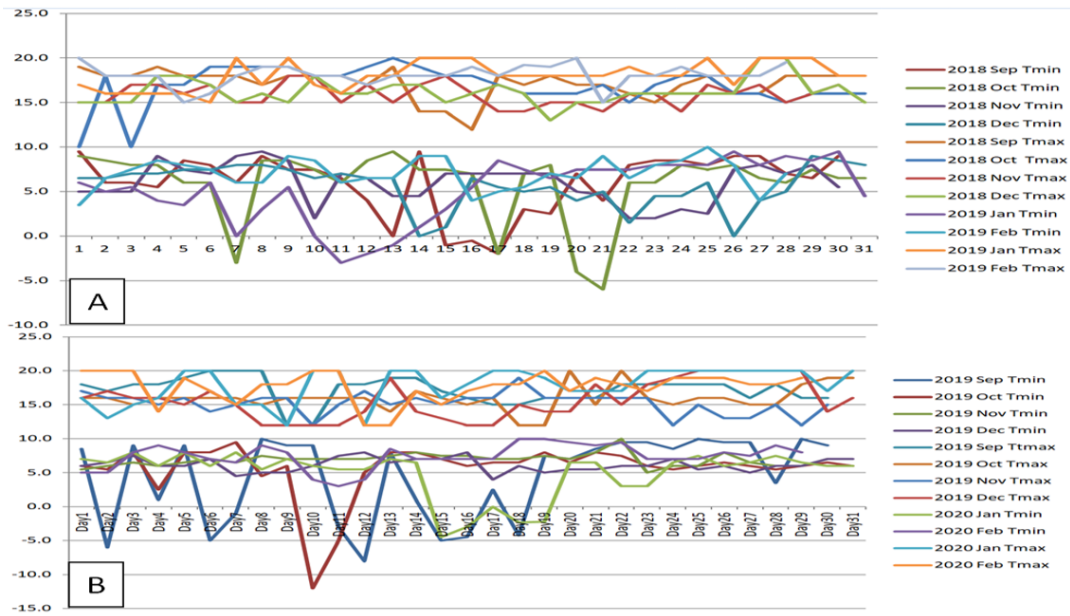

Figure 1: Daily maximum and minimum temperature of Bakelo, Debre Berhan during 2018/2019 (A) to 2019/2020 (B) growing seasons (Source: Debre Berhan Agricultural Research Center)

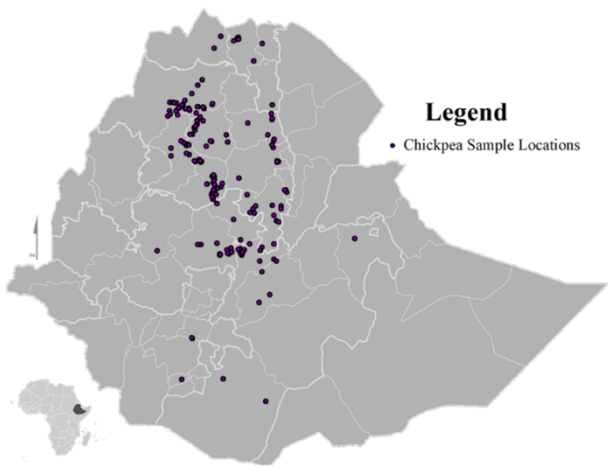

Figure 2: Map showing the geographical distribution of Ethiopian chickpea germplasm

2.3 EXPERIMENTAL DESIGN

Augmented design without replication was used. Each genotype was sown in two rows with 3 m row length and 0.2 m spacing between rows and 0.1 m between plants. Diammonium phosphate fertilizer (100 kg ha^{-1}) and other appropriate management practices were applied. Five individual plants were tagged randomly from each genotype per plot and they were used for morphological data collection. Recording agronomic characteristics were conducted following the procedure described by chickpea descriptor (IBPGR, ICRISAT and ICARDA 1993).

2.4 DATA COLLECTED

Qualitative and quantitative morphological traits were recorded as per described in Table 1.

Table 1: List of qualitative and quantitative characters recorded, their codes and descriptions

Characters	Description
Qualitative traits	
Stem/Foliage Pigmentation (SLM)	0 = No Anthocyanin, 1 = Low Anthocyanin 2 = Medium Anthocyanin, 3 = High Anthocyanin
Seed Color (SC)	1 = Black, 2 = Brown, 3 = White
Flower Color (FC)	0 = White, 1 = Pink
Quantitative traits	
Plant Height (cm) (PLH)	Average canopy height of five representative plants taken at maturity stage
Days to 50 % Flowering (DTF)	Number of days from sowing until 50 % of the plants have started to flower
Days to 50 % Podding (DTP)	Number of days from sowing until 50 % of the plants have started to podding
Days to 90 % Maturity (DTM)	Number of days from sowing until 90 % of the pods have matured and turned yellow
Number of Primary Branches (NPB)	Average number of basal primary branches per plant taken from five representative plants
Number Secondary Branches (NSB)	Average number of secondary branches per plant taken from five representative plants
Number of Fertile Pods per Plant (NIPPP)	Average number of fertile pods taken from five representative plants taken at maturity stage
Number of Infertile Pods per Plant (NIPPP)	Average number of infertile pods taken from five representative plants taken at maturity stage
Thousand Seed Mass (TSM)	Thousand seeds were counted and weighted at 12 % moisture content on a 0.1 g sensitive balance in milligram
Grain Yield (GY in kg ha^{-1})	Dried mass (kg) of seed per plot at 12 % moisture content
*Freezing tolerance rate (FTR)	Scored on 1-9 scale bases (Singh et al., 1989): where, 1 = No visible symptoms of damage; 2 = Highly tolerant, up to 10 % leaflets show damage; 3 = Tolerant, 11-20 % leaflets show damage; 4 = Moderately tolerant, 21-30 % leaflets and up to 20 % branches show withering and drying, but no killing; 5 = Intermediate, 41-60 % of leaflets and 21-40 % branches show withering and drying, up to 5 % plant killing; 6 = Moderately susceptible, 61-80 % leaflets and from 41-60 % branches show withering and drying, 6-25 % plant killing; 7 = Susceptible, 81-99 % leaflets and 41-80 % branches show withering and drying, 26-50 % plant killing; 8 = Highly susceptible, 100 % leaflets and 81-99 % branches show withering and drying, 51-99 % plant killing; and 9 = 100 % plant killing
Plant survival rate (SR)	Calculated by dividing the number of surviving plants after the frost period by the number of emerged plants after sowing was calculated (Heidarvand et al., 2011)
Seed shriveling score (SSS)	Visual measurement and estimating the kernel's condition (1 = plump, 3 = intermediate and 5 = shriveled

*= Frost score was recorded when susceptible checks showed sign for frost damages or completely died.

2.5 DATA ANALYSIS

The collected data for each trait were subjected to statistical analysis of variance using augmentedRCBD R Packages version 0.1.3 (Aravind et al., 2020). The analysis helps us to partition the variance into different sources (phenotypic, genotypic and environmental variance) and genetic parametrs to see if the difference among genotypes is statistically significant or not for each trait considered (Singh & Chaudhary, 1977). Pearson correlation coefficients between variable was estimated and tested for significance using MINTAB 10 statistical package (Wild, 2005)

3 RESULTS AND DISCUSSION

The performances of the chickpea genotypes in response to frost stress were assessed in natural condition and the results obtained are discussed.

3.1 THE EFFECT OF FROST ON AGRONOMIC TRAITS

ANOVA was performed for the two seasons separately because the intensity of frost stress was different for both years. There was a significant difference ($p < 0.01$) among genotypes for plant canopy height, number of primary branches, number of secondary branches, fertile pods per plant, infertile pods per plant, days to 50 % flowering, days to 50 % podding, days to 90 % maturity, thousand seed mass, and grain yield (Table 2) in 2018/2019 and 2019/2020 growing seasons. These differences in performance indicate the existences of variability among genotypes for frost tolerance. Similar finding was reported by Mir et al. (2019).

Based on Fisher's least significant difference (LSD) result indicated that there was a significant difference ($p < 0.05$) among genotypes for the mean value of agronomic traits examined in this study. A wide range value of the means was recorded for the traits recorded. The LSD means and range of values of the traits for chickpea genotypes examined is presented in Supplementary Tables S2 and S3 for further details. The LSD means value differences and the mean rage value of the traits further confirms the existence of variable responses to frost stress among genotypes. The responses of genotypes to the effect of frost stresses at each crop stage are discussed below because the genotypes responses to the frost damage were variable at each stage.

3.1.1 Seedling and vegetative stage

The frost stress occurred in both seasons and genotypes had shown uniform germination and seedling establishment (Fig 3A). The lowest temperature recorded during this stage was -2.0 °C in Sept 2018 and -8.0 in Sept 2019 growing seasons. All genotypes did not show any symptoms or damage in response to frost stress, which means that these genotypes had shown good tolerance to frost stress at seed germination and seedling development stages. However, most authors agreed that germination percentage and seedling development are sensitive to frost stress which results in poor crop stand establishment, and reduced seedling vigor with stunted seedlings (Croser et al., 2003; Maphosa et al., 2020; Srinivasan et al., 1998). During the vegetative stage, 43 (6.4 %) genotypes (One improved variety, one EBI genotypes and 54 ICARDA genotypes) were killed by frost (Fig 3B) in both growing seasons. The list of genotypes killed by frost is indicated in the Supplementary Table S4. Theses genotypes were identified as a highly susceptible to frost stress because they could not resist the frost stress when the minimum temperature of -6.0 °C and -12 °C were recorded in Oct 2018 and Oct 2019, respectively**.** These genotypes showed poor growth development, wilting, chlorosis, necrosis and finally death of the whole plant, which was the manifestation of frost injury. Similar observations were reported by Croser et al. (2003) and Mahajan & Tuteja (2005). The remaining genotypes had shown medium to good reactions to frost stress at vegetative stage because the impact of frost stress at this stage was minimal in both growing seasons.

3.1.2 Number of branches and plant height

The number of primary and secondary branches has been significantly affected by frost in both seasons where a wide range was recorded. The range of number of primary branches was 0 to 16.1 in 2018/2019 and 0 to 27.2 in 2019/2020 growing season and for number of secondary branches it was 0 to 25.6 in 2018 and 0 to 46.5 in 2019. The majority of the accession scored below five for primary and secondary branch in both growing seasons. However, 69 (10.3 %) and 71 (10.6 %) genotypes produced better number of primary branch (> 7) in 2018/2019 and 2019/2020 growing seasons, respectively. The response of genotypes to the effect of frost stress for plant height development was variable. A wide range of plant height was observed in both cropping seasons (20.3 to 58 cm in 2018/2019 and 17.2 to 57 cm in 2019/2020). One hundred two (15.2 %) and 89 (13.2 %) genotypes had a record of less than 35 cm plant height in 2018/2019 and 2019/2020 cropping season, respectively. Genotypes 132663 (58 cm) and 140294 (57.04 cm) had shown better plant height. In this experiment, most genotypes gave good positive reaction for plant height to the frost effect though frost significantly reduced plant height. This is probability because of the duration of time that frost occurred is not sufficient to have a negative impact to the plant development.

3.1.3 Reproductive stages

Seventeen genotypes (2.5 %) (Seven EBI genotypes and 10 from ICARDA) were killed by frost stress during reproductive stages (Fig 3C and 3D). Moreover, the effect of frost was clearly examined in the delay of number of days to flower, pod and mature in the remaining genotypes with different degree. The range of 47.7 to 87.54, 54.2 to 89.6 and 118.7 to 160 days was recorded for days to flower, days to pod and mature for 2018/2019 growing season, respectively, while 48 to 77.7, 55 to 99.6 and 99.9

to 171.2 days for 2019/2020 cropping season, respectively. The range of fertile pods per plant was 0 to 237.5 and 0 to 162.7 for 2018/12019 and 2019/2020 cropping seasons, respectively. The range of infertile pods per plant was 0 to 77.3 and 0 to 116 for 2018/2019 and 2019/2020 cropping seasons, respectively. The genotypes 227152-A (237.5) and 41301-B (162.7) produced the highest number of fertile pods in 2018/2019 and 2019/2020 cropping seasons, respectively (Fig 3F). The minimum temperature recordered during reparative stage especially at flowering and podding stages was below 5 °C in both seasons (Fig 1) which caused flower abortion and pod dropping for genotypes having poor response to frost stress. These frost symptoms were observed in most frost susceptible genotypes and they produced either empty pods or pods containing small shriveled seeds. Similar observation was reported by Gogoi et al. (2018) stating that temperature falls below 15 °C causes flower and pod abortions. Various authors agreed that the reproductive stage is more susceptible to frost stress than seedling stages because frost stress negatively affects pollen fertility, pod set, number of aborted flowers, total number of pods per plant, seed number, size and shape, rate and duration of seed filling which consequently reduced biomass and grain yield (Berger et al., 2012; Croser et al., 2003; Gogoi et al., 2018; Kumar et al., 2010; Nayyar et al., 2007; Srinivasan et al., 1999). Low temperature stress during reproductive development is responsible for the induction of flower abscission, pollen sterility, pollen tube distortion, ovule abortion and reduced fruit set leading to reduction in seed yield (Sharma & Nayyar, 2014).

3.1.4 Thousand seed mass and grain yield

Seed development of all genotypes was severely affected by frost because the minimum temperature of -3.0 °C and -4.5 °C were recorded during seed dvelopment stage in Jan 2019 and Jan 2020, respectively (Fig 1). The majority of the genotypes produced shrived seed (Fig 4). Most genotypes that performed well till seed development became affected at seed development stage. The range of 0 g to 300 g and 0 kg ha^{-1} to 2531 kg ha^{-1}were recorded for thousand seed mass and grain yield for 2018/2019 cropping season respectively, while for 2019/2020 cropping season the range was, 0 to 297 g and 0 kg ha^{-1} to 2604 kg ha^{-1}, respectively. Wu et al. (2014) indicated that the prolonged period of chilling range temperatures (0 °C to 12 °C) at any phenological stage of development in chickpea has detrimental effects on final seed yield. Low temperature has negative impact on yield and 15-20 % yield loss was estimated and temperature below 15 % during flowering leads to flower and pod abortion leading to poor yield (Croser et al., 2003). Frost stress affects the source-sink balance by markedly decreasing the source of assimilates for grain filling which, in turn, reduces potential yield (Maphosa et al., 2020). Chaturvedi et al. (2009) estimated a yield loss of 15-20 % has been associated with low temperature. Low temperature during vegetative stage leads to decreased vegetative growth, biomass production and yield in north India (Mir et al., 2019; Singh et al., 1993).

3.1.5 Seed color

The majority of the frost susceptible genotypes showed a seed color fade up. Some of the genotypes had shown plumped seed with fade up seed color. This indicates that frost causes seed size and seed discoloration in chickpea. Similar observation was made for faba bean (Sallam et al., 2015). This happened because frost affects the mobilization of plant resources in to seed setting (Croser et al., 2003).

3.2 PLANT SURVIVAL RATE (SR)

Frost tolerance was assessed using plant survival rate (SR) for 673 diverse chickpea germplasms for two growing seasons under field condition (Table 3). It was observed that the SR values ranged from 0.0 (60 genotypes) to 0.86 (genotypes 16341-A, 24159-C and 30290-A) and 0.0 (60 genotypes) to 0.87 (genotype 41167-C) for 2018/2019 and 2019/2020 growing seasons, respectively. The value of SR score for the two growing seasons showed variation because of the different duration and intensity of frost stress occurred in the different seasons. The frost intensity and length of occurrence were more sever in 2019/2020 growing season than in 2018/2019. So, high value of SR was recorded in 2018/2019 than in 2019/2020 growing season (Fig 1). One hundred fifty seven and 87 chickpea genotypes had shown above 0.8 SR score, while the remaining 516 and 586 genotypes were below 0.8 SR score for 2018/2019 and 2019/2020 growing seasons, respectively. Eighty five genotypes were consistently given SR score value above 0.8 in both growing seasons. In the experimental site, frost occurred consistently throughout the life cycle of the crop's development, and hence, the frost survival score were taken at the end of each crop stages. The fluctuation of minimum temperature of two different growing seasons exhibited a similar pattern of SR value change for all genotypes. Minimum temperature of 2019/2020 growing seasons was lower than that of 2018/2019 growing season. It is clear that the SR of chickpea is closely associated to the temperature changes. Similar patterns were observed also in field pea (Liu et al., 2017). This approach has been employed to screen frost tolerance in rapeseed/canola (Fiebelkorn & Rahman, 2016) and field pea (Liu et al., 2017).

Table 2: Mean square and mean for the tested traits of 673 (562 EBI genotypes, 83 exotic and 28 improved chickpea) genotypes grown at Bakelo, Debre Berhan, Ethiopia from 2018-2020 growing seasons (I for 2018/2019 and II for 2019/2020)

(I)											
Sources of Variation	Degree of freedom	Type III Mean Squares									
		PLH	NPB	NSB	FPPP	IPPP	DTF	DTP	DTM	TSM	GY
Block	9	0.6^{ns}	0.3^{ns}	1.6^{ns}	168.3^{ns}	35.9^{ns}	4.55^{ns}	14.9^{ns}	3.51^{ns}	5.0^{ns}	7224^{ns}
Treatment	612	23.5^{***}	2.2^{***}	4.9^{***}	429.4^{***}	47.6^{*}	8.15^{*}	17.4^{*}	28.57^{*}	814.8^{***}	154550^{***}
Treatment: check	2	0.99^{ns}	0.3^{ns}	0.6^{ns}	35.3^{ns}	277^{***}	41.6^{**}	61.9^{**}	21.43^{ns}	13.1^{ns}	174411^{***}
Treatment: test and test vs. Check	610	24***	2.2^{***}	4.9^{*}	430.7^{***}	46.8^{*}	4.04^{*}	17.2^{*}	28.59^{*}	817.4^{***}	154485^{***}
Residuals	18	0.78	0.43	0.53	84.8	21.6	4.82	7.8	11.59	6.8	8976
CV		2.32	13.32	20.06	22.88	41.34	4.16	4.57	2.56	3.07	8.72
Mean		38.35	4.89	3.65	40.31	10.9	52.72	61.22	133.0	87.11	1118.9
(II)											
Block	9	8.74	2.29^{ns}	0.9^{ns}	295^{ns}	59.6^{ns}	2.1^{ns}	34.4^{ns}	50^{ns}	10^{ns}	7435^{ns}
Treatment	612	38.4^{***}	2.2^{*}	3.6^{*}	513^{**}	180^{***}	22.5^{***}	29.45^{*}	49.13^{*}	1177^{***}	253659^{***}
Treatment: check	2	8.59^{ns}	0.8^{ns}	1.8^{ns}	211^{ns}	27^{ns}	7.03^{ns}	$103.^{**}$	65.1^{ns}	75.8^{*}	65909^{**}
Treatment: test and test vs. Check	610	38.5^{***}	2.2^{*}	3.6^{*}	514^{**}	180^{***}	22.6^{***}	29.21^{*}	49.08^{*}	1180^{***}	254275^{***}
Residuals	18	2.57	1.38	2.56	184	26.3	5	14.49	49.36	16.7	9573
CV (%)		3.89	21.47	41.33	25.47	16.0	4.09	5.29	5.36	6.78	14.0
Mean		41.39	5.49	3.87	53.76	31.75	54.72	72.19	130.66	61.39	720.0

Symbols for level of significance:'***' 0.001 '**' 0.01 '*' 0.05, ns is none significant, PLH = Plant Canopy Height (cm), NPB = Number of primary branches, NSB = Number secondary branches, FPPP = Fertile pods per plant, IPPP = Infertile pods per plant, DTF = Days to 50 % flowering, DTP = Days to 50 % podding, DTM = Days to 90 % maturity, TSM = Thousand seed mass, and GY = Grain yield in kg ha^{-1}

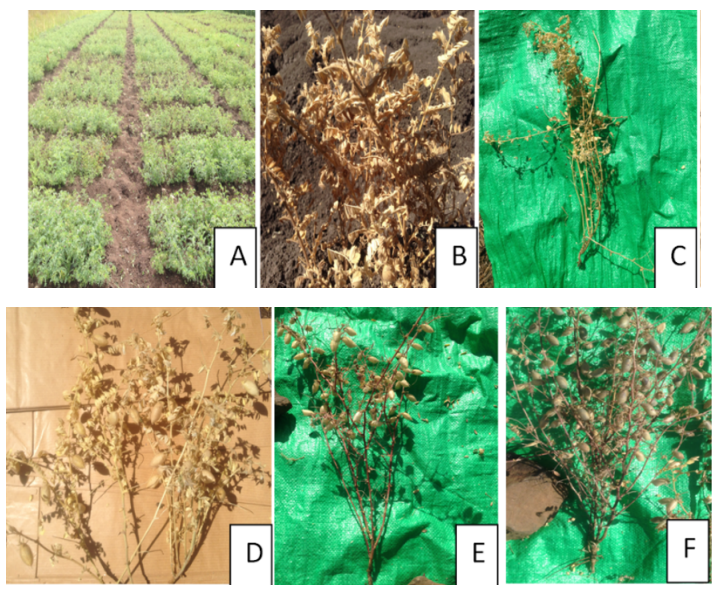

Figure 3: Frost response in chickpea at different growing stages: chickpea genotypes seedling coverage (A), plant death during pre-flowering stage (B), reduced pod setting (C and D) and better pod setting (E and F)

Table 3: Frost survival rate (SR) of 562 Ethiopian chickpea, 83 exotic and 28 improved chickpea genotypes tested at Bakelo, Debre Berhan, Ethiopia, 2018 to 2020 growing seasons

No	SR Rating	2017/2018		2019/2020		Common genotypes for both years	
		No of genotypes	Percentage	No of genotypes	Percentage	No of genotypes	Percentage
1	≥ 0.8	157	23.3	87	12.9	85	19.6
2	≥ 0.6 to < 0.8	273	40.6	199	29.6	155	35.8
3	≥ 0.4- < 0.6	136	20.2	213	31.7	96	22.2
4	≥ 0.2- < 0.4	33	4.9	60	8.9	23	5.3
5	< 0.2	74	11.0	114	16.9	74	17.1
Total		673		673		433	

Table 4: Freezing tolerance rate (FTR) of 673 (562 Ethiopian chickpea, 83 exotic and 28 improved) chickpea genotypes tested at Bakelo, Debre Berhan, Ethiopia from 2018 to 2020 growing seasons

No	FTR Rating	2017/2018		2019/2020		Common genotypes for both years	
		No of genotypes	Percentage	No of genotypes	Percentage	No of genotypes	Percentage
1	1	27	4.0	0	0		
2	2	32	4.8	27	4.0		
3	3	110	16.3	57	8.5		
Sub Total		169	25.1	84	12.5	83	15.5
4	4	261	38.8	154	22.9		
5	5	82	12.2	131	19.5		
6	6	50	7.4	118	17.5		
Sub Total		393	58.4	403	59.9	341	63.9
7	7	29	4.3	57	8.5		
8	8	20	3.0	25	3.7		
9	9	62	9.2	104	15.5		
Sub Total		111	16.5	186	27.6		
Grand Total		673		673		424	

3.3 FREEZING TOLERANCE RATE (FTR)

Freezing tolerance rate with a rating scale of 1-9 has been used for measuring cold stress injury during early vegetative stage or seedling stage in earlier studies (Singh et al. 1989). Based on FTR, 169 (1-3 score) and 84 (2-3 score) genotypes were described as tolerant to highly tolerant, while 504 (4-9) and 590 (4-9) were described as moderately tolerant to highly susceptible genotypes during 2018/2019 and 2019/2020 growing seasons, respectively (Table 4). Eighty three genotypes were rated within the score of 1-3 consistently in both growing season. In this experiment, it is observed that the majority of the genotypes that were resistant at seedling stages failed to resist frost that occured late at reproductive stage. From this result we can conclude that FTR score must be taken throughout the crop stages. Generally, single FTR score may work for areas where frost occurs once in the life cycle of the crop stages, however, in areas where, frost occurs consistently throughout the life cycle of the crop, FTR should be score frequently. In addition, genotypes that showed better FTR value gave either shriveled seeds or empty pods. So, FTR is not able to evaluate the capacity of frost resistance at reproductive stages and the suscep-

Table 5: Seed shriveling score (1-5) of 673 (562 Ethiopian chickpea, 83 exotic and 28 improved chickpea) genotypes tested at Bakelo, Debre Brehan, Ethiopia from 2018 to 2020 growing seasons

No	SSR Rate	2017/2018		2019/2020		Common genotypes for both years	
		No of genotypes	Percentage	No of genotypes	Percentage	No of genotypes	Percentage
1	1	145	21.6	47	7.0	33	12.6
2	2	128	19.0	83	12.3	33	12.6
3	3	126	18.7	154	22.9	42	16.0
4	4	177	26.3	194	28.8	78	29.8
5	5	97	14.4	195	29.0	76	29.0
Total		673		673		262	

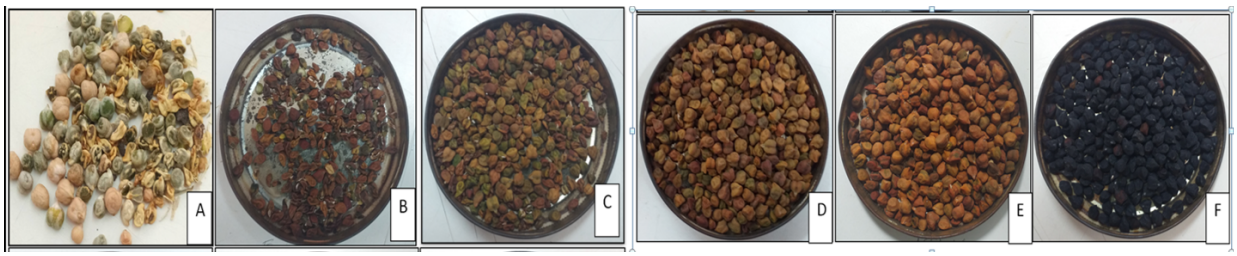

Figure 4: Seeds of chickpea genotypes showing different reaction to frost stress (A and B are very shriveled (Score of 5), C is Shriveled (Score of 4), D is intermediate (Score of 3), E is medium plumped (Score of 2) and F is plumped (Score of 1)

tible genotypes will be overlooked by this approach. FTR is the most important indices used for freezing screening for different crops tested at seedling stage (Badeck et al., 2015; Nezami et al., 2012; Srinivasan et al., 1998; Toker, 2005).

3.4 SEED SHRIVELING SCORE (SSS)

Visual assessment of seed damage by frost stress was done for all the genotypes for both seasons (Table 5). One hundred forty five and 47 genotypes produced plumped seeds (Score of 1: Fig 4E and 4F) in 2018/2019 and 2019/2020 cropping seasons, respectively, while the remaining genotypes gave medium to shriveled seeds (Fig 4A to 4D). Genotypes that were rated as frost resistant based on SR and FTR indices failed to produce plumped seeds, which means that all genotypes that had a better SR and FTR score did not produce plumped seed. However, all genotypes that produced plumped seed had a better SR and FTR value. From this result, it is possible to conclude that SR and FTR indices can indicate frost resistances at seedling or vegetative stages alone. Therefore, SR and FTR indices will not be applicable to screen genotypes for frost resistance at reproductive stage. Visual assessment of frost damaged seed has been applicable also to screen faba bean genotypes for frost resistance variability (Henriquez et al., 2018).

In general to select the frost tolerant promising genotypes, it is advisable to consider frost tolerance related traits and agronomic traits together. Genotypes that are consistently selected by all the parameters are considered as a promising frost tolerant genotype which can be directly taken by farmers or serve as a breeding material for further breeding activities. The selected frost tolerant genotypes will help to stabilize yield and expand the chickpea production areas into Ethiopian highland where chickpea production is not a common practice because of frost damage. In this study, 94 (51 black, 29 brown and 14 white) genotypes were selected as frost tolerant, the remaining genotypes as intermediate to susceptible. The promising frost resistant genotypes were selected with the following criteria i.e. Frost survival rate (>0.75), seed shriveling score (1-2), and freezing tolerance rate (1-4). The selected genotypes are listed in table 6.

Table 6: List of eighty two frost resistant chickpea genotypes selected based on SR (> 0.75), FTR (score of 1,2,3) and seed score (1 and 2)

No	Genotype	Seed Color	Source	No	Genotype	Seed Color	Source	No	Genotype	Seed Color	Source
1	16341-A	Black	EBI	33	208994-A	Brown	EBI	65	30293-A	Brown	EBI
2	207674	Black	EBI	34	235036-A	Brown	EBI	66	207739-B	Brown	EBI
3	30336-A	Black	EBI	35	209016-B	Black	EBI	67	71875	Brown	ICARDA
4	30336-B	Black	EBI	36	209022-A	Black	EBI	68	75095	Brown	ICARDA
5	41004-C	Black	EBI	37	209026-A	Black	EBI	69	140941	Brown	ICARDA
6	41036-B	Black	EBI	38	212589-B	Black	EBI	70	116451	Brown	ICARDA
7	41051-A	Black	EBI	39	212914-B	Black	EBI	71	126302	Brown	ICARDA
8	41081-A	Black	EBI	40	214731-B	Black	EBI	72	9427	Red	ICARDA
9	41107-B	Black	EBI	41	214734-A	Black	EBI	73	128699	White	ICARDA
10	41133-A	Black	EBI	42	215067-A	Black	EBI	74	9632	White	ICARDA
11	41167-C	Black	EBI	43	215190-A	Black	EBI	75	10163	White	ICARDA
12	41206-B	Black	EBI	44	215289-B	Black	EBI	76	140394	White	ICARDA
13	207608	Black	EBI	45	236196-B	Black	EBI	77	7339	White	ICARDA
14	207622	Black	EBI	46	236459-B	Black	EBI	78	70753	White	ICARDA
15	207638	Black	EBI	47	236479-C	Black	EBI	79	73395	White	ICARDA
16	207640	Black	EBI	48	237054-B	Black	EBI	80	69420	White	ICARDA
17	207648	Black	EBI	49	207686	Black	EBI	81	132663	White	ICARDA
18	207652	Black	EBI	50	207664-A	Black	EBI	82	9415	White	ICARDA
19	207668	Black	EBI	51	30349-B	Black	EBI	83	Yelebe	White	EARCs
20	207670	Black	EBI	52	30348-B	Black	EBI	84	Akaki	Red	EARCs
21	207684	Black	EBI	53	41127-B	Black	EBI	85	mariye	Red	EARCs
22	207688-A	Black	EBI	54	207746	Black	EBI	86	Natoli	Red	EARCs
23	207692	Black	EBI	55	207173-C	Black	EBI	87	Teketay	Red	EARCs
24	207712	Black	EBI	56	41075-C	Brown	EBI	88	kutaye	Brown	EARCs
25	207714	Black	EBI	57	41093-B	Brown	EBI	89	Teji	White	EARCs
26	207728-A	Black	EBI	58	41255-B	Brown	EBI	90	Shola	White	EARCs
27	207730	Black	EBI	59	207175-A	Brown	EBI	91	Worku	Red	EARCs
28	207748	Black	EBI	60	207635-C	Brown	EBI	92	Harbu	White	EARCs
29	208988-A	Red	EBI	61	30350-B	Red	EBI	93	Dalota	Brown	EARCs
30	209026-B	Red	EBI	62	41301-B	Red	EBI	94	Mastewal	Brown	EARCs
31	227152-B	Red	EBI	63	207766	Black	EBI				
32	30334-C	Red	EBI	64	207770	Black	EBI				

EBI = Ethiopian Biodiversity Institute, ICARDA is International International Center for Agricultural Research in the Dry Areas, EARCs = Ethiopian Agricultural Research Centers

Table 7: Phenotypic Pearson's correlation matrix for 9 traits in chickpea 673 (562 Ethiopian chickpea, 83 exotic and 28 improved chickpea) genotypes tested at Bakelo, Debre Berhan, Ethiopia from 2018/2019 (above diagonal) to 2019/2020 (below diagonal) growing seasons

	PLH	NPB	NSB	FPPP	IPPP	DTF	DTM	TSM	GY	SR	FTR	SSS	FC	SLP	SC
PLH	0	0.65**	0.43**	0.51**	-0.3**	0.13**	0.13**	0.64**	0.59**	0.68**	-0.66**	-0.48**	-0.52**	0.36**	-044**
NPB	0.64**	0	0.71**	0.68**	0.01**	0.12**	0.12**	0.35**	0.39**	0.43**	-0.40**	-0.31**	0.41**	0.28**	-0.33**
NSB	0.47**	0.68**	0	0.69**	0.04ns	0.13**	0.19**	0.18**	0.23**	0.25**	-0.25**	-0.18**	0.20**	0.16**	-0.17**
FPPP	0.52**	0.69**	0.69**	0	-0.0ns	-0.1ns	0.12**	0.28**	0.33**	0.37**	-0.36**	-0.3**	0.28ns	0.22**	-0.26**
IPPP	-0.2**	0.17**	0.19**	0.22**	0	0.08*	0.06ns	-0.4**	-0.7**	-0.6**	0.56**	0.70**	0.1ns	-0.3**	0.11**
DTF	0.01ns	0.06ns	0.15**	-0.2**	-0.1ns		0.28**	-0.0ns	-0.2**	-0.2**	0.16**	0.15**	-0.4**	-0.2**	0.30**
DTM	0.02ns	0.00ns	0.06ns	-0.2**	-0.1ns	0.30**	0	-0.0ns	-0.08*	-0.08*	0.03ns	0.01ns	-0.2**	-0.1ns	0.11**
TSM	0.47**	0.24**	0.22**	0.20**	-0.5**	0.14**	0.13**	0	0.69**	0.77**	-0.76**	-0.60**	-0.27**	0.32**	-0.27**
GY	0.42**	0.20**	0.18**	0.21**	-0.6**	0.0ns	0.00ns	0.72**	0	0.86**	-0.84**	-0.8**	0.43**	0.59**	-0.52**
SR	0.65**	0.4**	0.3**	0.38**	-0.4**	0.0ns	-0.1**	0.66**	0.73*	0	-0.90**	-0.79**	0.41**	0.47**	-0.47**
FTR	-0.6**	-0.36**	-0.3**	-0.3**	0.44**	-0.1ns	0.09*	-0.6**	-0.7**	-0.9**	0	0.77**	-0.37**	-0.6**	0.53**
SSS	-0.5**	-0.18**	-0.2**	-0.2**	0.54**	-0.1**	-0.1ns	-0.8**	-0.8**	-0.8**	0.75**	0	-0.32ns	-0.5**	0.44**
FC	0.57**	0.46ns	0.25**	0.41**	0.22**	-0.4**	-0.2**	0.11*	-0.21*	-0.3**	-0.20**	-0.11**	0	0.57**	-0.79**
SLP	0.43**	0.30**	0.18**	0.31**	-0.4**	-0.2**	-0.1ns	0.37**	0.48**	0.47**	-0.49**	-0.51**	0.60**	0	-0.80**
SC	-0.51*	-0.4**	-0.2**	-0.3**	0.11**	0.30**	0.18**	-0.2ns	-0.3**	-0.5**	0.40**	0.32**	-0.79**	-0.8**	0

ns = non significant; **=Correlation is significant at the 0.01 level (2-tailed); *=Correlation is significant at the 0.05 level (2-tailed), PLH = Plant Canopy Height (cm), NPB = Number of primary branches, NSB = Number secondary branches, FPPP = Fertile pods per plant, IPPP = Infertile pods per plant, DTF = Days to 50 % flowering, DTP = Days to 50 % podding, DTM = Days to 90 % maturity, TSM = Thousand Seed Mass, GY = Grain yield in kg ha^{-1}, SR = Frost survival rate, FTR = Frost tolerance rate, SSS = Seed shriveling score, FC = Flower Color, SLP = Stem/leaf pigmentation, and SC = Seed color

3.5 PHENOTYPIC CORRELATION COEFFICIENT

The phenotypic association of agronomic and frost tolerance related traits were analyzed for each genotype and the following result were obtained (Table 7). Most of the frost tolerance related traits have shown a strong significant relationship with agronomic traits. Grain yield was positively and significantly correlated (p < 0.01) with fertile pod per plant (0.33 and 0.21), thousand seed mass (0.69 and 0.72), SR (0.86 and 0.73), and stem/leaf pigmentation (0.59 and 0.48), while a strong negative correlation was seen for infertile pod per plant (-0.7 and -0.6), FTR (-0.70 and -0.6), SSS (-0.8 and -0.8), seed color (-0.52 and -0.30), and flower color (-0.43 and -0.21) for 2018/2019 and 2019/2020 growing seasons, respectively. It was observed that genotypes having strong stem/leaf pigmentation had shown a good performance in all agronomic traits and had also a better SR and FTR score. Similarly, flower and seed color had shown also a strong correlation with agronomic performances. Genotypes having pink flower and black seed color had better performances than the ones with white flower and white seed colored ones. From this result, the selection of genotypes having strong stem/leaf pigmentations and genotypes with black seeded chickpea types and pink flower would greatly assist plant breeders to develop frost resistant variety to reduce the risk of frost damages. The majority of black seeded chickpea performed well in agronomic traits and SR and FTR score was higher than brown and white seeded chickpea types. The majority of white seeded chickpea types were highly susceptible to frost stress. Brown seeded chickpea with strong stem/leaf pigmentation exhibited better reaction to frost stress than the one with brown seeded with week stem/leaf pigmentation. The result agree with previous findings in faba bean where genotypes with white flower being susceptible to frost stress, while tannin-containing genotypes and wild relatives are more tolerant (Henriquez et al., 2018; Inci & Toker, 2011). Frost stress causes accumulation of anthocyanins in the basal part of the stem, branches and leaves (Croser et al., 2003). Bhasker et al. (2018) indicated that the accumulation of anthocyanin due to high temperature has a positive relation with high grain yield because of the induction of antioxidant defense system. Frost damage has strong correlation with lower yield (Henriquez et al., 2018; Kanouni et al., 2009).

From this result it is possible to conclude that the presence of pigmentation induced by frost stress can be a good indicator for frost tolerance mechanism.

4 CONCLUSIONS

This experiment has shown that the degree of frost damage varied at different crop stages. The effect of frost was not seen on seed germination and seedling establishment. However, considerable frost damage was observed at vegetative and reproductive stages for most genotypes. The capacities of genotypes for frost tolerance were estimated using freezing tolerance rate (FTR) and frost survival rate (SR) and their agronomic performances. Eighty three and 85 genotypes were selected based on FTR and SR respectively. However, both indices are not able to evaluate frost resistance of the genotypes at reproductive stage, if the frost occurs consistently throughout the crop stages. Genotypes having good SR and FTR value produced shriveled seed and empty pods due to frost stress that occurred later at flowering and seed development stages. Therefore, to select the frost tolerant potential genotypes, it is advisable to consider SR and FTR values, pod setting, seed shriveling score, and grain yield together. Genotypes that are consistently selected by all these parameters are considered as promising frost tolerant genotypes. In addition, in areas where frost occurs consistently during the seedling and vegetative stages of the crop only, the selection of frost resistance at these stages by considering less FTR and high SR values are enough to select the frost resistant promising genotypes. The effect of frost stress to chickpea genotypes are variable depending on seed color type, presence and absence of stem/leaf pigmentation and different level of stem/leaf pigmentation. Chickpea genotypes with black seeded and/or having strong stem/leaf pigmentation performed well for frost stress reaction. From these observations, it is possible to conclude that stem/leaf pigmentation and black seeded color might be linked to a gene that confers frost resistance in chickpea. From this experiment, 94 genotypes were identified to be frost tolerant genotypes which can be taken by plant breeder for frost tolerant chickpea variety development program attesting that Ethiopian checkpea genotypes have a potential source for frost tolerance trait. Identification of the mechanism of stem/leaf pigmentation and black seed color for frost resistance is required. Also, identification of quantitative trait loci (QTLs) associated with gene controlling frost tolerances in chickpea is equally important.

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Diallel analysis of the duration of vegetation period in tomato (*Solanum lycopersicum* L.) with increased lycopene content in the fruit

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Diallel analysis of the duration of vegetation period in tomato (*Solanum lycopersicum* L.) with increased lycopene content in the fruit

Abstract: Five parental forms of tomato *(Solanum lycopersicum* L.) and twenty hybrids F_1, were studied which were obtained by the complete diallel scheme (5 x 5). For genetic analysis (by Hayman, 1954; Jinks,1954) was used line №477 *(sp,u)*, variety Alya *(sp)* with reduced duration of vegetation period and three collection samples with the high lycopene content in fruits: Dark Green *(hp-2^{dg}),* MO 112 *(hp)*, T-3627 *(B^{c})*. The effects of the general (GCA) and specific combining ability (SCA) of the duration of vegetation period were determined and established character of inheritance. According to the results of research, the duration of vegetation period is controlled by additive-dominant genetic system. Inheritance occurs by type of over dominance, and in dry and hot summers which led to the prolongation of the duration of vegetation period, there is a tendency to incomplete dominance, but it is apocryphal. In genetic control a major role play non-additive effects of genes. The best reliable effects of the general combining ability (GCA) had line №477 and variety Alya. They can be recommended for the creation of heterotic hybrids and varieties.

Key words: combining ability; diallel analysis; hybrid; duration of vegetation period; tomato

Dialelna analiza trajanja rastne dobe paradižnika (*Solanum lycopersicum* L.) s povečano vsebnostjo likopena v plodovih

Izvleček: V raziskavi je bilo preučevanih pet starševskih linij paradižnika *(Solanum lycopersicum* L.) in dvajset F_1 križancev, ki so bili pridobljeni po popolni dialelni shemi (5 x 5). Za genetsko analizo po metodah Haymana (1954) in Jinksa (1954) je bila uporabljena linija №477 *(sp,u)* in sorta Alya *(sp)* s skrajšanim trajanjem vegetacijske dobe in trije vzorci iz zbirke s povečano vsebnostjo likopena v plodovih in sicer: 'Dark Green' *(hp-2^{dg}),* MO 112 *(hp)*, in T-3627 *(B^{c})*. Določeni so bili učinki splošne (GCA) in specifične kombinacijske sposobnosti (SCA) za trajanje vegetacijske dobe in ustaljene znake dedovanja. Glede na izsledke raziskave je trajanje vegetacijske dobe nadzorovano z aditivno dominantnim genskim sistemom. Dedovanje se pojavlja z načinom naddominace, kar v suhih in vročih poletjih vodi do podaljšanja vegetacijske dobe. Obstaja tudi nagnenje k nepopolni dominanci, a tega ne moremo zagotovo potrditi. Pri genetski kontroli igrajo glavno vlogo neaditivni učinki genov. Najbolj zanesljive učinke splošne kombinacijske sposobnosti (GCA) je imela linija №477 in sorta Alya. Ti dve bi lahko priporočali za tvorbo heterotičnih križancev in sort.

Ključne besede: kombinacijska sposobnost; dialelna analiza; križanec; trajanje vegetacijske dobe; paradižnik

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1 INTRODUCTION

Tomato is one of the most important vegetable plants, which ranks first in the world on terms of area grown in both open and protected areas (Distefano et al., 2020). In EU 2.1 million hectares of land are used for growing vegetables (Bogevska et al., 2017). The largest producer is Italy (35.5 %). The average consumption of fresh tomato per one person is 39 kg on year (Cook, 2019). The fruits of this plant have high nutritional and dietary properties (Alenazi et al., 2020).

One of the main directions of tomato breeding is creation of varieties and hybrids with high content of carotenoids, in particular lycopene, which, unlike others, not only retains quality after heat treatment, but also improves them (Wai et al.,2020). Lycopene is a unique natural antioxidant that has anti-cancer properties (Timofeev et al., 1972; Sharma et al., 2019). This is a biologically active compound that can prevent damage to cells by so-called free radicals, almost three times more active than b-carotene. Lycopene is a natural remedy for the prevention of cardiovascular and cancer diseases (Li & Xu, 2014; Pouchieu et al., 2014; Tang et al., 2014; Li et al., 2018).

The samples of tomato with mutant genes *hp-2^{dg}, hp, B^{c}*, containing increased lycopene content in fruits, have a number of pleiotropic effects, including the weakening of the initial growth (Galpas et al., 2008; Samovol & Kondratenko, 2018; Diretto et al., 2020). As a result, the seeds germinate slowly, seedling growth is slowed and weakened, which leads to prolongation of the duration of vegetation period, namely, this -determines the distribution of culture (Zhuchenko, 1991; Dannehl et al., 2021).

The main task in creating the source material of tomatoes with high lycopene content in the fruit is to reduce the duration of vegetation period. This will allow expand range of genetic diversity of tomatoes to increase efficiency of breeding work (Cimo et al., 2020).

Assessment of combining ability of parental forms allows predict the results of future crosses and focus on the creation of promising breeding material. High heterosis is manifested in those hybrid combinations in which at least one of the components of crossing is a sample with high general combining ability (GCA). The most complete and comprehensive combining ability is assessed in diallel crosses. In the work was used method of diallel analysis of quantitative features (Yates, 1947; Hayman, 1954). It is used to quickly obtain data on the nature of the inheritance of quantitative traits that are controlled by groups of genes (polygenes) (Fedin, 1970).

2 MATERIAL AND METHODS

The purpose of this research work was to identify parental forms with high general and specific combining ability. Genetic analysis provided parameters and character or inheritance of the duration of vegetation period.

The research was conducted in 2017-2019 in the fields of breeding and seed crop rotation of Cherkasy research station of the National Scientific Center «Institute of Agriculture of the National Academy of Agrarian Sciences of Ukraine», located in the village Kholodnyansky (49^{0}11'N at 31^{0}52'E) Cherkasy region, Ukraine. For genetic analysis (Hayman, 1954; Jinks, 1954) were used the line №477 *(sp,u)* and variety Alya *(sp)* with reduced duration of vegetation period and three collection samples with high lycopene content in fruits: 'Dark Green' *(hp-2^{dg})*, 'MO 112' *(hp)* and 'T-3627' *(B^{c})*.Five parental forms of tomato and twenty F_1 hybrids were analysed which were obtained by the complete diallel scheme (5 x 5).

The research based on the method of one-factor experiments (Dospekhov, 1985). The duration of vegetation period (period of full germination (more than 75 % of plants) prior to beginning of ripening of fruits of the first raceme) was determined according to «Methodology for conducting expertise of potato plants and groups of vegetable, melon, spicy-tasting for the expiration in Ukraine». Evaluation of combining ability regarding the duration of vegetation period was performed according to first scheme (Griffing, 1956) with the matrix of crosses and test *p2 (*direct and reciprocal crosses + parental forms), where p - is the number of parental forms.

3 RESULTS AND DISCUSSION

The analysis of variance of a one - factor experiment made it possible to establish significant differences between hybrids. That is, of the duration of vegetation period is different, and on the combining ability can be expected differences in the studied samples, variety and line (general or specific). The sum of the squares in hybrids is due to genotypic differences (Table 1).

Parental line, variety, samples and hybrids differed in the magnitude of the expression of the trait (Table 2).

The longest duration of vegetation period were in samples 'Dark reen' (104.3-121.3 days), 'MO 112' (100.0-110.7 days) and 'T-3627' (97.0-112.3 days). Shortest duration of vegetation period were in line '№477' (94.0-102.0 days) and variety Alya (96-108.3). The average values in hybrids with the participation of samples 'T-3627', 'Dark Green' and 'MO 112' are the largest, and are 95.3-110.9 days; 97.7-114.5 days and 95.8-110.7 days respectively. For the participation of line №477 and variety Alya

Table 1: Analysis of variance of the duration of vegetation period

Years	Type of scattering	Sum of squares	Degree of freedom	Middle square	F calc.	F tabl.
2017	General	1228.0	74			
	Repetitions	0.8	2			
	Genotype	1161.3	24	48.4*	35.28	1.74
	Residual	65.8	48	1.4		
	LSD_{05}			2.0		
2018	General	918.0	74			
	Repetitions	6.6	2			
	Genotype	824.6	24	34.4*	19.01	1.74
	Residual	86.8	48	1.8		
	LSD_{05}			2.2		
2019	General	1766.7	74			
	Repetitions	12.4	2			
	Genotype	1539.3	24	64.1*	14.32	1.74
	Residual	215.0	48	4.5		
	LSD_{05}			3.5		

* Significant at 5 % level

Table 2: Average value of the duration of vegetation period in the parental line, variety, samples, *($\bar{x}p$)* and hybrids *($\bar{x}F_1$)*, days

Line, variety, samples	Years of research					
	2017		2018		2019	
	P	F_1	P	F_1	P	F_1
№477	94.0	93.0	97.3	98.5	102.0	105.2
Alya	96.0	94.2	99.3	100.3	108.3	109.3
Dark Green	105.3	97.7	104.3	102.0	121.3	114.5
MO 112	103.3	95.8	100.0	102.2	110.7	110.7
T-3627	97.0	95.9	103.7	103.5	112.3	110.0
$\bar{x}_f$	99.1	95.3	100.9	101.3	110.9	109.9
LSD_{05}	1.95		2.24		3.53	

values is lower than the averages group on the parental forms during the three years of research.

The results of analysis of variance of combining ability (Table 3) indicate on significant differences (general and specific).

In addition, a significant reciprocal effect over three years of research. In this case, when is a reciprocal effect, it can be eliminated by averaging the values of the trait in direct and inverse hybrids and take the same average values.

Studies have shown that by the duration of vegetation period the best (in this case the lowest, negative, because it is a best indicator for the trait - a reduced period of fruit ripening) significant effects of general combining ability (GCA) for three years of research had line №477 (from minus 4.70 to minus 2.34); during two years of research variety Alya (from minus 1.11 to minus 0.99) (Table 4). Samples that have significant positive effects GCA – 'Dark Green' (from 0.74 to 4.53) and 'MO 112' (from 0.46 to 0.84) contribute to the prolongation duration of vegetation period.

Table 3: Analysis of variance of the combining ability on the duration of vegetation period

Years	Type of scattering	Sum of squares	Degrees of freedom	Middle square	F calc.	F tabl.
2017	Hybrids	1161.3	24	48.4*	35.3	1.79
	GCA	131.1	4	32.8*	71.7	2.61
	SCA	153.2	10	15.3*	33.5	2.08
	Reciprocals	102.7	10	10.3*	22.5	2.08
	Residual	22.0	48	0.5*		
2018	Hybrids	807.6	24	33.6*	19.1	1.79
	GCA	145.8	4	36.5*	62.0	2.61
	SCA	47.2	10	4.7*	8.0	2.08
	Reciprocals	76.2	10	7.6*	13.0	2.08
	Residual	28.2	48	0.6*		
2019	Hybrids	1539.3	24	64.1*	14.3	1.79
	GCA	437.3	4	109.3*	73.2	2.61
	SCA	32.1	10	3.2*	2.2	2.08
	Reciprocals	43.7	10	4.4*	2.9	2.08
	Residual	71.7	48	1.5*		

* Significant at 5 % level

Table 4: Evaluation of the effects of general combining ability (GCA) of the duration of vegetation period

Line, variety, samples	Years		
	2017	2018	2019
№477	-2.34*	-2.76*	-4.70*
Alya	-1.11*	-0.99*	-0.67
Dark Green	2.43*	0.74*	4.53*
MO 112	0.46*	0.84*	0.80*
T-3627	0.56*	2.17*	0.03
LSD_{05}	0.38	0.44	0.69

* Significant at 5 % level

Significant differences of the specific combining ability (SCA) that was observed over the years of research indicated that some hybrid combinations within the variety differed significantly from the average value and in the inheritance of traits where non-additive effects of genes were involved. In order to identify samples and varieties with high or low specific combining ability (SCA), for each parent formthe variance for comparison with the overall average value were calculated (Table 5).

We found that high (negative) reliable values of the specific combining ability (SCA) for one year of research were in line №477 (minus 0.59), samples 'MO 112' (minus 0.36) and 'Dark Green' (minus 0.22). The best values (SCA) were in hybrid combinations: '№477' / 'Dark Green' (minus 2.73), 'Dark Green' / 'MO 112' (minus 2.19), '№477' / 'MO 112' (minus 1.59), '№ 477' / 'Alya' (minus 1.21), '№477' / 'T-3627' (minus 1.21), 'Alya' / 'Dark Green' (minus 0.96), 'Dark Green' / 'T-3627' (minus 1.83).

Comparison o the variance of effects of the general (δ_{g1}^2) and specific (δ_{Si}^2) combining ability determined that in line №477 (shortened of the duration of vegetation period, high negative significant effects of GCA) and in the sample 'Dark Green' (the longest duration of the vegetation period, low positive significant effects of GCA) years of research - the predominance of non-additive effects of genes in genetic control of the trait was observed during all three years of research.

For two years of research was established advantage of non-additive effects of genes in variety Alya and sample 'T-3627'. In sample 'MO 112' predominate additive effects of genes.

Table 5: Estimation of variances of general combining ability (GCA) and specific combining ability (SCA) for line, variety and samples on the duration of vegetation period of 2017-2019

Line, variety, sample	Years	Alya	Dark green	MO 112	T-3627	δ_{Si}^2	δ_{g1}^2
№ 477	2017	1.47*	-2.73*	-1.59*	-0,53	2.76*	5.41
	2018	-1.21*	-0.77	1.63*	-1.21*	1.10*	7.53
	2019	-1.40	-0.10	0.30	-0.27	-0.59	21.88
Alya	2017		-0.96*	-2.99*	-0.43	2.72*	1.17
	2018		0.13	-0.64	1.69*	0.75	0.89
	2019		-0.47	1.43*	0.70	0.06	0.24
Dark Green	2017			-2.19*	-0.71	3.07*	5.84
	2018			-0.21	-0.71	-0.15	0.46
	2019			0.07	-1.83*	-0.22	20.31
MO 112	2017				-0.33	3.76*	0.15
	2018				2.19*	1.54*	0.62
	2019				-0.93	-0.36	0.43
T-3627	2017					-0.08*	0.25
	2018					1.97*	4.62
	2019					0.08	-0.21
Average value	2017					2.45	
	2018					1.04	
	2019					-0.21	

* Significant at 5 % level

Note. δ_{Si}^2 - variance of the effect of specific combining ability; δ_{g1}^2 - variance of the effect of general combining ability.

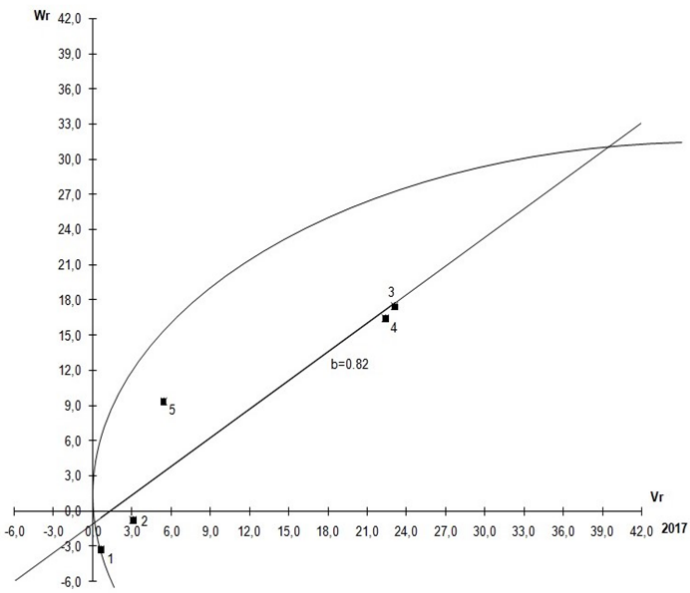

Figure 1: Graph of the dependence of *Wr* by *Vr*, 2017.
1 - Line №477; 2 - Variety Alya; 3 - Sample 'Dark Green'; 4 - Sample 'MO 112'; 5 - Sample 'T-3627'.

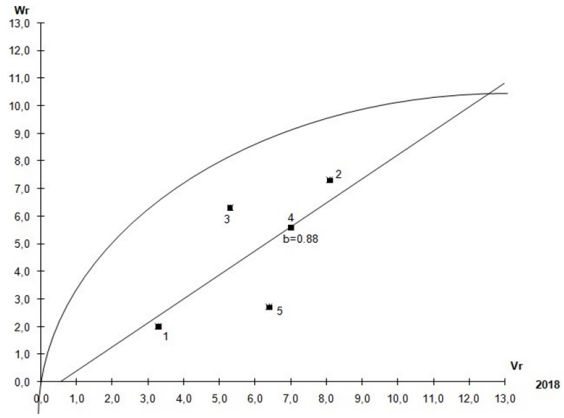

Figure 2: Graph of the dependence of *Wr* by *Vr*, 2018.
1 - Line №477; 2 - Variety Alya; 3 - Sample 'Dark Green'; 4 - Sample 'MO 112'; 5 - Sample 'T-3627'.

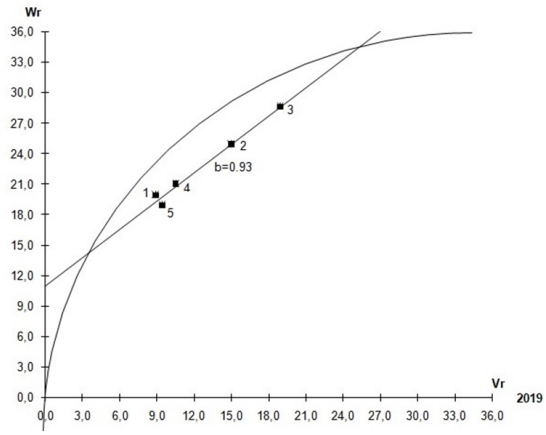

Figure 3: Graph of the dependence of *Wr* by *Vr*, 2017.
1 - Line №477; 2 - Variety Alya; 3 - Sample 'Dark Green'; 4 - Sample 'MO 112'; 5 - Sample 'T-3627'.

Thus, it was found that line №477 and variety Alya should be used to create heterotic hybrids and varieties.

Genetic analysis (Hayman, 1954) checks the homogeneity of the difference between covariance and variance of *Wr-Vr* through Student's t test did not reveal epistatic interaction of genes. Graphic analysis shows that the inheritanceof the trait duration of vegetation period involved additive and non-additive effects of genes (the regression coefficients are reliable and do not differ from one), that is, the regression line does not deviate from the unit slope line (Fig. 1-3).

This is confirmed high value of the components *D* and *H1* that characterize the variability of the trait, which is due to the additive and dominant effects of genes.

Values of *H1* and *H2* are unequal, from which it is possible to conclude that alleles that positively and negatively determine the trait are unevenly distributed between the parental forms. The uneven ratio of genes with positive and negative effects is indicates value of *H2/4H1*, which was 0.14-0.21.

The parameter $\frac{\sqrt{4DH1+F}}{\sqrt{4DH1-F}}$ in 2017 and 2019 varied in the range of 1.42-1.79, which indicates an excess of dominant alleles in the studied samples; in 2018 - 0.81 - recessive alleles.

The value of parameter *D*, which measures additive variability in the population, was lower in comparison with *H1*, which measures dominant variability over two years of research (Table 6). Values *H1>H2* indicates an unequal ratio of positive and negative effects.

Positive significant values of the indicator *F (F > 0)* in 2017 and 2019 indicate the predominance of dominant alleles in the studied line, variety and samples, in 2018 prevailed recessive alleles.

For two years of research, the average degree of dominance was 1.23-1.40. The regression line intersects the negative part of the axis *Wr*, so it can be argued about dominance in all loci. In 2019, in dry and hot summers degree of dominance was 0.35, there is a tendency to incomplete dominance, but it is apocryphal.

Table 6: Genetic parameters of the duration of vegetation period

Parameters	Years		
	2017	2018	2019
D	23.74 ± 3.11	8.27 ± 1.29	47.7 ± 0.47
F	16.59 ± 7.77	-2.19 ± 3.23	5.89 ± 1.18
H_1	35.87 ± 7.90	12.62 ± 3.29	5.95 ± 1.20
H_2	29.73 ± 7.62	8.26 ± 3.17	3.43 ± 1.16
h^2	526.87 ± 5.14	4.29 ± 2.14	35.04 ± 0.78
E	0.46 ± 1.27	0.59 ± 0.53	1.49 ± 0.19
H_1/D	1.51	1.53	0.13
$\sqrt{H_1}/D$	1.23	1.24	0.35
$H_2/4H_1$	0.21	0.16	0.14
$\frac{\sqrt{4DH1+F}}{\sqrt{4DH1-F}}$	1.79	0.81	1.42
h^2/H_2	17.72	0.52	10.21
Conditionally dominant (CD)	94.03	99.11	100.96
Conditionally recessive(CR)	108.35	102.52	125.47

Table 7: Estimation of the direction of dominance *(F)* by the duration of vegetation period of tomato for each parental form and their hybrids

Line,variety, samples	Years		
	2017	2018	2019
№477	59.38±10.58*	8.73±4.40*	18.62±1.61*
Alya	49.33±10.58*	-11.37±4.40*	-3.58±1.61*
Dark Green	-26.87±10.58*	-3.79±4.40*	-18.66±1.61*
MO112	-23.60±10.58*	-5.73±4.40*	13.39±1.61*
T-3627	24.71±10.58*	1.19±4.40*	19.67±1.61*

* Significant at 5 % level

Table 8: The results of correlation and regression analyzes of the duration of vegetation period

Indicator	2017	2018	2019
Correlation *(r)* between *Wr* and *Vr*	0.93	0.66	0.99
Regression (b_1) between *Wr* and *Vr*	0.82	0.81	0.93
Correlation *(r)* between $\bar{x}p$ and *Wr* + *Vr*	0.98	0.20	0.70
Regression (b_2) between $\bar{x}p$ and *Wr* + *Vr*	0.24	0.15	0.59

The parameter *h2/H2* indicates the number of groups of genes (more precisely, the number of effective factors) that show dominance and control the genotypic variation of the trait. It is determined in the case of a significant difference between parameters *h2/H2*. This difference existed in 2017 and 2019 that allowed calculate value of this indicator, which was 10.21-17.72. That is, studied samples differ in seventeen groups of genes that show the effect of dominance.

In table 7 the indicators of genetic component F are shown, which reflects the relative contribution of additive and dominant effects of genes in the phenotypic manifestation on the trait in F_1 hybrids.

Significant positive effects for three years of research were observed in line №477 and sample 'T-3627', which indicates the predominance of dominant alleles. In the sample 'Dark Green' for three years, sample 'MO 112' and variety Alya - for two years of research - in 2017 and 2018, 2018 and 2019 recpectively, prevailed recessive alleles.

High correlation coefficients between covariance *(Wr)* and variance *(Vr)*, regression coefficients b_1 between *Wr* and *Vr* indicate that the actual regression lines do not differ significantly from the unit slope (Table 8).

The correlation coefficient between the average values of parents ($\bar{x}p$) and sum (*Wr* + *Vr*) in 2017 and 2019 was 0.70-0.98.

The high positive value of correlation coefficient indicates the existence of a relationship between of the duration of vegetation period in line, variety and samples and the presence of recessive genes (parental forms with the longest duration of vegetation period have the largest number of recessive alleles). Indicators of the high positive correlation indicate that recessive genes determine the increase of trait, therefore prolongation of the duration of vegetation period is controlled by recessive alleles.

With participation of correlation coefficient between the mean values of parental forms (in parents ($\bar{x}p$) and sum *(Wr + Vr)* the theoretical values $W_{dom} + V_{dom}$ and $W_{rec} + V_{rec}$ for line, variety and samples were determined, which had dominant and recessive alleles.

The line, variety or sample that theoretically has all dominant alleles of the studied parental forms will have $W_{dom} + V_{dom}$ 94.04 (2017), 99.11 (2018) and 100.96 (2019). The theoretical value of parental form with the largest number of recessive genes was 108.35 (2017), 102.52 (2018) and 125.47 (2019).

More complete information about manifestation of dominant and recessive effects was obtained using regression graphs (Fig. 1-3). If the paternal forms have many dominant genes, then its offspring F_1 will have a level of trait near to it. In this case, the variance *(Vr)* will be small and this sample will coincide with the regression line, near to coordinate origin.

Therefore, variety that has many recessive genes, will be placed on the regression line at the top right, as his offspring from crossing with samples that containing many dominant genes will give a large value variation (Vr). In 2017, according to the result of assessment F_1, line №477 and variety Alya approached the point with maximum dominance. Sample 'Dark Green' which had 50 percent recessive alleles approached the point with the highest recessiveness.

In 2018, the position of samples on regression graph has changed; line №477 and sample 'T-3627' were located in zone with the highest dominance. Samples 'MO 112', 'Dark Green' and variety Alya were located in the middle area of regression line, near to zone with the greatest recessiveness.

In 2019, line №477, samples 'MO 112' and 'T-3627' were located in zone with the highest dominance. The position of variety Alya is located in the middle area of regression line; the position of sample 'Dark Green' that characterized prolongation of the duration of vegetation period is located near to zone with the greatest recessiveness.

4 CONCLUSIONS

Thus, it is established that the duration of vegetation period is controlled by additive-dominant genetic system. The main role in genetic control of the trait is in non-additive effects of genes. Inheritance occurs by the type of over dominance, and in dry and hot summers leeds to the prolongation of the duration of vegetation period, there is a tendency to incomplete dominance, but it is apocryphal. The direction of dominance varies from

dominance of genes that reduce manifestation of the trait to its absence.

The study showed that the duration of vegetation period is the best reliable effects of general combining ability (GCA) for three years of research in line №477 and in two years of research in variety Alya. They can be recommended for the creation of heterotic hybrids, as well as from the above-mentioned hybrid combinations with high values of specific combining ability (SCA) - varieties.

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High resistance of *Panicum miliaceum* L. to phenanthrene toxicity based on growth response and antioxidant system assessment

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High resistance of *Panicum miliaceum* L. to phenanthrene toxicity based on growth response and antioxidant system assessment

Abstract: Polycyclic aromatic hydrocarbons are a group of organic pollutants influencing different aspects of plants physiology. Physiological responses associated with the impact of phenanthrene (500, 1000, 1500, 2000 ppm) were analysed on *Panicum miliaceum* L. Seed germination was delayed in all treatments and 2000 ppm of phenanthrene (PHE) significantly retarded the germination rate (28 %) compared to control. The results revealed after 30 day of cultivation, only 1500 and 2000 ppm of PHE had negative impacts on growth parameters as well as photosynthetic pigment contents. Plants exposed to 500 and 1000 ppm of PHE showed an increase in the growth parameters without any symptoms of toxicity, indicating the high tolerance of seedlings to PHE. The activities of antioxidant enzymes were elevated in treated plants. In higher concentrations, H_2O_2 content also increased despite a reduction in malondialdehyde content. Furthermore, PHE had no effect on root phenol and shoot flavonoid contents and on shoot and root protein contents. Taken together, only higher concentrations of PHE triggered oxidative stress. It can be concluded PHE was not very toxic to *P. miliaceum* probably because of higher activity of antioxidant system involving in elimination of produced ROS even in plants treated by PHE higher concentrations.

Key words: polycyclic aromatic hydrocarbons; *Panicum miliaceum*; phenanthrene; physiological responses; toxicity

Velika odpornost navadnega prosa (*Panicum miliaceum* L.) na strupenost fenantrena temelji na rastnem odzivu in antioksidacijskem sistemu

Izvleček: Policiklični aromatski ogljikovodiki so skupina organskih onesnažil, ki vplivajo na različne vidike fiziologije rastlin. Fiziološki odzivi, povezani z vplivom fenantrena (PHE; 500, 1000, 1500, 2000 ppm) so bili analizirani na navadnem prosu (*Panicum miliaceum* L.). Kalitev semen je bila zapoznela pri vseh obravnavanjih, koncentracija fenantrena 2000 ppm je značilno zmanjšala hitrost kalitve (28 %) v primerjavi s kontrolo. Rezultati so pokazali, da se je negativni učinek fenantrena na rastne parametre in fotosintezena barvila v koncentracijah 1500 in 2000 ppm pokazal šele po 30 dnevih gojenja. Rastline, ki so bile izpostavljene koncentracijam 500 in 1000 ppm PHE so pokazale povečanje v rastnih parametrih, brez vsakršnih znakov zastrupitve, kar kaže na veliko tolerance teh rastlin na fenantren. V obravnavanih rastlinah se je povečala aktivnost antioksidacijskih encimov. Pri obravnavanjih z večjimi koncentracijami se je povečala tudi koncentracija H_2O_2, kljub zmanjšanju vsebnosti malondialdehida. Dodatno, PHE ni imel nobenega učinka na vsebnost fenolov v koreninah in flavonoidov v poganjkih, kot tudi ne na vsebnost beljakovin v koreninah in poganjkih. Zaključimo lahko, da fenantren ni bil zelo strupen za navadno proso, verjetno zaradi večje aktivnosti antioksidacijskega sistema, ki je preprečil tvorbo ROS, celo pri rastlinah obravnavanimi z večjimi koncentracijami PHE

Ključne besede: policiklični aromatski ogljikovodiki; *Panicum miliaceum*; fenantren; fiziološki odziv; strupenost

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1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) belong to persistent organic pollutants (POPs) consisting of 2–7 condensed aromatic rings that are arranged in various structural configurations. They have been detected in various concentrations in different environments. These pollutants are derived from either natural or anthropogenic activities mainly associated with industrialization and urbanization (Krzebietke et al., 2018; Pogorzelec & Piekarska, 2018; Wiłkomirski et al., 2018). PAHs are mostly known for being highly toxic, mutagenic, teratogenic, and carcinogenic (Tian et al., 2019). PAHs hydrophobic nature leads to their accumulation and enrichment in soils, which is the reason for the necessity of the remediation of contaminated sites (Pretorius et al., 2018). Their physicochemical characteristics are low solubility in water, low vapour pressure, and highly lipophilicity which results in their high mobility in environment (IARC, 1983). The USEPA has categorized 16 mainly studied PAHs as priority pollutants with occurrence in environment (Mojiri et al., 2019). Phenanthrene (PHE), as one of the priority compounds presented in US-EPA list, represents a typical low molecular weight (LMW) PAH with three fused benzene rings that exists at high levels in PAH polluted environments (Fu et al., 2018). Increased environmental pollution and anthropological disturbances to ecosystems led to the study of abiotic stress responses in plants and subsequent concern about remediation of PAHs and ecosystem restoration strategies (Alkio et al., 2005).

Accumulation capacity and toxicity of PAHs in biota have resulted in a concern about their fate and transport in plants (Hamdi et al., 2007). Plants, as a dominant component of ecosystems, generally are first organisms exposed to the PAHs. They can uptake PAHs through the roots from contaminated soils and subsequently transfer them into shoots (Oguntimehin et al., 2010; Kummerová et al., 2013). There have been plenty of studies concerning the toxicity of PAHs on plants. It appears that all stages of plant growth and development can be influenced by PAHs, such as morphological, cytological, genetical, and metabolic disorders (Tomar & Jajoo, 2014; Dupuy et al., 2016). Inhibition of seed germination, reduction in biomass production, induction of oxidative stress, and disruption in photosynthetic apparatus function are some proven effects of PAHs on plants (Li et al., 2008; Liu et al., 2009; Tomar & Jajoo, 2014). A study carried out by Ahammed et al. (2012) has suggested that growth inhibition and biomass reduction by PAHs can be due to induction of oxidative stress. The oxidative stress induced by PAHs and its connection with morphological disorders has been previously asserted by other studies, such as a study accomplished via *Arabidopsis thaliana* (L.) Heynh. exposed to PHE (Alkio et al., 2005). Plant morphology has been regarded as important indicators for toxicity measurements in some PAHs researches (Sverdrup et al., 2003; Kummerová & Kmentová, 2004; Tomar & Jajoo, 2014). However, biochemical changes can precede the growth and morphological changes. A better understanding of PAHs' influence on plant metabolic processes would provide data that is required for phytotoxicity assessment of these contaminants as well as selection of potential plants for the PAH phytoremediation in contaminated soils.

Panicum miliaceum L., also known as proso millet, broomcorn millet, etc., is an annual crop with thermophilic and photophilic characteristics which can adapt to various types of soils. Due to the resistance of *P. miliaceum* to salt, drought, and alkali stress, as well as its short growth cycle, it is usually grown as a remediation crop in various adverse conditions (Habiyaremye et al., 2017; Na et al., 2019). The previous studies on *P. miliaceum* have primarily described its tolerance/resistance to biotic and abiotic stresses (Hu et al., 2009). It has been reported that *P. miliaceum* is more tolerant to salt stress than foxtail millet, wheat, maize, or rice (Dong & Zheng, 2006; Liu et al., 2015). However, no report is available on the potential of *P. miliaceum* L. for evaluating its tolerance to PAHs and also its growth and physiological responses to PAHs' contamination. Considering the importance of this species as feed for livestock and birds and the subsequent entry of these compounds into the food chain, as well as a plant that is used directly in the human diet in some parts of the world (Sabir et al., 2011), this study was conducted to investigate the phytotoxicity of high concentrations of PHE on *P. miliaceum*. In other words, the evaluation of the biochemical and physiological responses of *P. miliaceum* plant and its resistance capability to PHE were the main purposes of this study.

2 MATERIAL AND METHODS

2.1 PLANT CULTIVATION AND TREATMENT

Experiment was carried out as a pot culture of plants using a completely randomized design with three replications for each treatment. Particular amounts of PHE were dissolved in ethanol and appreciate volume of PHE solution was added to perlite to give a final concentration of PHE (500, 1000, 1500 and, 2000 ppm). After thoroughly mixing, perlites were dried at room temperature (22-25 ˚C) to allow the ethanol evaporation for 72 h. Treated perlites were subdivided into 100 g portions in

plastic pots for plant cultivation. The aforesaid concentrations were selected due to the ineffectiveness of lower concentrations of PHE to plant based on initial screening experiments (Data not shown).

The seeds of *P. miliaceum* 'Pishahang' were purchased from Pakan Bazr (Isfahan, Iran) and stored at 4 °C until cultivation. Uniform and healthy seeds were selected and sterilized by soaking in 1 % (v/v) sodium-hypochlorite solution for 5 minutes, and rinsed thoroughly using sterile distilled water. Then, 20 disinfected seeds per pot (replicate) were planted in uncontaminated (control) and PHE-contained perlites. Then, they were germinated on watered perlites in a dark condition for three days at room temperature. Finally, pots with all germinated seeds were placed in growth chambers with controlled conditions (25-30 °C, 16/8 h light/dark photoperiod, light intensity of 75 µmol m^{-2} s^{-1} provided by common day light fluorescent lamps, and relative humidity of 60 %) for four weeks. The water content of the pots was adjusted to 100 % field capacity every two days using sterile distilled water. After 4 and 10 days, the water of pots was replaced with 50 % and 100 % Hoagland solution, respectively. Seed germination rate was recorded. The rate of seed germination was defined as the percentage of germinated seeds to the total number of seeds.

2.2 HARVESTING OF PLANTS AND ASSAYS

The experiment was conducted for 30 days, and then plants were harvested and divided into the roots and shoots. Some morphological and growth parameters (such as germination, dry and fresh mass, root and shoot length) were estimated in order to evaluation of eventual differences between treatments. Plant samples were completely washed with water, immediately dried on the towel paper, and transferred to 70 °C after determining of the fresh mass (FM). The dry mass (DM) of samples was measured after 72 h. Three replicates of each root and shoot samples of different treatments were utilized for examination of biochemical and physiological parameters.

2.3 MEASUREMENT OF PHOTOSYNTHETIC PIGMENTS CONTENT

The assessment of the photosynthetic pigment contents (chlorophyll a, b, total chlorophyll, and total carotenoids) was performed based on the method described by Hartmut (1987). In brief, a quantity of 0.1g of fresh leaf samples was homogenized with 5 ml of acetone using a mortar and pestle on ice bath. After 48 hours, mixtures were filtered using a number 42 Whatman filter paper and the absorbance of filtrates was recorded at 645, 663, and 470 nm by using a spectrophotometer (Analytic Jena, Specol 1500, Germany). The determined contents of chlorophyll a, b, total chlorophyll and carotenoids were expressed in µg g^{-1}M.

2.4 MEASUREMENT OF STRESS INDICATOR METABOLITES

2.4.1 Malondialdehyde

Malondialdehyde (MDA) content measured by a method described by Boominathan and Doran (2002). In order to preparation of extract, 0.1 g of plants fresh material was homogenate in trichloroacetic acid 0.1 % (TCA, Merck, Germany) and then centrifuged at 10000g for 5 min. Then, 0.5 ml of supernatants was mixed with 2 ml of 20 % TCA containing 0.5 % of 2-thiobarbituric acid (TBA, Merck, Germany) and transferred to hot water for 30 min at 95 °C. Mixtures cooled immediately and centrifuged at 10000g for 15 min. Finally, the absorbance of supernatants was recorded at 532 nm and MDA concentration were calculated according to a standard curve prepared using 3,1,1,3-tetraethoxy propane (0-100 nM). MDA content expressed as µmol g^{-1} FM.

2.4.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) assay was carried out according to Harinasut et al. (2003). Briefly, 0.1 g of plant fresh tissue was homogenized in TCA 0.1 % and centrifuged at 10000 *g* for 15 min. 0.5 ml of supernatant was immediately mixed with 0.5 ml of phosphate buffer (10 mM, pH = 7) and 1 ml potassium iodide (KI, 1 mM) and left at ambient temperature for 15 min. Then, the absorbance of extracts was recorded at 390 nm. The content of H_2O_2 was calculated using a standard curve prepared by using different concentrations of H_2O_2 (0-120 µmol) and expressed as µmol g^{-1} FM.

2.5 TOTAL PROTEIN CONTENT AND ACTIVITY OF ANTIOXIDANT ENZYMES

In order to preparation of extracts, 0.1 g of the plant fresh material was homogenized in phosphate-buffered solution (PBS, 50 mM, pH = 7) using mortar and pestle on ice bath and then centrifuged at 10000 *g* for 10 min at 4 °C. The supernatant was immediately used for quantification of the total soluble protein content using method described by Bradford (1976) as well as the activity of peroxidase (POD), Ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT). Each measurement was performed with three replications for each treatment.

The activity of POD (EC 1.11.1.7) was assayed by the method of Obinger et al. (1997). One unit of POD activity was defined as the enzyme amount capable of oxidizing of 1 µM guaiacol to tetraguaiacol per minute and the enzyme activity was expressed as U mg^{-1} protein.

The activity of APX (EC 1.11.1.11) was assayed according to of Boominathan and Doran (2002). One unit of enzyme activity is considered as the amount of enzyme required for production of 1 µl min^{-1} of ascorbate. APX activity expressed as U mg^{-1} protein.

SOD (EC 1.15.1.1) activity was acquired by determination of nitro-blue-tetrazolium (NBT) photoreduction inhibition rate by extracts (Winterbourn et al., 1976). One unit of enzyme activity is defined as the amount of enzyme that inhibits 50 % of photochemical reduction of NBT. SOD activity was expressed as U mg^{-1} protein.

CAT (E.C. 1.11.1.6) assay conducted by using the method described by Chance & Maehly (1955). One unit of enzyme activity was considered as the amount of enzyme required for the decomposition of 1 µM H_2O_2 per min. The enzyme activity was expressed as U mg^{-1} protein.

2.6 QUANTIFICATION OF NONENZYMATIC ANTIOXIDANTS (TOTAL PHENOL, FLAVONOIDS AND ANTHOCYANIN CONTENTS)

For measurement of total phenolic and flavonoid contents, 0.1 g of plant samples were homogenized in 5 ml methanol using mortar and pestle. Then, the homogenates were centrifuged at 10000 *g* for 5 min and supernatants used for the tests. Assessment of flavonoids content has been accomplished based on a reported method by Chang et al. (2002). Briefly, 1.5 ml of methanol, 100 µl of 10 % aluminum chloride, 100 µl of 1 M potassium acetate, and 2.8 ml of distilled water were added to 500 µl of each extract (supernatant). After 40 minutes, the absorbance was recorded at 415 nm compared to the control. Quercetin was used for the preparation of calibration curve (20-200 mg l^{-1}). The total flavonoid content of the extract was reported as milligram quercetin equivalents (QE) g^{-1} FM. The phenolic content has been evaluated by the method of Meda et al. (2005). 100 µl of the methanolic extract and 100 µl of Folin-Ciocalteu's reagent have been added to 2.8 ml distilled water, mixed thoroughly, and maintained for 6 min. Then, 2 ml of 20 % (w/v) sodium carbonate ($NaHCO_3$) was added and left at ambient temperature for 30 minutes in the dark. Finally, the absorbance was recorded at 720 nm by a spectrophotometer. The standard curve was prepared by using of different concentrations of gallic acid with the same procedure. The content of phenolics was calculated (mg ml^{-1}) by the measured absorbance based on calibration line equivalent to the gallic acid (mg of GA g^{-1} of FM) (Meda et al., 2005).

To measure the total anthocyanin content, 0.05 g of dried plant sample was pulverized with 5 ml of hydrochloric acid containing 1 % methanol in a porcelain mortar. The solution was kept in the refrigerator for 24 hours and then, centrifuged for 10 minutes at 10000 *g*. The absorbance of supernatant was measured at 530 and 657 nm against the control (hydrochloric acid containing 1 % methanol). Finally, the anthocyanin content was calculated using the following equation (Mita et al., 1997).

$$A = A_{530} - (0.25 \times A_{657})$$

Where, A is absorbance of the solution (subscripts indicate the wavelength at which the absorbance is measured).

2.7 DPPH RADICAL SCAVENGING ACTIVITY

The method of Miliauskas et al. (2004) was used for DPPH radical scavenging capacity of plant samples. In order to, 0.1 g of plant fresh sample was extracted by 5 ml of methanol and then, centrifuged at 10000 *g* for 5 min. The supernatant was used for the test. To conduct the assay, 100 µl of extract was reached to 2 ml volume using methanol and 2 ml 0.0004 DPPH methanolic solution was added. The mixture was immediately mixed and incubated for 30 minutes in the dark at ambient temperature. The absorbance of the solution was recorded at 517 nm against control (2 ml DPPH and 2 ml methanol) and radical scavenging activity was calculated using the following equation:

$$I\ \% = (A\ control - A\ sample) / A\ control \times 100$$

Where, A is absorbance of the solution.

2.8 DATA ANALYSIS

The obtained data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS software (version 22) and expressed as mean ± SD of three independent replications. Tukey's multiple range tests were applied for mean comparison and values of $p \leq 0.05$ were considered statistically significant. SPSS software was also applied to calculate the correlation coefficient (Pearson) between characteristics. Microsoft excel 2013 software was used for the preparation of figures.

3 RESULTS AND DISCUSSION

3.1 GERMINATION RATE

Seed germination was delayed by all PHE treatments for one day and the germination rate was decreased significantly as the concentration of PHE raised to 2000 ppm in comparison with the control ($p < 0.05$). Lower concentrations (500, 1000 ppm) of PHE had no effect on germination rate (Figure 1). Generally, seed germination is one of the most sensitive processes in the plants, using as a bioindicator for evaluation of pollutants effects on plants. Prevention of seed germination is one of the obvious toxic influence of PAHs (Henner et al., 1999; Somtrakoon & Chouychai, 2013) which has been frequently reported in plants such as soybean (Li et al., 2013), maize (Houshani et al., 2019), wheat (Wei et al., 2014), sunflower and alfalfa (Salehi-Lisar & Deljoo, 2015). Probably, the deterioration of seed embryo by PAHs is a main reason for the inhibition of seed germination (Reynoso-Cuevas et al., 2008; Kummerová et al., 2012).

3.2 GROWTH PARAMETERS

Plants exposed to PHE were morphologically more erect and up-righted at all levels in comparison with the control plants which were almost lying. The effect of PHE on growth parameters were depended on the type of parameter, plant organs as well as PHE concentration. After 30 days, root length only decreased at the highest concentration (2000 ppm) of PHE. Shoot length showed a significant increase in plants treated by 500, 1000 and 1500 ppm of PHE ($p < 0.05$), but did not show a significant change at 2000 ppm in comparison with the control (Figure 2a). Shoot FM increased significantly at 1000 and 1500 ppm, but showed a significant decrease at 2000 ppm of PHE ($p < 0.05$). However, it had no notable increment at 500 ppm (lowest level). Both 500 and 1000 ppm of PHE led to considerable increase in root FM, but 1500 and 2000 ppm concentrations caused no significant enhancement ($p < 0.05$) (Figure 2b). Root and shoot DM were not remarkably affected by PHE, except for shoot DM at 500 ppm of PHE which increased significantly ($p < 0.05$) (Figure 2c).

Our results showed that the highest treatment of PHE significantly increased fresh and dry weight ratios of root to shoot (R/S) ($p < 0.05$) (Figure 2d).

Growth parameters such as plant height, biomass, and leaf area represent a function of plants' growth (Li et al., 2013). The reduction of the growth indices in the presence of PAHs has been demonstrated previously in plants such as rice (Li et al., 2008), wheat (Tomar & Jajoo, 2014; Salehi-Lisar & Deljoo, 2015), maize and pea seedlings (Kummerová et al., 2012). In the mentioned studies, the applied concentrations of PAHs were mostly below 100 mg kg^{-1}. Rice plants had good resistance to slightly higher concentrations of PAHs (100 and 200 mg kg^{-1} of pyrene and PHE), while the addition of 400 mg kg^{-1} of pyrene and PHE resulted in some negative impacts on this plant growth (Li et al., 2008). In our work, intriguingly, *P. miliaceum* plants not only grew without any symptoms of toxicity at 500 and 1000 ppm of PHE, but also showed an increase in the growth parameters; indicating the high tolerance of *P. miliaceum* seedlings to PHE. Although the higher concentrations of PHE (1500 and 2000 ppm) reduced the germination rate of *P. miliaceum* seeds, it is presumed that 1500 ppm is the highest concentration of PHE that *P. miliaceum* could tolerate

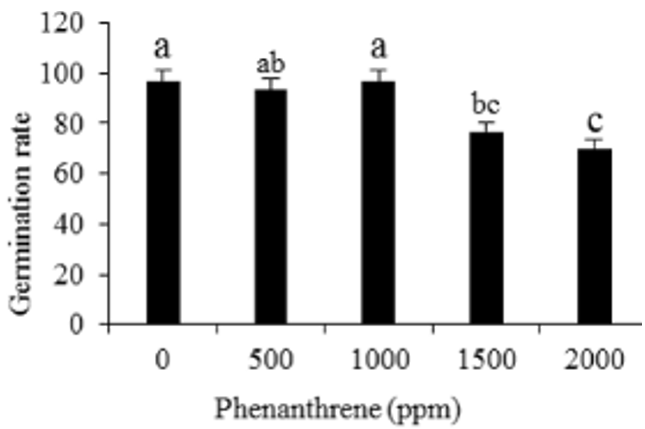

Figure 1: The effect of different concentrations of phenanthrene on seed germination rate of *P. miliaceum*. The level of confidence is 95% according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

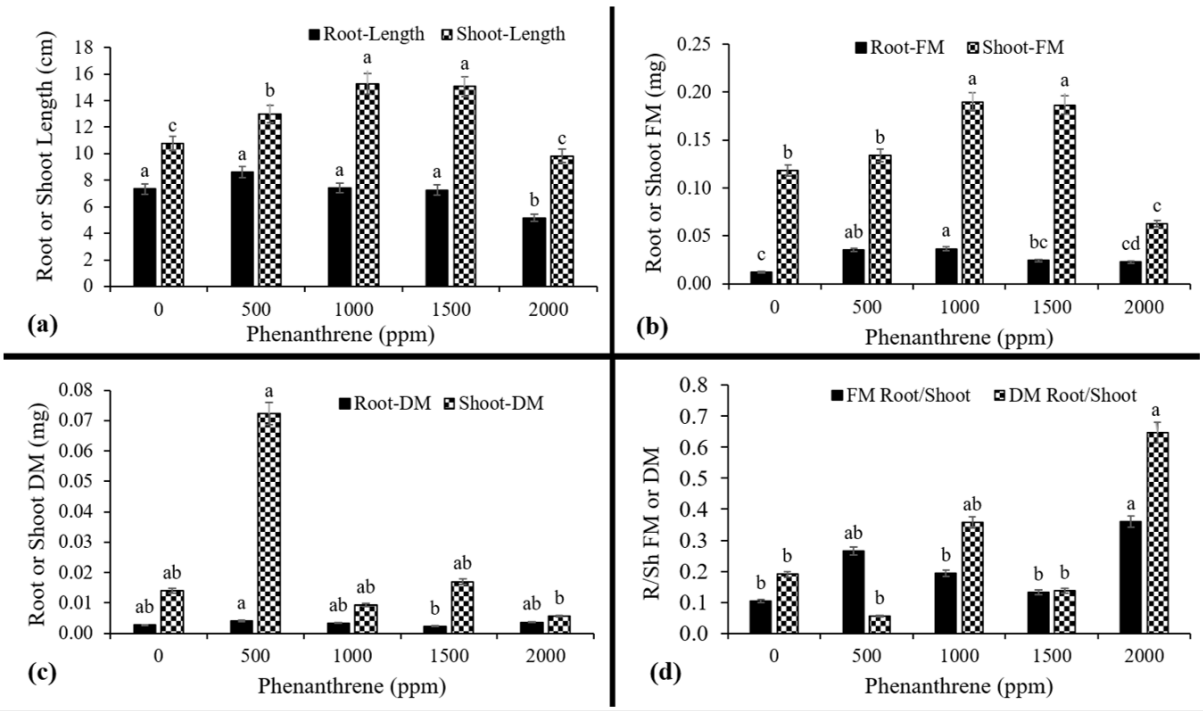

Figure 2: The effect of different concentrations of phenanthrene on *P. miliaceum* growth parameters a) root and shoot length, b) root and shoot fresh mass, c) root and shoot dry mass, and d) root to shoot fresh or dry mass ratios). The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

without negative effects on growth. At this concentration the growth parameters are either increased or similar to control plants. However, at 2000 ppm of PHE, shoot fresh mass was decreased by 50 %. In spite of this, *P. miliaceum* was able to survive in presence of 2000 ppm of PHE during the testing period. The previous studies on *P. miliaceum* have also primarily described its resistance to various adverse conditions compared to similar plants from Poaceae family such as foxtail millet, wheat, maize, or rice (Dong & Zheng, 2006; Hu et al., 2009; Liu et al., 2015).

The data showed a noticeable increase in R/S ratio of *P. miliaceum* exposed to the highest concentration of PHE (2000 ppm). Literature review revealed that it is sometimes important for plants to allocate carbon resources to roots under stress condition such as PAHs toxicity. Plant biomass allocation, as a similar response induced by PAHs, was previously reported in *Zea mays* L. and *Phragmites australis* (Cav.) Trin. ex Steud. (Nie et al., 2010; Dupuy et al., 2016). The same results with increasing R/S ratio were acquired in *Medicago sativa* L., clover and maize impressed by PAHs (Desalme et al., 2011; Dupuy et al., 2015; Salehi-Lisar & Deljoo, 2015; Afegbua & Batty, 2018). Altogether, the high R/S ratio along with a significant decrease in root length in present work is a reason for the increased root thickness. The increment in root thickness was also confirmed for *Pisum sativum* L. and *Z. mays* plants treated with fluoranthene (Kummerová et al., 2013). In maize, PHE induced the extensive suberization of endo- and exodermises as a protective response by the plant for reduction of PHE penetration into roots (Dupuy et al., 2016).

3.3 PHOTOSYNTHETIC PIGMENT CONTENTS

Chlorophyll a, chlorophyll b and total chlorophyll were remarkably reduced in the plants treated by PHE compared to the control ($p < 0.05$). The lowest content of chlorophyll a and b were observed in the plants exposed to 1500 and 2000 ppm of PHE, respectively (Figure 3 a & b). Moreover, the increase in chlorophyll a/b ratio at all levels (especially 2000 ppm) of PHE in comparison with the control was statistically significant ($p < 0.05$) (Figure 3c). Also, plants treated with 1500 ppm of PHE showed a notable decline in carotenoids content in comparison with the control ($p < 0.05$) (Figure 3d). It appears that chlorophyll b was more affected by PHE, but carotenoids were less sensitive compared to chlorophylls (Figure 3).

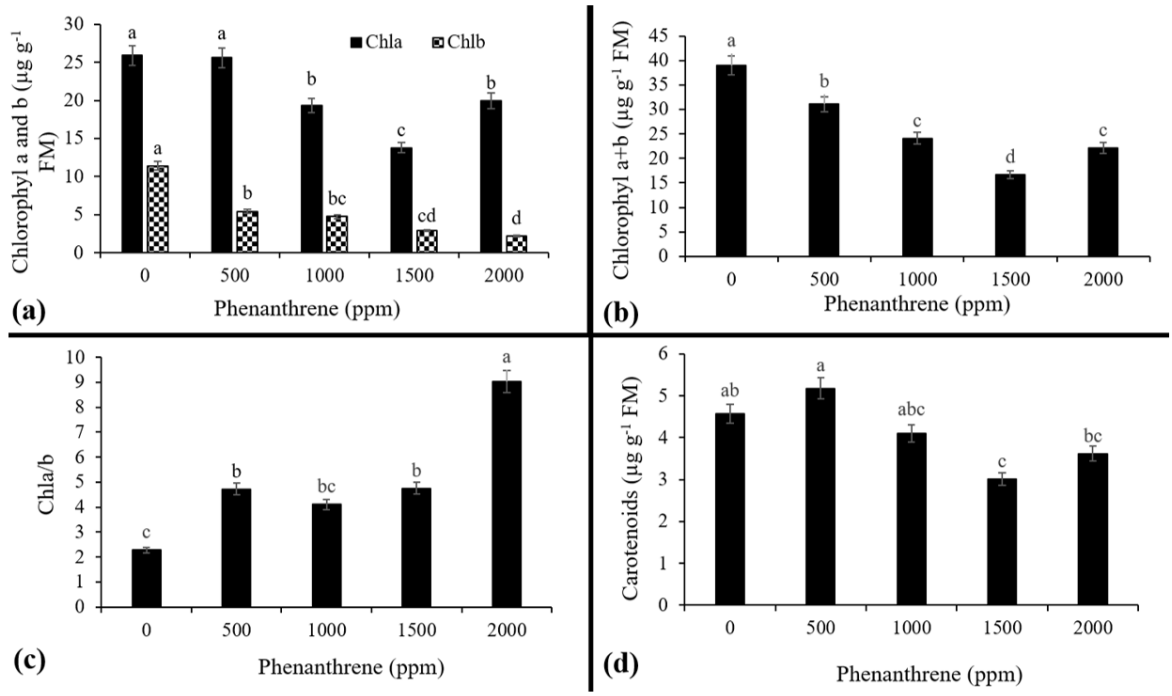

Figure 3: The effect of different concentrations of phenanthrene on photosynthetic pigments contents of *P. miliaceum* plants a) Chla and b, b) Chl a+b, c) Chla/b, and d) Carotenoids. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

According to the results, photosynthetic processes in *P. miliaceum* were probably inhibited by PAH. This was demonstrated by the decrease of Chl a, b and total chlorophyll, and by the increase in the Chl a/b ratio at all treatments. However, based on the data obtained by present work, growth parameters generally increased or at least maintained at control level, and only the highest concentration of PHE led to reduction in some traits. Biomass production formerly regarded as a reliable external indicator of internal status of plant photosynthesis processes (Tomar & Jajoo, 2014). Accordingly, it can be suggested that most likely changes in photosynthetic pigments content cannot be an appropriate indicator for assessing the sensitivity or resistance of *P. miliaceum* to PAHs contamination. This assumption was also supported by results of Salehi-Lisar & Deljoo (2015). Reduction of photosynthetic pigments content have been reported in *Arabidopsis thaliana* and wheat plants treated by PHE and fluorene, respectively (Liu et al., 2009; Tomar & Jajoo, 2014).

PAHs can cause oxidative stress in plants through induction of ROS production. In addition to structural role and light absorption, carotenoids can directly inactivate ROS or indirectly prevent the formation of ROS by quenching chlorophyll-elicitation (via xanthophyll cycle) and protect chloroplast membranes against oxidative stress (Ramel et al., 2012). Therefore, it seems that preservation of carotenoid content, especially at lower concentrations of PHE, has also contributed to plant tolerance for this pollutant.

3.4 MALONDIALDEHYDE AND HYDROGEN PEROXIDE CONTENTS

A considerable dose-dependent elevation in H_2O_2 content was observed after 30 days of plants treatment by PHE. As compared to the control and the other PHE treatments, plant treated by 2000 ppm of PHE showed a significant increase in root H_2O_2 content ($p < 0.05$). 1500 and 2000 ppm of PHE also caused a notable enhancement in shoot H_2O_2 content in comparison with the control plants ($p < 0.05$) (Figure 4a). 1000 ppm of PHE led to a significant increase in root MDA content, compared with the control and other PHE treatments ($p < 0.05$). Also, a significant reduction in shoot MDA content was observed in plants treated by 1000, 1500 and 2000 ppm compared to the control plants ($p < 0.05$) (Figure 4b).

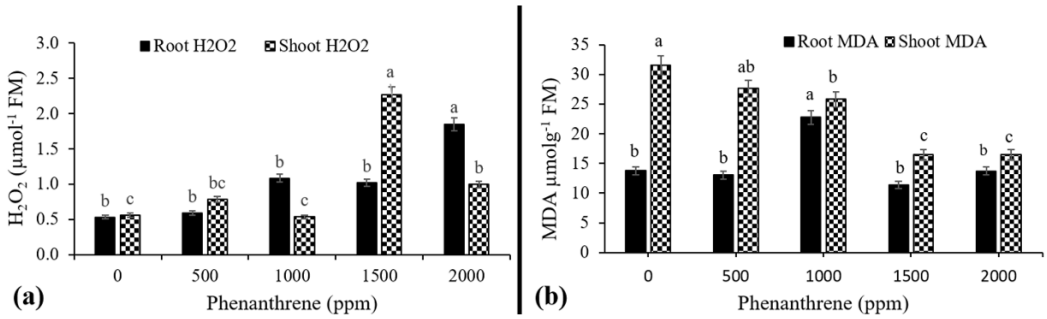

Figure 4: The effect of different concentrations of phenanthrene on H_2O_2 and MDA contents in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

In general, with increment in PHE concentrations, H_2O_2 content was raised in the shoots and roots of *P. miliaceum* compared to the control plants. The increased amount of H_2O_2 was previously reported in the seedlings of wheat (Wei et al., 2014) and *Arabidopsis thaliana* (Liu et al., 2009). The highest amount of this trait was obtained at 1500 and 2000 ppm of PHE for roots and shoots, respectively. However, the amount of MDA mounted to the highest level in 1000 ppm, and then dramatically dropped in higher concentrations of PHE in the roots ($p < 0.05$). In shoots, the amount of MDA was decreased compared to control. Taken together, given that even in non-stress conditions, there is always some H_2O_2 in plant tissues; here, it appears that the enhancement in H_2O_2 amount was not a big challenge for plant or its antioxidant system would be able scavenge produced H_2O_2. So, not only the content of MDA did not increase, it even decreased. According to the results, some growth parameters even increased at 1500 treatment. It can be suggested that at 2000 ppm of PHE plant allocated more photosynthetic products to the root, for its thickening in order to create barriers against PHE penetration. Analysis of PHE concentration using HPLC also showed a decrease in the penetration of PHE into the root in plants treated by 2000 ppm of PHE (Data not shown). In addition, a 50 % reduction in shoot FM and increased root FM, along with a significant reduction in its length, also supports this point of view. So, it can be assumed that the antioxidant system of the root is strong enough and has been able to reduce the stress effects of PHE on plants. On the other hand, given that at high concentrations of PHE, the accumulation of MDA was lower in roots, it indicates that the potency of PHE stress in *P. miliaceum* is not considerable. Therefore, it can be concluded that MDA accumulation due to oxidative stress could not be a reliable marker for evaluation of the negative effect of PHE on *P. miliaceum*.

3.5 TOTAL PROTEIN CONTENT

Generally, PHE showed no significant effect on shoot and root protein content, however at the highest concentration of PHE a significant fall in shoot protein was observed ($p < 0.05$) (Figure 5). Therefore, the decrease in growth observed at this concentration of PHE may also be due in part to the plants' low capacity to produce protein under PHE toxicity. However, it is also possible that the transfer of more photosynthetic products to the root, and hence, the decrease in shoot growth, has led to a decrease in plants protein content. It is well known that plants need both carbohydrates and proteins to grow. In addition, root thickening may act as a barrier to efficient and effective uptake of nutrients and in turn lead to reduced growth. Salehi-Lisar & Deljoo (2015) has also reported a decrease in shoot and root soluble protein in sunflower plants treated by fluorene.

3.6 ANTIOXIDANT ENZYMES ACTIVITY

The activity of antioxidant enzymes were assessed in *P. miliaceum* 30 days after seed cultivation. A dramatic increment was observed in the SOD, POD, CAT, and APX activities in plants treated by 1500 and 2000 ppm of PHE ($p < 0.05$) in both shoots and roots ($p < 0.05$). However, there was often a significant difference between 1500 and 2000 treatments and generally the highest activity of these enzymes observed at 2000 ppm of PHE, except for CAT activity in roots which was the highest in 1500 ppm of PHE ($p < 0.05$) (Figure 6a-d). In general, antioxidant enzymes' activities in roots were higher than those in shoots of the plants (Figure 6a-d).

ROS accumulation is a main factor involving in toxicity induction due to direct or an indirect outcome of the PAHs exposure. ROS stimulates the activity of anti-

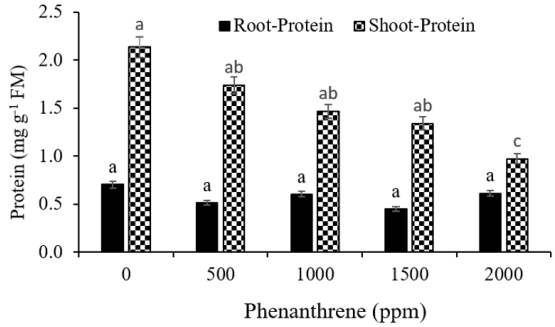

Figure 5: The effect of different concentrations of phenanthrene on protein content in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

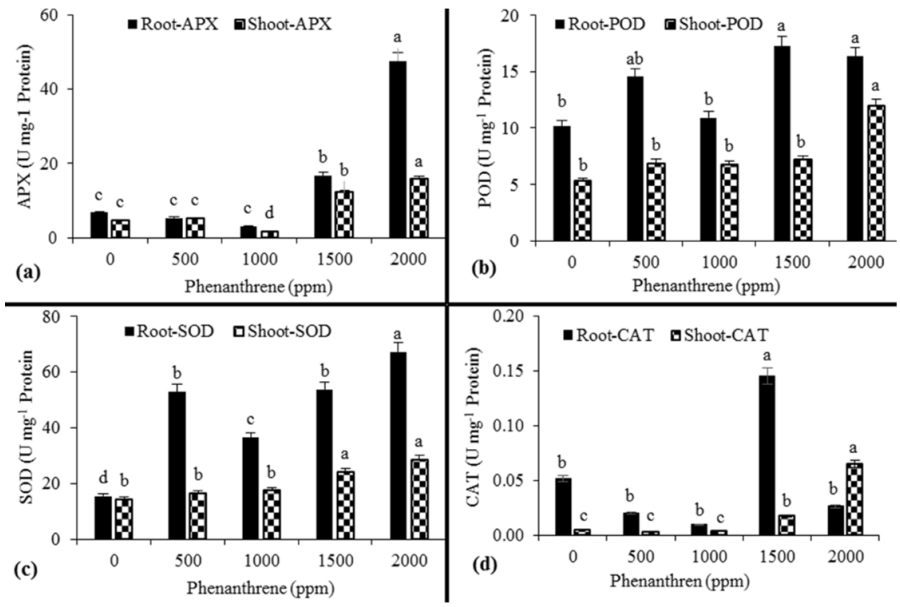

Figure 6: The effect of different concentrations of phenanthrene on antioxidant enzymes activity (U mg $^{-1}$ protein) in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

oxidant enzymes (e.g. SOD, POD, CAT, and APX). These enzymes are responsible for scavenging of produced ROS and their activities may indicate stress resistance potential of plants (Di Giulio, 1991). Increased activity of antioxidant enzymes by PAHs toxicity has been previously reported in maize (Houshani et al., 2019), wheat, sunflower, and alfalfa (Salehi-Lisar & Deljoo, 2015), rice (Li et al., 2008) treated by fluorene, pyrene and PHE, respectively. In present study, higher activity of antioxidant enzymes in plants treated by 1500 and 2000 ppm of PHE is a result of induced ROS generation by PHE. However, PHE induced a concentration-dependent oxidative stress and the activity of antioxidant enzymes did not changed significantly in the lower PHE treatments. The analysis of correlation showed negative correlation coefficient between MDA content and antioxidant enzyme activities in shoot (SOD r^2 = -0.893, POD r^2 = -0.590, CAT r^2 = -0.705, APX r^2 = -0.740) and root (SOD r^2 = -0.771, POD r2 = -0.717, CAT r^2 = -0.451, APX r^2 = -0.688) (Table 1). These findings indicate that antioxidant enzymes involved in ROS detoxification and plants resistance to oxidative stress are induced by PHE toxicity. Similar results were obtained for maize, alfalfa, sunflower, and wheat plants (Salehi-Lisar & Deljoo, 2015).

3.7 NONENZYMATIC ANTIOXIDANTS

PHE significantly increased anthocyanin and total phenol contents in shoots of plants treated by 500 and 2000 ppm and 1500 ppm, respectively, compared to the control and other PHE treatments ($p < 0.05$). PHE had no effect on root anthocyanin and total phenol contents. However, flavonoids contents elevated at 2000 ppm of PHE only in roots ($p < 0.05$) and no remarkable changes were detected in shoots (Table 2). This increment can be interpreted as a defence mechanism. Plants requiring a protective system involving enzymatic and non-enzymatic mechanisms for detoxification of ROS (Alscher et al., 1997). In this work, the amount of non-enzymatic antioxidants in shoot or root was raised probably in order to detoxification of accumulated oxidant metabolites at higher concentrations of PHE, along with increased antioxidant enzymes' activity, resulting in ROS scavenging and finally reduction in oxidative stress severity.

3.8 DPPH RADICAL SCAVENGING ACTIVITY

As seen in Figure 7, there were no significant changes in DPPH radical scavenging activity between treatments. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) a molecule containing a stable free radical widely used to study the free radical-scavenging activity of natural antioxidants (Brand-Williams et al., 1995). According to the results of this work it can be concluded that studied plant was able to maintain their antioxidant capacity as to control plants.

Table 1: Statistical analysis for correlation between the activity of antioxidant enzyme and MDA content in the shoot and root of *P. miliaceum* plant.

	APX Root	APX Shoot	SOD root	SOD Shoot	POD Root	POD Shoot	CAT Root	CAT Shoot	MDA Root	MDA Shoot
MDA Shoot	0.688^{**}-	0.740^{**}-	0.771^{**}-	0.893^{**}-	0.717^{**}-	0.590^{*}-	0.451^{ns}-	0.705^{**}-	0.192^{ns}	1
MDA Root	0.304^{ns}-	0.563^{*}-	0.299^{ns} -	0.142^{ns}-	0.532^{*}-	0.152^{ns}-	0.490^{ns}-	0.254^{ns}-	1	
CAT Shoot	0.978^{**}	0.875^{**}	0.679^{**}	0.801^{**}	0.527^{*}	0.784^{*}	0.022^{ns}-	1		
CAT Root	0.041^{ns}	0.196^{ns}	0.074^{ns}	0.238^{ns}	0.397^{ns}	0.130^{ns}-	1			
POD Shoot	0.688^{**}	0.733^{**}	0.791^{**}	0.747^{**}	0.538^{*}	1				
POD Root	0.554^{*}	0.767^{**}	0.816^{**}	0.730^{**}	1					
SOD Shoot	0.797^{**}	0.767^{**}	0.812^{**}	1						
SOD Root	0.641^{*}	0.772^{**}	1							
APX Root	0.873^{**}	1								
APX Shoot	1									

Notes: **Correlation is significant at 0.01 levels, *Correlation is significant at 0.05 levels, ns correlation is not significant.

Table 2: The effect of the different concentrations of phenanthrene on phenol (mg GAE g^{-1} FM), flavonoids (mg QE g^{-1} FM) and anthocyanin (mg QE g^{-1} FM) contents in the shoot and root of *P. miliaceum* plant.

Phenanthrene (ppm)	Root			Shoot		
	Phenol	Flavonoids	Anthocyanin	Phenol	Flavonoids	Anthocyanin
0	2.93^{a}±0.17	0.27^{b}±0.00	0.03^{a}±0.01	0.73^{b}±0.32	1.10^{a}±0.07	0.18^{b}±0.17
500	3.03^{a}±0.03	0.27^{b}±0.00	0.05^{a}±0.02	1.17^{b}±0.99	1.30^{a}±0.05	0.50^{a}±0.17
1000	1.95^{a}±0.58	0.28^{b}±0.00	0.04^{a}±0.01	1.22^{b}±0.16	1.20^{a}±0.04	0.16^{b}±0.17
1500	2.07^{a}±1.47	0.27^{b}±0.00	0.05^{a}±0.01	7.85^{a}±0.26	1.04^{a}±0.20	0.15^{b}±0.17
2000	2.06^{a}±0.29	0.30^{a}±0.01	0.02^{a}±0.00	1.03^{b}±0.12	1.14^{a}±0.06	0.39^{a}±0.06

The data represent the mean of three replications ± SD and similar upper case letters indicates no significant difference at $p < 0.05$.

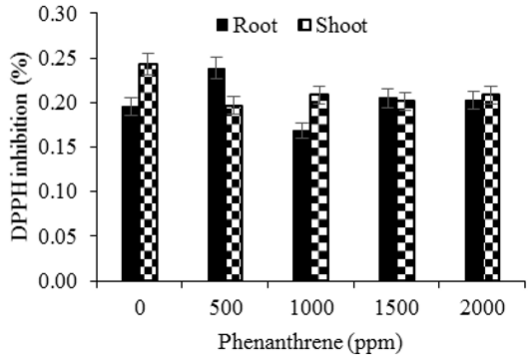

Figure 7: The effect of different concentrations of phenanthrene on DPPH radical scavenging activity in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD.

4 CONCLUSIONS

The measurements of some morphological parameters as well as cellular responses in the forms of enzymatic and non-enzymatic antioxidants' activities in *Panicum miliaceum* exposed to phenanthrene have been main objectives of presented work. PHE had a negative impact on germination rate, growth and pigment content of *P. miliaceum* only at higher concentrations (1500 and 2000 ppm). Therefore, in comparison to other studied plants *P. miliaceum* showed relatively very high tolerance to PHE toxicity, The results also showed a noticeable increment in antioxidant system activity due to the toxicity caused by PHE at higher concentrations as well as a significant elevation in non-enzymatic antioxidant contents that can be explained as a probable contributory system to the enzymes in order to ROS detoxifying. Taken all together, the antioxidant system of *P. miliaceum* was probably strong enough to reduce toxicity of produced ROS in significant amount, thus plant survived even in higher concentrations of PHE. It would be worthwhile to examine the capability of this plant for phytoremediation purposes through further research.

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Pesticide residues in bee pollen - validation of the gas chromatography-mass spectrometry multiresidual method and a survey of bee pollens from Slovenia

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Pesticide residues in bee pollen - validation of the gas chromatography-mass spectrometry multiresidual method and a survey of bee pollens from Slovenia

Abstract: A new analytical method for determining environmental pesticide residues in pollen was introduced and validated. The extraction was conducted using acetonitrile, the clean-up using Supelclean Ultra 2400 solid phase extraction cartridges, which contain Grapsphere, anion exchanger, C18 and zirconia-based sorbent, and the determination was conducted using gas chromatography coupled with mass spectrometry. The method was applied in practice. A total of 49 active substances (pesticides) were sought in 30 bee pollen samples gathered from Slovenian beekeepers from all 12 statistical regions of Slovenia. The fungicide azoxystrobin was the only active substance found and was found only in one sample with a concentration of < 0.05 mg kg^{-1}. The active substances sought were not detected in 96.7 % of the samples analysed. The risk assessment revealed that the analysed pollen samples do not represent an unacceptable risk for consumers. The results were compared with those from the literature and the outcome was that bee pollen from Slovenia contained a lower number of active substances at mainly lower contents as compared pollen from some other European countries.

Key words*:* bee pollen; GC-MS; pesticide residues; multiresidual method

Ostanki fitofarmacevtskih sredstev v cvetnem prahu - validacija multirezidualne metode s plinsko kromatografijo sklopljeno z masno spektrometrijo in preiskava cvetnega prahu iz Slovenije

Izvleček: Uvedli in validirali smo novo analizno metodo za določanje ostankov fitofarmacevtskih sredstev iz okolja. Ekstrakcijo smo izvedli z acetonitrilom, čiščenje z Supelclean Ultra 2400 koloncami za ekstrakcijo na trdni fazi, ki vsebujejo Grapsphere, anionski izmenjevalnik, C18 in sorbent na osnovi cirkonija, in določitev s plinsko kromatografijo sklopljeno z masno spektrometrijo. Metodo smo uporabili v praksi. V 30 vzorcih cvetnega prahu slovenskih čebelarjev iz vseh 12 statističnih regij Slovenije smo določali skupno 49 aktivnih spojin (pesticidov). Edina najdena aktivna snov je bil fungicid azoksistrobin in sicer le v enem vzorcu, pri koncentraciji < 0,05 mg kg^{-1} Iskanih aktivnih snovi nismo detektirali v 96,7 % analiziranih vzorcev. Z oceno tveganja smo ugotovili, da analizirani vzorci cvetnega prahu ne predstavljajo tveganja za potrošnika. Rezultate smo primerjali z literaturnimi podatki in ugotovili, da je cvetni prah v Sloveniji vseboval manjše število aktivnih spojin pri v glavnem nižjih vsebnostih fitofarmacevtskih ostankov kot cvetni prah iz nekaterih Evropskih dtžav.

Ključne besede: cvetni prah; GC-MS; ostanki fitofarmacevtskih sredstev; multirezidualna metoda

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1 INTRODUCTION

Bee pollen is a dietary supplement. It contains carbohydrates (mainly fructose, glucose and sucrose (13-55 %), proteins (10-40 %), lipids (1-13 %), and crude fibre (0.3-20 %)), minerals (mainly potassium, phosphorus, calcium, magnesium, zinc, manganese, iron and copper (2-6 %)), vitamins (0.005-5.6 mg kg^{-1}), and polyphenols (0.69-213.2 mg GAE g^{-1}) (Thakur and Nanda, 2020). Bee pollen has antioxidant activity, antimicrobial activity, anti-inflammatory activity, anticarcinogenic activity, cardioprotective effects, hepatoprotective effects, antiallergic activity and it boosts the immune system (Li et al., 2018). A diet supplemented with bee pollen strengthens muscles and improves the physical health of humans (Salles et al., 2014). Bee pollen also benefits those undertaking strenuous mental/physical work (Nakajima et al., 2009).

Honeybees fly up to 4.8 km from their apiary (Eckert, 1933) to collect pollen. When hives are located near agricultural fields, plants treated with plant protection products (PPP) are a possible source of contamination for bee pollen (Tosi et al., 2018). Honeybees may come into contact with PPP residues through the nectar, pollen or plant leaves of treated plants, or through air, soil and water where PPPs have drifted (Crenna et al., 2020).

Bee pollen is usually harvested by means of a trap fixed at the entrance of beehives (Thakur and Nanda, 2020). This type of pollen is called corbicular pollen. Some beekeepers also collect pollen from hives deposited in combs by bees. This type of pollen is called beebread.

Numerous analytical methods have been developed to analyse PPP residues in pollen. The more recent ones are based on the QuEChERS method, which has been introduced to analyse a wide range of PPP residues in fruit and vegetables (Anastassiades et al., 2003, Lehotay, 2007). In this method, acetonitrile is used as an organic solvent for the extraction. The advantage of acetonitrile is that it minimizes the co-extraction of lipids and proteins by precipitating the proteins (Wang et al., 2012) and limiting the lipid solubility (Lozano et al., 2014). This makes acetonitrile a suitable solvent for extracting PPP residues from pollen. In some cases (Tosi et al., 2018; Wiest et al., 2011), n-hexane was added to remove fatty acids and fatty acid esters.

The clean-up in the original QuEChERS method was conducted using primary secondary amine (PSA) sorbent and C18 (Anastassiades et al., 2003; Lehotay, 2007). In the case of pollen some authors used either PSA (Cabrera de Oliveria, 2016; Kasiotis et al., 2014), PSA and C18 sorbent (Mullin et al., 2010), PSA, C18 and graphitized carbon black (GCB) sorbent (David et al., 2016), or PSA, C18 and zirconia-based sorbents such as Z-Sep, which consists of a mixture of C18 and silica coated with zirconium dioxide sorbents (Hakme et al., 2017, Vázquez et al., 2015). In our laboratory we used Supelclean Ultra 2400 solid phase extraction (SPE) cartridges, which contain Grapsphere (graphitized spherical carbon), PSA, C18 and Z-Sep. PSA retains acidic interferences such as fatty acids. Grapsphere removes planar molecules such as pigments and at the same time enables better recovery of planar pesticides than GCB. The bottom layer of the cartridge contains Z-Sep, which removes oily residues and provides additional retention of some pigments (Stenerson, 2018). C18 retains lipids (Lehotay,2007). Thus, these SPE cartridges combine all the common clean-up procedures from the literature.

Determination of PPP residues can be performed using gas chromatography coupled with mass spectrometry (GC-MS) (Li et al.; 2015; Mullin et al.,2010; Raimets et al., 2020), GC coupled with tandem mass spectrometry (GC-MS/MS) (Cabrera de Oliveria, 2016), GC coupled with time-of flight mass spectrometry (GC-TOF) (Hakme et al., 2017), and/or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (David et al., 2016; Kasiotis et al., 2014; Raimets et al., 2020). Multiresidual methods are fit-for-purpose when their limits of quantification are lower or equal to the Maximum Residue Limits (MRLs) established for pollen in Regulation (EC) 396/2005. When the MRLs in this Regulation are set at the LOQ determined by the analytical method (this LOQ was gathered by different laboratories), an * is added to mark this fact. Many pesticides have an MRL and LOQ of 0.05 mg kg^{-1}, meaning that GC-MS is still suitable despite its smaller sensitivity than tandem mass detectors.

Numerous authors have analysed pesticide residues in pollen. García-Valcárcel et al. (2019) analysed 10 active substances in pollen samples in Spain. Vázquez et al. (2015) analysed 253 active substances in pollen samples in Spain. Hakme et al. (2017) tested pollen samples from Spain for 100 active substances. Wiest et al. (2011) introduced a method for determining 80 active substances in French pollen. Tosi et al. (2018) analysed 66 active substances in Italian pollen. Kasiotis et al. (2014) analysed 115 active substances in Greek pollen. Raimets et al. (2020) analysed 47 active substances in Estonian pollen. David et al. (2016) analysed 20 active substances in pollen from the United Kingdom. Many of active substances sought in these studies were introduced in our study as well. Our selection of active substances was based on both those authorised for use in Slovenia and those not authorised for use in Slovenia, the latter to cover misuse of PPP. Of those selected, 59 % were acaricides and/or insecticides, which may be the main reason for the death of bees.

The purpose of this paper is to present the multire-

sidual GC-MS method introduced for identifying 49 active substances in pollen using acetonitrile as the extraction solvent and Supelclean Ultra 2400 SPE cartridges for the clean-up. The validation parameters are summarised, as well as the practical use of the method on 30 samples of bee pollen gathered from Slovenian beekeepers. The contents of pesticide residues were compared with those from the literature. Finally, a risk assessment for consumers was conducted.

2 MATERIAL AND METHODS

2.1. MATERIALS

2.1.1 Chemicals

The certified standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany). The acetonitrile HPLC-grade (used for the extraction procedure) and acetone HPLC-grade (used for preparation of standards) were supplied by J.T.Baker (Deventer, Netherlands). All other chemicals used were supplied by Sigma-Aldrich (Steinheim, Germany). The water used was MilliQ deionised water. The Ultra 2400 3 ml SPE columns were supplied by Supelco (Bellefonte, USA).

2.1.2. Preparation of the solutions

Stock solutions in acetone of individual active substances were prepared with the concentrations of 625 µg pesticide ml^{-1}. From 49 stock solutions, two mixed solutions of all 49 active substances were prepared: one with a concentration of 5 µg ml^{-1} and the second at the LOQ of active substances. All solutions used to determine the linearity and the LOQs and to perform calibration during sample analysis were prepared from a mixed solution of 5 µg ml^{-1} with proper dilutions. For other validation parameters, a mixed solution with a concentration at the LOQ was used.

2.2. EXTRACTION PROCEDURE

The samples were analysed within a maximum period of 27 days after arrival at the laboratory. During that time, they were stored at -20 °C.

To 10 g of pollen in the beaker, 50 ml of acetonitrile was added. The mixture was homogenised for 2 minutes with a mixer. The mixture was left for 30 minutes so that the sediment settled on the bottom of the beaker. The liquid part was transferred to a 50 ml centrifuge tube and centrifuged for 10 minutes at 7000 rpm. The supernatant was filtered through 15 g anhydrous Na_2SO_4 and black strip filter paper into a 100 ml Soxhlet flask. Then 30 ml of acetonitrile was added to the sediment in the beaker. The mixture was homogenised for 2 minutes with a mixer and transferred to a 50 ml centrifuge tube. Centrifugation followed for 10 minutes at 7000 rpm. This supernatant was combined with the first one after it was filtered through 15 g of anhydrous Na_2SO_4 and black strip filter paper into a 100 ml Soxhlet flask. The Na_2SO_4 was rinsed with 15 ml of acetonitrile. Then acetonitrile in Soxhlet flask was evaporated to approximately 2 ml on a rotavapor and dried with nitrogen flow. The dry eluate was dissolved in 1 ml of acetonitrile using ultrasound. The extract was transferred onto a column of Ultra 2400 3 ml, preconditioned with 3 ml of acetonitrile. The SPE column was rinsed with 16 ml of acetonitrile. The flow rate was 3-4 ml min^{-1} under vacuum. The whole eluate (partly taken from the SPE column after the sample was applied to the SPE column and partly eluate created during rinsing of the SPE column) was combined in a beaker. The content of the beaker was transferred to a 100 ml Soxhlet flask. The beaker was rinsed twice with 5 mL of acetonitrile and the content was transferred to the Soxhlet flask. The acetonitrile was then evaporated to approximately 2 ml on a rotavapor and dried with nitrogen flow. The dry eluate was dissolved in 1 ml of acetone using ultrasound in order to prepare a sample. For matrix match standards, 1 ml of the working solutions with proper concentrations was added and dissolved using ultrasound.

2. 3. DETERMINATION

The samples were analysed using a gas chromatograph (Agilent Technologies 7890A, Shanghai, China) equipped with a Gerstel MPS2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) and a HP-5 MS UI column (Agilent Technologies, 30 m, 0.25 mm i.d., 0.25 µm film thickness) with a constant flow of helium at 1.2 ml min^{-1}. The GC oven was programmed as follows: 55 °C for 2 min, from 55 °C to 130 °C at 25 °C min^{-1}, held at 130 °C for 1 min, from 130 °C to 180 °C at 5 °C min^{-1}, held at 180 °C for 30 min, from 180 °C to 230 °C at 20 °C min^{-1}, held at 230 °C for 16 min, from 230 °C to 250 °C at 20 °C min^{-1}, held at 250 °C for 13 min, from 250 °C to 280 °C at 20 °C min^{-1}, held at 280 °C for 20 min. In order to determine the analytes, a mass spectrometer (Agilent Technologies 5975C, upgraded with a triple-axis detector, Palo Alto, CA, USA) was used. The temperature of the ion source was 230 °C, the auxiliary temperature was 280 °C and the quadrupole temperature was 150 °C. For qualitative determination, the retention time and

mass spectrum in the SIM were used. For each active substance, one target and two qualifier ions, presented in Table 2, were used. The calibration was performed to matrix match standards.

2. 4. VALIDATION OF METHODS

LOQ and linearity

The linearity was verified using the matrix match standards (two repetitions for one concentration level, four to six concentration levels for the calibration curve). The linearity and range were determined by linear regression, using the F test.

LOQs were estimated from the chromatograms of matrix match standards. LOQs were chosen at a minimum of S/N = 10.

MRLs for environmental pesticide residues are set in Regulation (EC) 396/2005. Where the MRLs are set at the LOQ determined using the analytical method (this LOQ was gathered by different laboratories) in the Regulation an * is added to mark this fact. Therefore, in cases where MRLs were marked with an *, our LOQs were set at those MRLs.

Precision

Blank pollen was bought in store and analysed to prove that it contains no pesticide residues. For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, the extracts of spiked blank pollen were analysed at LOQ. Within a period of 10 days, two parallel extracts were prepared each day for each concentration level. Each one was injected once. Then the standard deviation of the repeatability of the level and the standard deviation of reproducibility of the level were both calculated.

Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and the uncertainty of reproducibility were calculated by multiplying the standard deviation of repeatability and the standard deviation of reproducibility by the Student's t factor, for nine degrees of freedom and a 95 % confidence level ($t_{95;9}$ = 2.262).

$$U_r = t_{95;9} \times s_r \; ; \; U_R = t_{95;9} \times s_R$$

The measurement uncertainty for PPP residues should be 50 %, as proposed in SANTE/11813/2017. When validating, analysts must prove that their measurement uncertainty is below or equal to the proposed measurement uncertainty.

Accuracy

The accuracy was verified by checking the recoveries. The average of the recoveries from the tests for precision (10 days, 2 parallel samples each day) was calculated. According to the requirements for method validation procedures (SANTE/11813/2017), acceptable mean recoveries are those within the range of 70 % to 120 %, with an associated repeatability of RSDr ≤ 20 %.

According to the guidelines for single-laboratory validation (Alder et al. 2000), acceptable mean recoveries are as follows:

- at level > 0.01 mg kg^{-1} ≤ 0.1 mg kg^{-1}, acceptable mean recoveries are those within the range of 70 % to 120 %, with an associated repeatability RSDr ≤ 20 % and
- at level > 0.001 mg kg^{-1} ≤ 0.01 mg kg^{-1}, acceptable mean recoveries are those within the range of 60 % to 120 %, with an associated repeatability RSDr ≤ 30 %.

2. 5. CONSUMER RISK ASSESSMENT

Long-term exposure was calculated using the EFSA PRIMo model revision 3.1, accessible online at https://www.efsa.europa.eu/en/applications/pesticides/tools. Chronic consumer exposure was expressed in % of the ADI. The acceptable limit for long-term exposure is 100 % of the ADI.

2. 6. SAMPLING

A total of 30 bee pollen samples (none of them beebread) were collected in May and June 2020 from Slovenian beekeepers that produce apiculture products sold on the market. Samples were gathered from all 12 statistical regions in Slovenia. The sampling distribution is presented in Table 1. All samples originated from conventional production.

3 RESULTS AND DISCUSSION

3. 1. COMPARISON OF QUECHERS METHOD WITH OUR METHOD

In the original QuEChERS method, 10 ml of acetonitrile was added to 10 g of the sample (Anastassiades et al.; 2003). In our method this ratio was different: 80 ml of acetonitrile was added to 10 g of the sample. The reason for increasing the solvent volume was that when we tested the addition of 10 ml of acetonitrile to 10 g of pollen, the recoveries were 20-30 % lower.

Table 1: Number of pollen samples collected from different statistical regions in Slovenia in 2020

Statistical region	Number of samples
Gorenjska	2
Goriška	2
Jugovzhodna Slovenija	1
Koroška	2
Notranje kraška	3
Obalno kraška	2
Osrednja Slovenija	7
Podravska	3
Pomurska	2
Savinjska	3
Spodnje posavska	1
Zasavska	2
Sum	30

The clean-up in our method was not conducted with dispersive SPE as in the original QuEChERS method (Anastassiades et al.; 2003, Lehotay; 2007), but with Ultra 2400 3 ml SPE columns.

In the QuEChERS method, aliquots of extracts were cleaned-up, while in our method, the transference of the extracts was quantitative.

3. 2. VALIDATION OF METHOD

LOQ and linearity

The linear model is valid for all active substances presented in Table 2. Linearity was proven in the range of 0.01 mg kg^{-1} to 0.15 mg kg^{-1} for six active substances, in the range of 0.05 mg kg^{-1} to 0.12 mg kg^{-1} for one active substance and in the range of 0.05 mg kg^{-1} to 0.15 mg kg^{-1} for 42 active substances. R^2 ranged from 0.974 to 0.996.

The LOQs are presented in Table 2. Six active substances have an LOQ of 0.01 mg kg^{-1} and 43 of them 0.05 mg kg^{-1}. The LOQs are equal to MRLs set in Regulation (EC) 396/2005.

Accuracy

The results for the recoveries are given in Table 2. The recoveries at LOQs for the active substances scanned with GC-MS are in the range of 73.0 % to 93.4 %, with RSDs of 5.6 % to 17.7 %. More precisely, the recoveries at LOQs of 0.01 mg kg^{-1} are within the range of 75.4 % to 93.4 % with RSDs of 9.3 % to 17.7 % and the recoveries at LOQs of 0.05 mg kg^{-1} are within the range of 73.0 % to 88.2 % with RSDs of 5.6 % to 15.9 %.

All recoveries and RSDs are within the required ranges from the literature (Alder et al., 2000; SANTE/11813/2017).

Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and uncertainty of reproducibility were determined at contents equal to the LOQs. The results are presented in Table 2. Uncertainty of repeatability ranged from 0.001 mg kg^{-1} to 0.013 mg kg^{-1}, which is 10.0 % to 30.0 % of LOQ and uncertainty of reproducibility ranged from 0.002 mg kg^{-1} to 0.015 mg kg^{-1}, which is 12.0 % to 30.0 % of LOQ.

Table 2: Validation parameters, ions scanned, MRLs and activity type of active substances

Active substance	Activity type [a]	MRL[b] (mg kg^{-1})	Ions scanned[c] (m/z) T, Q_1, Q_2	Linearity range (mg kg^{-1})	R^2	LD (mg kg^{-1})	LOQ (mg kg^{-1})	Recovery (%)	RSD[d] (%)	U_r[e] (mg kg^{-1})	U_r[f] (%)	U_R[g] (mg kg^{-1})	U_R[h] (%)
acrinathrin	A, I	0.05*	181, 208, 289	0.05-0.15	0.991	0.015	0.05	87.8	13.8	0.010	20	0.014	28
azinphos-methyl	A, I	/	160, 132, 105	0.05-0.15	0.987	0.015	0.05	84.2	9.9	0.009	18	0.010	20
azoxystrobin	F	0.05*	344, 388, 345	0.05-0.15	0.987	0.015	0.05	85.3	11.8	0.011	22	0.011	22
bifenthrin	A, I	0.05*	181, 165, 166	0.05-0.15	0.992	0.015	0.05	76.6	8.0	0.010	20	0.010	20
boscalid	F	0.05*	140, 342, 142	0.05-0.15	0.984	0.015	0.05	85.0	11.9	0.009	18	0.012	24
carbaryl	A, I	0.05*	144, 115, 116	0.05-0.15	0.988	0.015	0.05	81.9	7.0	0.010	20	0.010	20
carbofuran	A, I	0.05*	164, 149, 131	0.05-0.15	0.992	0.015	0.05	80.6	8.2	0.007	14	0.008	16
chlorpropham	H	0.05*	213, 127, 154	0.05-0.15	0.983	0.015	0.05	74.9	8.0	0.007	14	0.007	14
chlorpyriphos	A, I	0.05*	314, 316, 197	0.05-0.15	0.982	0.015	0.05	76.0	8.5	0.010	20	0.010	20
chlorpyriphos-methyl	I	0.05*	286, 288, 125	0.05-0.15	0.996	0.015	0.05	77.6	7.0	0.010	20	0.010	20
clomazone	H	0.05*	125, 204, 127	0.05-0.15	0.988	0.015	0.05	75.2	10.9	0.006	12	0.009	18
cyhalotrin-lambda	I	0.05*	181, 197, 208	0.05-0.15	0.987	0.015	0.05	88.2	12.3	0.011	22	0.012	24
deltamethrin	I	0.05*	181, 251, 255	0.05-0.15	0.990	0.015	0.05	80.1	14.9	0.010	20	0.014	28
diazinon	A, I	0.01*	179, 304, 199	0.01-0.15	0.994	0.003	0.01	81.3	12.9	0.002	20	0.002	20
dichlofluanid	A, F	/	226, 123, 167	0.05-0.15	0.990	0.015	0.05	79.1	7.9	0.010	20	0.010	20
dimethachlor	H	0.05*	134, 197, 210	0.05-0.15	0.995	0.015	0.05	81.2	6.5	0.006	12	0.006	12
dimethoate	A, I	/	87, 229, 143	0.05-0.15	0.994	0.015	0.05	81.7	5.6	0.010	20	0.010	20
diphenylamine	F	0.05*	169, 167, 168	0.05-0.12	0.982	0.015	0.05	73.2	9.1	0.008	16	0.008	16
endosulfan-sulphate	A, I	0.01*	272, 274, 387	0.01-0.15	0.995	0.003	0.01	82.4	9.3	0.001	10	0.002	20
fenbuconazole	F	0.05*	198, 129, 125	0.05-0.15	0.989	0.015	0.05	78.9	15.9	0.013	26	0.014	28
fenitrothion	I	0.01*	277, 260, 109	0.01-0.15	0.981	0.003	0.01	84.7	14.7	0.002	20	0.003	30
flonicamid	I	0.05*	174, 146, 229	0.05-0.15	0.992	0.015	0.05	79.8	7.7	0.007	14	0.007	14
fludioxonil	F	0.05*	248, 154, 127	0.05-0.15	0.990	0.015	0.05	85.5	9.8	0.009	18	0.010	20
fluquinconazole	F	0.05*	340, 342, 108	0.05-0.15	0.989	0.015	0.05	82.6	10.7	0.010	20	0.010	20
HCH-alpha	I	0.01*	219, 181, 183	0.01-0.15	0.996	0.003	0.01	75.4	12.3	0.002	20	0.002	20

Active substance	Activity type [a]	MRL[b] (mg kg^{-1})	Ions scanned[c] (m/z) T, Q_1, Q_2	Linearity range (mg kg^{-1})	R^2	LD (mg kg^{-1})	LOQ (mg kg^{-1})	Recovery (%)	RSD[d] (%)	U_r[e] (mg kg^{-1})	U_r[f] (%)	U_R[g] (mg kg^{-1})	U_R[h] (%)
HCH-deltha	I	/	219, 181, 183	0.05-0.15	0.979	0.015	0.05	82.8	12.7	0.007	14	0.012	24
iprodione	F	0.05*	314, 316, 187	0.05-0.15	0.986	0.015	0.05	81.6	9.8	0.010	20	0.010	20
kresoxim-methyl	F	0.05*	116, 206, 131	0.05-0.15	0.992	0.015	0.05	84.5	9.3	0.008	16	0.009	18
mecarbam	A, I	0.05*	131, 159, 329	0.05-0.15	0.974	0.015	0.05	79.7	10.8	0.010	20	0.010	20
methacrifos	A, I	0.05*	208, 180, 240	0.05-0.15	0.993	0.015	0.05	73.0	8.4	0.006	12	0.007	14
metrafenone	F	0.05*	393, 408, 379	0.05-0.15	0.992	0.015	0.05	80.5	9.2	0.010	20	0.010	20
parathion	A, I	/	291, 292, 235	0.05-0.15	0.993	0.015	0.05	82.3	15.7	0.009	18	0.015	30
permethrin	A, I	/	183, 163, 165	0.05-0.15	0.989	0.015	0.05	78.3	10.2	0.008	16	0.008	16
phorate	A, I	0.01*	231, 260, 97	0.01-0.15	0.996	0.003	0.01	82.4	17.7	0.002	20	0.003	30
phosalone	A, I	0.01*	182, 367, 121	0.01-0.15	0.995	0.003	0.01	93.4	13.9	0.003	30	0.003	30
pirimicarb	I	0.05*	166, 238, 167	0.05-0.15	0.983	0.015	0.05	79.5	7.2	0.006	12	0.007	14
pirimiphos-methyl	A, I	0.05*	290, 305, 276	0.05-0.15	0.992	0.015	0.05	78.4	7.0	0.010	20	0.010	20
procymidone	F	0.05*	283, 285, 96	0.05-0.15	0.981	0.015	0.05	80.1	9.6	0.010	20	0.010	20
propyzamide	H	0.05*	173, 175, 145	0.05-0.15	0.993	0.015	0.05	78.3	9.8	0.007	14	0.009	18
pyridaphenthion	I	/	199, 340, 188	0.05-0.15	0.992	0.015	0.05	83.9	9.2	0.008	16	0.009	18
quinalphos	A, I	0.05*	146, 298, 157	0.05-0.15	0.992	0.015	0.05	81.2	7.8	0.010	20	0.010	20
quinoclamine	H	0.05*	207, 172, 209	0.05-0.15	0.990	0.015	0.05	76.9	12.8	0.010	20	0.010	20
tetradifon	A	0.05*	159, 229, 356	0.05-0.15	0.990	0.015	0.05	82.1	10.7	0.009	18	0.010	20
tolclofos-methyl	F	0.05*	265, 267, 250	0.05-0.15	0.994	0.015	0.05	78.4	7.9	0.010	20	0.010	20
tolylfluanid	F	0.05*	238, 137, 240	0.05-0.15	0.985	0.015	0.05	80.8	7.7	0.007	14	0.007	14
triadimefon	F	0.05*	208, 210, 181	0.05-0.15	0.993	0.015	0.05	80.4	9.0	0.007	14	0.008	16
triazophos	A, I	0.05*	161, 162, 285	0.05-0.15	0.982	0.015	0.05	84.1	10.4	0.010	20	0.010	20
trifloxystrobin	F	0.05*	116, 222, 186	0.05-0.15	0.992	0.015	0.05	83.7	8.7	0.008	16	0.008	16
vinclozolin	F	0.05*	285, 124, 187	0.05-0.15	0.995	0.015	0.05	83.9	8.1	0.008	16	0.008	16

[a] A = acaricide, I = insecticide, F = fungicide, H = herbicide
[b] Regulation (EC) 396/2005, * means that MRL is set at LOQ of analytical method
[c] T = target ion, Q = qualifier ion
[d] RSD was obtained during recovery analyses
[e,f] U_r = uncertainty of repeatability
[g,h] U_R = uncertainty of reproducibility

3. 3. SURVEY OF PESTICIDE RESIDUES IN BEE POLLEN SAMPLES

The Ministry of Agriculture, Forestry and Food reported that in Slovenia in 2020, 582 PPPs, containing 239 active substances, are authorised for use on different agricultural products. The Statistical Office announced that in 2018, 1,172 tons of active substances were sold in Slovenia, where we have 476,000 hectares of cultivated agricultural area. This suggests broad use of PPPs among farmers. Since bees collect pollen not only on flowers, acacia, spruce, sage, lime and chestnut but also on agricultural products treated with PPPs, such as oilseed rape, fruits, etc., we wanted to research if these kinds of pesticide residues are found in bee pollen. We were searching for authorised (33 % of active substances sought) and non-authorised active substances in Slovenia, to cover the possible misuse of PPPs.

Of the 30 bee pollen samples analysed, only one contained one active substance: azoxystrobin, with a concentration of < 0.05 mg kg^{-1}. This means that in 96.7 % of all samples analysed, no pesticide residues were detected. The MRL for azoxystrobin in pollen is 0.05 mg kg^{-1} and it was not exceeded. In Slovenia, azoxystrobin is authorised as a fungicide for use on oilseed rape, vine and ornamentals (among others) in 14 different PPPs. These are the plants on which bees collect pollen.

A consumer risk assessment was performed using the EFSA PRIMo model rev. 3.1, in which 36 national diets from EU countries are included. This model was used since Slovenia has not created a model of its own. The same model is used in the process of registration of PPPs in Slovenia. Since azoxystrobin was the only substance found and it was only found in one sample at a concentration of < LOQ, the LOQ for this substance was used as the input value in PRIMo model. It was compared to the Acceptable Daily Intake (ADI) of azoxystrobin (0.2 mg $(kg\ bw)^{-1}\ d^{-1}$). The calculations of chronic exposure for azoxystrobin showed that the highest was observed in the German diet for children. It represented 0.003 % of ADI. Since no Acute Reference Dose was set for azoxystrobin, no acute exposure was calculated. Based on these calculations, the conclusion was that the analysed bee pollen samples are of no cause for concern for consumers.

Our results were compared with the results from other scientific papers. Azoxystrobin was found in the Estonian pollen by Raimets et al. (2020) in 3.4 % of all samples analysed up to a concentration of 0.04 mg kg^{-1}. Tosi et al. (2018) wrote that azoxystrobin was found in 2.9 % of the Italian pollen samples analysed, with a maximum concentration of 0.054 mg kg^{-1}. Vázquez et al. (2015) reported that azoxystrobin was found in a concentration of up to 0.235 mg kg^{-1} in the Spanish pollen. Azoxystrobin was found in 3.3 % of the Slovenian pollen samples analysed, which is comparable to Estonia and Italy. The concentration of azoxystrobin found in Slovenia is comparable to that found in Estonia and Italy, but much lower than in Spain.

Other active substances analysed in our laboratory, namely acrinathrin, bifenthrin, boscalid, carbaryl, carbofuran, chlorpyrifos, clomazone, dimethoate, fenitrothion, fludioxonil, iprodione, lambda-cyhalothrin, permethrin, trifloxystrobin and vinclozoline, were not detected in Slovenian pollen, but were found in samples analysed in Estonia, France, Greece, Italy, Spain and the United Kingdom.

All active substances sought by our laboratory and positively identified in Europe were measured up to concentrations higher than our LDs. The exception is carbofuran, which was found at a concentration 10-times lower than our LD. Literature results for these active substances are presented in Table 3.

4 CONCLUSIONS

In our research, a method for determining pesticide residues originating from the environment in pollen was introduced and validated. The limit of detection was 0.003 mg kg^{-1} for 6 active substances and 0.01 mg kg^{-1} for 43 active substances. The limit of quantification was 0.01 mg kg^{-1} for 6 active substances and 0.05 mg kg^{-1} for 43 active substances. The calibration curves gave a linear response with R^2 0.974 to 0.996. The recoveries ranged from 73.0 % to 93.4 % with RSDs from 5.6 % to 17.7 %. The measurement uncertainty of repeatability ranged from 10 to 30 % and the measurement uncertainty of reproducibility from 12 to 30 %. The method was found to be fit for purpose of measuring possible breaches of MRL for 49 active substances.

The method was used to analyse 30 bee pollen samples gathered from Slovenian beekeepers, all from conventional production. A total of 49 active substances were sought, but only the fungicide azoxystrobin was found in only one of these samples. In 96.7 % of the samples analysed, the active substances sought were not detected. A risk assessment revealed that the Slovenian bee pollen samples are no cause for concern for consumers.

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Table 3: Literature results for active substances sought, but not found in our laboratory

Active substance	Limit of detection (mg kg $^{-1}$)	Max content (mg kg $^{-1}$)	Ratio of positive samples (%)	Country of origin	Reference
acrinathrin	not reported	0.458	20.0	Spain	Calatayud-Vernich et al., 2018
acrinathrin	0.015	0.055	not reported	Spain	Vázquez et al., 2015
bifenthrin	0.015	0.015	not reported	Spain	Vázquez et al., 2015
boscalid	0.0025	0.058	0.7	Italy	Tosi et al., 2018
boscalid	0.00012	0.021	52.0	United Kingdom	David et al., 2016
boscalid	0.0015	0.03	not reported	Spain	Vázquez et al., 2015
carbaryl	0.0007	0.015	8.0	France	Wiest et al., 2011
carbaryl	0.00025	0.001	0.2	Italy	Tosi et al., 2018
carbofuran	0.0004	0.002	2.0	France	Wiest et al., 2011
chlorpyrifos	not reported	0.05	14.0	Spain	Hakme et al., 2017
chlorpyrifos	0.001	0.3982	not reported	Spain	García-Valcárcel et al., 2019
chlorpyrifos	not reported	0.1	31.1	Spain	Calatayud-Vernich et al., 2018
chlorpyrifos	0.0015	0.07	not reported	Spain	Vázquez et al., 2015
chlorpyrifos	0.008	0.14	4.0	France	Wiest et al., 2011
chlorpyrifos	0.0032	0.046	not reported	Greece	Kasiotis et al., 2014
chlorpyrifos	0.001	0.179	30.3	Italy	Tosi et al., 2018
clomazone	not reported	0.02	5.0	Spain	Hakme et al., 2017
dimethoate	not reported	0.042	20.7	Estonia	Raimets et al., 2020
dimethoate	0.0015	0.015	not reported	Spain	Vázquez et al., 2015
dimethoate	not reported	0.022	8.9	Spain	Calatayud-Vernich et al., 2018
dimethoate	0.00025	0.163	7.9	Italy	Tosi et al., 2018
dimethoate	0.0028	0.1445	not reported	Greece	Kasiotis et al., 2014
dimethoate	0.0091	0.0182	1.0	France	Wiest et al., 2011
fenithrothion	not reported	0.014	2.2	Spain	Calatayud-Vernich et al., 2018
fludioxonil	0.0015	0.033	not reported	Spain	Vázquez et al., 2015
iprodione	0.0156	0.0195	1.0	France	Wiest et al., 2011
lambda-cyhalothrin	not reported	0.077	17.2	Estonia	Raimets et al., 2020
permethrin	not reported	0.034	5.0	Spain	Hakme et al., 2017
permethrin	0.0015	0.0035	not reported	Spain	Vázquez et al., 2015
trifloxystrobin	0.0086	0.058	not reported	Greece	Kasiotis et al., 2014
trifloxystrobin	0.00024	0.01	40.0	United Kingdom	David et al., 2016
trifloxystrobin	0.00025	0.046	5.6	Italy	Tosi et al., 2018
trifloxystrobin	0.0015	0.0154	not reported	Spain	Vázquez et al., 2015
vinclozoline	0.0015	0.07	2.0	France	Wiest et al., 2011

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Investigation on amitraz, coumaphos and thymol concentrations in honey produced by Slovenian beekeepers in 2020

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Investigation on amitraz, coumaphos and thymol concentrations in honey produced by Slovenian beekeepers in 2020

Abstract: A survey on concentrations of veterinary drug residues amitraz, coumaphos and thymol in honey, produced in year 2020 by Slovenian beekeepers, was conducted. 100 samples were analysed: 22 from organic and 78 from conventional production, with two analytical methods. In method for determination of coumaphos and thymol samples were extracted with acetone, petroleumether and dichlorometane. In method for determination of amitraz and its degradation products, samples were hydrolisated with HCl and NaOH, extractied with n-hexane and derivatisated with heptafluorobutyric anhydride. Determination in both methods was performed with gas chromatograph coupled with mass spectrometer. Measured concentrations of amitraz, coumaphos and thymol were in the range of 0.01-0.12 mg kg^{-1}, 0.02-0.06 mg kg^{-1} and 0.08-0.17 mg kg^{-1}, respectively. In 61 % of samples analysed no residues of amitraz, thymol and coumaphos were found. Data obtained were compared with the data from literature. Chronic and acute exposure were calculated for consumers. Maximum chronic exposure for amitraz and thymol was 0.1 % and 0.05 % of acceptable daily intake, respectively. Maximum acute exposure for amitraz and thymol was 4 % and 0.8 % of acute reference dose, respectively.

Key words: acaricide residues; GC-MS; amitraz; coumaphos; thymol; honey; consumer exposure

Raziskava o koncentracijah amitraza, kumafosa in timola v medu slovenskih čebelarjev v letu 2020

Izvleček: Izvedli smo raziskavo v kateri smo spremljali ostanke veterinarskih zdravil: amitraza, kumafosa in timola v medu, ki so ga slovenski čebelarji pridelali v letu 2020. Analizirali smo 100 vzorcev: 22 iz ekološke in 78 iz konvencionalne pridelave, z dvema analiznima metodama. Pri metodi za določanje kumafosa in timola smo vzorce ekstrahirali z acetonom, petroletrom in diklorometanom. Pri metodi za določanje amitraza in njegovih razpadnih produktov, smo vzorce hidrolizirali s HCl in NaOH, ekstrahirali z n-heksanom in jih derivatizirali z heptafluorobutiričnim anhidridom. Določevanje je pri obeh metodah potekalo s plinskim kromatografom sklopljenim z masnim spektrometrom. Izmerjene koncentracije amitraza, kumafosa in timola so bile v območju 0,01-0,12 mg kg^{-1}, 0,02-0,06 mg kg^{-1} oziroma 0,08-0,17 mg kg^{-1}. V 61 % analiziranih vzorcev, nismo določili amitraza, kumafosa in timola. Pridobljene podatke smo primerjali s podatki iz literature. Izračunali smo kronično in akutno izpostavljenost za potrošnike. Maksimalna kronična izpostavljenost za amitraz in timol je bila 0,1 % oziroma 0,05 % sprejemljivega dnevnega vnosa. Maksimalna akutna izpostavljenost za amitraz in timol pa je bila 4 % oziroma 0,8 % akutne referenčne doze.

Ključne besede: ostanki akaricidov: GC-MS; amitraz; kumafos; timol; med; izpostavljenost potrošnikov

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1 INTRODUCTION

Honey is produced by honey bees from nectar of plants, as well as from honey dew. It contains carbohydrates, water, traces of organic acids, enzymes, amino acids, pigments, pollen and wax (Anklam, 1998). Besides its nutritional value, honey is nowadays used as natural sweetener, which is very important for diabetics.

Unfortunatelly, honey bees have an enemy called *Varroa* mite, more precisely *Varroa destructor* Anderson & Trueman (2000) described, which represent a great threat to honey bee populations worldwide. Beekeepers can control the mite by using veterinary drugs, which contain amitraz, coumaphos, tau-fluvalinate or flumetrine. In case of organic production only thymol, menthol, eucalyptol, camphor, formic acid, lactic acid, acetic acid and oxalic acid can be used as laid down in Regulation (EC) 889/2008. Consequence is that veterinary drugs utilized for beehive treatments are incorporated into the honey and accumulate in other hive products (Fernandez-Muiño et al., 1995).

In organic production in Slovenia mainly thymol, formic acid, lactic acid and oxalic acid are used. No maximum residue levels (MRLs) are set for these substances in European Union. Thymol residues in honey are safe up to concentration 50 mg kg^{-1} (Bogdanov et al., 1998). But the taste threshold of thymol in honey is between 1.1 and 1.6 mg kg^{-1} (Bogdanov et al., 1998). Not to change natural taste of honey, Swiss set MRL for thymol at 0.8 mg kg^{-1}.

The most commonly used veterinary drugs in conventional production in Slovenia are the ones which contain amitraz and coumaphos. European Union MRLs for these two compounds are set in Regulation (EC) 37/2010. MRL for amitraz is 0.2 mg kg^{-1} and for coumaphos is 0.1 mg kg^{-1}. Since consumers demand safe products, with veterinary drug residues below MRLs, surveys of such residues are required.

Amitraz and coumaphos were found in honey by different authors: in Polish samples by Gaweł et al. (2019), in Spanish samples by Lozano et al. (2019) and in French samples by Wiest et al. (2011). Amitraz was also measured in Spanish honey samples by Juan-Borrás et al. (2016). Coumaphos was also found in Portugese market honey samples by Rial-Otero et al. (2007), in French honey samples by Martel et al. (2002), in Italy organic samples by Chiesa et al. (2016), in Italy honey samples by Del Carlo et al. (2010) and in Polish honey samples by Bargańska et al. (2013). Thymol residues in honey were measured more rarely than the ones for amitraz and coumaphos. Viñas et al. (2006) and Nozal et al. (2002) found thymol in Spanish honey samples and Bogdanov et al. (1998) found thymol in Swiss honey samples.

In present paper, measurements of all three most frequently used acaricides in honey of Slovenian beekeepers, collected in 2020 are presented. Only allowed compounds were tested, meaning that no potential usage of non registered compounds has been investigated. Also, comparison between honey from conventional production and organic production was conducted. Finally, risk assessment was calculated for analysed samples. Our hypothesis was that most frequently amitraz will be used among beekeepers since its degradation time in honey is 10 days (Korta et al., 2001), while for coumaphos it is 9 months (Korta et al., 2001). Thymol is not expected to be used frequently as well, since it can change natural taste of honey.

2 MATERIAL AND METHODS

2.1. SAMPLING

100 honey samples were collected in June, July and August 2020, from Slovenian beekeepers from all 12 statistical regions in Slovenia. Sampling distribution is presented in Table 1. 78 samples originated from conventional production, meaning that beekeepers reported use of amitraz, coumaphos, thymol, flumethrin, formic acid, lactic acid and/or oxalic acid to supress varroa and 22 samples from organic production, meaning that beekeepers reported only use of thymol, formic acid, lactic acid and/or oxalic acid to supress varroa.

2. 2.ANALYTICAL PROCEDURE

Amitraz and its degradation products (all metabolites containing the 2,4-dimethylaniline moiety)

After sample hydrolisation with 2N HCl and 2M NaOH, extraction was performed with n-hexane, followed by derivatisation with heptafluorobutyric anhydride (HFBA). Determination was conducted with gas chromatograph coupled with mass spectrometer (GC-MS). Method is in detail described elsewhere (Kmecl & Baša Česnik, 2011; Baša Česnik et al., 2019).

Coumaphos and thymol

Samples were dissolved in water and then extracted with mixture of acetone, petroleumether and dichlorometane at ratio 1:2:2 (v/v/v). Determination was conducted with GC-MS. Method is in detail described elsewhere (Baša Česnik et al., 2019).

Quality assurance

Each series of analyses included one or two spiked samples of commodity analysed. Recoveries were 70-120

Table 1: Sampling distribution

Statistical region	No. of samples from conventional production	No. of samples from organic production	Sum
Gorenjska	4	2	6
Goriška	9	2	11
Jugovzhodna Slovenija	4	3	7
Koroška	4	0	4
Notranje kraška	5	0	5
Obalno kraška	7	2	9
Osrednja Slovenija	8	3	11
Podravska	6	1	7
Pomurska	10	1	11
Savinjska	15	5	20
Spodnje posavska	4	2	6
Zasavska	2	1	3
Sum	78	22	100

%. In SANTE/11813/2017 requirement for recoveries is 60-140 %.

2. 3. RISK ASSESSMENT

Chronic exposure

The calculation of long-term exposure was performed with the EFSA PRIMo model revision 3.1, accessible on the internet at https://www.efsa.europa.eu/en/applications/pesticides/tools. The Supervised Trial Median Residue (STMR) was calculated from all samples analysed. It was compared to the Acceptable Daily Intake (ADI) of a single active substance. Chronic consumer exposure was expressed in % of the ADI. The acceptable limit for long-term exposure is 100 % of the ADI.

Acute exposure

The calculation of short-term exposure was performed with the EFSA PRIMo model revision 3.1, accessible on the internet at https://www.efsa.europa.eu/en/applications/pesticides/tools.

The Highest Residue (HR) was compared to the Acute Reference Dose (ARfD) of a single active substance. Acute consumer exposure was expressed in % of ARfD. The acceptable limit for short-term exposure is 100 % of the ARfD.

3 RESULTS AND DISCUSSION

In 43 samples from conventional production (55.1 %), no residues of amitraz, coumaphos and thymol were found. Amitraz, coumaphos and thymol residues were found in 29 (37.2 %), 7 (9.0%) and 3 (3.8 %) samples from conventional production respectively. Multiple residues in conventional production were found only in 4 samples (5.1 %): 3 samples contained residues of amitraz and coumaphos (3.8 %) and 1 sample contained residues of amitraz and thymol (1.3 %). No MRL exceedances were observed for any of the substances analysed. Results are presented in Table 2.

In 18 samples from organic production (81.8 %), no residues of amitraz, coumaphos and thymol were found. Amitraz, coumaphos and thymol residues were found in 2 (9.1 %), 1 (4.5 %) and 1 (4.5 %) samples from organic production respectively. It was expected that thymol would be present in larger amount of samples in organic production, but beekeepers obviously prefer the use of formic acid, and oxalic acid to supress varroa, probably because of fear to change sensory characteristics of honey. Multiple residues in organic production were not found. No MRL exceedances were observed for any of the substances analysed. Results are presented in Table 2.

The highest concentration of amitraz in conventional production was found in Pomurska region (0.12 mg kg^{-1}) and in organic production in Jugovzhodna Slovenija region (0.03 mg kg^{-1}).The highest concentration of coumaphos in conventional production was found in Osrednja Slovenija region and in organic production in Gorenjska region (0.02 mg kg^{-1}). The highest concentration of thymol in conventional production was found in Savinjska region (0.17 mg kg^{-1}) and in organic production in Spodnje posavska region (0.16 mg kg^{-1}). Results are presented in Table 3.

Table 2: Amitraz, coumaphos and thymol residues in honey samples in 2020

	amitraz	coumaphos	thymol
LOQ (mg kg^{-1})	0.01	0.009	0.07
MRL (mg kg^{-1})	0.2 (a)	0.1 (a)	/
conventional production			
Min concentration (mg kg^{-1})	0.01	0.02	0.08
Max concentration (mg kg^{-1})	0.12	0.06	0.17
Average (mg kg^{-1})	0.03	0.04	0.12
SD (mg kg^{-1})	0.03	0.02	0.04
No. of samples where residues were found	29	7	3
organic production			
Min concentration (mg kg^{-1})	0.01	0.02	0.16
Max concentration (mg kg^{-1})	0.03	0.02	0.16
Average (mg kg^{-1})	0.02	0.02	0.16
SD (mg kg^{-1})	0.02	/	/
No. of samples where residues were found	2	1	1

(a) Regulation (EC) 37/2010
LOQ means limit of quantification of the method
MRL means maximum residue limit
SD means standard deviation

Table 3: Range of concentrations in honey samples according to region in 2020

	Amitraz	Amitraz	Coumaphos	Coumaphos	Thymol	Thymol
	Conventional	Organic	Conventional	Organic	Conventional	Organic
	conc (mg kg^{-1})	conc (mg kg^{-1})	conc (mg kg^{-1})	conc (mg kg^{-1})	conc (mg kg^{-1})	conc (mg kg^{-1})
Gorenjska	0.02-0.07	n.d.	n.d.	0.02	n.d.	n.d.
Goriška	0.01-0.08	n.d.	n.d.	n.d.	0.08	n.d.
Jugovzhodna Slovenija	n.d.	0.03	n.d.	n.d.	n.d.	n.d.
Koroška	0.01	n.a.	0.02-0.04	n.a.	n.d.	n.a.
Notranje kraška	0.06	n.a.	n.d.	n.a.	n.d.	n.a.
Obalno kraška	0.01-0.02	n.d.	n.d.	n.d.	n.d.	n.d.
Osrednja Slovenija	0.02	n.d.	0.06	n.d.	n.d.	n.d.
Podravska	n.d.	n.d.	0.03-0.05	n.d.	n.d.	n.d.
Pomurska	0.01-0.12	0.01	0.02	n.d.	n.d.	n.d.
Savinjska	0.01-0.03	n.d.	0.04	n.d.	0.12-0.17	n.d.
Spodnje posavska	0.05-0.07	n.d.	n.d.	n.d.	n.d.	0.16
Zasavska	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.a means not analysed
n.d. means not determined

Table 4: Ratio of positive honey samples according to region in 2020

	Amitraz	Amitraz	Coumaphos	Coumaphos	Thymol	Thymol
	Conventional	Organic	Conventional	Organic	Conventional	Organic
	Ratio (%)	Ratio (%)	Ratio (%)	Ratio (%)	Ratio (%)	Ratio (%)
Gorenjska	**50.0**	0.0	0.0	**50.0**	0.0	0.0
Goriška	**33.3**	0.0	0.0	0.0	**11.1**	0.0
Jugovzhodna Slovenija	0.0	**33.3**	0.0	0.0	0.0	0.0
Koroška	**25.0**	n.a.	**50.0**	n.a.	0.0	n.a.
Notranje kraška	**20.0**	n.a.	0.0	n.a.	0.0	n.a.
Obalno kraška	**57.1**	0.0	0.0	0.0	0.0	0.0
Osrednja Slovenija	**12.5**	0.0	**12.5**	0.0	0.0	0.0
Podravska	0.0	0.0	**33.3**	0.0	0.0	0.0
Pomurska	**80.0**	**100.0**	**10.0**	0.0	0.0	0.0
Savinjska	**46.7**	0.0	**6.7**	0.0	**13.3**	0.0
Spodnje posavska	**50.0**	0.0	0.0	0.0	0.0	**50.0**
Zasavska	0.0	0.0	0.0	0.0	0.0	0.0

n.a. means not analysed

The highest ratio of positive samples (where active substance was found) for amitraz was observed in Pomurska region in conventional and organic production. Amitraz was not found in Podravska and Zasavska regions in conventional and organic production. Coumaphos was in conventional production most frequently found in Koroška region and in organic production in Gorenjska region. Coumaphos was not found in conventional and organic production in Goriška, Jugovzhodna Slovenija, Obalno kraška, Spodnje posavska and Zasavska regions. Thymol was in conventional production most frequently found in Savinjska region and in organic production in Spodnje posavska region. Thymol was not found in conventional and organic production in Gorenjska, Jugovzhodna Slovenija, Obalno kraška, Osrednja Slovenija, Podravska, Pomurska, and Zasavska regions. Results are presented in Table 4.

Amitraz was found in 31 % of samples analysed in year 2020. It was also the most frequently found in years 2016 (unpublished results), 2017 (Baša Česnik and Kmecl, 2017), 2018 (Baša Česnik and Kmecl, 2018) and 2019 (Baša Česnik and Kmecl 2019) in 24 %, 30 %, 28 % and 46 % of samples analysed respectively. Coumaphos was found in 8 % of samples analysed in year 2020. It was also the second most frequently found substance in years 2016 (unpublished results), 2017 (Baša Česnik and Kmecl, 2017), 2018 (Baša Česnik and Kmecl, 2018) and 2019 (Baša Česnik and Kmecl 2019) in 5 %, 10 %, 6 % and 7 % of samples analysed respectively. The least frequently found was thymol in 4 % of samples in 2020. It was also the least frequently found substance in years 2016 (unpublished results), 2017 (Baša Česnik and Kmecl, 2017), 2018 (Baša Česnik and Kmecl, 2018) and 2019 (Baša Česnik and Kmecl 2019) in 3 %, 1 %, 3 % and 4 % of samples analysed respectively.

Risk assessment for samples from year 2020 was conducted for amitraz and thymol only, since no ADI and ARfD are available for coumaphos. Chronic risk assessment for amitraz was conducted with ADI 0.003 mg $(kg\ bw)^{-1}d^{-1}$ and STMR 0.02 mg kg^{-1}. It resulted in 0.1 % of ADI maximum, for consumer group DE child. This represented maximum amitraz exposure through honey of 0.002 µg $(kg\ bw)^{-1}d^{-1}$. Acute risk assessment for amitraz was conducted with ARfD 0.01 mg $kg\ bw)^{-1}$ and HR 0.12 mg kg^{-1}. It resulted in 4 % of ARfD maximum, for consumer group children. This represented maximum amitraz exposure through honey of 0.43 µg $(kg\ bw)^{-1}$ with one meal. Chronic risk assessment for thymol was conducted with ADI 0.03 mg $(kg\ bw)^{-1}d^{-1}$ and STMR 0.14 mg kg^{-1}. It resulted in 0.05 % of ADI maximum, for consumer group DE child. This represented thymol exposure through honey of 0.01 µg $(kg\ bw)^{-1}d^{-1}$. Acute risk assessment for thymol was conducted with ARfD 0.08 mg $(kg\ bw)^{-1}$ and HR 0.17 mg kg^{-1}. It resulted in 0.8 % of ARfD maximum, for consumer group children. This represented maximum thymol exposure through honey of 0.61 µg

(kg bw)$^{-1}$with one meal. Low chronic exposure for consumers means that analysed honey can be consumed by all consumer groups: children, adults and elderly people every day of their life without risk that it would affect their health. Low acute exposure for consumers means that analysed honey can be consumed with one meal without risk that it would affect consumers health. Based on calculations it can be concluded that analysed honey represents no unacceptable risk for consumers.

In literature we found data for amitraz concentrations in honey from different authors. Lozano et al. (2019) found amitraz in Spanish honey samples up to concentration 0.648 mg kg^{-1}. Gaweł et al. (2019) found amitraz in Polish honey samples in concentrations up to 0.6 mgkg^{-1} Wiest et al. (2011) found amitraz in French honey samples up to concentration 0.116 mg kg^{-1}. Juan-Borrás et al. (2016) found amitraz in Spanish market honey samples up to concentration 0.05 mg kg^{-1}. Highest concentration found in 2020 in Slovenia (0.12 mg kg^{-1}) is comparable to French one, but lower than in Polish samples and Spanish samples measured by Lozano et al. (2019) and at the same time higher than in Spanish samples measured by Juan-Borrás et al. (2016).

Coumaphos concentrations in honey were even more frequently reported in literature than the ones for amitraz. Martel & Zeggane (2002) found coumaphos in French honey samples in concentrations up to level 0.26 mg kg^{-1}. Del Carlo et al. (2010) and Chiesa et al. (2016) found coumaphos in honey samples from Italy: the first one up to concentration 0.084 mg kg^{-1} and the second one up to concentration 0.00206 mg kg^{-1} (the last ones were organic samples). Gaweł et al. (2019) and Bargańska et al. (2013) found coumaphos in Polish honey: the first one in concentrations up to 0.039 mg kg^{-1} and the second one in concentrations up to 0.0167 mg kg^{-1}. Lozano et al. (2019) found coumaphos in Spanish honey samples up to concentration 0.036 mg kg^{-1}. Wiest et al. (2011) found coumaphos in French honey samples up to concentration 0.029 mg kg^{-1}. Juan-Borrás (2016) found coumaphos in Spanish market honey samples up to concentration 0.013 mg kg^{-1}. Rial-Otero et al. (2007) found coumaphos in Portugese market honey samples in concentrations up to 0.000015 mg kg^{-1}. The highest concentration found in 2020 in Slovenia (0.06 mg kg^{-1}) is lower than in French samples measured by Martel & Zeggane (2002) and Italian samples measured by Del Carlo et al. (2010) and at the same time higher than in Polish, Spain, Portugese samples, Italian samples measured by Chiesa et al. (2016) and French samples measured by Wiest et al. (2011).

In literature, thymol residues in honey were reported more rarely than for amitraz and coumaphos. Viñas et al. (2006) and Nozal et al. (2002) measured thymol in Spanish honey samples: the first one in concentrations up to 0.000346 mg kg^{-1} and the second one in concentrations up to 0.00036 mg kg^{-1}. Bogdanov et al. (1998) measured thymol in Swiss honey samples in concentrations up to 0.48 mg kg^{-1}. Highest concentration found in 2020 in Slovenia (0.17 mg kg^{-1}) is higher than in Spanish samples, but lower than in Swiss samples.

4 CONCLUSIONS

In 61 % of samples analysed no residues of amitraz, thymol and coumaphos were found. No MRL exceedances were observed in any of the samples. Amitraz was the most frequently found substance in honey produced in year 2020. We quantified it in 31 % of samples analysed. The second most frequently found substance was coumaphos, which was quantified in 8 % of samples. The least frequently found was thymol, which was quantified in 4 % of samples analysed. Risk assessment revealed that the analysed honey is safe for consumers. Concentrations of amitraz, coumaphos and thymol in Slovenian honey from 2020 are in range of data found in literature.

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Maize seedling emergence in response to climatic variability in a tropical rainforest area

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Maize seedling emergence in response to climatic variability in a tropical rainforest area

Abstract: Environmental factors causing low seedling emergence often observed in tropical maize (*Zea mays* L.) are poorly documented. This study was conducted to investigate the effects of weather factors on maize seedling emergence at the Obafemi Awolowo University Teaching and Research Farm (OAU TRF). Five maize varieties sown weekly, in 3-replicate RCBD experiments throughout the 2016 and 2017 cropping seasons, were used to monitor emergence percentage (E %), emergence index (EI) and emergence rate index (ERI). Climatic data were obtained from the automatic weather station located on the farm. Analysis of variance revealed highly significant ($P \leq 0.01$) environmental effect for all traits. Soil moisture (Sm), relative humidity, air temperature, heat unit and soil heat flux (SHF) showed significant ($P \leq 0.05$) correlation coefficients with all traits, but there was no relationship between the emergence traits and grain yield. Stepwise multiple regression and sequential path coefficient analyses indicated that increased Sm, rather than rainfall per se, increased the speed of emergence. Minimum air temperature and SHF with direct effects, and heat unit with indirect effect, negatively affected emergence the most. Relatively low Tmin and SHF, along with just enough Sm maximized seedling emergence in the rainforest agro-ecology of southwestern Nigeria.

Keywords: climatology; crop phenology; growth analysis; seedling vigour; *Zea mays* L.

Vpliv sprememb podnebja na vznik kalic koruze na območju tropskega deževnega gozda

Izvleček: Okoljski dejavniki, ki vplivajo na slab vznik koruze (*Zea mays* L.) so v tropskih razmerah pogosto opaženi a slabo dokumentirani. Ta raziskava je bila izvedena za preučevanje učinkov dejavnikov podnebja na vznik koruze na univerzitetnem učnem in raziskovalnem posestvu Obafemi Awolowo (OAU TRF), Nigerija. Pet sort koruze je bilo posejanih tedensko s tremi ponovitvami v popolnem naključnem bločnem poskusu (RCBD) v rastnih sezonah 2016 in 2017. Pri tem so bili spremljani odstotek kalitve (E %), indeks vznika (EI) in hitrost vznika (ERI). Klimatski podatki so bili pridobljeni iz avtomatske vremenske postaje na posestvu. Analiza variance je pokazala zelo značilne ($P \leq 0{,}01$) okoljske vplive na vse opazovane lastnosti. Vlažnost tal (Sm), relativna vlažnost zraka, temperatura zraka, privzem toplote in tok toplote v tleh (SHF) so imeli značilno korelacijo ($P \leq 0{,}05$) z vsemi opazovanimi lastnostmi, ugotovljena pa ni bila nobena povezava med lastnostimi kalitve in pridelkom zrnja. Stopenjska multipla regresija in kasnejša analiza posrednih in neposrednih vplivov na kalitev sta pokazali, da je imela nanjo večji vpliv vlažnost tal, ki je povečala hitrost kalitve kot pa sama količina padavin. Minimalna temperatura zraka in dotok toplote v tla sta imela največji neposredni vpliv na kalitev. Nizka temperature tal (Tmin) in majhen dotok toplote v tla (SHF) sta ob ravno zadostni vlažnosti tal (Sm) maksimalizirali vznik koruze v agro-ekoloških razmerah deževnega gozda jugozahodne Nigerije.

Ključne besede: klimatologija; fenologija poljščin; rastna analiza; vigor sejank; *Zea mays* L.

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1 INTRODUCTION

The demand for maize (*Zea mays* L.) grains in sub-Saharan Africa (SSA) has continued to increase because of its importance in human food, livestock feed, and industrial raw material. Maize accounts for more than 15 % of the total calorie intake by the population in SSA, along with its use in animal feed production and as raw material in some agro-allied industries. As noted by Talabi et al. (2017), better adaptation of maize crop to various agro-ecologies, responsiveness to fertilizer application, and relative ease of cultivation, processing and storage has greatly increased its popularity in West and Central Africa (WCA) over other traditional cereal crops, such as sorghum [*Sorghum bicolor* (L.) Moench] and pearl millet (*Penisetum typhoides* L.). There is, therefore, an urgent need for increased maize production in these regions to meet the constant demand for its grains. Environmental factors such as poor soil conditions, especially decreasing nitrogen level, drought, increased urbanization, poor or inaccurate agronomic practices, and poor climatic conditions have affected maize production, limiting it from attaining the desired production levels, despite maize crop improvements over the years from active breeding programs.

FAO (2016) puts maize production in Nigeria at about 11 MT/year compared to 384 MT/year obtained in the USA. A combination of improved varieties, improved agronomic practices and production intensification, including adaptation of maize production to climate through adequate knowledge of crop response to the variability of climatic conditions throughout the life cycle of the crop will be necessary to close this huge yield gap to a reasonable level. High emergence percentage followed by high seedling vigour and vigorous vegetative growth are necessary conditions for high final stand of the crop on the field which, in turn, leads to higher grain yield.

Maize, for all its positive features, depends on the environment for its survival; the climatic conditions constitute a very important component of the environment. Unfortunately, the information available on maize crop response to climate is very few and fewer still at the seedling stage, especially in the rainforest agro-ecology of Nigeria, and where such information exists, the depth and therefore the accuracy is often in question. This is due to the the use of rather outmoded climatology items of equipment for such studies compared with improved equipment with greater accuracy used in the developed world (Fakorede and Opeke 1985). Furthermore, there are often inadequacies in the number of climatic variables analyzed, or statistical analyses used, or both. Consequently, moisture availability has generally been seen as the most important variable influencing maize growth at seedling and flowering stages, even though there is paucity of information about maize crop response to other climatic variables such as temperature, heat unit and soil heat flux at the seedling stage of maize growth. Awosanmi et al. (2016) found significant effect of moisture stress on tropical maize seed yield in the rainforest ecology of Southwest Nigeria. Oke (2016) found that yield had significant positive correlations with total rainfall and air mean relative humidity and negative correlations with air temperature extremes in similar climate. Tunde et al. (2011) also found maize production to be highly inflenced by rainfall, air relative humidity, number of rainy days and air temperature at Ilorin, a Southern Guinea savanna location in Nigeria. Ammani et al. (2012) found that drought occuring at about one week before flowering to the grain filling stage caused significant reduction in yield when they evaluated the relationship between rainfall and maize crop production in the savannah ecologies. These studies have involved few climatic variables, mostly employed correlation for data analysis and none actually investigated crop response to climatic factors at the seedling stage. The study by Fakorede and Opeke (1985) employed several climatic variables and statistical analysis methods to elucidate the response of maize yield to weather factors in the rain forest of Nigeria, typified by Ile-Ife. They found significant negative correlation coefficients of effective rainfall with grain yield and air relative humidity even though the study was limited by the rather outmoded climatological equipment used. Studies conducted in advanced countries, such as that by Jong et al. (1982) concluded that solar radiation was the single most influencial climatic factor affecting yield and its components in maize even though only air temperature and solar radiation were the focus of analysis in the study. Dimpsey (1995) found 16 °C to 35 °C as the optimum germination range for maize seeds when he studied the effect of air temperature on the germination of different seeds in Australia. Alm et al. (1993) found a positive correlation between air temperature (from 10 °C to 25°°C) and seedling elongation of maize and soybean (*Glycine max* L).

Rainfall is the primary climatic factor that determines the timing of most agronomic practices, especially planting operations in Nigeria. False start of rainfall and unpredictable frequent occurrence of short duration drought during the growing season, both of which have become more severe over time, are now features of the rainforest agro-climatic zone of SW Nigeria typified by Ile-Ife, a location at 7° 28' N, 4° 33' E and 244 m asl in the zone (Fayose and Fakorede, 2021). Such weather anomalies have been attributed to climate change (Fakorede and Akinyemiju, 2003). The year 2020 received one of the smallest total rainfall amounts (< 700 mm) in recent

times, next to just above 700 mm received in 2017 at the location. Low amount of rainfall was received in June and July when frequent, heavy rainfall is normally expected. Reduced seedling emergence and low vigor, are usually associated with the weather anomalies that characterize maize especially those planted early in the zone. This has often resulted in missing stands, and the situation has been aggravated by climate change in recent times. It is therefore necessary to have adequate knowledge of crop response to climatic variables at all stages in order to be able to cope with the negative impacts of climate change.

The primary objective of this study was to identify the climatic factors influencing seedling emergence of maize under the natural climatic conditions of the rainforest agro-ecology of SW, Nigeria. A secondary objective was to determine the relationship of the seedling emergence traits with grain yield of maize.

2 MATERIAL AND METHODS

The study was conducted at the Teaching and Research Farm of Obafemi Awolowo University, Ile-Ife (OAU TRF) in the 2016 and 2017 early and late cropping seasons. OAU TRF is located at 7° 28' N, 4° 33' E and 244 m above sea level in the marginal areas of the rainforest agro-ecology of South Western Nigeria. As a typical rainforest agro-ecological location, Ile-Ife is characterized by two contrasting rainy seasons; the first or early season from about March/April to July and the second or late season from late August to October with a period of short dry spell that occurs in July/August, popularly referred to as the "August break" or "August dry spell". Total annual range of 740 mm to 2040 mm has been reported at the location, along with a mean maximum temperature range of 29 °C to 32 °C (Fakorede and Akinyemiju, 2003).

Five maize varieties adapted to the tropical rainforest environments were sown in three replicate-randomized complete block design. They include four open-pollinated varieties White DT STR SYN1.- TZL Comp. 1 – W, ACR 94 TZE Comp 5 C_3, TZL Comp. 4 DT F_2, TZL Comp. 1 C6/DT – SYN – 1 – W all of which were developed at the International Institute of Tropical Agriculture (IITA), Ibadan; and a hybrid, 'Oba Super 1', obtained from Premier Seeds, Zaria. All the varieties are white-grained, high yielding and have been released for commercial production in Nigeria and several other West and Central African (WCA) countries.

The maize was sown weekly from March to November each year. However, there were some weeks when sowing could not be done due to some extraneous factors; therefore, data from 54 dates (DOS) were analysed for seedling emergence and vigour and 42 dates were analysed for grain yield. Each plot contained four or six rows, which were 5 m long spaced 0.75 m apart; within row spacing was 0.5 m. Each plot was about 15 m^2 and 22.5 m^2 for the four and six-row plots, respectively. Three seeds were sown per hill. Prior to sowing, the experimental land was ploughed and harrowed, and the seed were treated with thiamethoxam, mefenoxam (metalaxyl-M) and difenoconazole, to control damage by soil-borne diseases and insect pests. Weeds were controlled by the application of post-sowing and pre-emergence of maize with primextra, which contains atrazine (2-chloro-4-(ethyl amino)-6-isopropylamino-s-triazine) and alachlor (N-(methyl-2-methoxy-ethyl)-2-ethyl-8-methyl-chloroacetanilide) as active ingredients at the rate of 5 l/ha. Emergence counts were made daily from five to nine days after sowing (DAS); from which emergence percentage (E%), emergence index (EI), and emergence rate index (ERI) were computed as follows (Fakorede and Agbana 1983):

$$E\,\% = \frac{Seedlings\ emerged\ in\ X\ \ DAP}{Total\ no.\ of\ seeds\ sown} * 100$$

$$EI = \frac{\sum(Plants\ emerged\ in\ a\ day)*(DAP)}{Plants\ emerged\ \ 9\ DAP}$$

$$ERI = \frac{EI}{E\%\ at\ 9DAP} * 100$$

Thinning was done immediately after emergence count at 9 DAS to two plants per stand giving an estimated plant population density of 53, 333 plants ha^{-1}. Fertilizer was applied immediately after thinning at the rate of 60 kg ha^{-1} each for N, P_2O_5 and K_2O. Further weed control was done using paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride), carefully applied as a post-emergence, non-selective and contact herbicide at the rate of 3.0 l ha^{-1}.

Data collected on grain yield were adjusted to 15 % moisture content. Minimum and maximum air temperatures and air relative humidity, rainfall, solar radiation, net radiation, wind speed, soil temperatures and soil heat flux were monitored at the automatic weather station (AWS), OAU MET Station, of the Atmospheric Physics Research Group located on the OAU TRF. The experimental plots were within 100 – 500 meters of the weather station mostly in plain sight except for the last four environment in 2016 that had a slightly dense vegetation inbetween. Most sensors (Table 1) at the station were manufactured by Campbell Scientific Inc., USA.

Table 1: Weather tracking sensors and their level of accuracy and output units at the automatic weather station of the OAU MET Station within the OAU TRF.

Weather variable	Tracking sensor	Accuracy	Output unit
Solar radiation	CS 300 Pyranometer	± 5 %	W m^{-2}
Net radiation	NR-LITE Radiometer	± 5 %	W m^{-2}
Air temperature/ Relative Humidity	HMP45 Temperature and Humidity Probe	± 0.4 °C ± 2 – 3 %	°C (Temperature) % (Relative humidity)
Soil temperature	Model 108 Soil Temperature Probe	± 0.3 °C to 0.7 °C	°C
Soil heat flux	Model HFP01 Soil Heat Flux Plate	-15 to 5 %	Wm^{-2}
Wind speed	A100L Cup Anemometer	± 0.1 m s^{-1}	m/s
Rainfall	TE525 Tipping Bucket Rain Gauge	± 1 % (for up to 1 inch per hour) -3 to 0 % (for 1 to 2 inches per hour) -5 to 0 % (for 2 to 3 inches per hour)	mm

They are popular for designing weather tracking systems that operate in vast array of climates including those specific for the tropical hot and humid climate with high level of sensitivity and precision. Heat unit was computed from the minimum and maximum temperature per day as given below:

$$HU = \sum_{i=1}^{n} \left(\frac{Xi^H + Xi^L}{2} \right) 10$$

where X_i^L is the daily minimum air temperature (°C), X_i^H is the daily maximum air temperature (°C) ($X_i^H = 30$ if $X_i^H > 30$°C, $X_i^H = X_i^H$ if $X_i^H \leq 30$°C), and 10°C is the base temperature (Abasi et al., 1985).

Prior to analysis, percentage data were transformed using the square root method; while soil moisture data were subjected to log ($\log_e$) transformation. Analysis of variance was done on all data using PROC GLM of Statistical Analysis System (SAS, 2000). The linear additive model for the ANOVA was: $Y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \lambda_k + \alpha\lambda_{(ik)} + \varepsilon_{ijk}$, in which Y_{ijk} is the observed measurement of the k^{th} genotype grown in the j^{th} rep under the i^{th} environment; μ is the grand mean; α_i is the main effect of the i^{th} environment, i = 1,2,…..,42 or 54; $\beta_{j(i)}$ is the effect of the j^{th} replication nested within the i^{th} environment, j = 1,2,3; λ_k is the effect of the k^{th} genotype, k = 1,2,….5; $\alpha\lambda_{(ik)}$ is the first order interaction of the i^{th} environment with the k^{th} genotype, and ε_{ijk} is the random error (residual) term. Furthermore, correlation and regression analyses were done to determine the trends in maize seedling response to weather variables. Emergence traits were regressed on weather variables in linear model: $Y = a + bX$ and polynomial model: $Y = a + b_i X^1 + ...bnX^n$, Y and X are the emergence traits and weather variable(s), respectively; a and b are the intercept and regression coefficient, respectively; n is the order of the polynomial (quadratic, cubic, quartic). Correlation analysis was done where the coefficient $\boldsymbol{r} = \frac{\Sigma(\boldsymbol{X}-\bar{x})(\boldsymbol{Y}-\bar{Y})}{\sqrt{[\Sigma(\boldsymbol{X}-\bar{x})2*\Sigma(\boldsymbol{Y}-\bar{Y})2]}}$.

Path analysis is an extension of multiple regression, particularly step-wise multiple regression analysis which, along with sequential path diagrams, was employed to elucidate the cause and effect relationships among traits. The Statistical Package for the Social Sciences (SPSS Inc, 2007) was used for the stepwise regression analyses to provide information on the path coefficients and the causal relationships required for the path diagrams. The procedure, which was described by Mohammadi et al. (2003), has been used by Badu-Apraku et al. (2014) and Talabi et al. (2017). The predictor variables which, in this case, were weather factors, were organized into first, second, and third order, based on their contributions to the total variation in emergence traits with minimized multi collinearity. To perform the stepwise regression analysis, emergence traits were regressed on climatic variables to identify the ones with significant contribu-

tions to the variation in the traits at $p \leq 0.05$, and they were categorized as first order variables. The first-order variables thereafter were each regressed on other climatic variables which were not in the first order category, to identify the climatic variables with significant contributions to emergence traits through the first-order variables. These variables were classified as second order variables. The same procedure was repeated to identify third order variables(s) and so on. The path coefficients were obtained from the standardized b-values of the stepwise regression analysis. The path coefficients were tested for significance using the standard errors at 0.05 probability level. Only traits having significant path coefficients were retained in each order.

3 RESULTS

Results from the combined ANOVA for grain yield and seedling emergence traits revealed highly significant effects ($p \leq 0.01$) for all sources of variation; that is, environment, variety and E x V interaction (Table 2). The effect of replication within environment was also highly significant for all traits except ERI. The coefficient of variation (CV) ranged from about 7 % for EI to about 81 % for ERI. Fairly high R^2 value was observed for grain yield (86 %) and higher still for E % at 5, 7 and 9 DAS (93 to 94 %), while ERI had the lowest R^2 value.

Rainfall had no significant correlation coefficients (r-values) with seedling emergence traits, unlike soil moisture (Sm) and air relative humidity (RH) which, in most cases, had statistically significant r-values with the traits (Table 3); the higher the Sm and RH, the higher the emergence percentage, and the faster the rate of emergence. Similarly, radiation had no significant r-values with seedling vigour traits, whereas air temperature and heat units (HU) were significantly correlated with the emergence traits; the higher the air temperature and HU, the lower the emergence percentage, and the slower the rate of emergence. A third group of sharp contrasts involved soil temperatures which, except in only two cases, were not significantly correlated with the emergence traits whereas the higher the soil heat flux (SHF), the lower the emergence percentage and rate of emergence (Table 3). As expected, wind speed had no relationship with emergence traits, although it showed a surprising significant negative relationship (r = -0.38 at $p \leq 0.05$) with ERI. Highly significant correlations were also observed among all seedling emergence and vigour traits but none had significant correlation with grain yield (Table 4).

Table 2: Mean squares from the ANOVA of seedling emergence traits of five maize varieties evaluated in 54 environments at the OAU TRF in 2016 and 2017 seasons.

Sources	DF	E %_5†	E %_7	E %_9	EI (Days)	ERI (Days)	DF	Yield (t ha^{-1})
Env (E)	53	2404.88***	4527.06***	3256.58***	3.55***	941.65***	41	7.61***
Rep/Env	108	100.42***	324.17***	268.92***	0.57***	127.98	84	0.49***
Variety (V)	4	7684.37***	15343.14***	14332.64***	7.33***	6680.45***	4	4.76***
E x V	212	201.47***	619.56***	607.57***	0.37***	475.86***	164	0.45***
Error	432	39.02	65.54	69.59	0.21	116.46	336	0.22
Total	809	282.58	613.08	516.49	0.56	298.69	629	0.83
CV,%		30.01	12.89	11.97	6.90	81.11		30.67
R^2		0.93	0.94	0.93	0.80	0.79		0.86

***- F statistic significant at 0.001 level of probability.
CV- Coefficient of variation
R^2- Coefficient of determination
† - E %_5 = emergence percentage at 5 days after sowing (DAS), E%_7 = emergence percentage at 7DASS, E %_9 = emergence percentage at 9DAS, EI = emergence index and ERI = emergence rate index.

Table 3: Correlation coefficients of seedling emergence traits of five maize varieties with climatic variables obtained for 54 environments at the OAU TRF in 2016 and 2017.

	E%_5†	E%_7	E%_9	EI	ERI
Soil temperature 1	0.16	-0.11	-0.05	0.06	-0.03
Soil temperature 2	0.08	-0.22	-0.16	0.13	0.11
Soil temperature 3	0.09	-0.26	-0.19	0.11	0.06
Soil temperature 4	0.06	-0.33*	-0.26	0.15	0.08
Soil temperature 5	0.03	-0.37*	-0.30	0.17	0.09
Soil heat flux	-0.49**	-0.64**	-0.77**	0.33*	0.44**
Soil moisture	0.38*	0.62**	0.54**	-0.43**	-0.48**
Rainfall	0.01	0.22	0.20	0.01	-0.24
Wind speed	0.04	0.19	0.27	-0.06	-0.38*
Mean air temperature	-0.41**	-0.79**	-0.76**	0.38*	0.43**
Minimum air temperature	-0.48**	-0.82**	-0.80**	0.38*	0.44**
Maximum air temperature	-0.39*	-0.67**	-0.64**	0.37*	0.33*
Heat unit	-0.47**	-0.81**	-0.80**	0.36*	0.44**
Mean relative humidity	0.30	0.66**	0.65**	-0.30*	-0.42**
Minimum humidity	0.04	0.35*	0.31*	-0.16	-0.26
Maximum humidiy	0.48**	0.67**	0.67**	-0.3	-0.34*
Net radiation	0.13	-0.07	-0.09	0.07	-0.06
Mean global radiation	0.09	-0.16	-0.09	0.03	-0.07
Total global radiation	0.09	-0.15	-0.10	0.02	-0.06

*,**- Significance at 0.05 and 0.01 level of probability, respectively.
† - See Table 1.
global solar radiation, net radiation and soil heat flux are in W m^{-2}; temperature is in °C, soil temperatures 1 – 5
are soil temperatures at different levels (2 cm, 5 cm, 10 cm, 20 cm and 50 cm for soil temperatures 1, 2, 3, 4 and
5, respectively), soil moisture is in m^3/m^3, rainfall is in mm, windspeed is in m/s, relative humidity is in %.

Table 4: Correlation coefficients of seedling emergence traits of five maize varieties with grain yield of 40 environments at the OAU TRF in 2016 and 2017.

	Mean E_5†	Mean E_7	Mean E_9	EI	ERI	Yield (t ha^{-1})
Mean E_5	1	0.70**	0.63**	-0.83**	-0.68**	0.21
Mean E_7		1	0.90**	-0.70**	-0.78**	0.24
Mean E_9			1	-0.43**	-0.80**	0.26
EI				1	0.58**	-0.10
ERI					1	-0.16
Yield (t/ha)						1

*,**- Significance at 0.05 and 0.01 level of probability, respectively.
† - See Table 1.

Table 5: Regression coefficients (b-values), coefficients of determination (R^2) and change in R^2 (ΔR^2) from the stepwise multiple regression of maize seedling emergence traits on climatic variables at the OAU TRF in 2016 and 2017.

Climatic variable	b-value	R^2	ΔR^2
	E%_5†		
Soil heat flux$^{\phi}$	-0.43	0.24	0.24
	E%_7		
Minimum air temperature	-20.92	0.67	0.67
Soil moisture	34.22	0.71	0.04
	E%_9		
Minimum air temperature	-6.52	0.62	0.62
Soil heat flux	-0.56	0.66	0.04
	EI		
Soil moisture	-8.06	0.2	0.2
	ERI		
Soil moisture	-154.57	0.23	0.23
Wind speed	-13.96	0.37	0.14
Average radiation	-0.07	0.43	0.07
Minimum humidity	-1.41	0.59	0.16
Rainfall	0.08	0.63	0.04

† - See Table 1.
ϕ - See Table 2

Results from the stepwise multiple regression (Table 5) showed that only SHF had a significant effect on E % at 5 DAS. The simple linear regression of E % at 5 DAS on SHF produced the prediction equation: $\hat{Y} = 19.44 - 0.46X$; ($R^2 = 0.24$)**.** This means that for every unit increase in SHF, emergence decreased by 0.46 % with a rather low, though statistically significant R^2 value. Stepwise multiple regression analysis showed minimum air temperature (X_1), followed by Sm (X_2) as the most important climatic factors controlling E % at 7 DAS. The multiple linear regression produced the prediction equation $\hat{Y} = 34.61 - 20.92X_1 + 34.22X_2$; ($R^2 = 0.71$) with minimum temperature accounting for 67 % of the 71 % variations captured by the regression model (Table 3). Emergence % at 9 DAS was significantly influenced by minimum air temperature (X_1) and SHF (X_2) with the prediction equation $\hat{Y} = 169.37 - 4.12X_1 - 0.51X_2$; $R^2 = 0.66$. Stepwise multiple regression also revealed that Sm was the only environmental factor that interacted with EI ($\hat{Y} = 7.89 - 7.91X$, $R^2 = 0.2$); while Sm (X_1), windspeed (X_2), average global radiation (X_3), minimum RH (X_4) and rainfall (X_5) significantly influenced ERI according to the equation $\hat{Y} = 198.79 - 158.25X_1 - 9.86X_2 - 0.29X_3 - 1.64X_4 - 0.08X_5$ ($R^2 = 0.63$). The summary of the influence of the climatic variables on the seedling emergence traits as obtained from stepwise multiple regression is contained in Table 5.

Although Sm had direct effect on E % at 7 DAS, temperature and the heat changes in the soil (SHF) seemed to have exerted greater influence on E % than soil moisture availability (Figures 1 – 4), because SHF had a direct effect on E % at 5 DAS, while minimum air temperature influenced E % at 7 DAS alongside Sm. This is the only significant effect (direct or indirect) of Sm on E %. Furthermore, E % at 9 DAS was again directly influenced by SHF and minimum air temperature. The secondary climatic variables that interacted with the primary variables to affect E % were mostly air temperature and radiation.

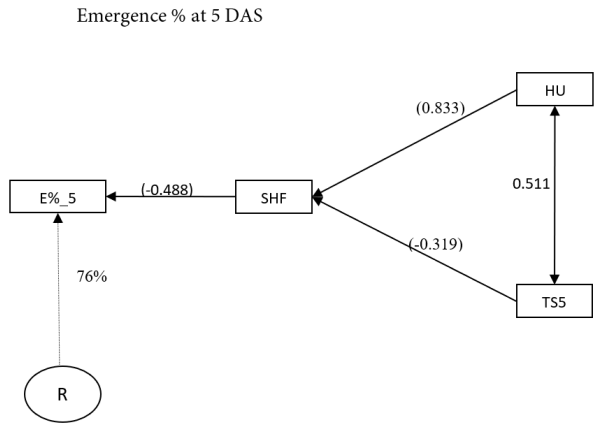

Figure 1: Sequential path-coefficient analysis diagram of climatic variables affecting emergence percent at 5 DAS. One directional arrows indicate direct effects while double arrows are correlation coefficients.

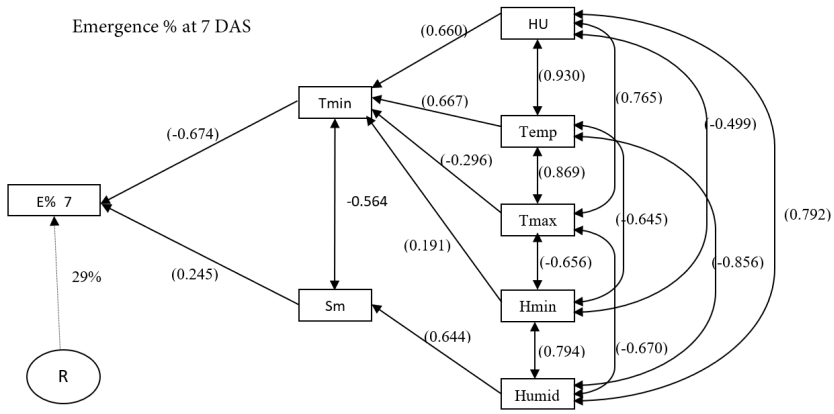

Figure 2: Sequential path-coefficient analysis diagram of climatic variables affecting emergence percent at 7 DAS. One directional arrows indicate direct effects while double arrows are correlation coefficients.

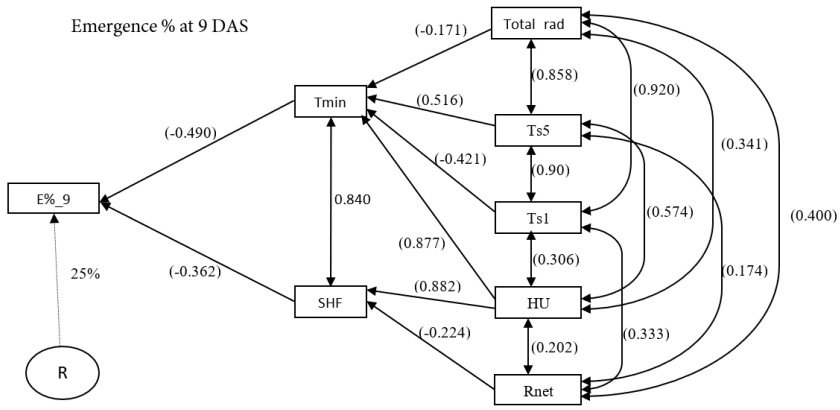

Figure 3: Sequential path-coefficient analysis diagram of climatic variables affecting emergence percent at 9 DAS. One directional arrows indicate direct effects while double arrows are correlation coefficients.

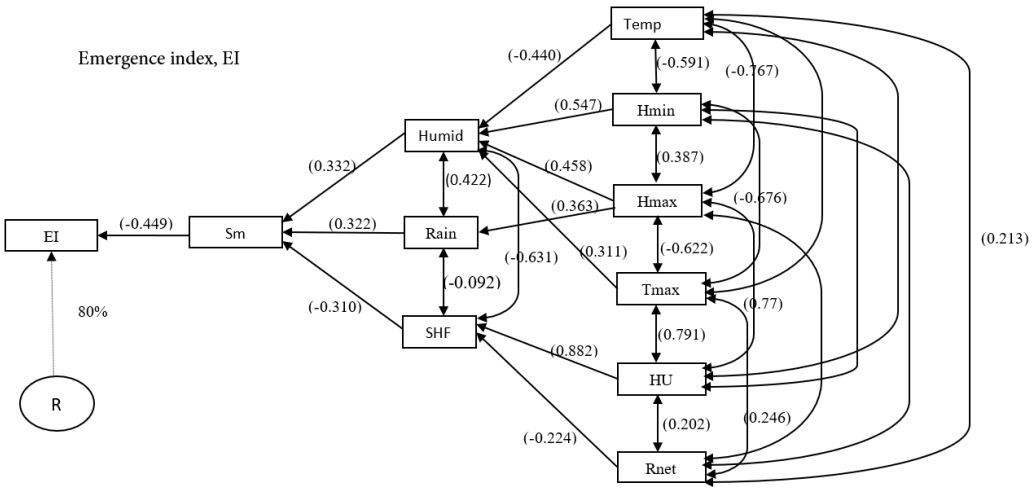

Figure 4: Sequential path-coefficient analysis diagram of climatic variables on emergence index. One directional arrows indicate direct effects while double arrows are correlation coefficients.

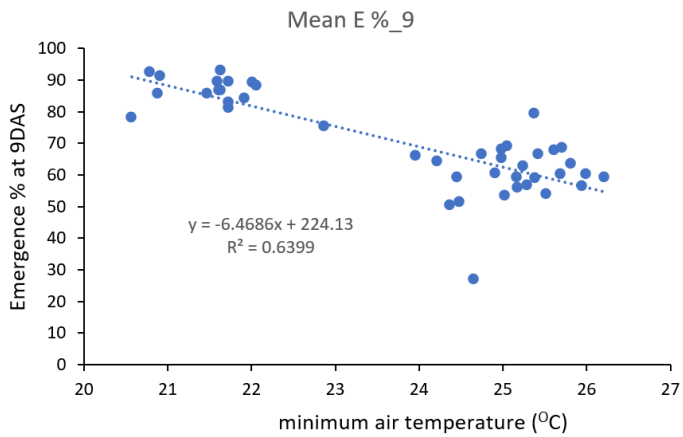

Figure 5: Response of emergence percentage to minimum air temperature for five maize varieties evaluated in 54 environments at the OAU TRF in 2016 and 2017 cropping seasons.

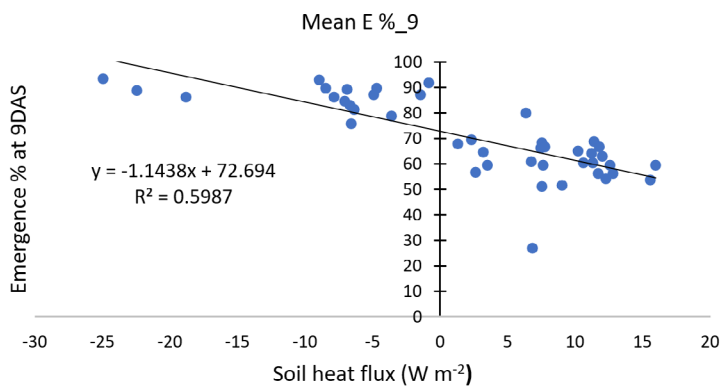

Figure 6: Response of emergence percentage to soil heat flux for five maize varieties evaluated in 54 environments at the OAU TRF in 2016 and 2017 cropping seasons.

However, Figure 4 showed that EI, which represents the speed of emergence, was directly influenced by Sm. The secondary (indirect) effect of SHF (P = -0.310) was again observed on EI alongside mean RH (P = 0.332) and rainfall (P = 0.322) (Figure 4). Emergence was found to occur at minimum air temperature range of 20 °C to 26 °C; and SHF range of -25 to 16 W m^{-2} but the optimum range was 21 to 22 °C for Tmin and -25 to 0 W m^{-2} for SHF (Figures 5 and 6).

4 DISCUSSION

The objective of this study was to investigate the effect of climatic factors, which are important factors of the environment, on maize seedling emergence in the rainforest agro-ecology of Southwestern Nigeria as typified by OAU TRF. Results of the ANOVA, showed highly significant mean squares for the environment source of variation for all seedling traits, accounting for up to 56 % of the total sum of squares in some cases. This suggests that the environment, which greatly influenced seedling emergence of maize varieties in this and other studies (see *inter alia* Fakorede, 1984; Fakorede and Agbana, 1983) needs serious research attention.

Emergence % in arable crops generally respond negatively to higher temperature, heat unit and soil heat flux (SHF); and positively to soil and air humidity. In the present study, E % had significant positive correlation with soil moisture but not with rainfall. This was contrary to expectation because rainfall was the only source of moisture at the location of this study and, therefore, the only source of the moisture available in the soil. However, other climatic factors with considerable influence on soil moisture such as temperature, humidity and heat flux as well as soil properties, such as the soil structure and water holding capacity, may have played a part in the observed results. It is not the amount of water received through rainfall, but the amount of soil moisture available for plant growth that is important. It would seem, therefore, that climatic factors that influence the amount of water retained in the soil, such as humidity and temperature, are more important to crops than the amount of water that enters the soil through rainfall. Emergence index and ERI generally showed an opposite trend to that observed in E %. This is expected because the negative correlations of EI and ERI with E % has been widely reported in the literature (Fakorede and Ojo, 1981; Fakorede and Agbana, 1983; Fayose and Fakorede, 2014). The relatively weaker correlations (compared to temperature variables) and the vastly low R^2 value where there was significant effect of Sm on seedling growth, suggests that Sm is not the most important environmental factor affecting maize seedling emergence in the rainforest. Contrary to the general belief, temperature and heat transfer in and around the seedlings could be more important if they reach a threshold (minimum air temperature above 23 °C, and soil heat flux above 0 W m^{-2} in this study) irrespective of the amount of moisture available in the soil.

Spurious correlation is also common where climatic variables are involved because of the complex interactions that exist among them. Fakorede and Opeke (1985) described spurious correlation as a relationship between two variables, *a* and *b*, in which r_{ab} results largely from the fact that *a* varies along with some other variable *c* which, indeed, is the true predictor of *b*. This is an aspect where most of the previous studies fell short as they depended largely on correlation for data analysis and interpretation. Therefore in this study, path coefficient analysis was used in addition to correlation and regression analyses to determine the true effect of climatic factors on maize seedling emergence. Results of path analyses implicated minimum air temperature and soil heat flux as influencing emergence much more than soil moisture per se. The two air temperature variables directly influenced E % negatively at 9 DAS and, in addition, soil heat flux had negative direct impact on E % at 5 DAS. This suggest that heat transferred at night caused an effect on maize growth at the early stage. Perhaps, the soil temperature gradient created by low air temperature caused the soil to radiate the heat accumulated during the day, thereby causing an effect on the maize seedlings in the process. The thermal exchange processes at the soil surface is dominated by the meteorological conditions and occur by radiation, conduction, and convection, with or without phase changes. The thermal soil properties are strongly dependent on water content and many processes occurring in soils are strongly influenced by temperature (Tokoro et al., 2016). Soil moisture had a direct positive impact on E % at 7 DAS even though the negative effect of minimum air temperature was even more pronounced. Results also revealed that, although soil moisture had a negative direct effect on EI, the ambit of influence might be limited because of the low R^2 (about 20 %), with soil heat flux negatively influencing EI at the secondary level of interaction. This suggests that increased soil moisture might speed up the rate of emergence if other variables were not interacting.

It is a known fact that moisture is necessary for germination. In the present study, seeds sown in March for the first environment in 2017 stayed in the soil for up to three weeks and only emerged following the first rainfall. Unfortunately, climatic variables are in constant interaction; therefore, the benefits of moisture for seedling growth is seemingly masked when it is not at a critical level. Also, 2017 had poorer seedling growth

performance compared to 2016, a result attributable to the air temperature/soil heat flux interaction, because of the smaller rainfall in 2017 (675 mm in 2017 vs more than 1000 mm in 2016) and consequently, higher air temperature (average value of 28.5 °C in 2017 vs 25.8 °C in 2016). One could theorize, therefore, that soil heat transfer occurring at night raises night air temperature around maize seedlings, thereby negatively affecting the seedlings, even though Sm remains at an adequate level; or that seedling growth will proceed at an optimum level with Sm level that is just sufficient, as long as soil heat and heat transfer are minimized especially in the early hours of the day. It is also noteworthy that most of the variables that affected E % indirectly via the first order variables were air temperature and radiation. Even though there was no direct relationships of the seedling emergence and vigour traits with yield, in agreement with some earlier studies (Fayose and Fakorede, 2014; Fakorede and Agbana, 1983; Fakorede and Ojo, 1981), it is however incontrovertible, that the seedling traits strongly influence yield, albeit, indirectly. For instance, severe and prolonged water stress at the seedling stage may damage the structure of the photosynthetic membrane resulting in lower chlorophyll content and thus, low radiation use efficiency (RUE, Song et al., 2019). Maize plant with such damage often did not show meaningful recovery irrespective of the amount of moisture supplied at the latter stages. Therefore, unrecoverable yield loss could occur if adequate attention is not paid to growth at the seedling stage. Also, high final stands, tolerance to infestation by pests and diseases, less likelihood of lodging, increased efficiency of interception of solar radiation for photosynthesis, and reduced need for weed control (when canopies touch) are some of the strong benefits of ensuring that climatic conditions are favourable for maize at the seedling stage that have been well documented in the literature (Li et al., 2015; Fayose et al., 2021).

5 CONCLUSIONS

Despite the wide recognition of the importance of soil moisture to seed germination and seedling emergence, soil heat flux, minimum air temperature and the interactions between them were more important, although Sm favours higher speed of emergence. Emergence occurred at minimum air temperature range of 20 °C to 26 °C; and SHF range of -25 to 16 W m^{-2} but the optimum air Tmin and SHF for emergence were 21 to 22 °C and -25 to 0 W m^{-2}, respectively. Also, soil moisture, rather than rainfall, is the important factor for maize seedling emergence, despite the general recognition of rainfall as an important factor for agriculture in the rainforest ecologies. Consequently, any agronomic practices that would conserve soil moisture would be of benefit to maize at the seedling stage.

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Susceptibility response of varieties and local populations of lupines to *Bruchus rufimanus* (Coleoptera: Chrysomelidae)

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Susceptibility response of varieties and local populations of lupines to *Bruchus rufimanus* (Coleoptera: Chrysomelidae)

Abstract: This study aimed to evaluate the susceptibility response of varieties and local populations of lupines to *Bruchus rufimanus* in multi-environment field tests. Seed damaged rate and susceptibility index were assessed in each environment and subjected to a heritability-adjusted genotype and genotype x environment biplot analysis. It was found that the susceptibility index of damaged seeds was positively related to precipitation amounts and humidity, and inversely to min and max temperatures. The seed damaged rate was positively related to temperatures but negatively to rain and humidity. The local polish population WAT and cultivars Pink Mutant, Solnechnii, and Bezimenii 1 had the lowest seed damaged rate and stable position across environments. Meanwhile, these cultivars showed a low susceptibility index and low variability. The discrepancy between the early phenological development of 'Pink Mutant', 'Solnechnii', and 'Bezimenii 1' and the life cycle of *B. rufimanus* was one of the reasons for manifested tolerance. Correlations between damaged seed and susceptibility index as well as the mass of 1000 seeds and sensitivity index were strongly positive and negative, respectively. 'WAT', 'Pink Mutant', 'Solnechnii', and 'Bezimenii 1' had a clear advantage in defending itself from *B. rufimanus* attack, which makes them particularly interesting for breeding purposes.

Key words: *Bruchus rufimanus*; *Lupinus albus;* HA-GGE biplot analysis; seed damaged rate; susceptibility index

Občutljivostni odziv sort in lokalnih polucij belega volčjega boba na bobarja (*Bruchus rufimanus* Bohemann, Coleoptera, Chrysomelidae)

Izvleček: Namen raziskave je bil ovrednoti občutljivostni odziv sort in lokalnih populacij belega volčjega boba na bobarja (*Bruchus rufimanus*) v poljskih poskusih v več okoljih. Stopnja poškodovanosti semen in občjutljivostni indeks sta bila ocenjena v vseh preučevanih okoljih in analizirana glede na dednost in vplive okolja. Ugotovljeno je bilo, da sta bila občutljivostni indeks in poškodovanost semen pozitivno povezana s količino padavin in vlažnostjo in negativno z minimalnimi in maksimalnimi temperaturami. Poškodovanost semen je bila pozitivno povezana s temperaturo in negativno s padavinami in vlažnostjo. Lokalna poljska populacija 'WAT' in sorte Pink Mutant, Solnechnii, in Bezimenii 1 so imele najmanj poškodovanih semen in so dobro uspevale v vseh preučevanih okoljih. Te sorte so imele tudi najmanše vrednosti občutljivostnega indeksa in majhno raznolikost. Neujemanje med zgodnjimi fenološkimi fazami razvoja sort Pink Mutant, Solnechnii in Bezimenii 1 in razvojnim krogom bobarja je bil eden od vzrokov za izkazano toleranco. Korelacije med poškodovanostjo semen in vrednostjo občutljivostnega indeksa kot tudi med maso 1000 semen in občutljivostnim indeksom so bile v prvem primeru močno pozitivne in v drugem negativne. Sorte WAT, Pink Mutant, Solnechnii in Bezimenii 1 so imele prednost v lastni obrambi pred napadom bobarja , zaradi česar so posebno zanimive za programe žlahtnjenja.

Ključne besede: *Bruchus rufimanus*; *Lupinus albus*; HA-GGE biplot analiza; poškodovanost semena; indeks občutljivosti

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1 INTRODUCTION

Broad bean beetle, *Bruchus rufimanus* Boheman, 1833 (Coleoptera: **Chrysomelidae)** is a common pest on faba beans (*Vicia faba* L.) all over Europe and worldwide (Roubinet, 2016). Bean beetle hosts, in addition to *V. faba*, are various genera *Vicia*, *Pisum* and *Lathyrus* (Delobel & Delobel, 2006; Ward, 2018).

Ramos & Fernández-Carrillo (2011) first reported that lupin plants were a new host of different species from *Bruchus* genus (*Bruchidius rubiginosus* (Desbrochers des Loges, 1869). Harris (1980) established that *Callosobruchus chinensis* Linnaeus, 1758 was an important lupin seed pest, but in a later study, the author found that *B. rufimanus* is one of the most destructive seed pests in lupine (Hurej & Twardowski Kozak, 2013).

B. rufimanus is univoltine insect. Adults emerge from overwintering sites and enter host crops to feed on pollen for several weeks, which females must terminate reproductive diapause. After that, females lay eggs on the pod surface. The larvae develop in the seeds and the adults emerge at harvest. Bruchids make a round output hole in seeds and go through it. Broad bean beetle move to sheltered winter sites, or they remain in the seed until the following year doing no further damage during storage.

The development duration, reproduction, damage degree and generation viability were determined largely by temperature in many insect species (Zhou Guo et al., 2010; Kutcherov, 2015; Hasan & Ansary, 2016). For example, changes in development and damage rate by temperature were reported regarding *Acanthoscelides obtectus* (Say, 1831) (Stewart et al., 2015). However, climatic conditions have a considerable impact on the attack and pest damage.

Control of *B. rufimanus* is primarily conducted by use of insecticides against adults before oviposition, at the stage of the mid-flowering and early pod-formation. Pyrethroids are one of the most used insecticides but managing adult pest attacks is difficult due to their mobility, and the lack of persistence of pyrethroids at high temperatures (Mansoor et al., 2015).

European restrictions and environmental concerns have increased the need for alternative measures. Site selection, crop rotation, cultivar and seed selection, sowing date and plant density are potential means to pest control. One of the effective alternative measures to beetle management are the identification of tolerant genotypes, integrate these genotypes in breeding programs, and to identify the genes involved in the tolerance mechanisms. In this regard, Szafirowska (2012) found that cultivars and their phenological development affect the activity of *B. rufimanus* and the quantity of damage. Southgate (1979) suggested that the seed size and portion remaining following Bruchinae larval feeding among different cultivars were important traits of germination capacity and damage extent. Roubinet (2016) observed differences in susceptibility between several cultivars of *Vicia faba* L. to *B. rufimanus* and the timing of flowering or pod formation turned out to be important factors influencing on the bruchid attack.

The application of alternative cropping strategies, specifically the use of different cultivars, is an efficacious and ecologically friendly approach to plant protection against main insect pests.

This study aimed to evaluate the susceptibility response of varieties and local populations of lupines to *Bruchus rufimanus* in multi-environment field tests.

2 MATERIAL AND METHODS

Field trial was conducted with 23 white lupine cultivars: Astra, Nahrquell, Ascar, BGR 6305, Shienfield Gard, WAT, Kijewskij Mutant, Hetman, Start, Amiga (originating from Poland), Garant (originating from Ukraine), Tel Keram, Bezimenii 1, Bezimenii 2, Pflugs Ultra, Termis Mestnii, Horizont, Solnechnii, Pink Mutant, Manovitskii, Barde, Dega, Desnyanskii (originating from Russia) during the period 2014-2016 at the Institute of Forage Crops (Pleven, Bulgaria). Sowing was made by hand, in optimum sowing time, according to the technology of cultivation. The experiment was laid out using a randomized block design. The studied genotypes were grown in an density of 50 plants m^{-2}. Plot units were twenty-three and each plot unit (5,50 m broad × 2 m length) in three replications included twelve rows spaced 50 cm apart.

The soil type is leached chernozem with $pH_{(KCl)}$ of 5.49 and content of total N was 34.30 mg/1000 g soil, $P_2 0_5$ was 3.72 mg/100 g soil and $K_2 0$ was 37.50 mg/100 g soil. The study was conducted without irrigation and introduced into the soil nitrogen-phosphorus fertilizers in the following amounts: nitrogen - 30, phosphorus - 60 kg active substance per 1 ha.

The period from germination to early flowering was determined for quantitative assessment, we used the coefficient of early-ripeness (Kuzmova, 2002) (1):

$$Cr = 1 + [\{Nc - Nmin\} / \{Nmax - Nmin\}] \qquad (1)$$

where: *Nc* is the duration of the period sowing - beginning of flowering for the particular cultivar; *Nmax* and *Nmin* are the maximum and minimum duration (in days) of the period sowing-beginning of flowering for all tested cultivars.

The values of the coefficient were as followed: for ultra-early ripening cultivars – from 1.00 to 1.17; for

early-ripening cultivars – 1.17 to 1.33; for medium-early ripening cultivars – 1.34 to 1.66 and for late-ripening ones > 1.66.

No chemical control of insect pests was conducted during the growing season. The degree of *Bruchus rufimanus* damaged seeds was determined after lupin harvesting. Bulk samples containing 1500 seeds were taken for each accession, and seed damaged rate (DR) was calculated by the following formula (2):

$$\%\ DR = Number\ of\ seeds\ damaged\ x\ 100/\ Total\ number\ of\ seeds \quad (2)$$

Susceptibility index (SI, %) was calculated by the following formula (3):

$$SI = (a - b) / a \times 100, \text{ where} \quad (3)$$

a : mass of 1000 healthy seeds;
b : mass of 1000 seeds damaged by the broad bean beetle

To eliminate interactions between variables and to include genotype and genotype x environment (GGE) interactions, a HA-GGE biplot analysis was carried out (Yan & Holland, 2010). Biplot graphs are suitable for simultaneous visualization of interacting factors and based mathematically on SVD (singular-value decomposition) models. They are used frequently, in a comparison of multiple genotypes in different environments (Rubiales et al., 2014; Sánchez-Martín et al., 2014). In this way, the best genotype will have the lowest values for the evaluated trait and stability through all the environments, and low G × E interactions.

To evaluate the influence of environmental factors on DR and SI, different climate variables were subjected to a Non-Metric Multidimensional Scaling (NMDS) ordination (Anderson, 2001). Data on the meteorological variables: rainfall, average air temperature, as well as average relative humidity were obtained from Pleven meteorological station for each environment. In order to focus on the occurrence of bruchids in the field, the climatic parameters used in the analysis ranged from March to June. To determine the relative impact of the selected climatic variables on the performance of DR and SI, canonical correspondence analysis (CCA) was carried out. The analysis was performed using the Paleontological Statistics Software Package (PAST) (Hammer et al., 2001). Relationships between damaged seeds and certain plant traits were tested using multiple regression analysis. The statistical processing of experimental data was conducted using the Statgraphics Plus software program.

A wide range of values for DR and SI were noted for the 23 lupin cultivars studied in the three environments. ANOVA (Table 1) revealed a significant effect of genotype (G), environment (E) and G × E in both variables, being the highest mean of a square for E, followed by G and the lowest for G × E.

3 RESULTS AND DISCUSSION

The meteorological conditions during the studied period were different (Figure 1) and had an impact on *Bruchus rufimanus* development, reproduction and damage rate. April, May and June months in 2015 were characterized by a higher average daily temperature (by 1.0 and 0.7 ^{0}C comparatively to 2014 and 2016) as well as a lower rainfall and air humidity (by 107.1 and 25,5 mm, and 9.7 and 6,.7 % humidity in comparison with 2014 and 2016). Those conditions led to an earlier appearance of bean beetle and their stronger attack compared to other years. The plants were in the sensitive stage of flowering and pod formation to bruchid infestation in May and the first ten days of June. At the same time, the plants suffered from a lack of moisture. During 2016, after sowing, the subsequent dry weather delayed seed germination. In April-June the higher temperatures accelerated the plant development and favored the broad bean beetle attack. The meteorological conditions during 2014 characterized by the highest amount of rainfall and relative humidity combined with low temperatures during the growing season. That suppressed infestation and damage rate of *B. rufimanus*.

A canonical correlation analysis helped to visualize the distinct relations of DR and SI components to climate variables (Figure 2). Whereas SI was positively related to bulk precipitation and humidity and inversely to Tmin and Tmax, the seed damaged rate was positively related to Tmin and Tmax but negatively to rain and humidity. Moreover, Tmin and Tmax were associated with the environmental 2 droughts (2015) and opposed to rain and humidity during the environmental 1 wet period (2014). Because of a negative effect of rainfall on DR, the seed damage decreasing at rainy seasons, while in driest environments - increasing. This might be due to the fact that rainfall might disturb bruchid oviposition and reduce egg viability (Roubinet, 2016). The opposite, rainfall and humidity had a positive effect, with SI increasing at higher values.

The HA-GGE biplot is the preferred GGE biplot for test environment and genotype evaluation (Yan & Holland, 2010). The GGE biplot presents the mean characteristic and stability, which gives us an essential visualization of the data (Yan, 2001; Yan & Rajcan, 2002). A GGE biplot is a biplot based on environment-centered data (Gabriel, 1971), which removes the environment's main effect and integrates the genotypic main effect with the

Table 1: Analysis of variance for *Bruchus rufimanus* seed damage rate (DR) and susceptibility index (SI) of the 23 lupin genotypes

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
			DR		
ENV	2	17878.48	8939.239*	3213.711	8.11E-10
REP(ENV)	6	16.690	2.782	58.494	8.03E-35
GEN	22	14129.08	642.231*	11.232	1.2E-11
ENV * GEN	44	2515.781	57.177 *	1202.361	9.9E-153
PC1	23	2511.448	109.193	2296.210	
PC2	21	4.333	0.206	4.340	
Residuals	132	6.277	0.048		
			SI		
ENV	2	2755.412	1377.706*	381.713	4.74E-07
REP(ENV)	6	21.656	3.609	33.620	2.21E-24
GEN	22	4587.940	208.543*	11.733	5.64E-12
ENV * GEN	44	782.079	17.775*	165.566	1.74E-96
PC1	23	678.050	29.480	274.600	-
PC2	21	104.029	4.954	46.140	-
Residuals	132	14.171	0.107	-	-

Legend: DF- degrees of freedom
Sum Sq - sum of the squared
Mean Sq - mean square
ENV – environments
REP(ENV) - replicates within each environment
GEN – genotype
ENV * GEN - term of genotype * environment interaction)
PC1 and PC2 - Principal component

* Significant at 0,0001 level probability

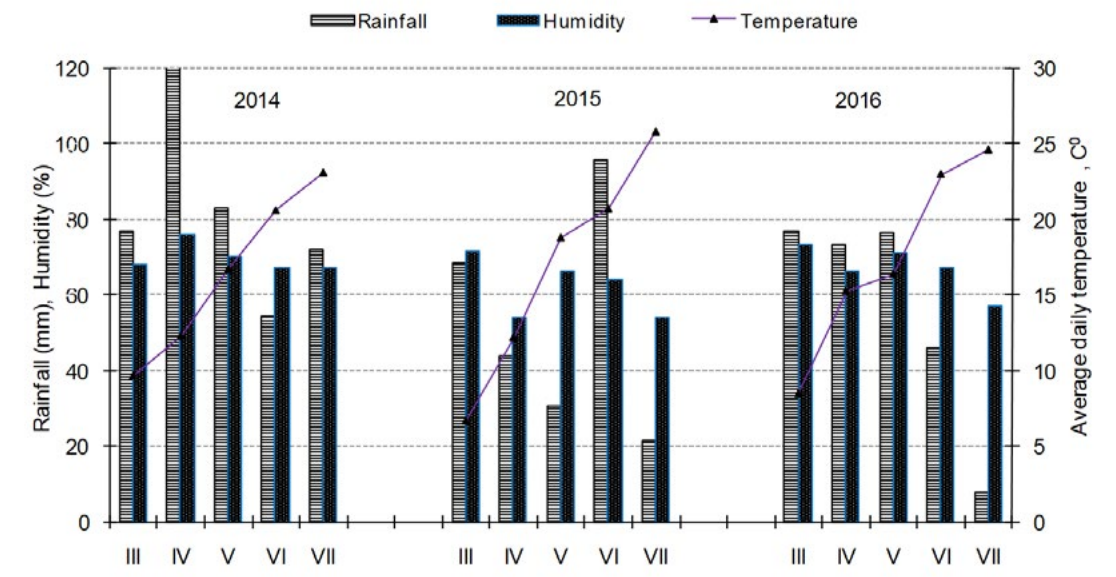


Fig. 1: Meteorological characteristic of the period 2014-2016

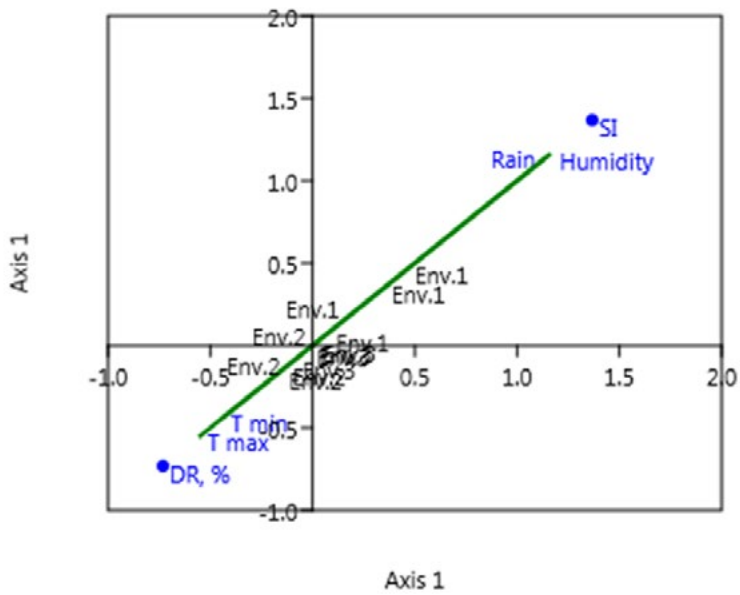


Fig. 2: CCA graph based on the correlation of DR and I of *Bruchus rufimanus* for 23 lupin cultivars according to several climatic parameters. The period analyzed was from April to June, Tmax = maximum temperature; Tmin = minimum temperature; DR = Seed damaged rate (%); SI, % = Susceptibility index

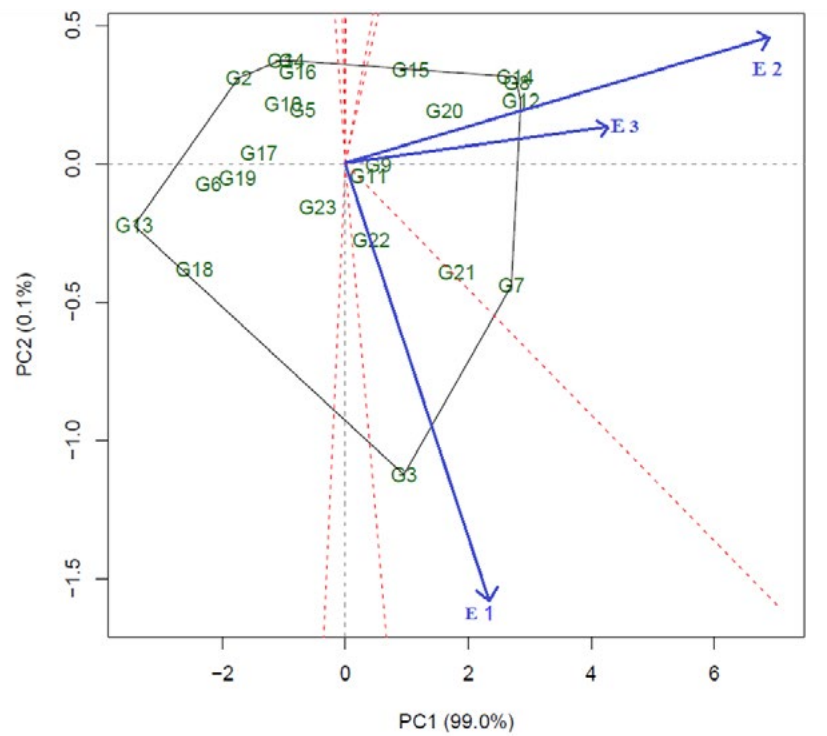


Fig. 3: The GGE biplot based on seed damaged rate (2014-2016). The genotypes are designated with the symbol "G" and the respective number from 1 to 23, as follow G1-Astra, G2-Nahrquell, G3-Ascar, G4-BGR 6305, G5-Shienfield Gard, G6-WAT, G7-Kijewskij Mutant, G8-Hetman, G9-Start, G10-Amiga, G11-Garant, G12-Tel Keram, G13-Bezimenii 1, G14-Bezimenii 2, G15-Pflugs Ultra, G16- Termis Mestnii, G17-Horizont, G18-Solnechnii, G19-Pink Mutant, G20-Manovitskii, G21-Barde, G22-Dega, G23-Desnyanskii. The years are designated with the letter E and number 1; 2; and 3 for 2014, 2015 and 2016, respectively, Note: G14 and G8 are heavily overlapped, as well as G1 and G4; G5 and G10

genotype-by-environment interaction effect of a genotype-by-environment dataset (Yanunt et al., 2000).

According to the results of GGE biplot analysis (Fig. 3), the difference in vector length among environments showed phenotypic variances within the environments. Based on the discrimination power (vector length) E1, followed by E2 were most discriminating, GGE biplot manifested clearly long vectors for E1 и E2 and shorter vector for E 3.

Although there are no strict relations, the goodness of approximation for the correlation coefficients by the angles is related to the goodness of fit of the biplot. Depending on the angle between two environment vector correlation is different. In that aspect, the environments were more or less positively correlated (acute angles). An exception was found between E1 and E2 environments which were not correlated (a right angle). In addition, within the environmental group, E 1 was apparently less associated with E3, while strongly positively correlated were E2 and E3.

In order to determine which of the 23 lupin genotypes studied were the least affected by bean beetle attack based on their representation in the biplots, the ranking of the genotypes (considering stability across the environments studied) for both variables assessed is shown in Table 2.

Thus, in the case of damaged seeds, the genotype with the lowest DR was G13 (6.3 %) despite exhibiting environmental interactions, followed by the genotypes G18 (10.9 %), G6 (11.8 %), G19 (14.0 %) and G17 (15.5 %), whose responses were more stable, as indicted by their location close to the axis 1. The results showed that genotypes G19, G17 and G6 were considered as the most stable being the ones closest to the midpoint of the boxplot and less preferred by *B. rufimanus*. Relatively stable and damage tolerant with little difference in each other, exhibited G1, G4 and G16, Genotype G2 had lower values for that trait but was more affected by the environment. The most susceptible genotypes (high DR, represented on the opposite side of the biplot) were G12 (35.8 %), G8 (34.7 %) and G14 (34.6 %).

The two principal components determined 99.1 % of the dispersion.

The GGE biplot based on SI (Fig. 4), analysis represented 96.2 %% of the total trait variation between two principal components. The environment with the shortest vector was E1, and the longest - E2. The most discriminative environment was E2 in which less rainfall was registered. Genotype 6 was the most responsive to that trait (the lowest value of SI, 5.6 %), and it was followed by G19, G18, G13 (7.4; 7.9 and 9.0 %, respectively) (see Table 2). A similar level of sensitivity showed G2 and G1 too, According to the ordinate, G6 was highly stable, followed by G19 within the group of the low susceptibility index. Lower variability had G18 and G13, G4 had a mean susceptibility index to the grand mean.

Table 2: Ranking of the twenty-three lupin genotypes with the lowest levels of *Bruchus rufimanus* seed damaged rate (DR) and susceptibility index (SI) (ascending order)

DR						SI					
1	G13	11	G5	21	G14	1	G6	11	G23	21	G7
2	G18	12	G23	22	G8	2	G19	12	G3	22	G12
3	G6	13	G11	23	G12	3	G18	13	G22	23	G14
4	G2	14	G22			4	G13	14	G11		
5	G19	15	G9			5	G2	15	G9		
6	G17	16	G3			6	G1	16	G5		
7	G1	17	G15			7	G17	17	G20		
8	G10	18	G20			8	G10	18	G21		
9	G4	19	G21			9	G4	19	G8		
10	G16	20	G7			10	G16	20	G15		

Stability throughout the environments has been taken into account by considering each genotype position in the biplots

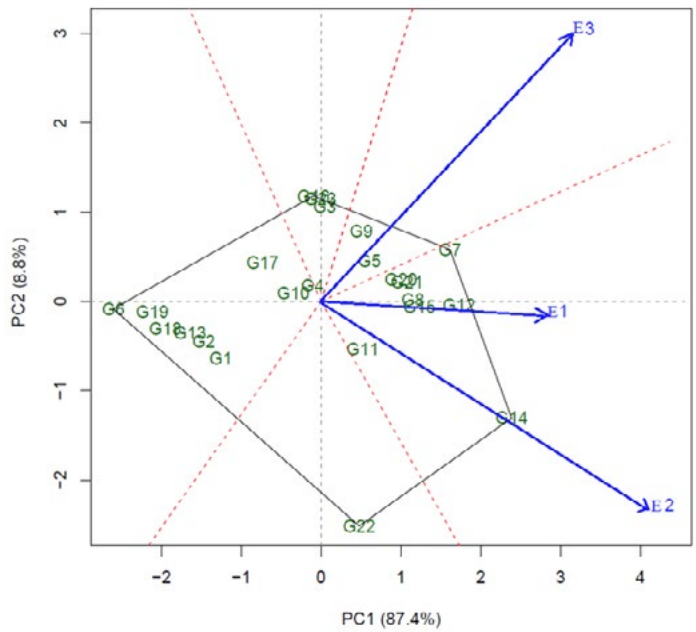


Fig. 4: The GGE biplot based on susceptibility index (2014-2016). The genotypes are designated with the symbol "G" and the respective number from 1 to 23, as follow G1-Astra, G2-Nahrquell, G3-Ascar, G4-BGR 6305, G5-Shienfield Gard, G6-WAT, G7-Kijewskij Mutant, G8-Hetman, G9-Start, G10-Amiga, G11-Garant, G12-Tel Keram, G13-Bezimenii 1, G14-Bezimenii 2, G15-Pflugs Ultra, G16- Termis Mestnii, G17-Horizont, G18-Solnechnii, G19-Pink Mutant, G20-Manovitskii, G21-Barde, G22-Dega, G23-Desnyanskii. The years are designated with the letter E and number 1; 2; and 3 for 2014, 2015 and 2016, respectively, Note: G23, G16 and G3 are heavily overlapped, as well as G21 and G20

The genotype presenting the highest value in that trait and identified like strong sensitive was G14, followed by F12 and G7. Furthermore, the genotype G14 was considerable variable (poor stability) together with G22. Also, G14 had the highest value in E2, which was the most favourable for its susceptibility.

Pearson correlations between DR and SI with genotype as a weighting variable (r = + 0.812, $p = 0.0001$) revealed a significantly high coefficient value, which suggests a strong association between both parameters.

The reduced DR and SI for G6, G19, G18 and G13 might be the result of the combination of different resistance mechanisms. The antixenosis mechanisms might be involved in the resistance of these genotypes by reducing the oviposition over their pods as the result of morphological, phenological or (and) chemical plant factors that adversely affect the insect behaviour. Such morphological traits hindering the penetration of the larvae could be related to a pod or seed coat thickness, seed mass, chemical compounds that hamper the penetration of pods or seeds (alkaloids in lupins) (Keneni et al., 2011). The discrepancy between the phenological development of the host plant and the life cycle of bean beetle could be a marker for tolerance too. In our case, several differences among the phenological development of the genotypes, affecting *B. rufimanus* damage, were observed (Fig. 5). After passing of the budding stage were found differences in the growing period length. Varieties Astra, Termis Mestnii and Barde were characterized with the lowest average duration of the period germination-beginning of flowering (37 days). 'Pink Mutant' (G19), 'Solnechnii' (G18), and 'Bezimenii 1' (G13) had a lower average duration of the period (38 days) and occupied an intermediate position. In the remaining stages of the growing season, the trend remained. The early cultivars (with early flowering) reached technical maturity on average after about 129-134 days and the late ones – for 140-148 days. Cultivars Ascar (G3), Termis Mestnii (G16), Barde (G21), as well as Pink Mutant (G19), Solnechnii (G18), and Bezimenii 1 (G13), could be included in the group of ultra-early ripening cultivars (the coefficient of early-ripeness of 1.00-1.14). Medium-early ripening cultivars were Astra (G1), Kijewskij Mutant (G7), Start (G9), BGR 6305 (G4), WAT (G6), Garant (G11), Tel Keram (G12), Bezimenii 2 (G14), Pflugs Ultra (G15) (coefficient of early-ripeness > 1.34) and the late-ripening one's - Hetman (G8), Shienfield Gard (G5) and Nahrquell (G2) (coefficient > 1.66).

Several cultivars of the ultra-early ripening group

stood out with considerably lower values of damage traits (DR and SI). For example, cultivars Pink Mutant, Solnechnii, and Bezimenii 1 had early flowering and slightly preference by bean beetle, while late-ripening 'Hetman' and 'Shienfield Gard' was considerably prefered by bruchids. The discrepancy between the early phenological development of those cultivars and the life cycle of *B. rufimanus* was one of the reasons for manifested tolerance.

There was published evidence of the influence of cultivar on damage caused to *Vicia faba* grain by *B. rufimanus* (Ebedah et al., 2006; Szafirowska, 2012). In those studies was suggested that plant architecture, flowering period and abundance, and the timing of pod formation were the key factors that influence the activity of *B. rufimanus*. According to Bruce et al. (2011), Ceballos et al. (2015), several plant characteristics could adversely affect insect behaviour. Authors found that some susceptible genotypes flowered later than the average, which could have contributed in some way to the escape of these pea plants from bruchid infestation. More recent research identified phenological tolerance in cultivars with an early flowering stage becoming unavailable to the weevils during the period when the attack is likely to be most severe (Bell & Crane, 2016).

On the other hand, results showed the mass of 1000 seeds strongly negatively correlated with the sensitivity index, r = −0.842. It was noticed that genotypes exceeding 300 g per 1000 seeds, such as G6 (322.2 g), G19 (317.1 g), G13 (308.2 g), and G18 (304.3 g) were distinguished by low susceptibility indexes (from 5.6 to 7.9 %). In contrast, genotypes with much smaller seeds like G14, G21, and G20 (173.2, 222.2, and 232.9, respectively) were characterized by higher SI values (from 19 to 23 %). Larger seeds are considerably richer in nutrients than small seeds, where larvae destroyed a large amount of them. For example, Mateus et al. (2011) reported that the attack by bruchids caused a significant reduction in seed mass, between 0.03 (large seeds) and 0.08 g (smaller seeds), depending on the genotypes/cultivars, corresponding to a decrease in nutrients available to the embryo development. In that aspect, the genotype 14, G21 and G20 were one of the cultivars with the highest susceptibility indexes as the larva destroyed most of the grain content for its feeding.

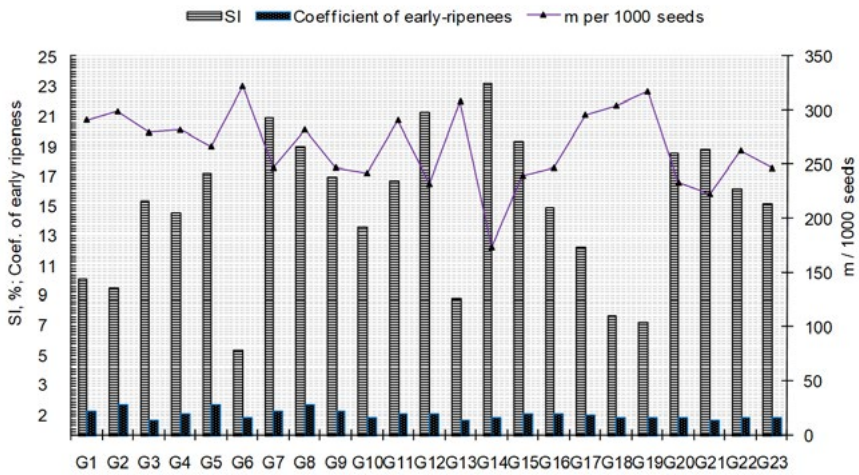
Also, antixenosis mechanisms might be involved in the tolerance of these genotypes by reducing the preference of bean beetle adults for feeding as the result of chemical plant factors that adversely affect insect behaviour. Probably, studied lupin cultivars may differ chemically to a great extent (in alkaloid content), and in that context, some species of them may even be toxic to some animals. The negative role of different alkaloids in cultivated lupins was indicated by Ströcker et al. (2013). The presence of such antinutrient substances in the genotype-host probably explain the preferences of bruchids.

About effect of some botanical oils, including lupin seeds on the granary weevil, *Sitophilus granarius* reported Makarem et al. (2017). According to authors, lupine oil protected the grain against weevils up to the 6th-week post-treatment achieving mortalities between 60.0 and 100 %. Meanwhile, the highest degree of inhibited oviposition and adult emergence was detected with a lupine oil treatment compared with other oils.

On the other hand, proteinase inhibitors are potential candidates for biocontrol of insect pests since insect digestive proteinases are promising targets towards control of various insects (Sharma et al., 2012). Proteases have been found to be effective against many Coleopteran (Elden, 2000). Scarafoni et al. (2008) reported for the inhibitory properties of a trypsin inhibitor from *Lupinus albus* L, a leguminous plant believed to be devoid of any protease inhibitor. Several protease inhibitors have been reported to exhibit inhibitory activity against insect proteases. Although the proteases were not evaluated in the present study, seed genotypes slightly affected by broad bean beetle had presumably protease inhibitors suppressing strongly its activity.

It is necessary to examine not only the individual effect of plant traits but also their mutual impact on the beetle damage. The applied regression analysis (ANOVA) in Table 3 showed that the interaction of plant traits had a significant effect on the damaged seed rate. The susceptibility index had the highest regression coefficient (r = 1.915) (Table 3, below). It had a significant positive effect. The coefficient of early-ripeness had a significantly strong effect on the *B. rufimanus* choice (r = −1.687) but correlated negatively. The mass of 1000 seeds had a low positive effect (r = 0.048) on the damaged seeds in the complex interaction between plant traits and seed damage rate.

According to the results above, G6, G19, G18 and G13 seems to have a clear advantage in defending itself from B. rufimanus attack. The low DR and SI make genotypes particularly interesting for breeding purposes because it probably presents a combination of different mechanisms like seed mass and phenological development adversely affect *B. rufimanus* behaviour. The possibility of combining these two types of resistance mechanisms have great importance because of the durability of the tolerance and successfully overcome an attack if one of these levels is broken.



Legend: SI- susceptibility index; G1-Astra, G2-Nahrquell, G3-Ascar, G4-BGR 6305, G5-Shienfield Gard, G6-WAT, G7-Kijewskij Mutant, G8-Hetman, G9-Start, G10-Amiga, G11-Garant, G12-Tel Keram, G13-Bezimenii 1, G14-Bezimenii 2, G15-Pflugs Ultra, G16- Termis Mestnii, G17-Horizont, G18-Solnechnii, G19-Pink Mutant, G20-Manovitskii, G21-Barde, G22-Dega, G23-Desnyanskii.

Fig. 5: Characteristics of lupin genotypes

Table 3: Regression coefficient of the damaged seed rate depending on some plant parameters for lupin genotypes

Source	*df*	*SS*	*MS*	*F*	Significance F
Regression	3	1319.330	439.780	33.140	0.051
Residual	19	252.143	12.270		
Total	22	1571.470			

Parameter	Coefficients	Standard Error	t Stat	P-value	Lower 95 %	Upper 95 %
Intercept	-17.145	15.206	-1.127	0.000	-48.970	14.681
SI	1.915	0.339	5.653	0.000	1.206	2.623
M of seeds	0.048	0.045	1.059	0.087	-0.047	0.142
CER	-1.687	2.843	-0.593	0.100	-7.639	4.264

Legend: SI- Susceptibility index, M of seeds- m per 1000 seeds, CER- Coefficient of early-ripeness

4 CONCLUSIONS

Bruchus rufimanus damage was affected by climate parameters. The susceptibility index of damaged seeds was positively related to precipitation amounts and humidity, and inversely to min and max temperatures. The seed damaged rate was positively related to temperatures but negatively to rain and humidity.

The local polish population WAT and cultivars Pink Mutant, Solnechnii, and Bezimenii 1 (G6, G19, G18 and G13, respectively) had the lowest seed damaged rate and stable position across environments. Meanwhile, these cultivars showed a low susceptibility index and low variability.

The discrepancy between the early phenological development of 'Pink Mutant', 'Solnechnii', and 'Bezimenii 1' and the life cycle of *B. rufimanus* was one of the reasons for manifested tolerance. Correlations between damaged seed and susceptibility index as well as the mass of 1000 seeds were strongly positive and negative, respectively.

Cultivars Pink Mutant, Solnechnii, Bezimenii 1 and local population WAT had a clear advantage in defending itself from *B. rufimanus* attack, which makes them particularly interesting for breeding purposes.

The matching of an early flowering with higher seed mass in cultivars could be used as markers for tolerance against broad bread weevil, and like an effective method for plant defence.

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Calcium lactate and salicylic acid foliar application influence eggplant growth and postharvest quality parameters

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Calcium lactate and salicylic acid foliar application influence eggplant growth and postharvest quality parameters

Abstract: Eggplant is one of the most popular and vital vegetable crops in the world. Various plant bio-regulators have been used in different crops to increase uptake of nutrients thereby leading to improvement in growth, flowering, fruit quality, storability and yield. The scope of this study was to evaluate the effects of calcium lactate and salicylic acid foliar application on growth parameters, physiological characteristics and shelf-life of eggplant fruit. Obtained results showed that the highest applied concentrations of calcium lactate (4 mM or 0.8 g l^{-1}) and salicylic acid (1.5 mM or 0.2 g l^{-1}) foliar application led to the highest values of measured growth parameters and yield. Applying of calcium lactate and salicylic acid foliar treatments could increase tissue firmness and ascorbic acid content of fruits. Foliar application of calcium lactate 4 mM (0.8 g l^{-1}) and salicylic acid 1 mM (0.13 g l^{-1}) was the best treatment to decrease percentage of fruit decay. In conclusion, our results showed that foliar application of calcium lactate and salicylic acid can be useful and inexpensive treatment to improve growth parameters, physiological characteristics and post-harvest properties of eggplant fruit.

Key words*:* eggplant; calcium sources; chlorophyll content; foliar spraying; post-harvest fruit characteristics; salicylic acid.

Foliarno dodajanje kalcijevega laktata in salicilne kisline vpliva na rast jajčevca in na obstojnost plodov pri shranjevanju

Izvleček: Jajčevec je v svetovnem merilu ena izmed najbolj popularnih plodovk. Pri pridelavi različnih kulturnih rastlin so bili uporabljeni razni bioregulatorji privzema hranil, kar bi vodilo k izboljšanju rasti, cvetenja, kakovosti plodov, povečanju pridelka in trajnosti pri shranjevanju. Namen te raziskave je bil ovrednostiti učinke foliarnega dodajanja kalcijevega laktata in salicilne kisline na rastne parametre, fiziološke lastnosti in trajanje plodov pri shranjevanju. Dobljeni izsledki so pokazali, da sta imeli največji foliarni dodajanji kalcijevega laktata (4 mM ali 0,8 g l^{-1}) in salicilne kisline (1,5 mM ali 0,2 g l^{-1}) največji učinek na vrednosti merjenih rastnih parametrov in velikosti pridelka. Foliarano obravnavanje s kalcijevim laktatom in salicilno kislino bi lahko povečalo čvrstost tkiv in vsebnost askorbinske kisline v plodovih. Foliarno dodajanje kalcijeva laktata 4 mM (0,8 g l^{-1}) in salicilne kisline 1 mM (0,13 g l^{-1}) je bilo najboljše obravnavanje za zmanjševanje odstotka propadlih plodov. Zaključimo lahko, da so ti izsledki pokazali, da bi lahko bilo foliarno dodajanje kalcijeva laktata in salicilne kisline uporaben in poceni postopek za izboljšanje rastnih parametrov, fiziološki lastnosti in lastnosti plodov jajčevca pri shranjevanju.

Ključne besede: jajčevec; vir kalcija; vsebnost klorofila; foliarno gnojenje; lastnosti plodov pri shranjevanju; salicilna kislina

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1 INTRODUCTION

Eggplant (*Solanum melongena* L.) is one of the most popular and vital vegetable crops in the world (Kaushik, 2019). Various plant bio-regulators have been used in different crops to increase uptake of nutrients thereby leading to improvement in growth, flowering, fruit quality, storability and yield (Ranjbar et al., 2017; Mustafavi et al., 2018; Ghahremani et al., 2020). However, recently new plant growth regulators like salicylic acid (SA) and calcium lactate (CL) have been found beneficial in maintaining balance between vegetative and reproductive growth andincreasing the uptake of nutrients thereby resulting in high yield of superior quality crops with prolonged storability and consistent bearing (Shaarawi et al., 2016). Calcium (Ca^{2+}) has been extensively studied both as an essential element and for its potential role in maintaining postharvest quality of fruit and vegetable crops by contributing to the linkage between pectic substances within the cell wall. CL treatment reduced the respiration rate and improved the firmness of persimmon slices (Youssef et al., 2017). SA is one of the groups of common phenolic compounds that are produced naturally by plants, which can act as endogenous plant growth regulator. Its application might be safe and more useful for plant growth improving. SA stimulates the growth and development of roots of the treated plants (by increasing of H^+-ATPase activity and root ATP content) thereby improving nutrient uptake (Ghassemi-Golezani and Farhangi-Abriz, 2018). Enhancement of chlorophyll and carotenoid pigments levels, photosynthetic rate and modifying the activity of some of the important enzymes are other roles assigned to SA. It induces specific changes in leaf anatomy and chloroplast structure (Uzunova and Popova, 2000). The effect of postharvest calcium chloride (CC) and SA applications on shelf-life and quality attributes of kiwifruits were evaluated by Kazemi et al. (2011). Results of this experiment showed that post-harvest SA and CC treatments prevented fruit softening and decreased mass loss of fruits. Also in the other experiment, the effect of pre-harvest CC application on post-harvest life and quality of peach fruits was studied. 0.5 %, 1.0 % and 1.5 % of CC solutions were sprayed on peach plants and CC 1.5 % resulted in maximum fruit firmness, sensory quality score and calcium content during the storage period.

The scope of the present research was to investigate the effects of CL and SA foliar application on growth parameters, several fruit quality attributes such as tissue firmness, ascorbic acid content and total soluble solids of fruits and some of the most important post-harvest properties of eggplant fruit such as titratable acidity, fruit mass loss, fruit decay and browning of pulp tissue.

2 MATERIALS AND METHODS

The experiment was conducted in University of Zanjan, Zanjan, Iran in 2018-2019. Soil samples of experimental farm were collected from a depth of 0 to 60 cm andthen analyzed. Table 1 shows the measured characteristics of experimental farm soil. Also, Table 2 shows quality and chemical properties of applied irrigation water.

Table 1: Physical and chemical properties of experimental farm soil

Clay (%)	Silt (%)	Sand (%)	Soil texture	Organic matter (%)	K (g kg^{-1})
37	38	25	Clay loam	0.94	0.2

Na (g kg^{-1})	Ca (g kg^{-1})	N (%)	EC (dS m^{-1})	pH
0.13	0.12	0.07	1.49	7.4

Table 2: Quality and chemical properties of applied irrigation water

SO_4^{2} (mg l^{-1})	HCO_3^- (mg l^{-1})	CO_3^{2-} (mg l^{-1})	Cl (mg l^{-1})	Mg (mg l^{-1})
550.5	159	0.0	435.3	241.6

Ca (mg l^{-1})	K (mg l^{-1})	Na (mg l^{-1})	EC (dS m^{-1})	pH
400	2.74	152	2.7	7.2

2.1 PLANT MATERIAL

Eggplant seeds (IR3121 cultivar) were sawn in peat moss at controlled condition (23 ± 2 °C temperature and 60 to 70 % relative humidity). Seedlings were transplanted to the field in 4-5 leaf stage (in May) at a distance of 60 cm between rows and 50 cm between plants. Seedlings were immediately irrigated after planting and then were watered by using of drip irrigation every 3 days. Weeds were controlled by hand weeding. Eggplant fruits were harvested at full ripening stage. Harvested fruits were stored at cold storage (10 °C temperature and 85 ± 5 % relative humidity) for 30 days. Post-harvest characteristics evaluation was performed during storage at 10-day intervals.

2.2 METHODS

2.2.1 Growth parameters and yield

Number of fruit per plant, diameter of fruit, height of plant, average mass of fruit, length of fruit, leaf area and yield were measured as growth parameters in harvest stage. Average mass of fruit was recorded by using digital scale (EK3000I). Leaf area was measured by using digital scanner (E84-10017) and imageJ software (V3).

2.2.2 Physiological characteristics

Total chlorophyll and carotenoid content, titratable acidity, tissue firmness, ascorbic acid and total soluble solids of fruits were measured as physiological characteristics. Total chlorophyll and carotenoid content of leaves was determined by measuring absorption with a spectrophotometer at 645 and 663 nm for chlorophyll content and 480 and 510 nm for carotenoid content (*spectrophotometer*-SAFAS UVmc2) as described by Arnon (1967). For evaluating of titratable acidity, tissue of eggplant fruit (10 g) was homogenized in 40 ml distilled water and filtered to extract the juice. 2 to 5 drops of phenolphthalein were added in this juice. A 10 ml aliquot was taken in a titration flask and titrated against 0.1N NaOH till permanent light pink color appeared. Three consecutive readings were taken from each replication of a treatment and percent acidity as malic acid was calculated by using the following formula:

% TA=[E×N×S×F/C]×100 (Raja et al. 2105). (E: Equivalent wt. of malic acid) (N: Normality of NaOH) (S: ml NaOH used) (F: vol. of aliquot taken) (C: wt. of sample).

Fruit firmness was measured with penetrometer (FT-327-48011-Alfonsine-Italy) and expressed pressure necessary to force a plunger of 11 mm size into the fruit (Arvanitoyannis et al., 2005). Ascorbic acid content of fruit was determined by applying of iodometric titration method according to Vanderslice et al. (1990). Total soluble solids was evaluated by using refractometer (ATAGO Brixo-32) and expressed as degrees brix (Paull and Chen, 1989).

2.2.3 Post-harvest characteristics

Following properties were analyzed to evaluate post-harvest characteristics of eggplant fruits (during storage): titratable acidity, tissue firmness, ascorbic acid, soluble solids content, fruit mass loss, fruit decay and browning of pulp tissue.

Fruit mass loss: Mass loss was determined by the following formula: Mass loss (%)=[(A-B)/A] × 100

Where A indicates the fruit mass at the time of harvest and B indicates the fruit mass after storage intervals (Huang et al., 2000).

Fruit decay: Fruit decay percent was estimated by visual scoring method, as described by Kader et al. (2010) on 1-4 scale, with reference points of: 4 = severe; 3 = moderate; 2 = slight; 1 = none. The score attribution depends on morphological effects such as color change, microorganism effects and smell.

Browning of pulp tissue: The color parameter L* indicates the lightness of color (0 = black and 100 = white). A Minolta Colorimeter model CR-300 was used to determine L*, and the readings were taken soon after slicing the central section of each fruit (thickness = 0.5 cm). All measurements were done on three fruits from each condition and by duplicate. The results were expressed as L_0, values higher than 86 denotes whitish pulp and values between 81 and 82 show only seed browning. Lightness near to 78 indicates an incipient browning of seed and pulp, while values below 73 denote considerable browning of seed and pulp (Ahmad et al., 2013).

2.3 STUDY DESIGN

Factorial experiment was laid out based on randomized complete blocks design (to evaluate growth parameters and physiological characteristics in harvest stage) and completely randomized design (to evaluate postharvest characteristics during storage) with three replications. The factors are foliar application of CL and SA in different concentrations including three levels for CL solution: 0 mM (control), 2 mM (0.4 g L^{-1}) and 4 mM (0.8 g L^{-1}) and three levels for SA solution: 0 mM (control), 1 mM (0.13 g L^{-1}) and 1.5 mM (0.2 g L^{-1}) and also during of storage in three levels including: 10, 20 and 30 days. CL and SA foliar application was carried out at 6-leaf stage for the first time and continued at 10-day intervals until harvest stage.

2.4 STATISTICAL ANALYSIS

Data were analyzed by analysis of variance (ANOVA) using the SAS software (V9). Mean comparisons were performed by Duncan's multiple range test at confidence level of 95 %.

3 RESULTS AND DISCUSSION

3.1 GROWTH PARAMETERS AND YIELD

According to ANOVA analysis, significant influence of CL and SA foliar application and interaction between them on all of measured growth parameters was found. Table 3 shows, the highest applied concentrations of CL (4 mM) and SA (1.5 mM) foliar application led to the highest values in all of measured growth parameters. Also, the highest value of yield (127.21 t ha^{-1}) was recorded in plants sprayed by CL 4 mM and SA 1.5 mM. Yield increased by 13.46 % at sprayed plants by CL 4 mM and SA 1 mM compared to CL 2 mM and SA 1 mM treated plants. Similar stimulatory effects of SA and different types of calcium sources (calcium oxide, calcium chloride, calcium chelate and calcium lactate) on different growth parameters were reported in tomato (Rab & Haq, 2012), strawberry (Kazemi, 2013a), cucumber (Kazemi, 2013b), cowpea (Mohamed & Basalah, 2015) and lettuce (Almeida et al., 2016; Khani et al., 2020). SA stimulates the growth and development of roots of the treated plants by increasing of H^+-ATPase enzyme activity and root ATP content (Ghassemi-Golezani and Farhangi-Abriz, 2018) thereby improving nutrient uptake. So, increasing of nutrient uptake rate can be as important reason for increasing of growth parameters and yield in SA treated plants. According to Hepler (1994), the effects of different calcium sources on growth parameters of different crops can be related to the fact that calcium ions (Ca^{2+}) appeared to participate in the regulation of different aspects of cell division. Calcium is one of the most important ions in formation of the mitotic spindle which directly affects cell division.

3.2 PHYSIOLOGICAL CHARACTERISTICS

All of the measured physiological characteristics were significantly affected by CL and SA foliar application and interaction between them. The highest chlorophyll content (1.32 mg g FM^{-1}) was related to sprayed plants by CL 4 mM and SA 1 mM and the lowest carotenoid content (0.36 mg g FM^{-1}) was obtained in control (sprayed plants by CL 0 mM and SA 0 mM) (Table 4). Foliar application of SA was found to increase the chlorophyll content in cowpea (Chandra & Bhatt, 1998), tomato (Kalarani et al., 2002), cucumber (Yildirim et al., 2008) and strawberry (Karlidag et al., 2009a, 2009b). Martin-Diana et al. (2005), reported that carotenoid levels were higher in CL-treated carrots than that in control samples at the end of 10 days storage. Results showed that, the highest and lowest values of total soluble solids were related to control fruits (harvested from sprayed plants by CL 0 mM and SA 0 mM) and harvested fruits from CL 4 mM and SA 0 mM sprayed plants, respectively. Foliar application of CL and SA led to a significant reduction in total soluble solids of fruits. Different results were reported about effect of calcium sources on total soluble solids of fruits, for instance, according to Akhtar et al. (2010), CC treatment could significantly increase total soluble solids in Loquat fruit but in contrast, Dong et al. (2004) reported that, total soluble solids of tomato reduced by employing of calcium treatment. Calcium sources and SA treatments lead to a decrease in respiration rate, ethylene biosynthesis and ripening of fruits, which in turn decrease the polysaccharide degradation in cell wall and cell membrane. So, decreasing of polysaccharide degradation can lead to a reduction of total soluble solids of fruits.

Titratable acidity increased by 2.86 % at harvested fruits from CL 4 mM and SA 1.5 mM sprayed plants compared to fruits of treated plants by CL 4 mM and SA 0 mM. Applying of CL and SA foliar treatment could increase tissue firmness and ascorbic acid content of fruits and CL 4 mM and SA 1.5 mM foliar spraying was the best treatment to increase tissue firmness and ascorbic acid content of eggplant fruits. Fruit softening results from cell wall degradation by cell wall hydrolases such as polygalactosidases, pectin methylesterases, b-galactosidase and xylanase along with cell membrane deterioration. As an ethylene inhibitor, SA delays fruit ripening and prevents fruit softening by reducing the activity of cell wall-degrading enzymes. Srivastava and Dwivedi (2000) reported that SA reduced polygalactosidases, xylanase, and cellulase enzyme activity in harvested banana fruits. Wang et al. (2006) reported that SA treatment can increase ascorbic acid content in fruit and vegetable crops by increasing of ascorbate peroxidase enzyme activity. Our finding with respect to the effect of CL and SA foliar application on ascorbic acid content is in line with those reported by Elvwan and Hamahyomy (2009). They observed an increase in ascorbic acid content of greenhouse pepper by employing of low concentration of SA foliar application.

Table 3: Effect of CL and SA foliar application on growth parameters and yield at harvest

SA concentration (mM)	CL concentration (mM)	Leaf area (mm)	Average mass of fruits (g)	Number of fruit per plant	Diameter of fruit (cm)	Length of fruit (cm)	Height of plant (cm)	Yield (t ha^{-1})
	0	185.4f	255.66f	11.23g	3.54f	19.35f	63.11h	95.57g
0	2	192.32d	262.66e	11.41f	3.65e	19.73ef	64.21g	99.72f
	4	196.12c	275.66c	12.03c	3.83d	20.12e	66.59e	110.06c
	0	191.59d	269.33d	11.64e	3.84d	21.47d	65.42f	104.16e
1	2	195.29c	273.66c	11.05h	3.94c	22.57bc	67.39e	100.31f
	4	199.19b	280b	12.43b	4.25b	23.28b	72.59c	115.89b
	0	188.64e	274c	11.82d	3.82d	21.60d	70.03d	107.48d
1.5	2	200.89b	282.66b	12.38b	3.91c	22.40c	74.18b	115.42b
	4	207.45a	297.33a	12.84a	4.42a	24.08a	81.77a	127.21a

Values in columns for same variable followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

Table 4: Effect of CL and SA foliar application on the content of metabolites and firmness at harvest

SA concentration (mM)	CL concentration (mM)	Chlorophyll content (mg g $FM^{-1)}$	Carotenoid content (mg g $FM^{-1)}$	Total soluble solids (0B)	Titratable acidity (%)	Tissue firmness (N)	Ascorbic acid content (mg 100 g Juice)
	0	0.80h	0.36h	5.07a	5.45e	3.23f	1.75e
0	2	0.93f	0.39g	4.87b	5.47de	2.76ef	1.70e
	4	1.25c	0.45d	4.18g	5.44e	2.64c	1.83d
	0	1.01e	0.42f	4.78c	5.49cd	3.00d	1.82d
1	2	1.05d	0.44e	4.67d	5.50bcd	3.37b	1.90c
	4	1.32a	0.51c	4.46e	5.52b	3.41ab	1.94bc
	0	0.87g	0.39g	4.82bc	5.52bc	2.92de	1.90c
1.5	2	0.94f	0.62a	4.53e	5.53b	3.29bc	1.99b
	4	1.29b	0.59b	4.34f	5.60a	3.54a	2.07a

Values in columns for same variable followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

3.3 POST-HARVEST CHARACTERISTICS

According to ANOVA analysis, all of the measured post-harvest characteristics except fruit decay were affected by interaction between CL and SA foliar application and duration of storage.

Figure 1 shows the effect of interaction between CL and SA treatment and duration of storage on total soluble solids, titratable acidity, tissue firmness and ascorbic acid content of fruits. Total soluble solids of fruits raised by increasing the time of storage from 10 to 20 days but a reverse trend (reduction) was detected in total soluble solids of fruits from 20 to 30 days of storage. In our opinion, change in ratio of respiration rate (consumption of sugars) to conversion of starch to sugar (production of sugars) would be a main reason on increasing of total soluble solids in first days of storage with a peak on 20 days of storage and then decreasing until 30 days after storage. The rate and speed of conversion of starch to sugar (production of sugars) is higher than respiration rate (consumption of sugars) in first days of storage and it can lead to a significant increase in total soluble solids of fruits

but the ratio of respiration rate to conversion of starch to sugar increased after 20 days of storage, so a significant decrease was detected in total soluble solids of fruits in the last days of storage. (Figure 1a). The highest value of titratable acidity (5.53 %) was obtained in harvested fruits from sprayed plants by CL 4 mM and SA 1.5 mM after 10 days of storage (Figure 1b). During storage, there is conversion of starch to sugar and the oxidation of organic acids to sugar which rapidly reduce the titratable acidity and increase total soluble solids of fruits (Campestre et al., 2002). Tissue firmness reduced by increasing the time of storage but harvested fruits from treated plants showed higher value of tissue firmness than that in control (Figure 1C). According to Mahajan et al. (2017), the reduction of fruit firmness during post-harvest stage is mainly caused due to the dissolution of the middle lamella, decreasing of cell-to-cell adhesion and the weakening of parenchyma cell walls as a result of the action of cell wall modifying enzymes leading to shriveling and softening. We guess the inhibitory effect of CL and SA on degrading enzymes activity can be as main reason for positive effect of foliar treatment on fruit tissue firmness

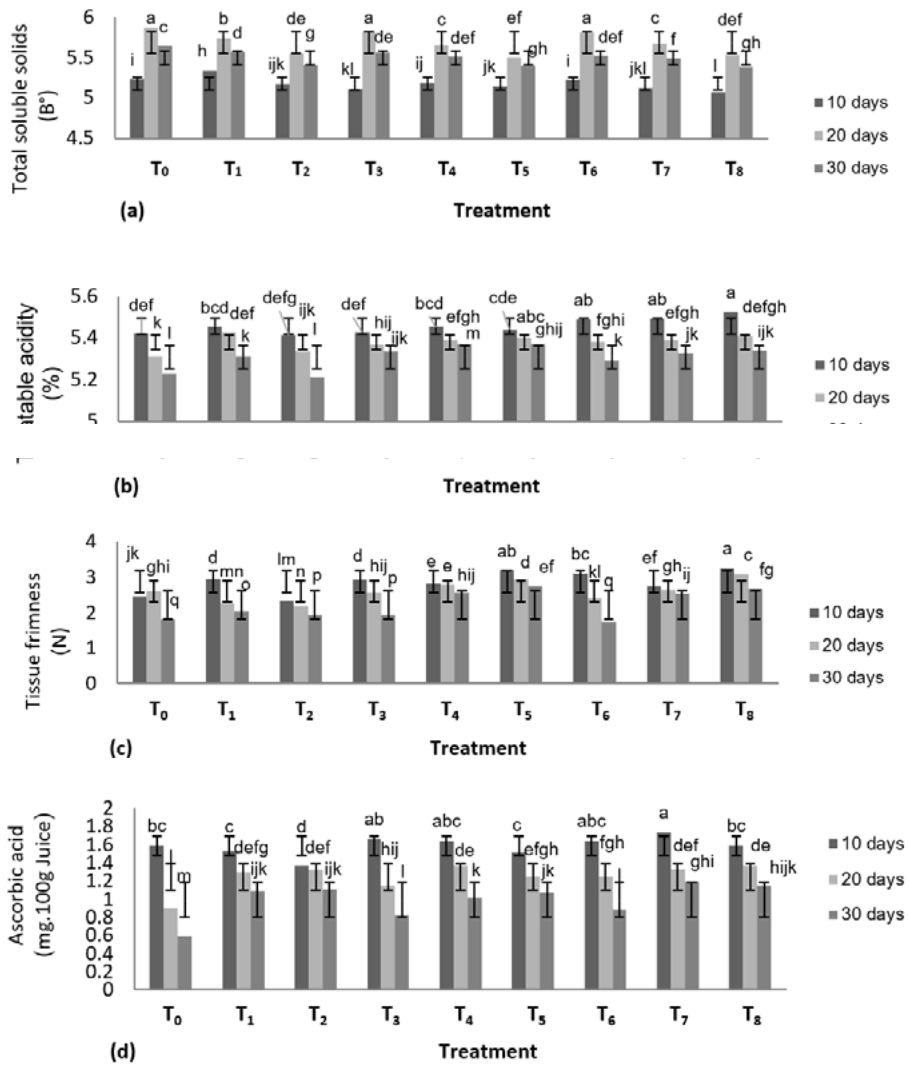

Figure 1: Effect of CL and SA foliar application and during of storage on: a total soluble solids, b titratable acidity, c tissue firmness and d ascorbic acid content
Values in columns for same variable followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

in this study. The highest and lowest values of ascorbic acid content were related to harvested fruits from CL 2 mM and SA 1.5 mM sprayed plants after 10 days of storage and control fruits after 30 days of storage (1.78 and 0.58 mg.100g juice, respectively) (Figure 1d). Our results showed that ascorbic acid content of fruits reduced by increasing of duration of storage but foliar treatment led to a reduction in ascorbic acid decreasing rate. According to Umebese and Bankole (2013), SA foliar application can increase nitrate reductase enzyme activity and this enhancement corresponds with the reduction of ascorbic acid decreasing rate.

Figure 2 shows the effect of CL and SA treatment and duration of storage on pulp tissue browning and fruit mass loss. The highest and lowest values of pulp tissue browning were related to control fruits after 30 days of storage and harvested fruits from CL 4 mM and SA 1.5 mM sprayed plants after 10 days of storage (89.27 and 67.16 % respectively) (Figure 2a).

The best treatment to minimize fruit mass loss was CL 4 mM and SA 1 mM foliar spraying after 10 days of storage (6.75 %) (Figure 2b). Our findings with respect to the effect of CL and SA treatment and duration of storage on fruit mass loss showed that control fruits recorded maximum fruit mass loss after 30 days of storage (19.54 %). In this study, increasing of duration of storage led to a raise in fruit mass loss. Fruit mass loss is basically related to water loss and this essentially due to transpiration, which accounts for 90 % of total mass loss and initially comes from the peel. Water loss adversely affects the quality and limits the economic post-harvest life of crops (Ennab et al., 2020). Our findings showed that foliar treatment led to a reduction in fruit mass loss. The result of this study is similar to the findings reported by Gupta et al. (2011). They reported that reduction in physiological mass loss in calcium sources treated fruits might be due to the maintenance of fruit firmness and tissue rigidity by decreasing the enzyme activity responsible for disintegration of cellular structure, which decreases the gaseous exchange.

According to ANOVA analysis, percent of fruit decay was conditioned by interaction between CL and SA foliar application and also main effect of duration of storage. Obtained results showed that the best treatment to decrease fruit decay was CL 4 mM and SA 1 mM foliar application (1.9 %) and 10 days storage led to the lowest

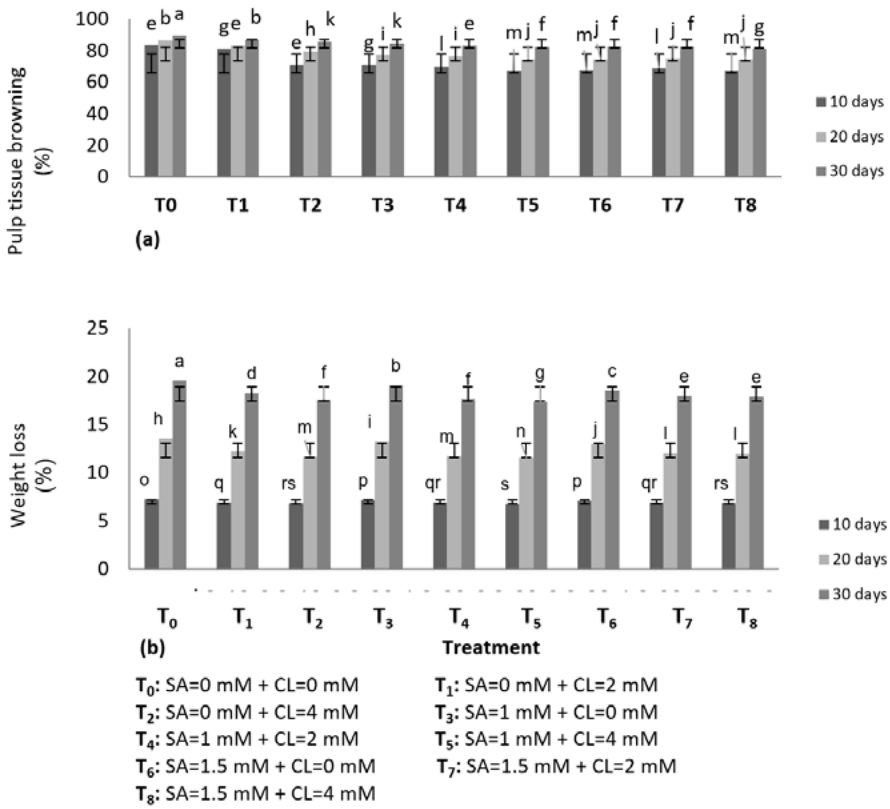

Figure 2: Effect of CL and SA foliar application and during of storage on: a browning percent and b mass loss
Values in columns for same variable followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

fruit decay percent (1.28 %) (Table 6). SA and calcium sources significantly reduced fruit decay of stored mandarins and sweet oranges due to enhancing the activity of antioxidant enzymes and improving resistance to fungal attack, the accumulation of H_2O_2 and defense-related metabolites like ornithine, threonine and polymethoxylated flavones (Zhu et al., 2016), and the anti-senescent effect that maintains fruit firmness, which eventually reduced microbial attack (Ahmed et al., 2013). Pre-harvest treatment of SA reduced post-harvest fruit decay and fungal diseases in melon (Huang et al., 2000), mango (Zainuri et al., 2001) and apple (Krishna et al., 2012).

Table 5: Effect of CL and SA foliar application and duration of storage on fruit decay after harvest

SA concentration (mM)	CL concentration (mM)	Fruit decay (%)	Duration of storage (day)	Fruit decay (%)
	0	2.58a	10	1.28c
0	2	2.15cd	20	2.20b
	4	2.03de	30	3.10a
	0	2.36b		
1	2	2.12cd		
	4	1.93e		
	0	2.18cd		
1.5	2	2.17cd		
	4	2.22bc		

Values in columns for same variable followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

4 CONCLUSION

Our results showed that foliar application of CL and SA can be useful and inexpensive treatment to improve growth parameters, physiological characteristics and post-harvest properties of eggplant fruit. The highest applied concentrations of CL and SA (4 mM and 1.5 mM) foliar application led to the highest values in all of measured growth parameters such as leaf area, mass, number, diameter and length of fruit, height of plant and yield. Foliar spray of eggplants by CL at 4 mM and 2 mM and also SA at 1 mM and 1.5 mM led to a significant increase in photosynthetic pigments. The highest tissue firmness and ascorbic acid content of eggplant fruit was obtained by highest concentration foliar application of CL and SA. Also, negative effects of increasing of storage time on post-harvest properties decreased by employing of CL and SA foliar application. Using of higher concentrations of SA and CL as well as applying of other plant bio-regulator in eggplant cultivation is recommended for future researches.

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Response of hemp (*Cannabis sativa* L.) to integrated application of chemical and manure fertilizers

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Response of hemp (*Cannabis sativa* L.) to integrated application of chemical and manure fertilizers

Abstract: The investigation of various nutrition systems in hemp plays an influential role in improving its production. An experiment was conducted in University of Birjand, Iran, during 2013-2014, in which manure (0, 10, 20, and 30 t.ha^{-1} of cow manure) was considered as the main plot and the combination of nitrogen (0, 50, and 100 kg N ha^{-1} as urea) with phosphorus (0 and 80 kg P ha^{-1} as triple superphosphate) fertilizers was considered as factorial in subplots. The type of soil fertility management had no significant effect on the percentage of female plants. Applying 20 t.ha^{-1} of manure plus 100 kg N ha^{-1} produced the highest biological yield, seed, and leaf extract. The highest oil content was obtained by applying a maximum of 50 kg N ha^{-1} without the use of phosphorus. The 30 t ha^{-1} manure plus 100 kg N ha^{-1} increased the leaf harvest index and decreased seed harvest index. Nitrogen consumption also increased the seed oil content and yield. Phosphorus increased the biomass and extracts of seed and leaves, also biological, seeds and oil yield. It seems hemp responds well to the combined application of nitrogen fertilizer and animal manure, while its response to P fertilization was limited.

Key words*:* cow manure; hemp; nitrogen; oil yield; phosphorus; seed yield

Odziv konoplje (*Cannabis sativa* L.) na hkratno uporabo mineralnih in organskih gnojil

Izvleček: Preučevanje različnih gnojilnih režimov igra pri konoplji pomembno vlogo za izboljšanje pridelave. Na univerzi v Birjandu (University of Birjand, Iran) je bil v letih 2013-2014 izveden gnojilni poskus, v katerem je bilo gnojenje s kravjim gnojem glavno obravnavanje (0, 10, 20, in 30 t ha^{-1}) in kombinirano gnojenje z dušikovimi (0, 50, in 100 kg N ha^{-1} kot urea) in fosforjevimi gnojili (0 in 80 kg P ha^{-1} kot trojni superfosfat) kot faktorski poskus na podploskvah. Način gnojenja ni imel značilnega vpliva na odstotek ženskih rastlin. Uporaba 20 t ha^{-1} kravjega gnoja z dodatkom 100 kg N ha^{-1} je dala največji biološki pridelek, pridelek semena in izvlečkov listov. Največja vsebnost olja je bila dosežena pri uporabi največ 50 kg N ha^{-1} brez dodatka fosforjevega gnojila. Uporaba 30 t ha^{-1} gnoja z dodatkom 100 kg N ha^{-1} je povečala žetveni indeks listov in zmanjšala žetveni indeks semena. Uporaba dušikovih gnojil je tudi povečala vsebnost olja v pridelku. Dodatek fosforjevih gnojil je povečal biomaso, vsebnost snovi v izvlečkih iz listov in semen, kot tudi biološki pridelek, pridelek semena in olja. Izgleda, da se konoplja odziva dobro na kombinirano gnojenje z mineralnimi dušikovimi gnojili in živinskimi gnojili, med tem, ko je njen odziv na gnojenje s fosforjevimi mineralnimi gnojili omejen.

Ključne besede: kravji gnoj; konoplja; dušikova gnojila; pridelek olja; fosforjeva gnojila; pridelek semena

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1 INTRODUCTION

Cannabis sativa L. is one of the oldest domesticated plants in human history and has been used since 10,000 years ago or more (Faux et al., 2013; Da Porto et al., 2014). Today, its cultivation is exposed to limitations due to the use of cannabis as a narcotic. Recently, the use of cannabis as a medicinal plant has been taken into consideration and the motive of this approach is to identify the effectiveness of whole plant cannabis in relieving some chronic diseases, although there is still debate about the medical benefits of cannabis worldwide (Madras, 2015). The seeds of this plant have a high nutritional value with a content of 22 to 35 % of oil (Peiretti, 2009), and has attracted the attention of nutritionists due to its high nutritional quality (Leizer et al., 2000; García-Tejero et al., 2014). There are high concentration of bioactive components, such as cannabinoids, in *Cannabis sativa* L., with nutritional value associated with health benefits, which can be isolated from different parts of the plant. While large amounts of Δ^9-THC accumulate in plant leaves, non-psychoactive cannabinoids are predominantly present in seeds (Fathordoobady et al., 2019). Meanwhile, extraction with ethanol is the preferred extraction method used for medicinal formulations (Thomas and Pollard, 2016).

The evaluation of different plant nutrition systems is one of the important needs in agronomic planning in order to achieve higher yields of plants such as hemp, as an oil or food crop. Nitrogen is one of the essential elements affecting the plants growth (Shams et al., 2013), which is required for proteins, enzymes, coenzymes, nucleic acids, cytochromes, and metabolic processes contributing to the synthesis and transfer of energy (Hoffman & Cleemput, 2004). In hemp, the application of 240 kg N ha^{-1} nitrogen was effective in increasing the plant biomass, stem dry mass, and inflorescence mass, compared to the control (without any fertilization) (Papastylianou et al., 2018). Poisa & Adamovics (2010) reported an increase in the applied nitrogen fertilizer increased the yield of hemp seed, while the content of seed oil decreased in such a way that the content of hemp seed oil decreased from 42 % in control plants to 40.5 % in in those plants that received 100 kg N ha^{-1}.

After nitrogen, phosphorus is the second most important nutrient for plant growth, which contributes to activating coenzymes producing amino acids (Hendawy & Khalid, 2011). Phosphorus is effective on the number of young cells in roots and stems and is highly needed in those places where cellular metabolism is greater and cells are dividing. Further, phosphorus plays a significant role in starting flowering, developing seeds and fruits, reducing diseases, increasing the quality of some crops, and root growth, especially the lateral roots, and developing fibrous root system (Hendawy et al., 2014; Kareem, 2013).

Although the use of chemical fertilizers for supplying nitrogen and phosphorus required by the plant has many benefits, there is an increasing demand for organic fertilizers, due to the soil and water pollution, and community health threats caused by synthetic chemical materials (Akande et al., 2010). The organic fertilizers (in particular, animal manures) have large amounts of organic matter, which could be used as a source of nutrients elements, especially nitrogen, phosphorus, and potassium (Cvetkov et al., 2010). Despite the benefits of using organic fertilizers, the chemical fertilizers cannot be removed from agricultural ecosystems and replaced by the organic fertilizers simultaneously, as the sustainability in agriculture is ensured by adequate income and food security. In this regard, the use of renewable and natural materials with organic sources, along with the optimal use of chemical fertilizers, can be of great importance in preserving fertility, biological building and activity, cation exchange capacity , water retention and ultimately, improving the physical and chemical structure of the soil (Ghosh et al., 2004). A large number of studies have been conducted on the integrated application of organic- with chemical fertilizers on different plants, including bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.) (Rurangwa et al., 2018), corn (*Zea mays* L.) (Arif et al., 2016), sunflower (*Helianthus annus* L.) (Akbari et al., 2011), and coriander (*Coriandrum sativum* L.) (Mallanagouda et al., 1995). Generally, nutritional studies on medicinal plants indicate the positive effect of organic fertilizers on the both quantitative and qualitative yields of medicinal plants. In saffron (*Crocus sativus* L.), for example, the highest stigma yield was reported 0.45 g m^{-2} in combined treatment of manure and chemical fertilizers (23 kg N ha^{-1} + 20 kg P_2O_5 ha^{-1} + 20 t ha^{-1} animal manure) (Amiri, 2009). The study of different fertilizer treatments on the German chamomile (*Matricaria chamomilla* L.) indicated that the combined application of manure and chemical fertilizers (23 kg N ha^{-1} + 23 kg P_2O_5 ha^{-1} + 7.5 t ha^{-1} cow manure) increased the biological yield (188.6 %), harvest index (18.69 %), and essential oil content (84.27 %), compared to the control (Shams et al., 2012).

It seems that the integrated application of appropriate amounts of organic and chemical fertilizers positively affect the oil yield of plants. For example, 50 % cow manure plus 50 % nitrogen in sunflower, produces the highest oil yield (1275.9 kg ha^{-1}), and 100 % application of cow manure produces the highest oil percentage (49.4 %) (Akbari et al., 2011). In another study, the use of 50 %

cow manure plus 50 % of chemical fertilizer (N, P and K at 220, 150 and 100 kg ha^{-1}, respectively), produces the highest yield (1482.4 kg ha^{-1}) and oil content (39.7 %) in sunflower (Esmaeilian et al., 2011). The combined application of organic fertilizers (15 t ha^{-1} vermicompost) with 100 kg N ha^{-1} was effective in increasing the seed yield and oil content of rapeseed (*Brassica napus* L.) (Zahedifard et al., 2014). In a study with chemical fertilizers (nitrogen and phosphorus), and bio-fertilizer, the combination of bio-fertilizer with 50 % of total nitrogen (200 kg ha^{-1} ammonium nitrate 33 %) and 50 % of total phosphorus (100 kg ha^{-1} of calcium superphosphate 15.5 %), produced the highest oil yield in the fennel (*Foeniculum vulgare* Mill.) (Dadkhah, 2012). It was reported that nitrogen fertilizer reduces the oil content of the juniper (*Juniperus communis* L.) (Hendawy et al., 2014), although it increases the oil yield in the thyme medicinal plant (*Thymus vulgaris* L.) (Baranauskienne et al., 2003).

To the best of our knowledge, the effect of the combined application of chemical and organic fertilizers on the yields of seed oil and extracts of different parts of the hemp has not yet been studied. Regarding the global trend towards the production and propagation of low-demand crops in sustainable agricultural systems and the importance of reducing chemical inputs in agriculture on community health, more studies are necessary on the impact of chemical and organic fertilizers on neglected and new crops such as hemp, to be introduced in sustainable farming systems especially in marginal lands. This valuable plant has not yet found its true position among cash crops due to legal constraints despite the thousands of years of planting hemp (Da Porto et al., 2014). The present study aimed to investigate the effect of chemical (nitrogen and phosphorus) and organic (cow manure) fertilizers on biological, seed, leaf extract, and oil yields of hemp in two cropping years.

2 MATERIAL AND METHODS

The integrated application of chemical (nitrogen, phosphorus) and organic (rotted cow manure) fertilizers on hemp (Khusf landrace) was investigated during an experiment in two consecutive cropping years (2013 and 2014) in the Research Farm of Faculty of Agriculture (32° 52′ N, 59° 12′ E, 1491 meters above the sea level), University of Birjand, Birjand, Iran. The seeds were obtained from smallholder farmers who grow this crop in their subsistence farming systems in the Khusf (a small town near Birjand, eastern Iran). Plant height and the length of main inflorescence of this local variety are 150 and 22 cm, respectively, with 25-20 nodes in the main stem, on average. The stem diameter is 2-3 cm and the height of the first flowering node is 80 cm. Its growth period lasts 160 to 180 days (Riahi et al., 2016)

Tables 1 and 2 represent the trends of temperature and rainfall during experiment and the characteristics of soil of experimental site and applied manure of each year, respectively. In order to determine the physical and chemical characteristics of the soil in each year, the five samples were taken prior to planting from five points through experimental site within a depth of 0-20 cm. The samples were mixed and a sub-sample was taken. The electrical conductivity of the saturated extract and pH of soil and manure were measured. The nitrogen content of the soil and manure was calculated by using the Kjeldahl method (Jackson, 1958). Further, the carbon and the phosphorous of the soil and manure were measured by using Walkley-Black (Walkley and Black, 1934) and spectrophotometry (Bouyoucos, 1951), respectively. In addition, the soil texture was determined based on Bicas's hydrometer method.

The experiment was conducted in a factorial split-plot arrangement with three replications. The manure (0, 10, 20, and 30 t ha-1 of rotted cow manure) was considered as the main plot and the combination of nitrogen (0, 50, and 100 kg N ha^{-1} as urea) with phosphorus (0 and 80 kg P_2O_5 ha^{-1} as triple superphosphate) fertilizers was considered as factorial in subplots. Generally, 100 kg ha^{-1} of potassium sulfate (50 kg K_2O ha^{-1}) was used for all plots before sowing. All phosphorus fertilizers and half of the nitrogen fertilizers were applied before sowing below the seed planting depth. After thinning, the other half of the nitrogen fertilizer treatments (except control) were applied.

In both years, sowing was on plots that were under fallow the previous year. The manure and potassium fertilizer were spread on the soil surface and then mixed into the soil during soil preparation for sowing. The plot size consisted of 5 rows with row length of 4 m. In each plot the hemp was sown with a density of six pl m^{-2} (with 60 and 30 cm space between rows and on the rows) in five rows with a depth of 3-4 cm. The seeds were hand sown with high density beside row bed on June 7, 2013, and May 5, 2014, and then emerged plants were thinned in two stages (when plants had 2-4 leaves and two weeks later) to achieve the final density. The irrigation interval was adjusted to 10 days. During the experiment, no herbicides, fungicides, and fertilizers (except for fertilizer treatments) were used, and all weeds were controlled by twice hand weeding early in plant growth. At final harvest, the side rows and one meter at all row ends were discarded to avoid border effects. The whole plants were harvested on November 11, 2013, and October 7, 2014, when 50 % of the grains were hard.

Table 1: Average precipitation and temperature during the growth season of hemp in 2013 and 2014

		May	June	July	August	September	October	November
2013	Average temperature (°C)	21.1	26.7	27.2	27.2	23.6	20.9	10.4
	Average precipitation (mm)	24.8	0.2	0	0	0	1.1	4.4
2014	Average temperature (°C)	22.3	25.7	28.5	25.3	22.4	19.6	13.4
	Average precipitation (mm)	3.8	0.4	0	0	0	6.2	11.2

Table 2: Physicochemical properties of the soil (0-20 cm depth) and animal manure used in 2013 and 2014

Year		pH	EC	Organic carbon	N	C/N ratio	P	Texture	Silt	Clay	Sand
			(dS m^{-1})	------- (%) -------			(ppm)		---------- (%) ----------		
2013	Soil	7.98	9.75	0.52	0.06	8.66	10.3	Loamy sandy clay	26	20	54
	Manure	8.5	6.9	12.6	0.66	19.09	1240	-	-	-	-
2014	Soil	7.79	7.48	0.31	0.04	7.75	9.8	Loamy sandy clay	26	22	52
	Manure	8.01	6.03	12.4	0.58	21.15	1180	-	-	-	-

2.1 MEASURED CHARACTERISTICS

In this experiment, biological and seed yield, thousand seeds mass, leaf and seed harvest indices, the yield of leaf and seed extract, percentage and yield of seed oil, and specific leaf area (SLA) were measured. Considering the same planting density in all plots, the number of all female plants per plot was counted.

At final harvest, whole plant were harvested from three m^2, and after completely drying in shading conditions and free air, were weighed on a scale (± 0.01 g) to measure biological yields of female plants. After that, seeds were separated and weighted to calculate seed yields. Five replicates of 100 seeds then were weighted with a 0.001 g scale to determine the mass of 1000 seeds. Also the seed and leaf harvest indices were calculated for female plants based on the leaf and seed dry yields to the biological yield ratio, respectively.

Another random sample was taken from one square meter of each plot to measure the leaf area and dry mass of female plants, and then SLA was calculated. The seed and leaves of these plants also used to measure their extracts. In this regard, we used ethanol to prepare an alcoholic extract of leaves and seeds (Khan et al., 2014). To do this, the leaves and seeds were separately grinded and 50 g of powdered material, after adding ethanol 70 %, were placed for 48 hours in a shaker incubator at 25 °C and the speed of 100 rpm. At the end of this period, the solution was filtered by Watten's No. 1 filter paper. In order to remove the solvent from the extract, the samples were placed in a 40 °C oven for 48 hours. The obtained extract had no alcohol and contained a sticky and green substance. Then, the extracts were weighed with a 0.001 gram scale.

The extraction of seed oil was performed with the soxhlet method according to the AOAC (1990). A sample of 10 g of seeds flour was extracted using 100 ml hexane as solvent, for 2 h. The solvent was removed with a rotary evaporator and the residue was placed in oven at 100 °C for 4 h and then transferred to a desiccator and weighed up to constant value. The seed oil percentage was obtained by equation [1]:

$$Fat\,(\%) = \frac{m3 - m2}{m1} \times 100 \qquad (1)$$

Where m_1 represents the initial mass of the sample (g), m_2 indicates the initial mass of the container (g), and m_3 is the secondary mass of the container (container + oil). The oil yield was simply obtained by multiplying seed yield in the oil percentage.

The specific leaf area ($cm^2\ g^{-1}$) was calculated from the equation [2] (Amanullah et al., 2007):

$$SLA = \frac{LA}{LW} \qquad (2)$$

Where LA represents the leaf area ($cm^2\ pl^{-1}$) and LM indicates the leaf mass per plant ($g\ pl^{-1}$). For this purpose,

in the seed filling stage, female plants were harvested from one m^2 of each plot, and their leaves were separated. Leaf area was determined using a leaf area meter (Delta-T Devices, UK).

2.2 STATISTICAL ANALYSIS

In the present study, check the normality of data, plotting, and analysis of regressions, as well as the correlations of traits were done by IBM SPSS Statistics 22 software. Before the combined analysis of the data, Bartlett's test was used to ensure the uniformity of the test error variance. Data analysis was performed using SAS 9.2 software considering the effect of year as random and the effect of experimental treatments for desired traits as constant. Comparison of meanings was done by FLSD test at 5% probability level.

3 RESULTS AND DISCUSSION

3.1 RESULTS

Table 3 shows the results of analysis of variance of traits. As shown in Table 4, the studied traits were not significantly different between two years. The fertilizer treatments had no significant effect on the percentage of female plants in the hemp. However, an increase in the manure level and also applying 50 kg N ha^{-1} increased the percentage of female plants. Bigger increase in the nitrogen fertilizer level reduced the percentage of female plants, which was not significant (Table 4).

By comparing biological yield among different manure levels, the most beneficial effect of manure on increasing biological yield was achieved from 10 to 20 t ha^{-1} applied manure, that was on average 148.94 kg ha^{-1} biological yield per each one t ha^{-1} of more manure; although a further increase in the manure amount reduced its effectiveness in increasing the biological yield (Table 4). An increase in the nitrogen amounts also reduced its effect on enhancing biological yield, such that the increase in biological yield per each kg more nitrogen between 0-50 kg N ha^{-1} (44.4 kg ha^{-1}) was more than 50-100 kg N ha^{-1} (13.61 kg N ha^{-1}) (Table 4).

The highest biological yield was obtained in the combined treatment of 20 t ha^{-1} manure plus 100 kg N ha^{-1}, which did not show any significant difference with the combined treatment of 30 t ha^{-1} manure plus 50 kg N ha^{-1} (Table 5). Without the applying manure, for increasing each kg N ha^{-1} from the 0 to 50 and 50 to 100 kg N ha^{-1}, the biological yield increased, on average by 29.4 and 21.4 kg ha^{-1}, respectively. When 10 t ha^{-1} cow manure was used, the increase in the biological yield was 35.22 and 17.9 kg ha^{-1} per each kg N in 0-50 and 50-100 kg ha^{-1} applied N, respectively. These increases in biological yield for 20 t ha^{-1} of manure were 54.28 and 20 kg ha^{-1}, respectively. At the level of 30 t ha^{-1} manure, the biological yield increased by an average of 57.25 kg ha^{-1} per each kg N ha^{-1} in the range from 0 to 50 kg N ha^{-1} while it decreased by 18.38 % per each kg of nitrogen in the range 50-100 kg N ha^{-1}. This indicates the use of manure has been effective in increasing the effectiveness of nitrogen fertilizer. In each level of manure, the efficiency of adding first 50 kg N ha^{-1} (0-50 kg N ha^{-1}) to improve biological yield was more than that of the second 50 kg (50-100 kg N ha^{-1}), confirming that the response of biological yield to the amount of consumed nitrogen follows law of diminishing returns. The results also indicated that an increase in the nitrogen levels to 100 kg ha^{-1} could have a negative effect on the biological yield of hemp for levels of more than 20 t ha^{-1} of manure (Table 5).

Based on the results, the beneficial effect of nitrogen on the seed yield increased under the influence of manure, and accordingly, no significant difference was observed between 0 and 50 kg N ha^{-1} when manure was not used. However, manure was applied at rates of 10, 20, and 30 t ha^{-1}, the use of 50 kg N ha^{-1} could significantly increase the seed yield by 29.33 %, 33.9 %, and 33.75 %, respectively, compared to non-application of nitrogen (Table 5). The highest seed yield was achieved when 20 t ha^{-1} manure was used along with 100 kg N ha^{-1} , which increased by 40.95 % compared to when the manure was not used. Further, there was no significant difference between the combined applications of 20 t ha^{-1} of manure plus 100 kg N ha^{-1} with 20 t ha^{-1} manure with 50 kg N ha^{-1}, and 30 t ha^{-1} manure plus 50 kg N ha^{-1} (Table 5). It is worth noting that an increase in the nitrogen fertilizer level increased the seed yield at 0, 10, and 20 t ha^{-1} of manure, while the use of 100 kg N ha^{-1} could not positively affect the seed yield when combined with 30 t ha-1 of manure. This combined treatment reduced the seed yield by 17.11 %, compared to a combined use of 30 t ha^{-1} manure plus 50 kg N ha^{-1} (Table 5).

The response of seed yield to manure was followed a second-order function under the influence of phosphorus consumption, upon which, when phosphorus was applied, the highest seed yield was obtained with the application of 20 t ha^{-1} manure; however when phosphorus was not consumed, the response of the seed yield to manure was linear (Figure 1). Therefore, in the case of phosphorus application, the use of more than 20 t ha^{-1} manure reduced the effectiveness of manure to increase the seed yield, while, in the case of non-application of phosphorus, as the amount of manure increased, seed yield also

Table 3: The Results of combined analysis of variance of measured traits (mean squares) in cannabis during 2013 and 2014 as affected by manure, nitrogen and phosphorus fertilizers

		Mean squares									
Source of Variation	df	female plant	Biological yield	Seed yield	Seed mass	Leaf harvest index	Seed harvest index	Leaf extract yield	Seed extract yield	Seed oil	Seed oil yield
Year (Y)	1	107.22^{ns}	478005.7^{ns}	16197.14^{ns}	0.18^{ns}	38.10^{ns}	1.25^{ns}	78.54^{ns}	11.30^{ns}	4.34^{ns}	2.87^{ns}
Replication (Y)	4	33.29	694989.4	11751.22	2.01	77.79	18.50	2.59	1.70	2.67	11.02
Animal manure (M)	3	17.00^{ns}	47084929.5**	1735544.27**	35.38**	203.46**	64.39**	23.29*	3.19*	48.58**	1816.24**
Y × M	3	1.34^{ns}	289722.6^{ns}	115785.15^{ns}	2.09^{ns}	73.29^{ns}	26.37^{ns}	0.20^{ns}	0.04^{ns}	3.82^{ns}	69.87^{ns}
Error a	12	27.84	680624.2^{ns}	88101.52^{ns}	10.79^{ns}	51.50	18.12	3.90^{ns}	0.62^{ns}	4.75^{ns}	57.61^{ns}
Nitrogen (N)	2	30.97^{ns}	109253454**	3896713.79**	79.64**	260.07**	141.72**	78.42**	8.35**	29.21*	2585.62**
Phosphorus (P)	1	35.16^{ns}	29378275.4**	1575995.26**	150.06**	37.40^{ns}	5.70^{ns}	6.37*	0.77*	4.34^{ns}	805.65**
M×N	6	17.05^{ns}	3472068.2**	179094.96*	8.36^{ns}	30.02^{ns}	11.00^{ns}	1.68**	0.27^{ns}	11.42^{ns}	157.32^{ns}
M×P	3	32.07^{ns}	492938.4^{ns}	232450*	1.31^{ns}	88.15^{ns}	46.48^{ns}	5.99^{ns}	2.72^{ns}	3.37^{ns}	203.11^{ns}
N×P	2	8.14^{ns}	679811.2^{ns}	73550.68^{ns}	0.36^{ns}	12.93^{ns}	5.04^{ns}	0.49^{ns}	0.67^{ns}	27.17*	218.10^{ns}
M×N×P	6	47.04^{ns}	871692.2^{ns}	79057.64^{ns}	3.19^{ns}	45.24^{ns}	27.13^{ns}	5.39^{ns}	1.79^{ns}	2.90^{ns}	70.21^{ns}
Y×N	2	8.41^{ns}	1419729.1^{ns}	145898.51^{ns}	7.06^{ns}	58.72^{ns}	8.23^{ns}	5.53^{ns}	1.25^{ns}	7.96^{ns}	98.09^{ns}
Y×P	1	3.62^{ns}	9586.7^{ns}	3344.92^{ns}	0.17^{ns}	38.61^{ns}	1.84^{ns}	0.69^{ns}	1.09^{ns}	10.56^{ns}	27.68^{ns}
Y×M×N	6	63.81^{ns}	613936.5^{ns}	162175.15^{ns}	8.68^{ns}	9.37^{ns}	27.62^{ns}	0.97^{ns}	0.46^{ns}	1.30^{ns}	135.78^{ns}
Y×M×P	3	81.13^{ns}	1351238.3^{ns}	1322860.66^{ns}	2.71^{ns}	9.98^{ns}	9.87^{ns}	7.23^{ns}	1.14^{ns}	3.67^{ns}	147.53^{ns}
Y×N×P	2	3.09^{ns}	810736.0^{ns}	125542.09^{ns}	0.04^{ns}	29.55^{ns}	35.51^{ns}	2.65^{ns}	0.39^{ns}	21.06^{ns}	119.29^{ns}
Y×M×N×P	6	73.76^{ns}	605625.4^{ns}	42360.76^{ns}	5.11^{ns}	7.50^{ns}	12.21^{ns}	4.91^{ns}	1.61^{ns}	12.25^{ns}	113.10^{ns}
Error b	80	45.02	757591.5	78291.42	6.93	45.04	20.57	8.24	0.97	7.68	79.63
Coefficient of variation (%)		12.12	12.11	15.49	14.13	12.13	17.64	14.82	12.60	10.21	18.20

ns: no significant difference, * and ** significant difference at the level of one and five percent, respectively

Table 4: Simple effects of animal manure, nitrogen and phosphorus on measured traits in hemp. For fertilization treatments, numbers are means of three replication ±SEM

Treatments	Level	female plant (%)	Biological yield (kg ha^{-1})	Seed yield (kg ha^{-1})	Seed mass (g 1000 seed)	Leaf harvest index (%)	Seed harvest index (%)	Leaf extract yield (kg ha^{-1})	Seed extract yield (kg ha^{-1})	Seed oil (%)	Seed oil yield (kg ha^{-1})
Year	2013	54.48^{a}	7242.34^{a}	1795.29^{a}	18.58^{a}	54.79^{a}	25.61^{a}	787.40^{a}	88.83^{a}	27.30^{a}	488.77^{a}
	2014	56.20^{a}	7127.11^{a}	1816.50^{a}	18.66^{a}	55.82^{a}	25.74^{a}	807.32^{a}	78.14^{a}	26.95^{a}	491.59^{a}
Animal manure (t ha^{-1})	0	54.62^{a}	5858.80^{c}	1544.87^{d}	17.47^{b}	52.76^{c}	27.03^{a}	599.50^{b}	66.52^{b}	25.52^{b}	394.02^{c}
	10	55.00^{a}	6602.00^{b}	1710.41^{c}	18.13^{ab}	53.88^{bc}	26.31^{a}	685.19^{b}	74.95^{b}	28.22^{a}	483.94^{b}
	20	55.54^{a}	8091.40^{a}	2027.85^{a}	19.47^{a}	56.72^{ab}	25.56^{ab}	939.89^{a}	98.67^{a}	27.66^{a}	558.77^{a}
	30	56.21^{a}	8186.70^{a}	1940.47^{b}	19.44^{a}	57.84^{a}	23.91^{b}	964.88^{a}	93.81^{a}	27.11^{a}	524.01^{ab}
Nitrogen fertilizer (kg ha^{-1})	0	54.72^{a}	5488.20^{b}	1475.00^{b}	17.17^{b}	52.79^{b}	27.45^{a}	538.02^{c}	61.88^{b}	27.33^{ab}	406.08^{b}
	50	56.25^{a}	7690.30^{a}	1946.55^{a}	19.08^{ab}	55.73^{a}	25.63^{ab}	872.65^{b}	86.75^{ab}	27.79^{a}	541.27^{a}
	100	55.06^{a}	8375.70^{a}	1993.15^{a}	19.63^{a}	57.38^{a}	24.02^{b}	981.43^{a}	100.57^{a}	26.27^{b}	523.20^{a}
Phosphorus fertilizer (kg ha^{-1})	0	55.84^{a}	6733.05^{b}	1701.28^{b}	17.61^{b}	54.79^{a}	25.90^{a}	734.54^{b}	76.76^{b}	27.30^{a}	466.53^{b}
	80	54.85^{a}	7636.41^{a}	1910.51^{a}	19.65^{a}	55.81^{a}	25.50^{a}	860.19^{a}	90.21^{a}	26.95^{a}	513.83^{a}

Table 5: Mean comparisons for interaction effects of animal manure and nitrogen levels on measured traits in hemp. For fertilization treatments, numbers are means of three replication ±SEM

Animal manure (t ha^{-1})	Nitrogen fertilizer (kg ha^{-1})	Biological yield (kg ha^{-1})	Seed yield(kg ha^{-1})	Leaf extract yield kg ha^{-1})
0	0	4522.29 ± 85.10^{i}	1159.98 ± 20.67^{e}	41.05 ± 17.23^{f}
	50	5992.23 ± 68.64^{fgh}	1455.86 ± 25.51^{cde}	63.20 ± 13.14^{def}
	100	7061.83 ± 56.72^{de}	1718.76 ± 19.05^{bc}	75.59 ± 11.80^{cd}
10	0	5129.13 ± 78.41^{hi}	1273.54 ± 11.81^{de}	48.68 ± 13.25^{ef}
	50	6890.58 ± 92.57^{def}	1647.19 ± 30.48^{c}	68.93 ± 13.81^{cde}
	100	7786.29 ± 86.14^{cd}	1710.48 ± 29.07^{c}	87.93 ± 14.09^{bc}
20	0	5848.32 ± 65.88^{gh}	1519.01 ± 23.02^{cd}	57.30 ± 10.26^{def}
	50	8562.79 ± 86.22^{bc}	2034.00 ± 31.75^{ab}	101.98 ± 16.79^{ab}
	100	9563.08 ± 120.53^{a}	2130.54 ± 29.25^{a}	122.67 ± 19.96^{a}
30	0	6453.02 ± 90.05^{efg}	1559.47 ± 32.94^{cd}	63.06 ± 11.45^{def}
	50	9315.63 ± 52.46^{ab}	2085.84 ± 19.64^{a}	115.57 ± 12.98^{a}
	100	8396.21 ± 121.13^{bc}	1728.82 ± 30.54^{bc}	106.36 ± 29.36^{ab}

In each column, the values that share at least one letter have no significant differences according to LSD test at 5 percent of probability.

increased, although there was not a significant difference between of 20 and 30 t ha^{-1} manure (Figure 1).

With the use of 20 and 30 t ha^{-1} manure, the grain mass increased by 11.44 and 11.26 %, respectively, compared to the control. The most beneficial effect of manure was achieved in the range of 10 to 20 t ha^{-1} (0.11 g per ton of manure, on average), although the more increase in manure reduced the effectiveness of manure on seed

mass (Table 4). The application of 100 kg N ha^{-1} increased the seed mass by 14.27 % compared with the control. Despite the increase of the seed mass under the influence of the nitrogen, the results indicated that the average effectiveness of nitrogen on the seed mass in the range of 0 to 50 kg N ha^{-1} (0.04 g per kg N ha^{-1}), was more than 50 to 100 kg N ha^{-1} (0.01 g per kg N ha^{-1}) (Table 4).

Based on a linear relationship, an increase in the leaf harvest index in hemp was consistent with decreasing the seed harvest index (Figure 2). The application of manure was effective in increasing the leaf harvest index and the levels of 20 and 30 t ha^{-1} manure, compared to the control, increased the leaf harvest index by 7.5 and 9.62 %, respectively. However, the use of 30 t ha^{-1} of manure reduced the seed harvest index by 8.87 %, as compared with the control (Table 4). Thus, it seems applying 30 t ha-1 of manure stimulate assimilate partitioning to plant leaves and thereby reduced allocation ratio to seeds.

It is worth noting that the use of nitrogen fertilizer in hemp also increased the leaf harvest index, as 50 and 100 kg N ha^{-1} increased this index by 5.56 and 8.7 %, respectively, compared with the control treatment, while using 100 kg N ha^{-1} resulted in a 12.49 % reduction in the seed harvest index, compared to its non-application (Table 4). So, like the highest level of manure, the level of 100 kg N ha^{-1} reduced allocation of assimilates to the seeds by more biomass partitioning to the plant leaves.

The highest and the lowest leaf extract yields were obtained with integrated application of 20 t ha-1 manure with 100 kg N ha^{-1} and in no-fertilizer treatment, respectively (Table 5). The use of nitrogen alone (without manure) reduced the efficiency of nitrogen in increasing the yield of leaf extract, as in the ranges of 0-50 and 50-100 kg N ha^{-1}, the average yield of hemp leaf extract increased by 0.44 and 0.27 kg per kg N applied, respectively. These values were 0.40 and 0.38 kg leaf extract per kg N applied for the level of 10 t ha^{-1} manure and 0.89 and 0.41 kg leaf extract per kg N applied for the level of 20 t ha^{-1} manure, respectively. With 30 t ha^{-1} manure, the leaf extract yield increased by 1.05 kg per kg N applied in the range of 0-50 kg N ha^{-1}, while it decreased by 0.18 kg per kg N applied from 50 to 100 kg N ha^{-1} (Table 5). The results suggested that at all levels of manure, the effectiveness of nitrogen on the leaves extract yield was more in the first 50 kg than that of the second one. The combination of the highest levels of manure and nitrogen had a negative effect on the yield of hemp leaf extract.

There was a relation between yield of hemp leaf extract with specific leaf area (SLA), as an increase in the manure level reduced the SLA and increased the yield of leaf extract. Further, the SLA of most treatments was in the range of 20 to 40 cm^2 g^{-1}. (Figure 3).

The 20 and 30 t ha^{-1} manure increased seed extract yield by 48.33 and 41.02 %, respectively, in comparison with the non-application of manure (Table 4). The level of 20 t ha^{-1} of manure was the most effective in increasing the yield of seed extract, since the yield of seed extract increased by 16.55 and 31.74 kg t^{-1} manure applied in the range of 0-10 and 10-20 t ha^{-1} manure, respectively. Nevertheless, the yield of seed extract was reduced by 8.73 kg t^{-1} manure applied from 20 to 30 t ha^{-1} manure (Table 4).

Seed extract yield increased in response to higher nitrogen fertilizer levels, so applying 50 and 100 kg N ha^{-1} increased the seed extract yield by 40.19 and 62.52 %, compared to the control (non-application of nitrogen), respectively (Table 4). The positive effect of nitrogen utility on seed extract yield decreased by increasing the nitrogen levels, so the amount of increase in the seed extract per nitrogen consumed in the range of 0-50 kg N ha^{-1} (0.5 kg kg^{-1} nitrogen) was more than that of 50-100 kg N ha^{-1} (0.28 0.5 kg kg^{-1} nitrogen) (Table 4).

The use of manure led to higher seed oil percentage,

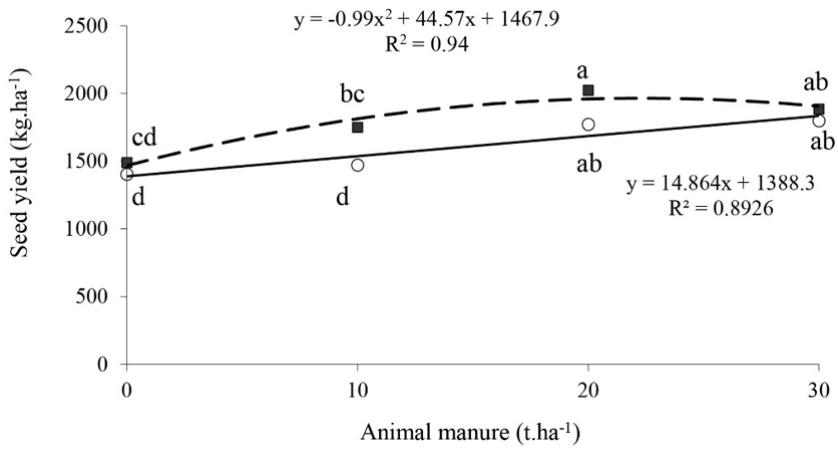

Figure 1: Response of hemp seed yield to animal manure at 0 (○) and 80 (■) kg P ha^{-1}. The points that share at least one letter have no significant differences according to FLSD test at 5 percent of probability.

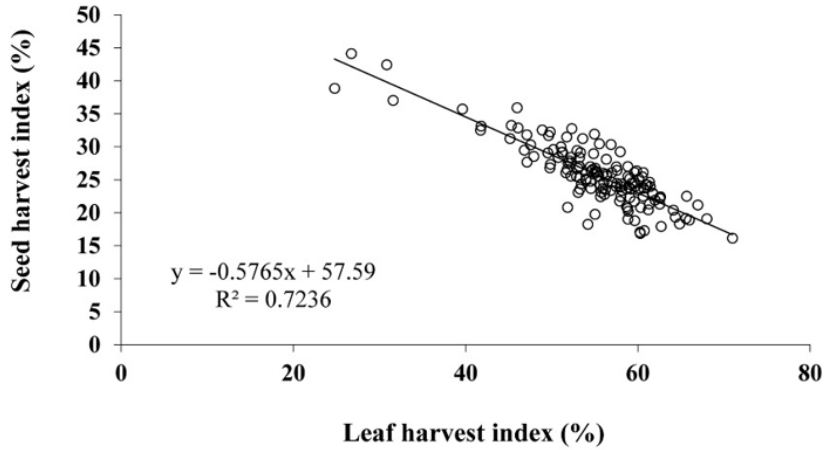

Figure 2: Changes of seed harvest index (%) to leaf harvest index (%) in hemp.

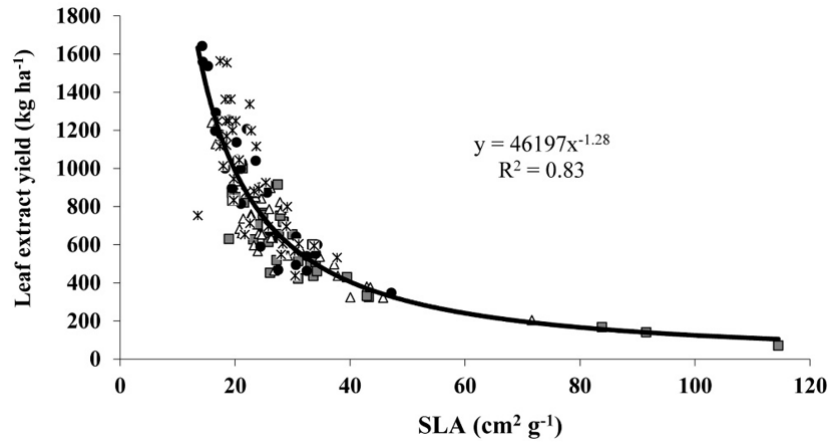

Figure 3: Changes of Leaf extract yield (kg ha^{-1}) to SLA (cm^2 g^{-1}) for 0 (■), 10 (△), 20 (●) and 30 (∗) ton animal manure ha^{-1}

so that applying 10, 20, and 30 t ha^{-1} of manure increased seed oil by 10.58 %, 8.38 % and 6.23 %, respectively, compared to the control. An increase in manure level by more than 10 t ha^{-1} decreased the profitability of manure to increase the percentage of seed oil (Table 4).

The highest percentage of seed oil (28.85 %) was achieved by applying 50 kg N ha^{-1} and not using phosphorus (Figure 4). When no phosphorus was used, an increase in the nitrogen levels up to 50 kg N ha^{-1} improved its efficiency to enhance the seed oil percentage, although efficiency was reduced with more nitrogen consumption, so that, in the absence of phosphorus application, the level of 100 kg N ha^{-1} decreased the percentage of seed oil by 4.77 %, compared to the 50 kg N ha^{-1}. When phosphorus was used, the nitrogen effectiveness on the seed oil percentage decreased by increasing its level, however there was not any significant differences between 50 and 100 kg N ha^{-1} with in terms of seed oil percentage (Figure 4).

The slopes of regression lines fitted between the oil yields versus the seed yields were significant ($p \leq 0.01$), indicating any increase in the seed yield is accompanied with the oil yield, the use of manure, especially at levels of 10 and 20 t ha^{-1} (compared with control), was more effective in increasing the yield of oil than grain yield (Figure 5).All levels of manure were effective in increasing the yield of seed oil as 10, 20 and 30 t ha^{-1} of manure increased the seed oil yield by 22.82 %, 41.81 %, and 33 %, respectively, compared to the control. Compared to the application of 20 t ha^{-1} of manure, the 30 t ha^{-1} manure caused a slight and non-significant decline in seed oil yield (Table 4).

The 50 and 100 kg N ha^{-1} levels improved the seed oil yield by 33.30 and 28.84 %, respectively, compared to

the control and with the application of 100 kg N ha^{-1} the yield of the hemp seed oil showed a slight and non-significant decrease (Table 4).

The consumption of 80 kg P ha-1 increased the biological yield (13.41 %), seed mass (11.6 %) and the seed oil yield (10.13 %), compared to the control (no phosphorus application) (Table 4). The results also indicated that the phosphorus improved the yield of leaf and seed extracts by 17.9 and 17.52 %, respectively. The phosphorus was more effective in increasing the leaf extract yield than seed extract, so that phosphorus increased the yield of leaf and seed extracts by 1.57 and 0.17 kg per kg P, respectively (Table 4).

3.2. DISCUSSION

In this experiment, the effect of year on the measured traits was not significant (Table 4). The average temperature for the cropping seasons was approximately the same for two years (the average temperature in 2013 and 2014 was 22.66 and 23.96 °C, respectively) (Table 1). A little superiority which was observed in 2014 in some measured traits, such as the seed yield, seed mass, leaf and seed harvest indices, the yields of seed and plant oil extracts, is probably due to more suitable germination temperature in this year compared to 2013. The optimum temperature for germination of hemp seeds is 20 °C (Albu and Marti, 2008) and the lower temperature in May 2014 (22.3 °C), compared to the June 2013 (26.7 °C, Table 1), which are the planting times in this experiment, caused germination and early growth of the plant in 2014 to be occurred in a more appropriate condition. The more rainfall in June and October 2014, compared to

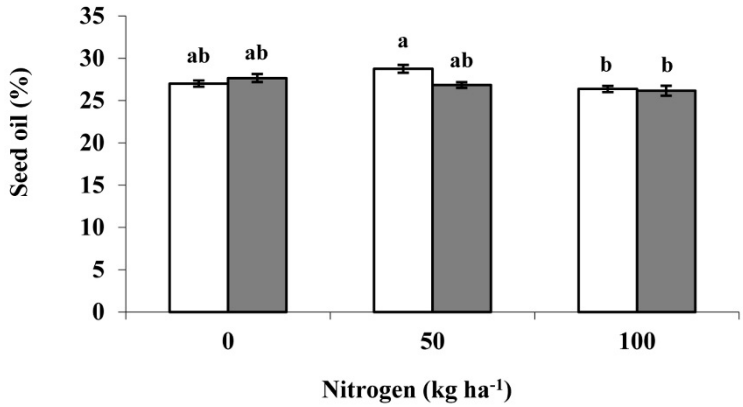

Figure 4: Response of hemp seed oil percentage to applied nitrogen at 0 (white columns) and 80 (dark columns) kg P ha^{-1}. The columns that share at least one letter have no significant differences according to FLSD test at 5 percent of probability. Vertical bars on columns indicate SEM.

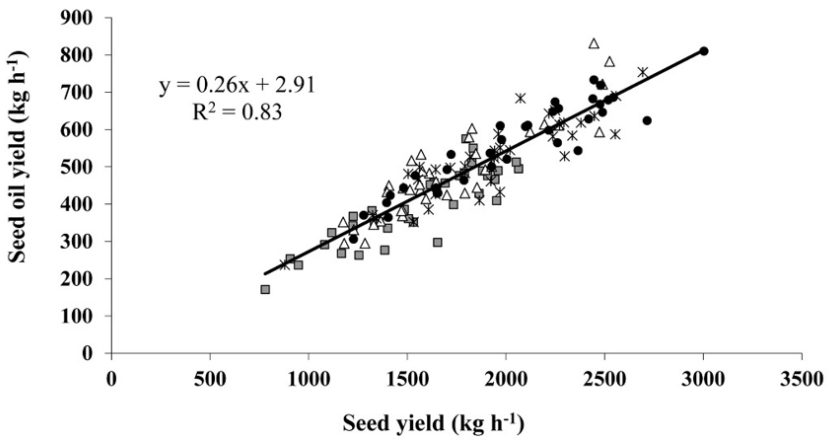

Figure 5: Changes of seed oil yield (kg ha^{-1}) to seed yield (kg ha^{-1}) for 0 (■), 10 (△), 20 (●) and 30 (∗) ton animal manure ha^{-1}

2013, was also effective in improving the measured traits in this year (Table 1). Also the higher soil EC of the experimental site in 2013 could be one of the reasons for the overall decline in traits in this year compared to 2014 (Table 2).

Chemical agents and environmental parameters have been identified are among the factors affecting the expression of sex genes in hemp (Milewicz and Sawicki, 2012). Nitrogen is more or less effective on incidence of male sex in hemp (Truta et al., 2007); but to increase the female sex ratio in population, it is necessary to increase the nitrogen level (Hall et al., 2013). Generally, in this study, an increase in the levels of manure and nitrogen slightly increased the percentage of female plants, compared to their control; which was not significant (Table 4). This is probably due to the use of moderate levels of nitrogen fertilizer in integration with manure application, and the effect of these moderate levels of fertilizers on increasing female sex in hemp. The use of phosphorus reduced the percentage of female plants in hemp, although its effect was not significant in this study (Table 4). In an experiment on sweet gale (*Myrica gale* L.), the application of phosphorus also reduced the abundance of female plants (Chang and Martin, 2014).

The increase of the biological, seed, and leaf extract yields under the combined effect of manure and the nitrogen fertilizer, compared to the application of manure alone, indicated the synergistic effect of manure and nitrogen (Table 5). The increase of plant yield in the integrated system of plant nutrition is due to the availability of the soil nitrogen when needed by plant. When chemical and organic fertilizers are used in combination with each other, the nutrients in the chemical fertilizers are quickly released and help the initial establishment of the plant. On the contrary the mineralization of organic fertilizer is gradually done during the growing season, which helps to achieve higher plant yields (Ayoola et al., 2007). More than 70 nitrogenous compounds, including alkaloids, amides, lignanamide and proteins (edestin, zeatin and zeatin nucleoside), enzymes (edestinase, glucosidase, polyphenoloxydase, peptidase, peroxidase and adenosine-5-phosphatase), and amino acids have been recognized in hemp (Bernneisen, 2007). The synthesis of these nitrogenous compounds existent in the hemp extract is influenced by the amount of nutrients absorbed by the plant, especially nitrogen.

The combined application of 30 t ha^{-1} of manure, with high levels of nitrogen had a negative effect on biological and seed yields (Table 5). In fact, adding manure was effective in increasing nitrogen absorption by plants. Despite the higher levels of phosphorus compared to the nitrogen in the most manures, plants absorb nitrogen about 2.4 to 4.5 times more than the phosphorus through animal manures (Mullins, 2009). The pools of nitrogen absorbed through the manure or chemical fertilizers by the plant, as protein or amino acids, requires carbon inputs to provide the structure of the carbon skeleton and energy supply (Cheng et al., 2004) and this can reduce the growth and yield of the plant. The nitrogen has a complex effect on the metabolism of plant carbohydrates. Sometimes it significantly increases the production of carbohydrates and in some cases reduces it significantly (Murata, 1969). Nitrogen requires some metabolites of Krebs cycle to stabilize amino acids. The continuation of this cycle involves the use of carbohydrates and their derivatives. The reduction of nitrite and nitrate also requires a reducing power derived from photosynthesis and respiration. If this power is provided through respiration, it will reduce plant's carbohydrates and, if it is provided through the photosynthesis, a less amount of CO_2 is reduced and converted to carbohydrates (Minotti et al., 1969).

Based on the results, an increase in the levels of manure and nitrogen increased the leaf harvest index, while the seed harvest index decreased significantly at 30 t ha^{-1} manure and also 100 kg N ha^{-1}, compared to their controls (Table 4). Thus, it seems that the high levels of manure and nitrogen disturb the partitioning balance between leaves and seeds of hemp plant by further allocation to leaves and thus stimulated the vegetative growth of the plant. However, when lower levels of manure or nitrogen were applied, a more balanced trend was observed in assimilates partitioning between the seed and the leaf. Maobe et al. (2010) also reported that the effect of nitrogen on increasing the photosynthesis rate in vegetative parts of the plant was effective in increasing dry matter accumulation and the ratio of vegetative parts to the plant seed and accordingly, reducing the seed harvest index. The existence of a negative and high correlation between the leaf harvest index and the seed yield index ($r= -0.851$, $p < 0.01$) suggests that an increase in assimilates allocation to the leaf does not necessarily lead to an increase in biomass partitioning to the seed at the reproductive stage in the hemp (Figure 2).

It seems that the addition of phosphorus fertilizer to the manure, up to 20 t ha^{-1}, has a positive effect on supplying the nutrient requirements of plant, especially the phosphorus, and increases the seed yield. Applying more manure may diminish the positive effect of phosphorus fertilizer because the plant's needs have already been achieved (Figure 1). The results of manure analysis revealed high levels of phosphorus in manure in both years of the experiment (Table 2). Therefore, manure seems to be effective in supplying phosphorus needed by the plant. Generally, it is not necessary to use higher fertilizer (a combined application of 30 t ha^{-1} with 80 kg

P ha^{-1}) to increase grain yield and even cause loss of fertilizer and increase the cost of fertilizer supply. Through the application of soluble forms of phosphorus (e.g. triple superphosphate), the P ions reacts with Ca, Fe or Al ions, and then may convert to an insoluble form, or adsorb on clay particles in the soil. In this regard, the use of animal manure may be a better source to supply plant's phosphorus needs. The manures can store phosphorus in their adsorption sites and provide plants with its soluble forms during growth season. Because of this, using animal manures is recommended in acidic and calcareous soils for optimal supply of phosphorus required by the plants (Abolfazli et al., 2010).

The seed mass showed a positive reaction to application of manure, nitrogen, and phosphorus fertilizers (Table 4). Increasing seed mass is one of the effective factors in increasing the seed yield (Akongwubel et al., 2012). Further, a positive and significant correlation was observed between the seed mass and the seed yield (0.424, $p < 0.01$) in this study. In another study on isabgol (*Plantago ovata* Forsk), an increase in seed mass and seed yield were reported by adding organic matter (Yadav et al., 2002). In fact, the seed mass is determined during its filling period, and providing sufficient photosynthetic materials at this stage, reduces the competitive effect of seeds to get these materials, which is effective in increasing the seeds mass (Reed et al., 1988).

Increasing the level of manure, especially at the levels of 20 and 30 t ha^{-1}, was effective in increasing the yield of leaf extract and hemp seed (Table 4). The highest leaf extract yield was obtained in the combined treatment of 20 t ha^{-1} manure along with 100 kg ha^{-1} nitrogen (Table 5). The leaf and seed extract yield had the highest correlation with the biological (0.915, $p < 0.01$) and seed yield (0.771, $p < 0.01$), respectively. It seems that the leaf extract yield was more sensitive to adding nitrogen fertilizer than seed extract yield. While the application of 50 kg ha^{-1} nitrogen significantly increased the yield of hemp leaf extract, a significant increase in the yield of seed extract was obtained by using 100 kg ha^{-1} nitrogen (Table 4).

By increasing the level of manure, SLA decreased and the yield of leaf extract increased (Figure 3). In other words, with decreasing SLA, leaf thickness increased (Dantas et al., 2017), and the synthesis of effective compounds in the extract of hemp leaves increased by increasing leaf dry mass (compared to leaf area).

In this experiment, with an increase in manure levels in excess of 10 t ha^{-1}, the rate of increase in seed oil decreased (Table 4). The highest seed and oil yield was obtained at 20 t ha^{-1}. The use of 20 t ha^{-1} of manure (compared to 10 t ha^{-1} of this fertilizer), increased grain yield more than reducing the percentage of seed oil, which increased the yield of seed oil (Table 4).

It seems that low nitrogen levels are necessary to increase the oil content and higher levels of this fertilizer, reduce the percentage of seed oil (Table 4). The reaction of the seed oil content to nitrogen was different in the case of application and non-application of phosphorus. With the application of phosphorus, the reaction of seed oil content decreased to nitrogen. In the absence of phosphorus application, consumption of 50 kg N ha^{-1} increased and application of 100 kg N ha^{-1} of this fertilizer reduced the content of seed oil (Figure 4). The higher nitrogen absorption under phosphorus treatment increases the nitrogen content of plant (Graciano et al., 2006) and consequently reduces the percentage of seed oil, because the amount of carbohydrates needed to synthesize the protein is less than oil. Therefore, with the use of nitrogen, more carbohydrates are used to synthesize amino acids and proteins, and consequently the synthesis of fatty acids and oils are reduced (Rathke et al., 2005). On the other hand, the results of this experiment indicated the main effect of phosphorus on reducing the oil content, although no significant difference was observed (Table 4).

The high correlation coefficient of the oil yield with the seed yield (0.913, $p < 0.01$) revealed that the oil yield was more affected by seed yield, compared to the oil content, it had less effect. In other words, an increase in the seed yield, increased the oil yield. In the other studies also a positive and significant correlation was observed between the oil yield and the seed yield (Basalma, 2007; Marjanovic-Jeromela et al., 2008; Flajšman et al., 2019). It seems that with application of 100 kg ha^{-1} nitrogen, the seed oil content was decreased compared to control treatment, but a 35.12 % increase in seed yield, compensated the reduction of oil percent and increased the oil yield (Table 4). In this experiment, the application of phosphorus and 100 kg ha^{-1} nitrogen was effective in increasing the oil and biological yield of hemp. Based on the results of Rajesware Rao et al. (1989), the application of phosphorus and 100 kg ha^{-1} nitrogen was effective in increasing oil and biomass yield of the *Artimisia pallens* Wall. ex DC.. Despite the slight reduction in the percentage of seed oil due to the consumption of phosphorus, the oil yield increased because of increasing seed yield of the plant (Table 4). Phosphorus can increase the oil content of plants by increasing the photosynthesis and the enzyme activity (Mohammadi et al., 2013).

4 CONCLUSIONS

The results suggested that adjusting the amount of organic and chemical fertilizers are important, depending on the purpose of the application for planting hemp. The combined application of 20 t ha^{-1} of manure along

with 100 kg ha^{-1} of nitrogen, produced the highest biological, seed and leaf extract yield. In order to increase the yield of seed oil, 20 t ha^{-1} manure, as well as 100 kg ha^{-1} of nitrogen is suitable and the application of higher amounts of fertilizer will increase costs and waste of fertilizer. In conclusion, given that the applied manure adds some nutrients to the soil that can be made available to the plant, it seems that hemp to respond well to nitrogen supplied by the combined use of chemical and animal fertilizers, in while its response to phosphorus fertilization is limited. Therefore, the application of animal manure not only reduces the need for chemical fertilizers consumption by satisfying part of the plant demand for nitrogen, but also has positive effects on improving soil properties, and therefore combined application of manure and chemical fertilizers is strongly recommended for the sustainability of cannabis planting systems.

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Gene action for grain protein content in durum wheat

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Gene action for grain protein content in durum wheat

Abstract: The aim of this study was to determine the gene action and combining ability of durum wheat for grain protein content. During the three year period a diallel cross was carried out with five modern parents of durum wheat – 'Victoria', 'Deni', 'Superdur', 'Progres' and 'Predel'. Ten hybrid combinations and the parents were grown in the experimental field of the Field Crops Institute, Chirpan. The experiment was performed by the randomize block method design in three replications. It was found that in the inheritance of grain protein content dominance and overdominance in positive and negative directions were observed. Statistical processing of the results showed that both additive and non-additive genetic effects have influenced on inheritance. Non-additive gene effects (SCA) had a greater role in inheritance. This suggests that an effective selection for this trait could begin in later generations. The combining ability analysis has identified two good general combinators (Predel and Superdur varieties) that could be used as donors to increase the values of the trait protein content in grain. Several crosses showing positive and significant SCA effects have also been identified, suitable for achieving reliable transgressive genotypes.

Key words: gene action; combining ability; GCA effects; SCA effects; grain protein content; durum wheat

Delovanje genov za vsebnost beljakovin v zrnih trde (durum) pšenice

Izvleček: Namen raziskave je bil določiti vpliv delovanja genov in kombinacijske sposobnosti trde (durum) pšenice na vsebnost beljakovin v zrnih. V obdobju treh let so bila izvedena dialelna križanja s petimi sodobnimi starševskimi sortami trde pšenice-– Victoria, Deni, Superdur, Progres in Predel. Deset križancev in njihovi starši so bili gojeni na poskusnem polju Inštituta za poljščine v Čirpanu (Field Crops Institute, Chirpan). Poskus je bil izveden kot naključni bločni poskus s tremi ponovitvami. Ugotovljeno je bilo, da sta pri dedovanju vsebnosti beljakovin v zrnju udeležni dominanca in naddominanca v pozitivni in negativni smeri. Statistična obdelava podatkov je pokazala, da so na dedovanje vplivali aditivni in neaditivni genski vplivi. Neaditivni genski učinki so imeli pri dedovanju večjo vlogo. To nakazuje, da bi se učinkovita selekcija za to lastnost lahko začela že v prejšnjih generacijah. Z analizo kombinacijske sposobnosti sta bila prepoznana dva dobra splošna kombinatorja ('Predel' in 'Superdur'), ki bi ju lahko uporabili kot donatorja za povečanje vsebnosti beljakovin v zrnih. Številna križanja kažejo pozitivne in značilne neaditivne genske učinke (SCA), ki so primerni za doseganje zanesljivih transgresivnih genotipov.

Ključne besede: delovanje genov; kombinacijska sposobnost; aditivni genski učinki; neaditivni genski učinki; vsebnost beljakovin v zrnju; trda (durum) pšenica

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1 INTRODUCTION

Durum or pasta wheat is the only tetraploid species of commercial importance that is cultivated in a number of countries, and in some of them it is essential. Durum wheat is an important crop for the preparation of pasta, bulgur, couscous and other products (Ahmad, 2015). The protein content of the grain is of particular importance for the quality of durum wheat and its products. High values of this trait have a positive relationship with the yield of semolina from the grain and are desired by the milling industry. The use of plant proteins in human food is of great importance for healthy human nutrition. The protein content in the grain is a very important indicator of wheat related to its nutritional value and technological qualities of the products (Blanco et al., 2006). Protein content is highly influenced by the genotype and content of available nitrogen in the soil, as well as humidity and temperature during the growing season (Campbell et al., 1977; Fowler et al., 1990; Ames et al., 2003). According to Clarke et al. (1990) and Fowler et al. (1990) the highest protein content of the grain is due to the cultivation of varieties that can withstand higher rates of nitrogen fertilization. The improvement of the varieties must meet the requirements for the production of high quality products. This leads to an accelerated improvement of individual traits. Knowledge of the gene action of the trait is useful for the development of a high quality durum wheat variety. Diallel crosses are used to establish gene action and mechanisms for inheriting quantitative traits. It is a reliable method for selecting parents and providing a comprehensive assessment of their hybrid combinations. This analysis is suitable for studying quantitative traits in early generations. It requires relatively more work, but gives a very effective estimate. More detailed information can be obtained from diallel crosses with one generation F_1. The other important advantage is that the estimates obtained in F_1 can be confirmed by testing the second generation F_2. In diallel analysis, general combining ability (GCA) is associated with genes that have additive effects and describes the ability of parents to pass on their traits to hybrid generation. Specific combining ability (SCA) is the deviation from additive effects caused by dominance and epistasis. The establishment of gene action (additive and non-additive) is of great importance for determining the strategy for achieving more meaningful and rapid results in the development of new varieties. In a properly designed breeding program, knowledge of gene action is the key that will maximize the effectiveness of improvement breeding work.

A number of authors have studied gene action in inheritance of grain protein content in wheat. Lysa (2009), Akram et al. (2011), Yao et al. (2014), Pansuriya et al. (2014), Tiwari et al. (2015) have reported significant participation of both additive (GCA) and non-additive (SCA) gene effects in the inheritance of the studied trait. According to the ratio of GCA / SCA variance, additive gene effects ($\sigma_g^2 > \sigma_s^2$) have played a greater role in inheritance. This suggests that this trait could be significantly more easily improved and it is possible for an effective selection of genotypes to be applied in earlier segregated generations. Other researchers (El-Habbad et al., 1996; Pansuriya et al., 2014; Tiwari et al., 2015) found preponderance of non-additive genetic effects, while Zahid et al. (2007) and Al-Naggar et al. (2015) noted the preponderance of additive gene effects. From the preponderance of the non-additive genetic effects reported by some authors, it could be assumed that dominance had a greater influence on this trait. Determining the degree of dominance would help to establish the possibility of purposeful work in the breeding program. Here, a link can be made with the use of specific results, aimed at reaching transgressive forms or creating heterosis varieties. According to Fonseca & Patterson (1968), Martin & Talbert (1995), Elfadl et al. (2006) dominance and overdominance correspond to the theoretical foundations of heterosis and suggest that it is the result of allelic and non-allelic interactions between the genetic material of the parents. Dominance in the inheritance of the trait was reported by Patel et al. (2018). The diversity of the results obtained is evidence of the great influence of the genotypes used and the environmental conditions in the inheritance of the trait. This suggests that modern domestic and foreign varieties of durum wheat should be included in the study and their genetic capabilities should be traced and determined. Plant breeding is the art and science of changing the heredity of plants and improving them for the benefit of human.

The aim of this research was to study the genetic difference between genotypes, as well as the type of gene action (additive and non-additive) in the inheritance of protein content in the grain. By determining the general and specific combining ability, we expected to identify the parents for successful combinations and to obtained promising genotypes in the earliest segregated generations. This will be of great benefit in optimizing the breeding process for durum wheat in terms of grain protein content.

2 MATERIAL AND METHODS

The study was conducted in the experimental field

of the Field crops institute in Chirpan, Bulgaria in three consecutive years (2014-2016) using the standard, local cultivation technology. The soil type is Eutric Vertisols (by FAO), characterized by medium organic matter (1.5-2.4 %), with slightly acid to neutral soil reaction. The experiments were sown in the optimal period for durum wheat in Bulgaria in time-frame October 20-30. The genotypes heading time was in time-frame May 8-16. The plants were taken (harvested) in time-frame July 7-10 in full maturity. Meteorological conditions during the three-year period of the study were characterized by higher temperature than the multi-annual norm. The first two harvest years of 2014 and 2015 were favourable in terms of soil moisture and rainfall higher than the average for many years. The third harvest year was characterized as the hottest and at the same time with 20 % less precipitation.

A half diallel cross was performed with the including of five modern varieties of durum wheat: Victoria (BG), Deni (BG), Superdur (AT), Progres (BG), Predel (BG). The three-year period has allowed three generations of F_1 and two generations of F_2 to be grown. The parents, F_1 and F_2 hybrids were sown under field conditions by the block method design in three replications. Each parent or F_1 was sown in two rows, and each F_2 was sown in five rows; each row was 2 m long; spaces between rows were 20 cm and 5 cm between plants. In full maturity a total of 20 plants from F_1 and parents and 30 plants from F_2 were taken randomly from each replication every year. Part of the seeds were used for the sowing of F_2. With the remaining seeds, a technological analysis was performed for estimation of grain protein content. The grain protein concentration (GPC, %) was estimated by measuring of N according to the Kjeldahl method. The following formula was used: Protein, % = N (% DM) x 5.7 to convert the N content to protein content (BDS ISO 20483:2014).

The results obtained were statistically processed by applying the method 2 model I of Griffing (1956) with software product of Mark D. Burow and James G. Coors 1994 (Burow & Coors, 1994). The same program was used for the analysis of variance. The general combining ability (GCA) of the parents and the specific combining ability (SCA) of the crosses were determined. The degrees of dominance in the individual hybrid combinations were calculated according to Ognyanova (1975). On the results for mean of parents and their hybrid combination was conducted Duncan`s test for multiple comparing the means at the detected significant differences ($p < 0.05$) (Duncan, 1955). Statistica 10 software program was used for the two analyzes performed above.

3 RESULTS AND DISCUSSION

The mean values for the trait grain protein content of the parents for the three years F_1 and both F_2 generations are presented in Table 1. It can be seen that there was a significant variation in the years of study, also and significant differences between mean values. The most favourable for grain protein content was 2014, while the most unfavourable was 2015. The parents had values from 13.75 % for the variety Victoria (F_1-2015) to 18.53 % for the variety Superdur (F_1-2014). The table also presents the average values of the hybrid combinations by years and generations. The highest value was found for the combination Victoria X Deni - 18.50 % (F_1-2014), and the lowest - 13.52 % for Victoria X Progres (F_2-2015). The same table includes the corresponding indices showing the ways and direction of inheritance. They show that there was a great diversity in inheritance. In the F_1 generation in 2014 positive overdominance (towards the better parent) prevailed. In 2015, there were more manifestations of intermediate inheritance, but there also were those with dominance and over-dominance to the weaker parent. In 2016 dominance and overdominance in both directions were observed. In the F_2 generation, several manifestations of overdominance were seen in a positive direction, but in most cases it was in a negative direction. In inheritance of the grain protein dominance and overdominance in both positive and negative directions were observed. Preponderance of dominance and overdominance for the trait grain protein content was established by other authors (Kumar & Maloo, 2011; Desale & Mehta, 2013; Patel et al., 2018).

Table 2 represents the analysis of variance for genotypes, general combining ability (GCA) and specific combining ability (SCA). Significant differences between genotypes were observed for all test cases. This makes it possible to conduct a diallel analysis for an in-depth study of the genetic causes controlling the trait grain protein content. The sums of the squares of the genotypes were in each case the largest and determine that the genotypes had the largest contribution to the overall variation of the studied trait. The values of the mean squares for genotypes, GCA and SCA were statistically significant for the three harvest years in both generations (table 2). Therefore, both additive gene effects (GCA) and non-additive gene effects (SCA) were involved in the inheritance of the trait. The results correspond to those obtained by a number of other authors (Barnard et al., 2002; Joshi et al., 2004; Lysa, 2009; Akram et al., 2011; Yao et al., 2014; Pansuriya et al., 2014; Tiwari et al., 2015; Patel et al., 2018).

Table 1: Mean values and indexes of inheritance for trait grain protein content (%).

Parents	Code	2014 y.	2015 y.	2016 y.	2015 y.	2016 y.
Victoria	11	14.74a	13.75ab	14.47a	13.75ab	14.47a
Deni	22	16.25abc	14.67bcd	16.27cde	14.67bcd	16.27cde
Superdur	33	18.53d	14.59abcd	16.43de	14.59bcd	16.43cde
Progres	44	15.35a	15.04d	15.09abc	15.04cd	15.09abc
Predel	55	17.70bcd	15.31d	16.21cde	15.31d	16.21cde
Hybrid combinations	Code	F_1-2014 y.	F_1-2015 y.	F_1-2016 y.	F_2-2015 y.	F_2-2016 y.
Victoria x Deni	12	$^{od+}$18.50d	$^{cd-}$13.89ab	$^{cd-}$14.55a	^{i}14.24abc	^{i}15.36abcd
Victoria x Superdur	13	^{i}16.57abcd	$^{cd-}$13.76ab	$^{od+}$16.78e	$^{od-}$13.65ab	$^{pd+}$16.11bcde
Victoria x Progres	14	$^{od+}$16.09ab	^{i}14.13abc	$^{od+}$15.39abcd	$^{od-}$13.52a	$^{od+}$15.54abcde
Victoria x Predel	15	$^{cd+}$17.43bcd	$^{cd-}$13.97ab	$^{cd+}$16.03bcde	$^{od-}$13.68ab	$^{cd-}$14.77ab
Deni x Superdur	23	^{i}17.56bcd	$^{od-}$13.76a	$^{od-}$15.52abcd	$^{od-}$14.48abcd	$^{od-}$15.49abcde
Deni x Progres	24	$^{od+}$17.28bcd	$^{cd+}$15.13d	$^{od-}$14.87ab	$^{od-}$14.18abc	$^{od-}$14.50a
Deni x Predel	25	$^{od+}$18.13cd	^{i}15.13d	$^{od-}$15.17abcd	$^{cd+}$15.18cd	$^{od-}$15.41abcde
Superdur x Progres	34	$^{pd+}$17.81bcd	$^{cd+}$15.18d	^{i}15.68abcde	$^{cd+}$14.99cd	$^{cd-}$15.14abc
Superdur x Predel	35	$^{cd-}$17.69bcd	^{i}14.96cd	$^{od-}$15.71abcde	$^{od-}$14.40abcd	$^{od+}$16.59de
Progress x Predel	45	$^{cd+}$17.95bcd	$^{od-}$14.56abcd	$^{od+}$16.03bcde	$^{od-}$14.19abc	$^{od+}$16.80e
M ± m		17.17±0.29	14.52±0.15	15.61±0.17	14.39±0.14	15.61±0.19

Mean values (in each column), followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test (DMRT). Indexes: i-intermediate, cd-complete dominance, pd-partial dominance, od-overdominance, minus-decrease, plus-increase

The ratio of GCA and SCA variances indicates a predominance of non-additive gene effects over additive ones in all cases except F_1-2015, where additives predominated. The same results, for the predominance of non-additive genetic effects, have been reported by a number of other authors (Perenzin et al., 1992; Singh et al., 2004; Joshi et al., 2004; Nazeer et al., 2011; Kumar, 2012; Khodadadi et al., 2012; Ahmad et al., 2017; Patel et al., 2018). On the other hand, the predominance of additive genetic effects in inheritance has been reported by other researchers (El-Habbad et al., 1996; Bnejdi & El-Gazzah, 2010; Sadeghi et al., 2012; Tiwari et al., 2015; Al-Naggar et al., 2015). The predominance of non-additive gene effects suggests that an effective selection in the breeding for grain protein content in durum wheat, in our set of parents, should be conducted in later segregated generations. This is associated with a reduced influence of non-additive genetic effects in later segregated generations.

Table 3 represents the values for parental GCA and hybrid crosses SCA. Predel variety manifested itself as a good general combiner for increasing the grain protein content. It had positive and significant GCA values for all test cases. This variety contained more genes with additive effects. Of interest was the variety Superdur, which in three of the five cases had significant and positive values of GCA. These genotypes could be successfully used in breeding of durum wheat to increase the values of the trait grain protein content. As a bad general combiner for the trait was Victoria variety showed significant negative values for all cases of research and lead to a decrease in the protein content in the hybrids obtained with it. The other parent varieties occupied an intermediate position. There was no observed clear outlined good combination of SCA effects in the hybrid crosses. In different years there was no hybrid combination with three or more significant effects in one direction. Of interest in this situation was the cross 'Victoria' X 'Superdur', which had two significant positive values for SCA. This combination was a cross between a parent with a negative GCA and a parent with a positive GCA. Other crosses with high SCA effects for two years were 'Progres' X 'Predel' and 'Superdur' X 'Progres'. Researchers Kumar & Maloo (2012) and Singh et al. (2012) reported that not all crosses with high SCA effects were obtained from the crosses of a 'Good'

Table 2: ANOVA by years for Genotypes, General combining ability (GCA), Specific combining ability (SCA) and relation to variances of GCA and SCA ($6_g^2 / 6_s^2$) for grain protein content

Year	Source of variation	Sum of squares	Mean squares	Significant (*,**,***)
F_1-2014	Genotype	53.769	3.841	***
	GCA	26.434	6.608	***
	SCA	27.336	2.734	***
	Error	29.506	1.054	
	$6_g^2 / 6_s^2$	0.32		
F_1-2015	Genotype	14.672	1.048	***
	GCA	10.023	2.506	***
	SCA	4.649	0.465	***
	Error	6.347	0.227	
	$6_g^2 / 6_s^2$	1.22		
F_1-2016	Genotype	20.008	1.429	***
	GCA	7.872	1.968	***
	SCA	12.13	1.214	***
	Error	11.713	0.418	
	$6_g^2 / 6_s^2$	0.11		
F_2-2015	Genotype	14.091	1.007	***
	GCA	8.012	2.003	***
	SCA	6.079	0.608	***
	Error	7.919	0.283	
	$6_g^2 / 6_s^2$	0.6		
F_2-2016	Genotype	23.806	1.700	***
	GCA	9.805	2.451	***
	SCA	14.001	1.400	***
	Error	15.219	0.544	
	$6_g^2 / 6_s^2$	0.17		

* - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$; n.s. – no significant

X 'Good' GCA combiner. Particularly, crosses with high SCA effects were obtained from crosses between 'Bad' X 'Bad' and 'Bad' X 'Good' general combiner. These researchers claimed that such manifestations were due to the involvement of dominance or epistatic gene effects.

Gami et al. (2011) and Tiwari et al. (2015) have determined that crosses with high SCA may be more likely to be sources of transgression. Transgressive lines on a given trait can be a source for creating high-nutrition varieties of durum wheat. According to them, assessments of gene action and variation explain the genetic potential of materials and contribute to breeding progress in durum wheat quality. The analysis of combining ability shows that non-additive genetic effects (dominance and epistasis) has played a major role in the inheritance of the trait grain protein content in durum wheat. Two good combiners have been identified to increase the grain protein content: 'Predel' and 'Superdur'. Furthermore as a promising combination was found the cross 'Victoria' X 'Superdur'. Varieties Predel and Superdur have been defined as good general combiners by other quantitative characteristics in our previous studies (Dragov & Dechev, 2015; Dragov, 2017; Dragov, 2020).

The study provided information on two of the most important moments in a successful breeding program - choosing parents for crossing and leading a purposeful selection on this trait. The choice of parents for crossbreeding is the basis for obtaining good results from a breeding program. Hybridization is a

Table 3: Values for general combining ability (GCA) of parents and specific combining ability (SCA) of crosses for grain protein content

Code	2014 y.	2015 y.	2016 y.	2015 y.	2016 y.
Parents / Error	±0.31	±0.14	±0.19	±0.16	±0.22
11 Victoria	-0.70*	-0.55*	-0.28*	-0.53*	-0.42*
22 Deni	0.13 n.s.	0.01 n.s.	-0.14 n.s.	0.15 n.s.	-0.05 n.s.
33 Superdur	0.52*	-0.04 n.s.	0.40*	0.05 n.s.	0.36*
44 Progres	-0.45*	0.27*	-0.21*	0.08 n.s.	-0.21 n.s.
55 Predel	0.50*	0.30*	0.24*	0.24*	0.33*
Hybrid combinations	F_1-2014 y.	F_1-2015 y.	F_1-2016 y.	F_2-2015 y.	F_2-2016 y.
Crosses / Error	±0.70	±0.32	±0.44	±0.36	±0.50
12 Victoria x Deni	1.9*	-0.09 n.s.	-0.62*	0.23 n.s.	0.22 n.s.
13 Victoria x Superdur	-0.41 n.s.	-0.16 n.s.	1.03*	-0.25 n.s.	0.55*
14 Victoria x Progres	0.08 n.s.	-0.11 n.s.	0.28 n.s.	-0.42*	0.56*
15 Victoria x Predel	0.46 n.s.	-0.30 n.s.	0.45*	-0.42*	-0.75*
23 Deni x Superdur	-0.26 n.s.	-0.73*	-0.35 n.s.	-0.11 n.s.	-0.42 n.s.
24 Deni x Progres	0.43 n.s.	0.31 n.s.	-0.37 n.s.	-0.44*	-0.84*
25 Deni x Predel	0.31 n.s.	0.28 n.s.	-0.53*	0.38*	-0.47 n.s.
34 Superdur x Progres	0.57 n.s.	0.41*	-0.12 n.s.	0.46*	-0.61*
35 Superdur x Predel	-0.51 n.s.	0.18 n.s.	-0.55*	-0.28 n.s.	0.28 n.s.
45 Progress x Predel	0.72*	-0.53*	0.39 n.s.	-0.53*	1.07*

* - $p \leq 0.05$; n.s. – no significant

basic method for increasing genetic diversity and obtaining valuable genotypes in segregating generations. When choosing parents, the following should be taken into account: genetic distance, adaptation potential and combining ability. Greater genetic variation and the possibility of transgressions were obtained from crosses with higher SCA effects. In turn, crosses with high SCA effects were obtained by crossing parents with high GCA effects with parents with medium or low GCA effects. This indicates that parents with high GCA effects should always be present in the hybridization scheme. In our set of parents in the diallel cross, two varieties: Superdur and Predel were established as the most valuable parents. The use of these varieties in the future hybridization program would show good results in segregated generations.

Successful selection in the early segregated generations F_2, F_3 is suitable for traits in which inheritance control is determined by additive gene effects. It should be noted that for the studied trait there was a significant participation of the additive genetic effects in our case. An effective selection in later segregated generations should be recommended for traits in which non-additive genetic effects predominate. Therefore, an effective selection on this trait should be applied in the later segregated generations, when the influence of the non-additive (dominance) decreases and the additivity increases. The significant influence of additive and non-additive gene effects found in our study suggests that the use of both types of gene effects is necessary to improve the trait. In the studied trait in F_1 in one year the inheritance was mainly controlled by additive gene effects while in the other two it was mainly by non-additive ones. The selection in different environmental conditions (years) would have a positive impact on breeding improvement work. This is due to the accumulation of different useful genes in different years.

4 CONCLUSIONS

In the inheritance of grain protein content there was complete dominance and overdominance, both in a positive and in a negative direction. Additive and non-additive genetic effects had a significant influence on the inheritance of this trait. Therefore, to maximize the

grain protein content of durum wheat, a system that includes both types of gene effects at the same time should be used. The ratio of variances indicates that non-additive genetic effects prevailed over additive ones in most cases. This result shows that it is necessary for an effective selection to start in the later segregated generations where dominance decreases and additivity increases. The study identified two good combiners that increased the values of the trait: Predel and Superdur varieties. Crosses with these two genotypes suggest opportunities for promising hybrids. The hybrid combination 'Victoria' X 'Superdur' is also of selection interest according to the demonstrated significant values for SCA.

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Early detection of sources of resistance to the fall armyworm in some tropically-adapted maize varieties in Southern Nigeria

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Early detection of sources of resistance to the fall armyworm in some tropically-adapted maize varieties in Southern Nigeria

Abstract: The outbreak of fall armyworm (FAW), *Spodoptera frugiperda,* in Nigeria since 2016 had caused serious socio-economic problem to farmers. Twenty maize varieties adapted to the agro-ecologies of Nigeria were evaluated in five environments to identify varieties with resistance to the FAW for possible improvement and deployment. The evaluations were under artificial and natural infestation between 2017 and 2018. Data were collected weekly after infestation on severity and incidence of FAW and plant height. All trials were terminated at six weeks after sowing. Varieties SUWAN 1 SR, BR LNTP-Y C_6, AMA TZBR-W C_4 and TZBR ELD 4 C_2 are good sources of resistance to FAW which could be used in improvement program.

Key words: crop improvement; fall armyworm; insect infestation, maize; pest resistance, Africa.

Zgodnje odkrivanje na ameriško koruzno sovko (*Spodoptera frugiperda* [J. E. Smith, 1797]) odpornih in tropom prilagojenih sort koruze v južni Nigeriji

Izvleček: Močan pojav ameriške koruzne sovke (*Spodoptera frugiperda* [J. E. Smith, 1797]) kmetom v Nigeriji od leta 2016 povzroča resne socio-ekonomske težave. V pričujoči raziskavi je bilo ovrednotenih dvajset sort koruze, prilagojenih agro-ekološkim razmeram v Nigeriji. Na petih lokacijah so preučevali odpornost sort na škodljivca, z namenom njihovega izboljšanja in uvajanja v pridelavo. Ocenjevanja so potekala v letih 2017 in 2018 v naravnih razmerah in v rastlinjaku. Podatki o pojavu škodljivca, obsegu poškodb zaradi gosenic in višini rastlin so se zbirali tedensko. Vsi poskusi so bili končani šest tednov po setvi. Sorte kot so SUWAN 1 SR, BR LNTP-Y C_6, AMA TZBR-W C_4 in TZBR ELD 4 C_2 so se izkazale kot dober vir odpornosti na škodljivca in bi lahko bile uporabljene v programih za izboljšanje učinkovitosti zatiranja ameriške koruzne sovke.

Ključne besede: izboljšanje poljščin; ameriška koruzna sovka; napadenost z žuželkami; koruza; odpornost na škodljivce; Afrika.

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1 INTRODUCTION

Maize (*Zea mays* L.) yield is limited by several biotic and abiotic stresses. Insect pests are one of the major biotic stresses contributing to yield losses of crops in the field with high socio-economic impact. Various control measures are employed which include use of chemicals, cultural control and use of bio-pesticides. For instance, entomopathogenic nematodes have been reported to significantly reduce number of larvae of Colorado potato beetles, but high cost of the agent limits its use (Laznik et al., 2010). Stem borers used to be the common insect pests of maize especially in the forest zone of Nigeria causing between 20-40 % yield losses (Oloyede-Kamiyo et al., 2011). The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) also of the family Lepidoptera, has been in Africa for approximately 10 years but in the early 2016, there was an outbreak of the pest on maize in southwest Nigeria. A survey carried out in 2016 by Institute of Agricultural Research and Training (I.A.R.&T), Ibadan showed that the pest is widespread, especially in the southwest and some southern states. By the end of that year, it has spread to the northern part of the country causing majority of farmers to abandon their farms. The larvae of the pest were observed to have caused as high as 100 % damage on maize fields, attacking virtually all the growth stages of the maize crop, from the vegetative stage to cob formation. The menace of this pest has caused loss of millions of dollars especially to commercial farmers who invest with loan from banks, hence posing a serious economic threat to the food security of the country. The report commissioned by the Department for International Development, indicates that the arrival of fall armyworm in Africa has the potential to cause maize yield losses in a range of 8.3 to 20.6 million tonnes per annum, in the absence of any control measure in just 12 maize-producing countries. (Abrahams et al., 2017). FAW has recorded long history of resistance to pesticides and GM toxins (Huesing, 2017). Several insecticides have been tested on the insect since its arrival in Nigeria in 2016 with little success. The heavy dose of different types of insecticide used has long-term effect on the health of maize farmers and even the end-users. Development of resistance lines is very crucial to combat the menace and eliminate the health hazard of persistent use of insecticides. In developing FAW resistant maize varieties, there is need to identify resistant source from the existing varieties. Some promising lines have been identified and validated by CIMMYT and KALRO, Kiboko, Kenya (Prasanna, 2018). There is need to identify varieties with resistance to the FAW among the varieties adapted to the agro-ecologies of Nigeria for possible improvement and deployment.

This study therefore aims at detecting maize varieties with resistance to the FAW at early growth stage among the adapted varieties in Southern Nigeria.

2 MATERIAL AND METHODS

Twenty open-pollinated maize varieties were evaluated in five environments at different out-stations of the Institute of Agricultural Research and Training (IAR&T), Ibadan, Nigeria, under natural and artificial FAW infestation between 2017 and 2018. The varieties were sourced from the International Institute of Tropical Agriculture (IITA), and Institute of Agricultural Research and Training (IAR&T), Ibadan, Nigeria. Some of varieties used have undergone cycles of recurrent selection for resistance to stem borer species, *Sesamia calamistis* (Hampson) and *Eldana saccharina* (Walker). The maize varieties used with their attributes are presented in Table 1.

Out of the five environments, one was under artificial infestation in the screen house in Ibadan (Lat.7° 24′7.06″N, Long. 3° 55′2.33″E, 225m above sea level) in November 2017. The remaining four environments were under natural infestation on the field at Amakama in the humid rain forest (Lat. 5° 26′40″N, long. 7° 28′ 49″E, 154.25 m above sea level), Ikenne in rain forest (Lat. 6° 51′57 ″N, Long. 3° 42′55″E, 70 m above sea level), and early and late seasons in Ibadan in derived savanna of Nigeria. The field evaluated commenced in April 2018 (early season in Ibadan), June 2018 in Ikenne and Amakama, and in October 2018 for late season in Ibadan. The experiment was laid out in a randomized complete design in three replications. Each plot on the field was a two-row plot of 5 m long with plant spacing of 75 cm between rows and 50 cm within rows with two plants per stand. No insecticide was used to control *S. frugiperda*, being the only major pest around. However, other agronomic practices were carried out appropriately. Pre-emergence herbicide, comprising mixture of atrazine (Southern AG) and paraquat (Syngenta, United states) was used a day after planting, with one manual weeding at four weeks after planting. NPK fertilizer and urea were applied at 10 days and 4 weeks after planting, respectively at the rate of 60 kg N ha^{-1} each. For the artificial infestation in the screen house, each plant was infested with average of six first instar larvae at two weeks after planting using camel hair brush.

Data collection started in the screen house at a week after infestation, while data commenced on the field at a week after the first notice of infestation on the plants. Plant height was taken in centimeter on five tagged plants per plot using ruler. Severity of infestation was rated per plot on a scale of 1-9 based on the level of feeding on the

leaves, presence of frass, and overall effects on the plants (Prasanna, 2018). Incidence of infestation was taken by counting the number of plants infested by the pest per plot and expressed as percentage of plant stands per plot. All data were taken weekly till termination of the experiment. The trials were terminated at six weeks after sowing (WAP).

Percentage data were transformed using arcsine before analysis. Means were separated using least significant difference (LSD). Combined analysis of variance was performed using SAS, version 9.2. Rank Summation Index (RSI) of Mulumba and Mock (1978) was used to rank the varieties according to their level of resistance using severity and incidence at 1 and 4 weeks after infestation as selection criteria. Principal component analysis was conducted to determine the contribution of the traits to the observed variation. The traits contributing most were then used to perform cluster analysis. Similarities were measured based on Euclidean distance.

Table 1: Maize varieties used for the study with their attributes

s/n	Name	Kernel colour	*Source	*Attribute
1	TZBR COMP 1-Y C_3	Yellow	IITA	Stem borer resistant
2	SUWAN-1-SR-Y	Yellow	IITA	Streak resistant
3	TZBR COMP-1-W C_2	White	IITA	Stem borer resistant
4	TZPB-SR-W	White	IITA	Streak resistant
5	TZBR COMP-2-Y C_3	Yellow	IITA	Stem borer resistant
6	ART/98/SW1	Yellow	IAR&T	High protein maize
7	TZBR ELD 4-Y C_2	Yellow	IITA	Resistant to stem borer (Eldana sp.)
8	ART/98/SW6-OB	White	IAR&T	Quality protein maize
9	TZBR COMP-2-W C_2	White	IITA	Stem borer resistant
10	TZE-COMP5	White	IITA	Striga resistant
11	BR9928 DMRSR	Yellow	IAR&T	Resistant to stem borers, downy mildew and streak
12	ART/98/ILE-1-OB	White	IAR&T	Quality protein maize
13	AMA TZBR-Y C_1	Yellow	IITA	Stem borer resistant
14	DMR-ESR-Y	Yellow	IAR&T	Downy mildew and Streak resistance
15	BR LNTP-Y C_6	Yellow	IITA	Stem borer resistant, tolerant to low soil nitrogen
16	PRO-VIT. A	Yellow	IAR&T	Provitamin A enriched
17	AMA TZBR-W C_4	White	IITA	Stem borer resistant
18	TZE BR-ELD3-W	White	IITA	Resistant to stem borer (Eldana sp.)
19	TZBR ELD 4-W C_2	White	IITA	Resistant to stem borer (Eldana sp.)
20	BR9943 DMRSR	White	IAR&T	Resistant to stem borers, downy mildew and streak

*The attributes are from the names of the varieties. The source indicated the producers of the varieties.

TZBR: Tropical Zea Borer Resistance; SR: Streak Resistance; DMRSR: Downy Mildew Resistance Streak Resistance; ELD: Eldana; IITA: International Institute of Tropical Agriculture; IAR&T: Institute of Agric. Research and Training

3 RESULTS

The weekly FAW severity and incidence level is presented in Table 2. Severity was high at early stage (1-2 weeks after infestation) but reduced as the plant grow older. FAW incidence varied among the maize varieties. It increased over time in some, while it reduced in some. The level of severity and incidence was more pronounced under artificial infestation than natural infestation. Severity was the least in 'TZBR ELD 4-W C_2' under natural infestation, and in 'TZBR COMP2-Y C_3' under artificial infestation. FAW incidence reduced drastically in 'BR9943 DMRSR' from 1-4 weeks after infestation (23.6 %, 15.6 %, 5.8 % and 4.2 % respectively) under natural infestation, and in 'TZPB SR-W' under artificial infestation (83.3 %, 58.3 %, 16.7 % and 0 % respectively).

Mean square of variety was significant for severity and plant height at one week after infestation under artificial infestation (Table 3), while under natural infestation (Table 4), mean square of environment and mean square of variety were significant for almost all the traits. Mean squares of environment by variety interaction was also significant for severity at 3 weeks after infestation, incidence at 2 and 4 weeks after infestation, and plant height at 1and 3 weeks after infestation (Table 4).

The top 25 % maize variety selected using RSI is presented in Table 5. Three of the five maize varieties selected under artificial infestation are stemborer resistant varieties. The BR LNTP-Y C_6 selected as the best resistant variety under artificial infestation has been tested at IITA to be resistant to the FAW. 'ART/98/SW6-OB' is a quality protein maize.

Variety developed by IAR&T, while SUWAN 1 SR is an old variety, resistant to streak. All the maize selected under natural infestation are stem borer resistant varieties except 'SUWAN 1 SR-W. SUWAN 1 SR-W', 'AMATZBR-W C_4' and 'TZBR ELD 4' were commonly selected under both conditions although the white version of 'TZBR ELD 4' was selected under natural infestation.

The result of principal component analysis revealed that PCA 1, 2 and 3 accounted for 85.5 % and 87.9 % of the variations observed under artificial and natural infestation respectively (Table not shown). The variables responsible for the observed variation under artificial infestation were incidence at 1, 2, 3 and 4 weeks after infestation, while incidence at 1, 2, 3 and 4 weeks after infestation and plant height at 3 and 4 weeks after infestation were responsible for the variations under natural infestation.

At 50 % similarity distance, 9 distinct groups were formed under artificial infestation (Figure 1). Varieties TZBR Comp 1-W (G1), BR LNTP-Y C_6 (G5), SUWAN 1 SR (G6), BR9928 DMRSR (G7) and TZPB SR (G9) stood alone in their groups. 'ART/98/SW6-OB' and 'TZBR ELD 4-Y C_2' clustered together in a group (G4). Other varieties clustered in three different groups. However, at 50 % similarity distance, six distinct groups were formed under natural infestation (Figure 2). Varieties BR9943D-MRSR (G1), TZEBR ELD 3-W (G3), AMATZBR-Y C_1 (G5) stood alone in their groups. 'BR LNTP-Y C_6', 'AMATZBR-W C_4', 'DMRESR-Y', 'TZBR Comp-Y C_3' and 'TZBR ELD 4-W C_2' clustered together in a group (G2), while others clustered in two other groups.

It is worth noting here that the top varieties selected by RSI under artificial infestation fell in the best groups in the dendrogram (G4, 5 & 6). Similar result was observed under natural infestation. The varieties selected by RSI clustered in G1 and G2 in the dendrogram in cluster analysis.

4 DISCUSSION

The economic effect of the fall armyworm could be determined on the field at early growth stage of maize plant through random sampling on the field, location of infestations in the field, larval size, and where the larvae are feeding on the plant. Hence, levels of infestation at this stage, especially under artificial infestation or hot spots suggest the inherent resistance of each maize variety to the pest. In the present study, it was observed that severity and incidence level reduced over time in some of the varieties although at different pace. The resistance check, 'BR LNTP-Y C_6' had its severity and incidence level reduced to 1 and 8 % respectively, after 4 weeks of infestation. This was comparable to what was observed in some other varieties such as ART/98/ SW6-OB, TZPB SR and TZBR ELD 4-Y C_2. Ni et al. (2011) had similar observation in some of the germplasms evaluated. This observation suggested that the varieties had the ability to tolerate/overcome the effects of FAW. Hence, substantial level of resistance to the fall armyworm was indicated in them. Williams et al. (1998) reported that maize that is resistant to FAW sustained less leaf-feeding damage, and larvae feeding on resistant maize grew more slowly. Some promising CIMMYT maize inbreds identified and validated in Kiboko, Kenya had their leaf damage ratings between 2.0 and 6.0 (Prasanna, 2018). This rating was similar to what was obtained in the present study.

The significant mean squares of environment, variety and the environment by variety interaction for most of the traits under natural infestation was similar to those observed by Giaveno et al. (2004), Giaveno and Ferrero (2003) and Ni et al. (2011). The significant environment by variety interaction observed for incidence and severity of FAW could be due to erratic performance across

Table 2: Mean performance of the 20 maize varieties for resistant traits under FAW infestation in the five environments between 2017 and 2018

Entry	Severity 1WAI (1-9)		Severity 2WAI (1-9)		Severity 3WAI (1-9)		Severity 4WAI (1-9)		Incidence 1WAI (%)		Incidence 2WAI (%)		Incidence 3WAI (%)		Incidence 4WAI (%)	
	NI	AI	NI	AI	NI	AI	NI	AI	NI	AI	NI	AI	NI	AI	NI	AI
AMA TZBR-W C4	3.67	4.33	3.11	4.00	1.67	2.00	1.67	1.33	25.62	100.00	18.67	83.33	14.54	58.33	16.67	16.67
AMA TZBR-Y C1	4.50	4.67	3.56	5.33	1.83	2.67	3.67	2.00	37.46	91.67	22.98	91.67	22.54	75.00	45.83	33.33
ART/98/SW1-Y	3.83	5.33	3.88	4.67	2.80	3.00	3.00	1.33	30.75	91.67	33.47	83.33	31.17	58.33	29.17	16.67
ART/98/SW6-OB-W	4.50	5.67	4.11	4.67	2.83	2.00	4.33	1.00	33.89	83.33	38.16	83.33	29.52	50.00	41.67	0.00
BR 9943 DMRSR	3.17	5.33	2.56	3.67	1.60	2.00	1.67	1.33	23.57	91.67	15.57	83.33	5.78	50.00	4.17	25.00
BR LNTP-Y C6	4.00	4.67	3.00	3.33	2.00	2.00	2.00	1.00	23.06	75.00	19.27	66.67	11.56	58.33	16.67	8.33
BR9928 DMRSR	3.50	6.00	3.00	3.33	2.50	2.00	2.67	1.33	29.63	58.33	25.95	50.00	17.90	33.33	33.33	16.67
DMR-ESR-Y	3.67	5.33	3.56	4.67	2.17	2.33	2.00	1.00	42.53	100.00	30.75	83.33	20.97	66.67	16.67	8.33
ART/98/ILE-1-OB	5.20	5.00	3.78	4.67	2.33	3.33	3.00	1.33	33.47	100.00	31.00	100.00	24.56	83.33	29.17	33.33
PRO-VIT. A	4.17	4.67	3.22	4.00	2.00	2.67	2.00	2.33	38.08	75.00	23.18	50.00	15.85	41.67	25.00	33.33
SUWAN-1-SR-Y	3.00	4.33	2.89	4.00	2.17	2.67	2.33	1.67	16.04	50.00	17.76	75.00	26.57	66.67	17.46	25.00
TZBR COMP-1-W C2	3.67	4.67	3.67	4.00	3.00	2.67	2.00	2.33	22.44	83.33	17.67	75.00	28.12	66.67	25.72	50.00
TZBR COMP-2-W C2	4.17	6.67	4.00	5.00	3.33	2.33	3.33	1.67	33.78	100.00	35.95	75.00	27.28	50.00	37.50	8.33
TZBR COMP-2-Y C3	3.00	3.00	3.00	3.33	3.33	1.33	4.67	2.00	26.07	75.00	26.85	58.33	31.77	25.00	45.83	41.67
TZBR COMP 1-Y C3	3.20	4.33	3.00	4.67	1.33	2.33	1.67	1.67	24.38	91.67	16.10	66.67	13.76	41.67	10.00	25.00
TZBR ELD 4-W C2	2.17	4.67	2.22	4.00	1.50	1.33	1.67	1.33	25.94	66.67	24.06	66.67	17.63	33.33	12.50	33.33
TZBR ELD 4-Y C2	2.67	4.33	2.78	4.33	2.50	2.33	4.00	1.00	23.30	100.00	22.71	91.67	29.87	41.67	50.00	0.00
TZE BR-ELD3-W	3.50	4.00	3.13	4.00	2.00	1.33	2.00	2.00	26.39	75.00	17.61	58.33	0.00	25.00	16.67	41.67
TZE-COMP5	3.50	6.33	3.29	5.00	1.50	2.67	3.33	1.33	26.19	100.00	29.40	91.67	21.43	50.00	29.17	25.00
TZPB-SR-W	4.00	6.00	3.89	4.00	3.17	0.67	3.33	1.00	27.95	83.33	39.77	58.33	24.73	16.67	42.59	0.00
Mean	3.64	5.00	3.28	4.23	2.30	2.18	2.72	1.50	28.58	84.58	25.41	74.58	20.83	49.58	27.29	22.08
SE	0.27	0.15	0.19	0.17	0.12	0.15	0.19	0.09	2.98	2.92	2.12	3.23	2.29	3.73	3.43	3.31
LSD (0.05)	2.68	1.54	2.33	2.41	1.35	1.90	1.56	1.03	30.04	33.50	32.55	41.54	42.0	44.89	39.2	40.65
cv (%)	45.24	18.70	43.72	34.47	36.21	52.70	35.40	41.46	64.70	23.96	78.84	33.70	108.18	54.77	70.98	111.40

WAI: weeks after infestation; Scale 1-9: 1 no infestation, 9 severe infestation; SE: Standard error; CV: coefficient of variation
NI: Natural infestation; AI: Artificial infestation

Table 3: Mean squares from analysis of variance for the traits studied under artificial infestation in Ibadan in 2017

Source	df	INC 1	INC 2	INC 3	INC 4	SVR 1	SVR 2	SVR 3	SVR 4	PH 1	PH 2	PH 3	PH 4
Variety	19	0.17	0.13	0.18	0.16	2.31**	1.02	1.25	0.58	13.24*	29.1	42.26	52.11
Rep	2	0.20	0.07	0.39	0.44	2.62	3.22	1.52	0.65	1.01	25.54	18.60	38.90
Error	38	0.10	0.15	0.14	0.13	0.86	2.13	1.32	0.39	7.08	16.93	26.23	39.41

SVR 1,2,3&4: FAW severity (scale1-9) at 1,2,3&4 weeks after infestation; INC 1, 2 , 3 & 4: FAW incidence (%) at 1, 2, 3 & 4 weeks after infestation; PH 1, 2, 3 & 4: Plant height at 1, 2, 3 & 4 weeks after infestation; df: degree of freedom; *,** : Significant at p = 0.05 and 0.01 respectively

Table 4:. Mean squares from analysis of variance for the traits studied under natural infestation in the four environments in 2018

Source	df	SVR 1	SVR 2	SVR 3	SVR 4	INC 1	INC 2
Env	3	701.53**	362.98**	4.54**	--	22.54**	8.37**
Variety (V)	19	2.40	2.2	9.98**	20.42**	0.08	0.19*
Rep (Env)	8	2.44	2.03	2.15**	2.75**	0.64**	0..31**
Env x V	57	2.2	1.92	2.16**	0.001	0.10	0.16**
Error	152	2.71	2.06	0.69	0.92	0.10	0.09

Source	INC 3	INC 4	PH 1	PH 2	PH 3	PH 4
Env	5.78**	20.67**	1416.84**	8009.34**	12091.77**	24084.52**
Variety (V)	0.74**	0.75**	83.92**	16.29	624.76**	120.10*
Rep (Env)	0.14	0.24**	3.12	375.66**	54.89**	1032.16**
Env x V	0.13	0.25**	6.10*	27.65	60.41**	83.09
Error	0.11	0.09	3.26	20.57	23.97	76.48

SVR 1, 2, 3 & 4: FAW severity (scale1-9) at 1, 2, 3 & 4 weeks after infestation; INC 1, 2, 3 & 4: FAW incidence (%) at 1, 2, 3 & 4 weeks after infestation; PH 1, 2, 3 & 4: Plant height at 1, 2, 3 & 4 weeks after infestation; df: degree of freedom; *,** : Significant at p = 0.05 and 0.01 respectively

Table 5: The top maize varieties selected under artificial and natural

s/n	Artificial infestation	Natural infestation*
1	BR LNTP-Y C6	BR 9943 DMRSR
2	ART/98/SW6-OB-W	TZBR ELD 4-W C2
3	SUWAN-1-SR-Y	TZBR COMP 1-Y C3
4	AMA TZBR-W C4	AMA TZBR-W C4
5	TZBR ELD 4-Y C2	SUWAN 1-SR Y

FAW infestation using RSI (25 % selection intensity)
*Selection under natural infestation is based on the pooled data for the four environments

environments (Giaveno et al., 2004) or variation of the buildup of the pest in different environments used for this study. The significant mean squares of variety showed high level of variability among the varieties for resistance to FAW. Oliveira et al. (2018) working on popcorn under fall armyworm reported significant differences among genotypes for both nutritional and physical traits.

The result of RSI corroborates the outcome of cluster analysis under both conditions. Three of the top 5 selected varieties under artificial infestation using RSI are stem borer resistant and also belong to the best groups under cluster analysis. Under natural environment, four of the selected varieties are stem borer resistant. Some of the varieties selected by RSI clustered in the same group with the resistant check 'BR LNTP-Y C_6' while some stood alone in distinct groups. This study suggested some levels of relationship between stem borer resistance and FAW resistance. Hence, multiple insect resistance could be developed in these varieties. Ni et al. (2011) recorded similar observation in the western corn rootworm resistant variety, CRW3(S1) C6 which showed resistance to the FAW. Previous reports on multiple insect resistance has been limited to similar plant tissues, such as multiple leaf-feeding insects (Wilson et al., 1995; Abel et al., 2000), and multiple ear-feeding insects and ear-colonizing diseases (Ni et al., 2007; Ni et al., 2008).

It is worthy to note that 'ART/98/SW6-OB' and 'SUWAN 1-SR' selected by RSI, and also grouped with the resistant check in cluster analysis are non-stem borer resistance varieties. 'ART/98/SW6-OB' is a quality protein maize developed by the Institute of Agricultural research and Training (IAR&T), while 'SUWAN 1 SR' is a streak resistant maize variety.

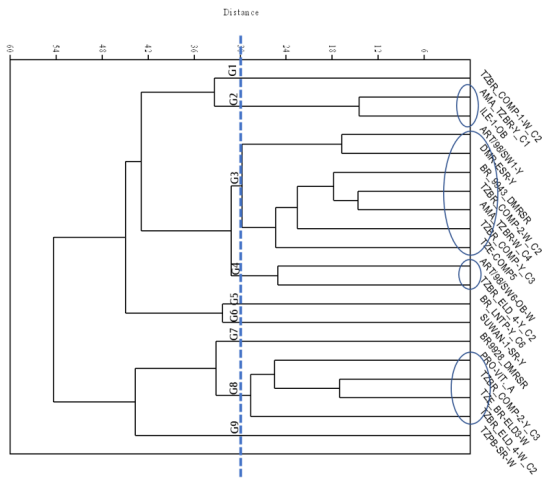

Figure 1: Dendrogram of the 20 maize varieties under artificial fall armyworm infestation based on Euclidean similarity distance

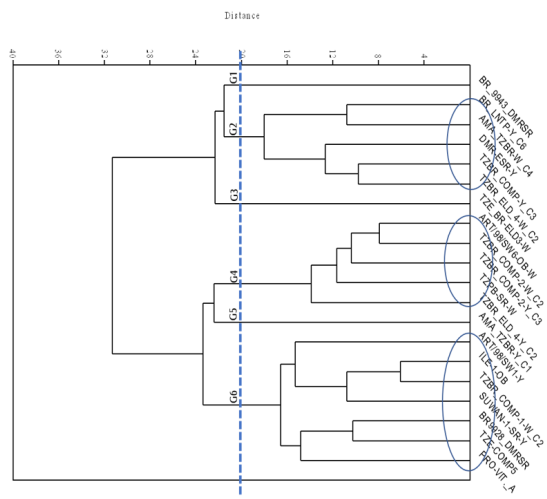

Figure 2: Dendrogram of the 20 maize varieties under natural FAW infestation based on Euclidean similarity distance

5 CONCLUSION

This study suggested that there is a relationship between resistance to stem borer and FAW. It also revealed that varieties BR LNTP-Y C_6, AMA TZBR-W C_4 and TZBR ELD 4 C_2, ART/98/SW6-OB and SUWAN 1-SR are good resistant source which could be used in a breeding program for resistance to FAW.

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Essential oil content, chamazulene content and antioxidative properties of *Achillea millefolium* agg. extracts from Slovenia

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Essential oil content, chamazulene content and antioxidative properties of *Achillea millefolium* agg. extracts from Slovenia

Abstract: The study aimed to clarify some biochemical properties, important for the phytopharmaceutical use of yarrow from the *A. millefolium* agg.. The study comprised 41 populations from Slovenia. The most abundant taxa were included: *Achillea millefolium* L., *A. roseoalba* Ehrend.*, A. collina* (Wirtg.) Becker ex Rchb.*, A. distans* Waldst. & Kit. ex Willd.*, A. pannonica* Scheele*, A. pratensis* Saukel & R.Länger and *A. nobilis* L. Assessment of essential oil content with the steam distillation method showed no significant difference between taxa. Essential oil content was the lowest in *A. collina* (6.50 ml kg^{-1} of dry matter), followed by *A. pannonica* (7.75 ml kg^{-1}), *A. distans* (8.50 ml kg^{-1}), *A. nobilis* (9.40 ml kg^{-1}), *A. pratensis* (9.65 ml kg^{-1}), *A. nobilis* × *A. millefolium* (12.25 ml kg^{-1}), *A. roseoalba* (12.75 ml kg^{-1}) and *A. millefolium* (13.50 ml kg^{-1}). The content of azulenes was determined by photometrical measurement of chamazulene in essential oil extracts. Chamazulene was only present in the diploid taxon and one tetraploid taxon, i.e., *A. roseoalba* (0.16 % of dry plant mass) and *A. collina* (0.05 %). The differences in antioxidative capacity of extracts from different taxa were not statistically significant, so we can assume that specific antioxidative capacity is not bound to a specific taxon or ploidy level.

Key words: *Achillea*; yarrow; chamazulene; essential oils; antioxidants

Vsebnost eteričnih olj in hamazulena ter antioksidativne lastnosti izvlečkov taksonov *Achillea millefolium* agg. v Sloveniji

Izvleček: Raziskava je skušala razjasniti nekatere biokemijske lastnosti, pomembne za uporabo različnih vrst rmana (*Achillea millefolium* agg.). V raziskavo je bilo vključenih 41 populacij rmana iz Slovenije. Zajete so bile najpogostejše vrste: *Achillea millefolium* L., *A. roseoalba* Ehrend.*, A. collina* (Wirtg.) Becker ex Rchb.*, A. distans* Waldst. & Kit. ex Willd.*, A. pannonica* Scheele, *A. pratensis* Saukel & R.Länger in *A. nobilis* L. Vsebnost eteričnih olj, določena z metodo parne destilacije, ni pokazala statistično značilnih razlik med taksoni. Vsebnost eteričnih olj je bila najmanjša pri *A. collina* (6,50 ml kg^{-1} suhe snovi), sledijo *A. pannonica* (7,75 ml kg^{-1}), *A. distans* (8,50 ml kg^{-1}), *A. nobilis* (9,40 ml kg^{-1}), *A. pratensis* (9,65 ml kg^{-1}), *A. nobilis* × *A. millefolium* (12,25 ml kg^{-1}), *A. roseoalba* (12,75 ml kg^{-1}) in *A. millefolium* (13,50 ml kg^{-1}). Vsebnost azulenov je bila določena s fotometričnimi meritvami hamazulena v izvlečku eteričnih olj. Hamazulen je bil prisoten le pri diploidni vrsti in eni tetraploidni vrsti, to sta *A. roseoalba* (0,16 % suhe snovi) in *A. collina* (0,05 %). Razlike v antioksidativni kapaciteti izvlečkov različnih taksonov niso bile statistično različne, zato lahko sklepamo, da antioksidativne lastnosti niso vezane na določen takson ali ploidnostno stopnjo.

Ključne besede: *Achillea*; rman; hamazulen; eterična olja; antioksidanti

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1 INTRODUCTION

The genus *Achillea* (yarrow) belongs to the family Asteraceae and subfamily Anthemideae, and currently includes around 130 species (Guo et al., 2004; Ehrendorfer & Guo, 2020). Its center of diversity is southeastern Europe (Ehrendorfer & Guo, 2006), although its representatives are spread all over Eurasia and the North American continent. Some species, for example *Achillea millefolium*, were spread throughout the northern hemisphere by humans. The genus shows great ecological plasticity, with different species inhabiting dry desert areas, subalpine mountainous regions and anthropogenically modified ruderal habitats.

Different species of yarrow, used in folk medicine and phytopharmaceutical products, originate from natural populations, collected in natural habitats, or from cultivation (Vitkova et a., 2005; Edreva et al., 2017; Edreva et al., 2019). They are used as antiphlogistics, antispasmodics, hemostatics, stomachics and holagogues (Kastner et al., 1995; Ali et al., 2017). While the content of phytopharmaceutically important compounds in plants also depends on the type of habitat and climatic conditions, it is presumed to be primarily genetically conditioned, and as such limited to specific taxa. Because of that, understanding the genus systematics is not only of academic, but also of practical importance.

Among the bioactive components in yarrow, essential oils are the most important in therms of medicinal effects (Franz, 2007). The content of essential oils in dry above-ground plant parts is about 0.2-1 % (Nemeth, 2005). They include 6-19 % of chamazulene and more than 100 other components, among them monoterpenes and sesquiterpenes. The content and composition of essential oils are influenced by genetic, ontogenetic (Farhadi et al., 2020) and environmental factors (Stahl, 1952; Deufel, 1954; Radulovich et al., 2007). The differences are not only reflected in the essential oils, extracted from inflorescences, but also from the leaves (Judzentiene & Mockute, 2005). Differences in essential oil composition are also known among taxa of different ploidy levels (Hofmann et al., 1992) and populations from different geographical provinces (Haziri et al., 2010).

The most important groups of sesquiterpene lactones found in yarrow include azulenogenic and non-azulenogenic guanolides, guanolide-endo-peroxides, 3-oxy-guanolides, eudezmanolides, longipin, and germacrenes. The basic azulenogenic guanolide in yarrow is achillicin (Kastner et al., 1995). Below-ground plant parts are characterized by their ability to synthesize and accumulate alkamides with specific olefinic and acetylenic patterns, which substitutes the synthesis of polia-cetylenic compounds, otherwise characteristic of the Anthemideae (Greger and Hofer, 1989).

Yarrow, *A. millefolium* s.l., is one of the first documented medicinal plants in Europe (Wagenitz, 1979). The drug Herba Milefolii is listed in the pharmacopoeias of many European countries. However, the European Pharmacopoeia (2004) explicitly mentions only *Achillea millefolium* L., a specific taxon from the *Achillea millefolium* agg. Many sources suggest there is no differentiation between individual taxa of the aggregat when collecting yarrow for use in folk medicine (Saukel & Länger, 1992). Moreover, it is known from literature that the hexaploid taxon *A. millefolium* s. str. usually does not contain proazulenes at all, although its content is the ground criterion for inclusion in pharmacopoeias (Dabrowska, 1972; Oswiecimska, 1968, 1974). On the other hand, proazulenes are commonly found in di- and tetraploid species of the *A. millefolium* s.l. (Bugge, 1991; Adler et al., 1994). It is generally accepted that the diploid taxa *A. asplenifolia* and *A. roseoalba* do contain proazulenes, but *A. setacea* does not, despite also being diploid. Among tetraploid taxa, only *A. collina* produces proazulenes, but *A. pratensis* and *A. nobilis* do not. Most sources also agree that the hexaploid *A. millefolium* and octoploid *A. pannonica* do not synthesize proazulenes.

The aim of the present study was to extend the current knowledge on the phytochemical constituents in the *A. millefolium* agg. in Slovenia. The study included 41 yarrow populations from 41 locations all over Slovenia. The most abundant taxa were included: *Achillea millefolium* L., *A. roseoalba* Ehrend., *A. collina* (Wirtg.) Becker ex Rchb., *A. distans* Waldst. & Kit. ex Willd., *A. pannonica* Scheele, *A. nobilis* L. and *A. pratensis* Saukel & R. Länger. The goal was to estimate the content of essential oils in above-ground plant parts and test for the presence and content of proazulenes. Additionally, the study quantified antioxidative activity of extracts from collected taxa as another property, important for use in folk medicine.

2 MATERIAL AND METHODS

2.1 COLLECTION AND PREPARATION OF PLANT MATERIAL

Plant material was collected from 41 locations across Slovenia. All known basic ploidy levels of *Achillea millefolium* agg. were included. The taxon *A. roseoalba* Ehrend., which grows in humid lowland meadows, is diploid (2n = 18). Taxa *A. collina* (Wirtg.) Becker ex Rchb., *A. nobilis* L. and *A. pratensis* Saukel & R. Länger are tetraploid (2n = 36), *A. millefolium* L. and *A. distans*

Waldst. & Kit. ex Willd. are hexaploid (2n = 54) and *A. panonica* Scheele is octoploid (2n = 72).

Plant material was collected and prepared in the same manner for all further analyses. 500 g to 2000 g of fresh above-ground plant material was collected at each site. The quantity particularly depended on the size and abundance of the plants of each taxon in a population. In taxa where plants are large, a few dozen plants were sufficient, but where they are smaller, a few hundred were collected. At each site, plants were harvested as close to each other as possible. Due to the large amount of material collected, it was impossible to ensure that it all came from the same individual. When different morphological variants were present at the same site, only plants of the same morphological type were collected. Additional specimens for morphological measurements were collected at each site and stored in a herbarium.

The plants were cleaned of any foreign plant material, tied into small bundles, and hung in a dry, dark and airy space, where they dried at room temperature for two to three days. The upper parts of the air-dried plants with inflorescences, healthy green leaves and the attached parts of the stem were cut off, cut into approximately 10 cm long pieces and stored in paper bags. The dry plant material was stored at room temperature until further processing in a dark, dry room.

2.2 EXTRACTION OF ESSENTIAL OIL AND PROAZULENES

Extraction of essential oils and proazulenes was performed in accordance with the 5th edition of the European Pharmacopoeia (2004) using 20 g of cut drug, a 1000 ml round-bottomed flask and 500 ml of a mixture of 1 volume of water and 9 volumes of ethylene glycol as the distillation liquid. 0.2 ml of xylene in the graduated tube was added to takeup the essential oil. The destilation time was 2 hours.

2.3 ESTIMATION OF CHAMAZULENE CONTENT IN ESSENTIAL OIL

The content of chamazulene in the essential oil was determined photometrically in accordance with the 5th edition of the European Pharmacopoeia (2004). After distillation, the xylene with dissolved essential oil, and with as little distillation liquid as possible, was transferred into a 50 ml volumetric flask. Photometric measurement of absorbance was performed on a Perkin Elmer spectrophotometer, Lambda 25 UV / VIS Spectrometer at 608 nm.

2.4 PREPARATION OF EXTRACTS FOR ANTIOXIDATIVE PROPERTIES ESSAY

Plant samples, prepared in step 2.1, were shredded and mixed by hand to homogenize. Approximately 50 g of each sample was prepared for grinding. The instruction of the European Pharmacopoeia (2004) that the drug should not contain more than 5 % of stems with a diameter exceeding 3 mm, or more than 2 % of other foreign components, was followed.

For extraction, 0.5 g of ground plant material was weighed and added to 5 ml of solvent in a glass centrifuge. 80 % methanol (a mixture of methanol and demineralized water in a volume ratio of 80 : 20) was used as solvent. Samples were stored in colorless bottles in the freezer at -18 °C.

2.5 MEASUREMENT OF ANTIOXIDATIVE PROPERTIES OF EXTRACTS

Antioxidant activity cannot be measured directly, but the inhibitory effect of antioxidants in oxidation can, using a wide range of methods. Efficiency of oxidation can be determined by measuring any of the factors in the oxidation process – the substrate, the oxidant or the intermediate and final products of oxidation (Antolovich et al., 2002). One commonly used method is based on the use of the stable free radical diphenyl picryl hydrazyl (DPPH) (Molyneux, 2004; Yordanov et al., 1997). The results of a DPPH tests were presented by the inhibition coefficient (IC), expressing DPPH inhibition in % and through TEAC (Trolox Equivalent Antioxidant Capacity) or antioxidant capacity in Trolox equivalents in TE units (Trolox Equivalent), i.e., in mM TE per 100 g of tested material.

2.6 STATISTICS

Descriptive statistics and plot production was performed using Statistica, Data Analysis Software System (StatSoft Inc., USA).

3 RESULTS AND DISCUSSION

3.1 ESSENTIAL OIL AND CHAMAZULENE CONTENT

Measurement of essential oil content with steam distillation using the Clevenger apparatus showed no significant differences among taxa. Essential oil content

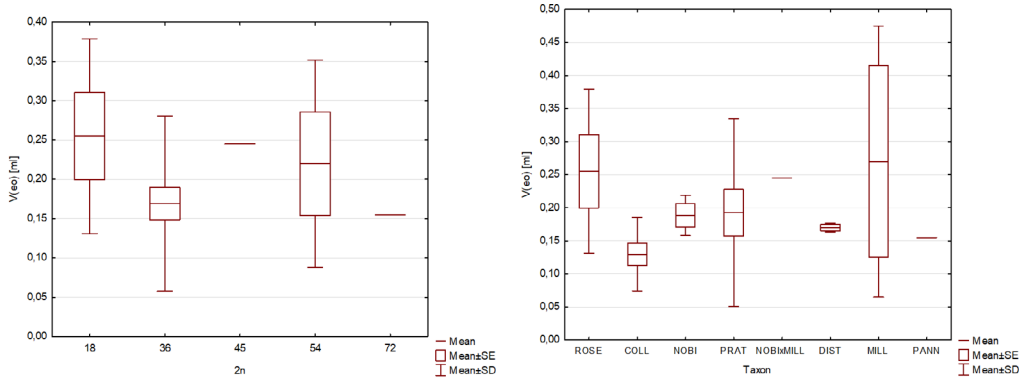

Figure 1: Essential oil volume in plant extracts by ploidy level and by taxon, expressed as ml per 20 g dry matter.

Table 1: Descriptive statistics and statistical significance of differences in average essential oil volume in plant extract among ploidy levels, expressed ml per 20 g of dry matter.

Ploidy	Average [ml]	Sig.	Min. [ml]	Max. [ml]	SD [ml]	SE [ml]
2n = 72	0.155	a	0.155	0.155		
2n = 36	0.169	a	0.055	0.555	0.112	0.020
2n = 54	0.220	a	0.125	0.415	0.132	0.066
2n = 45	0.245	a	0.245	0.245		
2n 18	0.255	a	0.125	0.445	0.124	0.055

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

Table 2: Descriptive statistics and statistical significance of differences in average essential oil volume in plant extract among taxa, expressed in ml per 20 g of dry matter.

Taxon	Average [ml]	Sig.	Min. [ml]	Max. [ml]	SD [ml]	SE[ml]
COLL	0.130	a	0.075	0.275	0.056	0.017
PANN	0.155	a	0.155	0.155		
DIST	0.170	a	0.165	0.175	0.007	0.005
NOBI	0.188	a	0.155	0.215	0.031	0.018
PRAT	0.193	a	0.055	0.555	0.142	0.035
NOBIxMILL	0.245	a	0.245	0.245		
ROSE	0.255	a	0.125	0.445	0.124	0.055
MILL	0.270	a	0.125	0.415	0.205	0.145

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

was the lowest in *A. collina*, with 6.50 ml per kg of dry matter (s. d. 2.80 ml), followed by *A. pannonica* with 7.75 ml kg^{-1}, *A. distans* with 8.50 ml kg^{-1} (s. d. 0.35 ml), *A. nobilis* with 9.40 ml kg^{-1} (s. d. 1.55 ml), *A. pratensis* with 9.65 ml kg^{-1} (s. d. 7.1 ml), *A. nobilis* × *A. millefolium* with 12.25 ml kg^{-1} and *A. roseoalba* with 12.75 ml kg^{-1} (s. d. 6.20 ml). The highest essential oil content was estimated in *A. millefolium*, with 13.50 ml kg^{-1} of dry matter (s. d. 10.25 ml).

Total essential oil content was consistent with existing data (Gharibi et al., 2015), while maximum differences between species were just approximately two-fold, much less than some other studies report. Orav et al. (2006) found nine-fold variance in essential oil yield in yarrow samples, and even twenty-seven-fold differences have been reported from *Achillea* samples from Iran (Rahimmalek et al., 2009). Consistent essential oil content in our study may be explained by the fact that the present study only included species from the *A. millefolium* agg., whereas other studies also took into account some taxonomically less related species. In addition, care was taken to only use the inflorescences and uppermost leaves, with as little stems as possible, since some studies showed large differences in essential oil content between the two plant parts, e. g. 0.65 % (v/w) in flowers and 0.0125 % (v/w) in stems (Bocevska & Sovova, 2007). The oil yield in all our samples conformed to the European pharmacopoeia 5.0 (2004) standard which is not less than 0.2 %.

Proazulenes, measured through chamazulene, were only present in *A. roseoalba* and *A. collina*. This is, to some extent, consistent with existing literature, suggesting only diploid and tetraploid taxa are proazulenogenic (Gherase et al., 2003; Nemeth et al., 2007; Konakchiev et al., 2005), although some researchers claim that azulenes can be found in all ploidy levels, albeit in different proportions (Kindlovits et al., 2012). However, the data on proazulene presence is quite contradictory (Nemeth, 2005). Even so, in our research, only the diploid *A. roseoalba* consistently contained chamazulene (with population differences ranging from 2.648 % of dry mass to 0.351 % of dry mass). No difference in chamazulene content was detected between white-flowering and pink-flowering diploid individuals from the same population. In contrast, chamazulene content in the tetraploid *A. collina* was less consistent, and not significantly different from other taxa, except *A. roseoalba*. Chamazulene content found in different populations ranged from 0.171 % of dry mass to 0.003 % of dry mass.

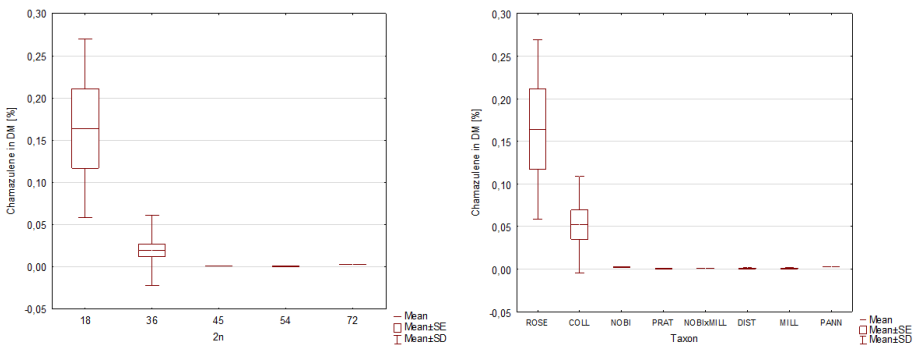

Figure 2: Chamazulene content in % of dry matter by ploidy level and by taxon.

Table 3: Descriptive statistics and statistical significance of differences in average chamazulene content in % of dry matter among ploidy levels.

Ploidy	Average [%]	Sig.	Min. [%]	Max. [%]	SD [%]	SE [%]
2n = 54	0.001	a	0.0002	0.0014	0.0006	0.0003
2n = 45	0.001	a	0.0010	0.0010		
2n = 72	0.003	a	0.0028	0.0028		
2n = 36	0.020	a	0.0000	0.1706	0.0418	0.0076
2n = 18	0.164	b	0.0357	0.2742	0.1055	0.0472

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

Table 4: Descriptive statistics and statistical significance of differences in average chamazulene content in % of dry matter among taxa.

Taxon	Average [%]	Sig.	Min. [%]	Max. [%]	SD [%]	SE [%]
PRAT	0.001	a	0.000	0.002	0.001	0.000
DIST	0.001	a	0.000	0.001	0.001	0.001
MILL	0.001	a	0.000	0.001	0.001	0.000
NOBIxMILL	0.001	a	0.001	0.001		
NOBI	0.002	a	0.002	0.003	0.001	0.000
PANN	0.003	a	0.003	0.003		
COLL	0.052	a	0.003	0.171	0.057	0.017
ROSE	0.164	b	0.036	0.274	0.106	0.047

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

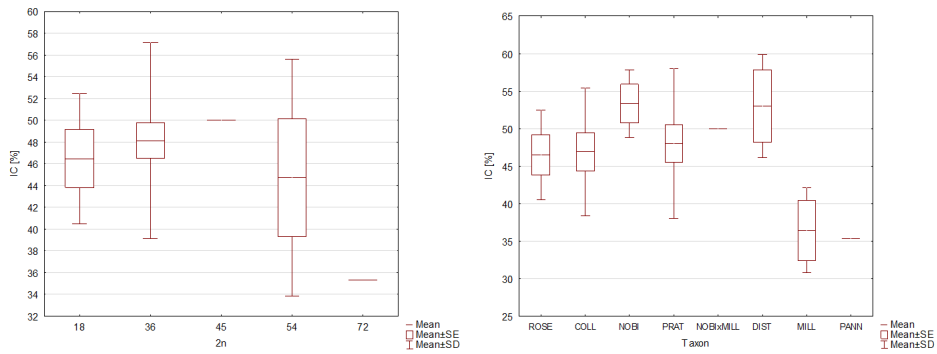

Figure 3: DPPH inhibition coefficient (IC) in % by ploidy level and by taxon.

Table 5: Descriptive statistics and statistical significance of differences in average DPPH inhibition coefficients (IC) in % among ploidy levels.

Ploidy	Average [%]	Sig.	Min. [%]	Max. [%]	SD [%]	SE [%]
2n = 72	35.34	a	35.34	35.34		
2n = 54	44.72	a	32.43	57.86	10.86	5.43
2n = 18	46.49	a	40.94	55.26	5.99	2.68
2n = 36	48.14	a	25.87	65.29	9.02	1.65
2n = 45	50.00	a	50.00	50.00		

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

Table 6: Descriptive statistics and statistical significance of differences in average DPPH inhibition coefficients (IC) in % among taxa.

Taxon	Average [%]	Sig.	Min. [%]	Max. [%]	SD [%]	SE [%]
PANN	35.34	a	35.34	35.34		
MILL	36.43	a	32.43	40.44	5.66	4.00
ROSE	46.49	a	40.94	55.26	5.99	2.68
COLL	46.91	a	34.83	65.29	8.52	2.57
PRAT	48.02	a	25.87	61.85	9.98	2.49
NOBI x MILL	50.00	a	50.00	50.00		
DIST	53.02	a	48.17	57.86	6.86	4.85
NOBI	53.34	a	49.92	58.43	4.49	2.59

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

Table 7: Descriptive statistics and statistical significance of differences in Trolox Equivalent Antioxidant Capacity (TEAC) in µM TE per 100 g of dry plant matter among ploidy levels.

Ploidy	Average [µM]	Sig.	Min. [µM]	Max. [µM]	SD [µM]	SE [µM]
2n = 72	42.54	a	42.54	42.54		
2n = 54	54.65	a	38.78	71.60	14.02	7.01
2n = 18	56.93	a	49.77	68.25	7.73	3.46
2n = 36	59.06	a	30.32	81.17	11.63	2.12
2n = 45	61.46	a	61.46	61.46		

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

Table 8: Descriptive statistics and statistical significance of differences in Trolox equivalent antioxidant capacity (TEAC) in µM TE per 100 g of dry plant matter among taxa.

Taxon	Average [µM]	Sig.	Min. [µM]	Max. [µM]	SD [µM]	SE [µM]
PANN	42.54	a	42.54	42.54		
MILL	43.95	a	38.78	49.12	7.31	5.17
ROSE	56.93	a	49.77	68.25	7.73	3.46
COLL	57.47	a	41.88	81.17	11.00	3.32
PRAT	58.89	a	30.32	76.74	12.87	3.22
NOBIxMILL	61.46	a	61.46	61.46		
DIST	65.34	a	59.09	71.60	8.84	6.25
NOBI	65.76	a	61.35	72.32	5.79	3.35

Note: same letters in column Sig. denote cases with no statistically significant difference (Duncan test, $p \leq 0.05$)

3.2 ANTIOXIDATIVE ACTIVITY OF THE EXTRACTS

The differences in antioxidative capacity were not statistically significant among extracts from plants with different ploidy levels and of different taxa. The results showed a large range of antioxidant efficacy in the samples. The DPPH radical inhibition coefficient (IC) ranged from 25.87 % in a population of *A. pratensis*, to 65.29 % in a population of *A. collina*. The highest detected value of Trolox equivalent antioxidative capacity (TEAC) was more than twice as high as the lowest. The values ranged from 81.17 µM in an *A. collina* population to 30.32 µM in an *A. pratensis* population (both tetraploid). The distribution of IC values was relatively continuous, with no obvious groupings. Based on the results, it can be assumed that specific antioxidative capacity is not associated with a specific taxon or ploidy level. Since the amount of antioxidants, as well as proazulenes, as shown by Stahl (1952), can be affected by environmental conditions and stress, or can even be related to the plant communities in which yarrow grows (Michler & Arnold, 1999; Radušiene & Gudaityte, 2005), it might be worth exploring the correlation between environmental conditions, in which the sampled plants grew, and their antioxidative activity.

4 CONCLUSIONS

Due to the importance of yarrow from the *Achillea millefolium* agg. in folk medicine and phytopharmaceuticals on one side, and great genotypical and phenotypical plasticity of the aggregate on the other, distinguishing among individual taxa is crucial. It is known, for instance, that taxa with different ploidy levels exhibit different abilities for proazulenic compound synthesis. The influence of environmental conditions and stress at the growing site is also important (Gudaityte, 2008), although some authors did not find any correlation (Nemeth, 2007). No such evaluation of the most abundant taxa from the *A. millefolium* agg. has so far been done in Slovenia.

The present study showed that the ability of proazulenic compound synthesis in Slovenian taxa greatly corresponds to the general patterns. The highest chamazulene content was found in the only diploid taxon included in the study, *A. roseoalba*. Although the differences in the content among individual populations were quite large, ranging from 2.65 % to 0.35 % of dry plant matter, it was the only taxon with consistent chamazulene presence. The only other taxon, where chamazulene was found, was the tetraploid *A. collina*. Here, chamazulene content never exceeded 0.17 % of dry plant matter. We can conclude that only *A. roseoalba*, occurring predominantly in wet meadows and slightly acidic fens (Dunkel et. al., 2011; Saukel, 2008), is worth being collected as a source of chamazulene.

There was a lot of variability in essential oil content among samples. No significant differences among taxa or ploidy levels could be found, perhaps also due to the small number of samples. Still, it appears that when picking yarrow for its essential oils, all taxa are similarly suitable for collection. The composition of essential oils, which was not tested here, however, most probably differs among taxa (Yener et al., 2020).

Similar conclusions were obtained from the assay of antioxidative properties. Antioxidative activity of the extracts showed no significant differences among taxa, but variability within taxa was large. One can speculate that antioxidative capacity is not determined only genetically, but largely depends on environmental and stress conditions.

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Nutrition and Covid-19 epidemic

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Nutrition and Covid-19 epidemic

Abstract: Proper nutrition is an essential part of an individual's defence against numerous diseases including coronavirus disease SARS-CoV-2 (Covid-19). Nutritional status of individual is affected by several factors such as age, sex, health status, physical activity, life style and medications. Optimal nutrition and dietary nutrient intake impact the immune system, therefore the sustainable way to survive in current context is to strengthen the immune system. Inadequate intake of energy, protein, and specific micronutrients are associated with depressed immune function and increased susceptibility to infection. Predominantly vital for the encouraging of immune function are elements selenium, iron and zinc and vitamins A, D, C, E, B_6, B_9 (folate) and B_{12} as well as omega-3 polyunsaturated fatty acids. Thus, during this time it is important to take care of nutritional habits, following a healthy and balanced nutritional pattern containing a high amount of elements, antioxidants and vitamins. It is also recommended, that individuals should be mindful of physical activity, known to be associated with all-cause mortality. Regular physical activity also improves mental health and overall feelings of wellbeing. Thus, now in the time of epidemic, more than ever, wider access to healthy foods should be a top priority for governments around the world

Key words: nutrition; Covid-19; immune system; nutrients; vitamins; elements; antioxidants; omega-3 polyunsaturated fatty acids

Prehrana in epidemija Covid-19

Izvleček: Pravilna prehrana je pomemben del posameznikove obrambe pred številnimi boleznimi, vključno pred koronavirusno boleznijo SARS-CoV-2 (Covid-19). Na prehranski status posameznika vpliva več dejavnikov, kot so starost, spol, zdravstveno stanje, telesna dejavnosti, življenjski slog in uživanje zdravil. Optimalna prehrana in z njo vnos hranil vplivata na imunski sistem, zato je trajnostni način preživetja v sedanjih okoliščinah krepitev imunskega sistema. Neustrezen vnos energije, beljakovin in določenih mikrohranil je povezan z oslabljenim delovanjem imunskega sistema in povečano dovzetnostjo za okužbe. Za spodbujanje imunske funkcije so pomembni predvem elementi selen, železo in cink ter vitamini A, D, C, E, B_6, B_9 (folati) in B_{12} ter večkrat nenasičene maščobne kisline omega-3. V obdobju epidemije je zato še toliko bolj pomembno skrbeti za zdravo in uravnoteženo prehrano, ki vsebuje dovolj elementov, antioksidantov in vitaminov.Priporočljivo je tudi, da se posamezniki zavedajo pomena redne telesne dejavnosti, za katero je znano, da zmanjšuje tveganje za smrtnost zaradi različnih vzrokov. Redna telesna dejavnost ima tudi ugoden vpliv na duševno zdravje in splošno počutje. Širši dostop do zdrave hrane bi v danih okoliščinah morala biti ena izmed prednostnih nalog vlad po vsem svetu.

Ključne besede: prehrana; Covid-19; imunski sistem; hranila; vitamin; elementi; antioksidanti; večkrat nenasičene maščobne kisline omega-3

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1 INTRODUCTION

At the time of this writing, the Covid-19 pandemic will have infected more than 125 million people and taken the lives of nearly 2,800,000 individuals world-wide. Data for Slovenia (2 million inhabitant) in the moment are not encouraging, nearly 210,000 infected since the beginning and 4,280 lives taken till 25th of March, 2021.

Proper nutrition, with the aim to maintain immune function is an essential part of an individual's defence against Covid-19. Optimal nutrition and dietary nutrient intake impact the immune system through gene expression, cell activation, and signalling molecules modification. In addition, various dietary ingredients are determinants of gut microbial composition and subsequently shape the immune responses in the body (Aman & Masood, 2020). Adequate intake of energy, protein, and specific micronutrients are associated with depressed immune function and increased susceptibility to infection. Predominantly vital for the strengthening of immune function are elements selenium, iron and zinc and vitamins A, D, E, C, B_6 and B_{12} and omega-3 polyunsaturated fatty acids (Naja & Hamadeh, 2020)

Therefore, the key to maintaining an effective immune system is to avoid deficiencies of the energy and nutrients that play an essential role in immune cell triggering, interaction, differentiation, or functional expression (Barazzoni et al., 2020).

Covid-19 does not treat the whole population equally, differences are due to genetics and lifestyle. World Health Organization exposed, that people who eat a well-balanced diet tend to be healthier with stronger immune system and lower risk of chronic noncommunicable diseases and infectious diseases (WHO, 2020a). Undernourished people have weaker immune system, and may be at greater risk of severe illness due to the virus. At the same time, poor metabolic health, including obesity and diabetes, is strongly linked to worse Covid-19 outcomes, including risk of hospitalisation and death (Global ..., 2020). The high consumption rate of diets high in saturated fats, sugars, and refined carbohydrates (collectively called Western diet), and low levels of dietary fibre, unsaturated fats and antioxidants worldwide, contribute to the prevalence of obesity and type 2 diabetes, and could place these populations at an increased risk for severe Covid-19 pathology and mortality. Typical western diet consumption activates the innate immune system and impairs adaptive immunity, leading to chronic inflammation and impaired host defence against viruses (Butler et al., 2020)

There is not enough scientific evidence whether people with diabetes are more likely to get Covid-19 than the general population. General opinion is that people with diabetes are more likely to have serious complications from Covid-19. As seen from observations from Italy 30 % of deceased people due to Covid-19 had diabetes (Antonio et al., 2020). Reports from Lombardy also suggest that anti diabetes medicines worsen the course of Covid-19 disease (Antonio et al., 2020).

In a future virus pandemic, we might face a "double burden" of malnutrition, when both undernutrition and overnutrition will promote severity of disease (Barazzoni et al., 2020).

This article explores the importance of nutrition to boost immunity and gives some professional and authentic dietary guidelines about nutrition and food safety to better withstand Covid-19. The food safety, food management, access to food and many other important topics related to Covid-19 and nutrition are not the issue of this article.

2 NUTRITION AND COVID-19

Nutritional deficiencies of energy, protein, and specific micronutrients are associated with depressed immune function and increased susceptibility to infection. A proper planned diet, comprised of well-balanced nutrients is crucial to health, supports normal B and T immune cell functions for optimal disease-reducing immunity. In the case of Covid-19, the goal of nutrition is to reduce infection and disease progression while improving recovery during the course of the disease (Jaggers et al., 2020).

2.1 FRUITS AND VEGETABLES

Food and Agriculture Organization put down seven healthy eating tips to face the Covid-19 crisis, first of them is dedicated how to strengthen our immune system through a proper diet. Focus should be put in consumption of at least five servings a day of fruits and vegetables, because they contain a lot of micronutrients, which can boost immune function. Some of these micronutrients such as vitamin C, A, C, E and beta-carotene are antioxidants that increase the number of T-cell subsets, enhance lymphocyte response to mitogen, increase interleukin-2 production, potentiate natural killer cell activity, and increase response to influenza virus vaccine compared with placebo (FAO, 2020; Muscogiuri et al., 2020).

2.2 FATS AND OILS

World Health Organization recommended to consume unsaturated fats, which are found in oils (sunflow-

er, olive, soy, canola and corn), fish, avocado and nuts, rather than saturated fats from fatty meat, butter, coconut oil, cream, cheese, ghee and lard (WHO, 2020a).

Excessive saturated fats consumption can induce a lipotoxic state and activate the innate immune system via activation of toll-like receptor 4 expressed on macrophages, dendritic cells, and neutrophils. This triggers activation of canonical inflammatory signalling pathways that produce proinflammatory mediators and other effectors of the innate immune system (Rogero et al., 2020). Furthermore, consumption of a high fat diet in mice increased macrophage infiltration to lung tissue, specifically in the alveoli, which is especially relevant to Covid-19 patients given the high rate of infection among lung alveolar epithelial cells and the involvement of lung tissue inflammation and alveolar damage in Covid-19 pathology (Butler & Barrientos 2020).

2.2.1 Role of omega-3 fatty acids in immune system

Omega-3 fatty acids are unsaturated long chain fatty acids known to decrease inflammation, which seems to be critical also for Covid-19 patients. Among them, eicosapentaenoic (EPA) and docosahexaenoic (DHA) are considered the most potential in inhibiting inflammation, could ameliorate some patients need for intensive care unit admission and have stimulating effect on immune system (Shakoor et al., 2020). Omega-3 fatty acids might inhibit growth of influenza virus, they are suggested to increase the oxygenation in Covid-19 patients (Barazzoni et al., 2020). From that point of view, patient therapy must consider omega-3 fatty acids as a co-therapy in Covid-19 (Rogero et al., 2020). Both, EPA and DHA are found in fish and fish oils. Cod liver oil is of special interest from nutritional point of view due to the high content of natural vitamin D_3 and vitamin A (retinol).

2.3 VITAMINS AND ELEMENTS

There is currently no guidance on micronutrient supplementation for the prevention of Covid-19 in healthy individuals or for the treatment of Covid-19. Wherever possible, micronutrient intakes should come from a nutritionally balanced and diverse diet, including fruits, vegetables and animal source foods (WHO, 2020b).

2.3.1 Vitamin A and carotene

Vitamin A has been defined as "anti-infective" vitamin since many of the body's defences against infection depend on its adequate supply. Vitamin A deficiency is involved in measles and diarrhoea and measles can become severe in vitamin A-deficient children (Barazzoni et al., 2020; Solomons, 2012). In experimental models, the effect of infection with infectious bronchitis virus, a kind of coronaviruses, was more pronounced in chickens, fed with a diet marginally deficient in vitamin A than in those fed a diet adequate in vitamin A. It has also been reported that vitamin A supplementation in humans reduced morbidity and mortality in different infectious diseases, such as measles, diarrheal disease, measles related pneumonia, malaria and HIV/AIDS infection (Barazzoni et al., 2020).

The richest animal sources of vitamin A in the human diet are fish liver oils, liver, other organ meats, cream, butter, and fortified milks. Certain tropical fatty fruits are the richest sources of provitamin A (Solomons, 2012). Beta carotene (provitamin A) is most abundant in sweet potatoes, carrots and green leafy vegetables (Muscogiuri et al., 2020).

2.3.2 Role of vitamin C in immune system

There is no evidence found that supplements can cure or »boost« the immune system except vitamin C, which is one of the best way to improve immune system. Vitamin C is one of the major constituents of water soluble vitamins which tends to contribute to a strong immune system (Aman et al., 2020). The daily recommended dietary allowance for vitamin C is 110 mg/day for men and 95 mg/day for women (NIJZ, 2020).

Vitamin C, water soluble antioxidant acts by scavenging damaging reactive oxygen species, thus protecting the tissues from oxidative damage and dysfunction. It is known for long as a protective factor for infectious diseases acting as an antioxidant through inactivation of free radicals and thus protecting proteins, lipids and nucleotides against oxidative damage (Shakoor et al., 2020). It accumulates in leucocytes reaching 50-100 fold higher concentration as compared to its plasma content, but it is depleted fast in case of infection (Shakoor et al., 2020).

Patients with asthma and pneumonia are known to have low vitamin C content in plasma (Hunt et al., 1994). Among others, vitamin C also reduces pro-inflammatory cytokines and increases anti-inflammatory cytokines; administration of 1 g of vitamin C per day increases the anti-inflammatory cytokines (Shakoor et al., 2020). Covid-19 patients are very susceptible to pneumonia, intravenous administration of high vitamin improved inflammatory respiration parameters (Hiedra et al., 2020).

Sources of vitamins C include red peppers, oranges, strawberries, broccoli, mangoes, lemons, and other fruits and vegetables (Muscogiuri et al., 2020).

2.3.3 Role of vitamin D in immune system

Vitamin D deficiency in winter has been reported to be associated to viral epidemics. Adequate vitamin

D status reduces the risk of developing several chronic diseases such as cancers, cardiovascular disease, diabetes mellitus, and hypertension that significantly increase risk of death from respiratory tract infections than otherwise healthy individuals. Further, vitamin D protects respiratory tract preserving tight junctions, destroying enveloped viruses through induction of cathelicidin and defending, and decreasing production of proinflammatory cytokines by the innate immune system, therefore reducing the risk of a cytokine storm leading to pneumonia (Muscogiuri et al., 2020).

Vitamin D seems to be tightly connected to the outcome of Covid-19 disease (Shakoor et al., 2020). Shortage of vitamin D is more pronounced in older people, at increased body weight, at men, hypertension, in higher geographic latitude and under conditions of higher coagulation (Shakoor et al., 2020). Older population is known to have chronic increased pro-inflammatory condition which render older people more susceptible to chronic diseases (Ferrucci et al., 2018). Vitamin D shortage pose a higher risk of community acquired pneumonia as has been reported in 8 studies including a total of 20966 patients as cited by Zhou et al., (2019). Sufficient consumption of vitamin D inhibits the synthesis of pro-inflammatory cytokines and limit the respiration stress connected to fatal outcome due to Covid-19 (Shakoor et al., 2020).

In the future, investigations will confirm whether insufficient vitamin D status more specifically characterizes Covid-19 patients and is associated to their outcome. In support to this hypothesis, decreased vitamin D levels in calves have been reported to enhance risk for bovine coronavirus infection (Barazzoni et al., 2020).

Since the time spent outdoor and consequently to the sun exposure is limited, especially during winter, it is encouraged to get more vitamin D from diet. Foods containing vitamin D include fish, liver, egg yolk and foods with supplemented vitamin D and food supplements (Muscogiuri et al., 2020).

2.3.4 Role of zinc in immune system

Essential trace element that is crucial for the maintenance of immune function is zinc. It has been reported that zinc inhibited severe acute respiratory syndrome (SARS) coronavirus RNA-dependent RNA polymerase template binding and elongation in Vero-E6 cells. Although oysters contain the most zinc per serving, the most common food to get zinc include poultry, red meat, nuts, pumpkin seeds, sesame seeds, beans, and lentils (Muscogiuri et al., 2020). The primary, relatively rich, plant source of zinc are whole-grain cereals. Zinc is mostly contained in the bran and germ portions, thus, nearly 80 % of the total zinc in these foodstuffs can be lost in the wheat milling process (Holt et al., 2012).

Zinc is a microelement involved in numerous biological processes including the immune response to virus infections (Shakoor et al., 2020). Shortage of zinc increases pro-inflammatory cytokines, permeability of epithelial cells in lungs (Shakoor et al., 2020). Increased intake of zinc results in higher number of T cells that inhibit synthesis, replication and transcription of coronavirus (Te Velthuis et al., 2010).

Due to the above mentioned facts, administration of zinc to Covid-19 patients resulted in an improved infection symptoms of lower respiration tract (Finzi 2020).

2.3.5 Role of Vitamin B_{12} in immune system

Serum vitamin B_{12} also called as cobalamin is a crucial micronutrient in many aspects of healthy metabolism. It plays an important role in maintaining nerve tissue health, brain function and red blood cell synthesis (Naik et al., 2020).

In Singapore cohort study, Tan et al. (2020) reported that a combination of vitamin D, magnesium and vitamin B_{12} lessen the need for oxygen therapy and/or intensive care support.

Humans obtain vitamin B_{12} from products of animal origin including meat, fish, shellfish, dairy products and eggs (Naik et al., 2020).

2.3.6 Role of vitamin E and selenium in immune system

Vitamin E and selenium play an important role in antioxidative system, shortage of any of them might change immune response against viruses (Shakoor et al., 2020). The content of selenium in the diet is influenced by geographical location of production (Terry & Diamond, 2012). Chinese researchers have proved the correlation between the content of selenium in soil from Chinese provinces and the course of the Covid-19 disease (Zhang et al., 2020). From that point of view the cure rate of Covid-19 patients inside Hubei Province, known as province with low soil selenium content, was significantly lower as compared to other provinces (Zhang et al., 2020). Selenium intake in humans originates principally from the consumption of meat, eggs and fish, which contain high levels of selenium in relation to other foods, ranging from 180 to 800 ng/g. Most plants do not accumulate high levels of selenium, with some exceptions like the crops from *Brassica* genus, which includes broccoli and kale, garlic, mushrooms and brazil nuts, which contain the highest levels of bioavailable selenium (Terry & Diamond, 2012).

Beside selenium alone, administration of selenium combined with vitamin E improved the immune response against respiratory infections (Wu et al., 2019). Rather than any of vitamin E isomers alone, the mixture of all four isomers proved to be more efficient than

α-tocopherol alone due to the availability of more receptors (Liu et al., 2002).

The major dietary sources of vitamin E are vegetable oils (soybean, sunflower, corn, wheat germ, and walnut), nuts, seeds, spinach, and broccoli (Muscogiuri et al., 2020).

2.3.7 Role of folate in immune system

Folate play an important role in the synthesis, repair and methylation of DNA, cellular division and in the maturation of red blood cells.

Inadequate supplementation of folic acid results in abnormally large red blood cells that do not work properly. This results in an increased red cell distribution width (RDW), a blood parameter associated with folate deficiency anaemia, which can cause tiredness and other symptoms (Batool et al., 2013; Im et al. 2020).

Studies investigating folate concentration in plasma are scares. A significantly lower serum folate has been reported in Israel for patients with severe Covid-19 infection (Itelman et al., 2020).

In their review authors Acosta-Elias et al., (2020) hypothesize that pregnant women are less likely to acquire Covid-19 infection while those infected have a higher chance of being asymptomatic (Acosta-Elias & Espinosa-Tanguma 2020).

Wiltshire et al. (2020) recommend a supplementation of folic acid at 5 mg as a therapeutic option for pulmonary hypertension and severe hypoxaemia as well as for patients affected by severe Covid-19 pneumonia.

A number of plant and animal foods are rich sources of folate, including spinach, kale, broccoli, avocado, citrus fruits, eggs, and beef liver.

2.4 HYDRATION

World Health Organization and Food and Agriculture Organization recommended to drink at least two litres of water a day, as the best choice, to stay hydrated and support our immune system. Water transports nutrients and compounds in blood, regulates our body temperature, gets rid of waste, and lubricates and cushions joints (WHO, 2020a; FAO, 2020).

3 RECENT FINDINGS REGARDING THE MOST COMMON NUTRITIONAL DEFFICIENCIES OF COVID-19 PATIENTS

Im et al. (2020) investigated the contents of vitamin B_1, B_6, B_{12}, vitamin D (25-hydroxyvitamin D), folate, selenium and zinc levels in 50 hospitalized patients with Covid-19. Covid-19 patients were deficient primarily in vitamin D (76.0 %) and selenium (42.0 %), while 6.0 % of patients showed deficiency in both vitamin B_6 and folate. Mechanically ventilated patients showed even higher deficiency in vitamin D (80.0 %) and 100 % deficiency in selenium (Im et al., 2020). Haematological parameter RDW is an indicator of red blood cells size, normal reference range of RDW-CV (Red Cell Distribution Width) in human red blood cells is 11.6–14.8 %. RDW is considered as biomarker indicative of cardiovascular disease (Borné et al., 2011). Higher RDW values are indication for inflammation and oxidative stress (Emans et al., 2011). Authors Batool et al., (2013) investigated the relationship between RDW and anaemia. They found 88.0 % of older people with iron deficiency to have RDW higher than 14.8 %. On the other hand 44.0 % of patients with vitamin B_{12} deficiency and 57 % with folate deficiency had RDW higher than 14.8 %. In their recent investigation authors Foy et al. (2020) found elevated RDW(>14.5 %) associated with an increased mortality risk in patients of all ages in 4 hospitals in Boston, USA. They found mortality rate of 11 % for patients with normal RDW and 31 % of patients with an elevated RDW. The literature data regarding the control of RDW by healthy lifestyle are scarce. In their attempt to clarify the impact of lifestyle on RDW, Loprinzi et al., (2015) studied dietary data collection and accelerometer-determined physical activity. They found physical activity inversely associated with RDW but not healthy eating. The limitation of this study is that nutritionally important components were not analysed precisely but it was estimated using 2 recall surveys. However, there are sufficient indications that food rich in iron, vitamin B_{12} and folate and probably other nutrients lower the RDW. Regarding physical activity, endurance sport seems to be beneficial in lowering RDW (Alis et al., 2015). Like for overall mortality, RDW seems to be a valuable predictor of mortality also for Covid-19 patients.

4 CONCLUSION

Individuals should be aware of healthy eating habits to reduce susceptibility to and long-term complications from Covid-19. Thus, now more than ever, wider access to healthy foods should be a top priority since people nutritional status strongly impact the outcome of Covid-19 patients. Recent nutritional status of Covid-19 patients shows a substantial deficiency in some nutritionally important compounds.

Eating a variety of nutritionally dense food is the recommended way to get nutrients we need. In some circumstances adding dietary supplements are recommended for specific groups like babies, pregnant women,

elderly and the people with weak immune system on one side as well as for people diagnosed with specific diseases. Dietary supplements are also recommended for people with inadequate nutrition or when nutrition is not the adequate source of specific nutrients. Before taking dietary supplements it is recommended to talk with the doctor or pharmacist.

It seems that Mediterranean diet pattern could represent a healthy nutritional pattern to be followed under Covid-19 circumstances. Key ingredients of Mediterranean cuisine include fresh fruits and vegetables, fish, protein-rich legumes, olive oil and whole grains with moderate amounts of wine and red meat.

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Metode za merjenje vsebnosti vode v tleh

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Methods for measuring soil water content

Abstract: Water has a significant influence on fundamental biophysical processes in the soil. It is one of the limiting factors for plant growth, which is why monitoring the water content in the field is particularly important in agriculture. In this article we present the methods currently used to measure the soil water content. We have described their functional principles, advantages, disadvantages and possible applications. Due to their widespread use in agriculture, we have focused on dielectric sensors, which are classified as electromagnetic methods. We have investigated the influence of soil properties on measurements with dielectric sensors and described possible methods for soil-specific calibration. In agriculture and environmental sciences, measurements of soil water content are particularly important for irrigation management. Irrigation based on measurements enables us to optimize the use of water resources and reduce the negative impact on the environment. For the correct functioning of such sensors it is necessary to check the suitability of the factory calibration function. Special attention is required when installing the sensors, as the presence of air gaps causes errors in the measurements.

Key words*:* measurement; water content; soil; precision irrigation; dielectric sensors; calibration; TDR; FD

Metode za merjenje vsebnosti vode v tleh

Izvleček: Voda ima pomemben učinek na temeljne biofizikalne procese v tleh. Je eden izmed omejitvenih dejavnikov rasti rastlin, zato je spremljanje vsebnosti vode v tleh posebej pomembno v kmetijstvu. V prispevku predstavljamo trenutno uporabljane metode za meritve vsebnosti vode v tleh, njihove principe delovanja, prednosti, slabosti in načine uporabe. Zaradi razširjenosti uporabe v kmetijstvu in okoljskih znanostih smo se osredotočili predvsem na merilnike, ki merijo dielektričnost tal in jih uvrščamo v skupino elektromagnetnih metod. Raziskali smo vpliv talnih lastnosti na meritve z merilniki, ki merijo dielektričnost tal in opisali možne načine talno specifične kalibracije. Meritve vsebnosti vode v tleh so v kmetijstvu pomembne predvsem za uravnavanje obrokov namakanja. Z namakanjem na podlagi meritev lahko optimiziramo izrabo vodnih virov in zmanjšamo negativne učinke na okolje. Za pravilno delovanje tovrstnih merilnikov je potrebno preveriti ustreznost tovarniške kalibracijske funkcije. Posebno pozornost je potrebno posvetiti vgrajevanju merilnikov, saj prisotnost zračnih prostorov povzroča napake v meritvah.

Ključne besede: meritve; vsebnost vode; tla; natančno namakanje; merilniki; kalibracija; TDR; FD

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1 UVOD

Vsebnost vode v tleh je ključni člen hidrološkega cikla, ki nadzira izmenjavo vode med atmosfero in podzemno vodo ter vpliva na večino fizikalnih, kemijskih in bioloških procesov, ki se pojavljajo v tleh (Zupanc in sod., 2020). Voda v tleh deluje kot mazivo in vezivo med talnimi delci, zato ima učinek na njihovo strukturno stabilnost. Velika toplotna prevodnost vode omogoča zmernost v dnevnih in sezonskih nihanjih temperature na površju tal. Kemijsko voda omogoča transport raztopljenih anorganskih snovi in suspendiranih organskih delcev, ki so vključeni v procese razvoja in degradacije tal. Količina vode ima pomemben vpliv na mnogo biofizikalnih procesov v tleh, vpliva na kaljenje semen, rast in mineralno prehrano rastlin ter mikrobno razgradnjo organske snovi (Topp & Ferré, 2002; Bittelli, 2011). Poznavanje variabilnosti vsebnosti vode na ravni parcele, je pomembno pri upravljanju kmetijskih površin v smislu maksimalnega povečanja pridelka ter zmanjševanja negativnih učinkov uporabe gnojil in fitofarmacevtskih sredstev na kakovost podzemne vode. Razumevanje in nadzorovanje variabilnosti vsebnosti vode v tleh je ključno pri izboljševanju strategij upravljanja namakanja z obzirom na rastlinsko pridelavo in optimalno izkoriščanje vodnih virov (Vereecken in sod., 2014). Upravljanje namakanja na podlagi meritev vsebnosti vode v tleh postaja v kmetijstvu vedno bolj razširjeno (Fares & Alva, 2000; Nemali & van Iersel, 2006; Zotarelli in sod., 2011; Sharma in sod., 2017; Li in sod., 2018; Souza in sod., 2019).

Namen prispevka je pregled relevantne svetovne literature s področja merilnih metod vsebnosti vode v tleh, pregled njihove uporabnost za namakanje ter strnjena predstavitev teme v slovenskem jeziku. Prispevek je namenjen vsem, ki se pri svojem raziskovalnem ali strokovnem delu soočajo s potrebo po spremljanju stanja vode v tleh.

2 METODE IN MATERIALI

2.1 METODE ANALIZE VIROV

Prispevek je nastal na podlagi pregleda literature 95 znanstvenih prispevkov, pridobljenih iz podatkovnih zbirk Web of Science in Google Scholar. Zbiranje literature je bilo sestavljeno iz treh vsebinskih delov, iz pregleda merilnih metod, kalibracije merilnikov, ki merijo dielektričnost tal in praktične uporabnosti meritev pri upravljanju namakanja. Najprej bomo predstavili različne metode meritev vsebnosti vode v tleh. Raziskali bomo njihovo zgodovinsko ozadje, pojasnili princip delovanja, prednosti in slabosti delovanja merilne metode in izpostavili tržno dostopne ter v raziskavah pogosto uporabljene modele merilnikov. V rezultatih bomo strnjeno, v obliki preglednice, povzeli glavne značilnosti različnih merilnih metod. V diskusiji se bomo, zaradi največje razširjenosti uporabe, osredotočili na merilnike, ki merijo dielektričnost tal in jih uvrščamo med elektromagnetne metode. Raziskali bomo vpliv spremenljivih talnih lastnosti na točnost meritev in opisali postopke talno specifične kalibracije tovrstnih merilnikov. Pregledali bomo njihovo uporabnost pri upravljanju namakanja.

2.2 NEPOSREDNE METODE MERITEV VSEBNOSTI VODE V TLEH

Neposredne metode določanja vsebnosti vode v vzorcu tal temeljijo na odstranitvi in meritvah količine iz vzorca odstranjene vode, ki jo lahko odstranimo s segrevanjem, ekstrakcijo in nadomestitvijo s topilom ali s kvantitativnimi meritvami reakcijskih produktov. Gravimetrično količino vode v tleh določamo po standardu (ISO 11465, 1993), kjer je vsebnost vode opredeljena kot razmerje med maso vode v vzorcu in maso suhega vzorca. Za gravimetrično določitev vsebnosti vode vzorec mineralnih tal sušimo na 105 ˚C do konstantne mase (v praksi je ta dosežena po vsaj 24 urah sušenja). Volumsko vsebnost vode v vzorcu tal, izračunamo z enačbama 1 in 2 (Topp & Ferré, 2002). Določanje vsebnosti vode po gravimetrični metodi je enostavno z vidika uporabe in potrebne opreme, vendar je tudi destruktivno, časovno zamudno in ne omogoča časovne vrste dinamičnih meritev (Dobriyal in sod., 2012).

$$\rho_{\text{tal}} = \frac{m_{\text{ss}}}{V_{\text{tal}}} \quad (1)$$

$$\theta(\%) = \left(\frac{\boldsymbol{m}_{\text{sv}} - \boldsymbol{m}_{\text{ss}}}{\boldsymbol{m}_{\text{ss}}}\right) \times \frac{\boldsymbol{\rho}_{\text{tal}}}{\boldsymbol{\rho}_{\text{vode}}} \times 100\ \% \quad (2)$$

ρ_{tal} je gostota tal (g cm^{-3}), m_{ss} je masa suhih tal (g), V_{tal} je volumen tal (cm^3), θ je volumski odstotek vode v tleh (vol. %), m_{sv} je masa svežega vzorca tal (g) in ρ_{vode} je gostota vode (g cm^{-3}).

2.3 POSREDNE METODE MERITEV VSEBNOSTI VODE V TLEH

S posrednimi metodami ne merimo dejanske vsebnosti vode v tleh, temveč neko drugo talno spremenljivko, ki se spreminja v odvisnosti od vsebnosti vode. Zato je potrebno med merjeno talno spremenljivko in vsebnostjo vode v tleh vzpostaviti razmerje, ki je opisano s kalibracijsko enačbo. Glavna prednost posrednih meritev vsebnosti vode v tleh je v tem, da niso destruktivne, mnoge izmed njih omogočajo avtomatično beleženje in shranjevanje izmerjenih podatkov (Muñoz-Carpena, 2004; Hignett & Evett, 2008). Med najpogosteje uporabljene posredne metode merjenja vsebnosti vode v tleh

uvrščamo: metodo nevtronskega sipanja, daljinsko zaznavanje podpovršinske vsebnosti vode v tleh in elektromagnetne metode.

2.3.1 Metoda nevtronskega sipanja

Gardner & Kirkham (1952) sta za meritve vsebnosti vode v tleh opisala metodo nevtronskega sipanja. Naprava oddaja hitre nevtrone iz razpadajočega radioaktivnega vira in zaznava gostoto upočasnjenih nevtronov okoli merilnika. Voda je glavni vir vodika v večini tal, zato je zaznana gostota nevtronov proporcionalna volumnu vsebnosti vode v tleh. Uporaba se opušča zaradi varnostnih razlogov, prisotnosti nizko radioaktivnega sevanja in s tem povezane kompleksne administracije, ki jo je potrebno zagotoviti za upravljanje merilnega instrumenta (Evett, 2000; Muñoz-Carpena, 2004). V preteklosti so metodo pogosto uporabljali kot referenčno pri kalibraciji takrat novih metod, ki temeljijo na meritvah dielektričnosti (Sheets & Hendrickx, 1995; Gaskin & Miller, 1996; Hanson & Peters, 2000).

2.3.2 Daljinsko zaznavanje podpovršinske vsebnosti vode

Od leta 1972 različne misije Landsat satelitov zagotavljajo pregledne in ponavljajoče multispektralne podatke o površju Zemlje (Lauer in sod., 1997). Prednost daljinskega zaznavanja je v meritvah na velikih površinah z enim merilnim instrumentom iz premične platforme (letalo ali satelit), kar je cenovno ugodno in izključuje napake, povzročene z variabilnostjo med merilniki. Pri daljinskemu zaznavanju vsebnosti vode pod površjem gre za optično zaznavanje, kamor uvrščamo metode, ki temeljijo na odbojnosti ali toplotne infrardeče metode ter metode mikrovalovnega zaznavanja vsebnosti vode pod površjem, t. i. pasivno ali aktivno zaznavanje. Nizke mikrovalovne frekvence so najprimernejše za daljinsko zaznavanje podpovršinske vsebnosti vode v tleh, saj se učinek vegetacije in hrapavosti površine zmanjša, poveča pa se globina prodora. Pri omenjenih frekvencah so oblaki in padavine praktično prosojni (Robinson in sod., 2008; Petropoulos in sod., 2015). Daljinsko pridobljene podatke je potrebno kalibrirati in ovrednotiti z *in-situ* meritvami vsebnosti vode pod površino (Rowlandson in sod., 2013; Gherboudj in sod., 2017).

2.3.3 Elektromagnetne metode

Njihova uporaba se je začela v 70. letih prejšnjega stoletja, z razvojem merilne tehnike TDR (Fellner-Feldegg, 1969). Elektromagnetne oziroma dielektrične metode so v praksi najpogosteje uporabljene posredne metode za določitev vsebnosti vode v tleh. Temeljijo na meritvah relativne dielektričnosti (ε_r), ki je sestavljena iz realnega (ε_r') in navideznega dela (ε_r''). Relativna dielektričnost določa hitrost potovanja elektromagnetnega vala ali impulza skozi tla. V združenem, poroznem mediju, kot so tla, ki jih sestavljajo trdna faza tal, zrak in voda, je dielektričnost določena na podlagi relativnega prispevka vsake izmed komponent. Relativna dielektričnost tekoče vode pri 20 °C (ε_r = 80) je mnogo večja od ostalih komponent, trdna faza tal (ε_r = 2 - 5) in zrak (ε_r = 1), zato je skupna dielektričnost primarno povzročena zaradi prisotnosti vode v tekočem agregatnem stanju (Topp & Ferré, 2002; Muñoz-Carpena, 2004). Relativna dielektričnost vode je sicer temperaturno pogojena in je za območje od 0,1 do 99 °C opisana z enačbo (Malmberg & Maryott, 1956):

$$\varepsilon_{r\,(\text{vode})} = 87{,}740 - 0{,}40008\,T + 9{,}398\,(10^{-4})\,T^2 - 1{,}410\,(10^{-6})\,T^3 \quad (3)$$

Topp in sod. (1980) so razvili empirično enačbo, ki opisuje razmerje med očitno relativno dielektričnostjo (ε_r) izmerjeno s TDR merilnim sistemom in vsebnostjo vode v tleh, ki je veljavna za večino mineralnih tal pri vsebnostih vode na območju med 0,1 in 0,5 $m^3\ m^{-3}$:

$$\theta_{\text{vode}} = -5{,}3 \times 10^{-2} + 2{,}92 \times 10^{-2}\,\varepsilon_r - 5{,}5 \times 10^{-4}\,\varepsilon_r^{\,2} + 4{,}3 \times 10^{-6}\,\varepsilon_r^{\,3} \quad (4)$$

Kasnejše izboljšave kalibracijskega razmerja na osnovi podatka o dielektričnosti zahtevajo predhodno poznavanje lastnosti tal, kot so tekstura, organska snov in gostota tal (Malicki in sod., 1996). Topp & Reynolds (1998) sta predlagala enačbo, ki predstavlja linearno razmerje med korenom realne dielektričnosti in volumsko vsebnostjo vode v tleh.

$$\theta_{\text{vode}} = 0{,}115\,\sqrt{\varepsilon_r'} - 0{,}176 \quad (5)$$

Linearno kalibracijsko razmerje ima pred polinomskim določene prednosti. Omogoča neposredno povezavo z dielektričnim mešanim modelom, ki omogoča fizično interpretacijo merjene lastnosti tal. Omogoča razvoj preproste, dvotočkovne kalibracije za izbran medij ter vodi do manj potencialnih napak ob ekstrapolaciji vsebnosti vode izven meja določenega polinomskega razmerja (Topp & Reynolds, 1998). Kljub razvitim splošnim kalibracijskim enačbam je potrebno izpostaviti, da metode, ki merijo relativno dielektričnost, merijo le približek. Zato imajo spremenljive lastnosti tal, kot so električna prevodnost, tekstura in gostota, v večji ali manjši meri vedno vpliv na meritve (Topp & Ferré, 2002). Spodaj opisane elektromagnetne metode uporabljajo empirično kalibrirana razmerja med volumsko vsebnostjo vode in signalom merilnika (čas, frekvenca, električna upornost oziroma impedanca, valovna faza) (Muñoz-Carpena, 2004).

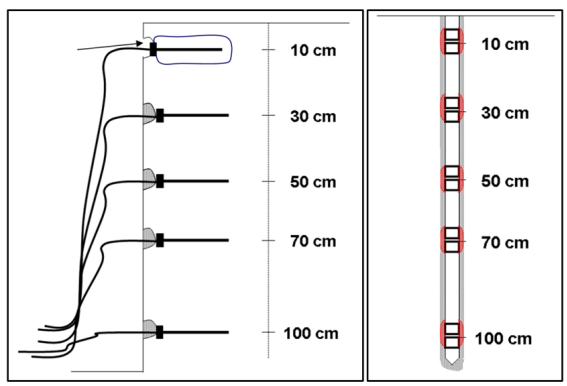
2.3.3.1 Merjenje odboja v časovnem prostoru (Time Domain Reflectometry - TDR)

Metoda z meritvami odboja v časovnem prostoru je bila prvotno uporabljena v telekomunikacijski industriji, za identifikacijo lokacij prekinitev v kablu. Hitrost širjenja signala s tipičnim odbojem na točki prekinitve kabla omogoča določitev lokacije poškodbe z uporabo analize časa potovanja (Noborio, 2001; Jones in sod., 2002). Fellner-Feldegg, (1969) je prvi opisal uporabo TDR tehnike za meritve električnih lastnosti materialov. Topp in sod. (1980) so prvi ugotovili empirično razmerje med relativno dielektričnostjo in volumsko vsebnostjo vode v tleh z različnimi teksturami in jo opisali s t. i. "Topp-ovo enačbo", prikazano v enačbi 4. Večina trenutno uporabljenih TDR merilnih sistemov po transmisijski liniji pošilja hitro rastoče, natančno časovno določene elektromagnetne impulze. Časovni zamik med odbojem impulza od začetka do konca transmisijske linije je uporabljen za določitev hitrosti širitve signala po tleh. Relativna dielektričnost tal v odvisnosti od vsebnosti vode nadzira hitrost širjenja signala. TDR merilni sistem potrebuje napravo, ki za potovanje po transmisijski liniji generira impulze visokih frekvenc, običajno 1 GHz. Visoke frekvence omogočajo odziv, ki je manj odvisen od talno značilnih lastnosti, kot so tekstura, gostota in temperatura. Merilnik je običajno sestavljen iz dveh do treh paralelnih kovinskih vilic, ki se jih vstavi v tla, kjer služijo kot valovodi. Sočasno TDR sistem uporablja tudi napravo, ki meri in digitalizira energijo transmisijske linije v intervalih običajno pod 300 pikosekund. Glavna slabost TDR merilnih sistemov je visoka cena zaradi kompleksne elektronike in izguba odboja v zelo slanih tleh (Noborio, 2001; Topp & Ferré, 2002; Muñoz-Carpena, 2004; Lekshmi in sod., 2014). Proizvajalec IMKO (Micromodultechnik Gmbh, Ettlingen, Germany), je razvil alternativni instrument, ki uporablja hitro naraščajoči časovni impulz, kot konvencionalni TDR sistem, vendar ne zajame in interpretira valovne oblike, da bi določili čas potovanja po merilniku. Naprava izračuna psevdo potovalni čas (t_p), ki ga je potrebno povezati z ε_r, kalibracijsko razmerje zagotovi proizvajalec. Merilna naprava se imenuje TRIME® - Time Domain Reflectometry with Intelligent Micromodule Elements (Evett & Parkin, 2005; IMKO, 1996, cit. po Dettmann & Bechtold, 2018).

2.3.3.2 Meritve v frekvenčnem prostoru (Frequency Domain - FD)

1) Kapacitivnost

Merilne elektrode merilnikov, ki temeljijo na principu kapacitivnosti, delujejo na način, da tla na njih delujejo kot dielektrik v kondenzatorju v kapacitivno - induktivnem resonančnem vezju. Induktivnost v vezju je fiksna, nato iz izmerjene resonančne frekvence določimo relativno dielektričnost tal. Podobno kot pri TDR, se relativna dielektričnost uporabi za določitev vsebnosti vode v tleh (Topp & Ferré, 2002). Elektrode kapacitivnostnih merilnikov so lahko v obliki para vzporednega vilic za izvajanje točkovnih meritev. Drug tip merilnih naprav je cevaste oblike, na kateri so vertikalno nameščene merilne elektrode, napravo se na terenu vstavi v tla zakopano cev in omogoča hkratne meritve na več globinah. Ob nameščanju cevi lahko pride do motenj v tleh okoli cevi, nastanejo lahko zračni žepi, ki povzročajo napake v meritvah. Merilniki običajno delujejo z delovno frekvenco pod 100 MHz. Pri tako nizkih frekvencah se dielektričnost tal spreminja in je ocena vsebnosti vode bolj podvržena spremenljivim lastnostim tal (Starr & Paltineanu, 1998; Muñoz-Carpena, 2004; Evett in sod., 2006; Matula



Slika 1: Primerjava vgradnje točkovnih merilnikov (levo) in več globinskih merilnikov, ki se jih vstavi v dostopno cev (desno) (Zupanc in sod., 2009)

Figure 1: Comparison of the installation of point sensors (left) and multilevel sensors inserted into the access tube (right) (Zupanc et al., 2009)

in sod., 2016; Roberti in sod., 2018). Slika 1 prikazuje v tla vgrajene točkovne merilnike (levo) in več globinski merilnik, vstavljen v dostopno cev (desno).

2) Merjenje odboja v frekvenčnem prostoru (Frequency Domain Reflectometry - FDR)

Pri meritvah odboja v frekvenčnem prostoru je frekvenca oscilatorja nadzorovana v določenem območju za določitev resonančne frekvence, pri kateri je amplituda največja, kar se uporablja za določitev vsebnosti vode v tleh. Kljub podobnemu principu delovanja metodi kapacitivnosti, FDR metoda zbira podatke iz širokega nabora frekvenc (Dean in sod., 1987; Lekshmi in sod., 2014).

2.3.3.3 Merjenje odboja v amplitudnem prostoru (Amplitude Domain Reflectometry - ADR)

1) Kompleksna upornost - Impedanca

Signal oscilatorja se širi po transmisijski liniji vilic merilnika. Če se impedanca vilic razlikuje od tiste v transmisijski liniji, se del naključnega signala odbije nazaj po liniji proti izvoru signala. Odbiti del ovira vpadni signal in povzroči napetostni stoječi val na transmisijski liniji, kar predstavlja spremembo amplitude vzdolž linije (Gaskin & Miller, 1996). Impedančni merilniki uporabljajo oscilator za generacijo sinusnega signala v obliki elektromagnetnega vala fiksne frekvence (npr. 100 MHz), ki se uporablja pri koaksialni transmisijski liniji. Merilniki so običajno sestavljeni iz več paralelnih kovinskih vilic (Muñoz-Carpena, 2004). V literaturi pogosto uporabljena impedančna merilnika sta ThetaProbe (Delta-T Devices Ltd., Cambrige, GB) in HydraProbe (Stevens Water Monitoring System Inc., Portland, USA) slednji ločeno meri realni (ε_r') in navidezni (ε_r'') del relativne dielektričnosti (Seyfried in sod., 2005; Vaz in sod., 2013; Ojo in sod., 2015; Matula in sod., 2016). Nemali in sod. (2007) so primerjali delovanje ECH_2O (Decagon Devices Inc., Pullman, WA, USA) merilnika, ki deluje na podlagi kapacitivnosti in ThetaProbe ML2X (Delta-T Devices), ki deluje po principu impedance. Ugotovili so, da imata električna prevodnost in temperatura tal velik vpliv na meritve z ECH_2O, medtem ko vpliva na meritve s ThetaProbe niso zaznali. Podobno Fares in sod. (2011) poročajo večjo natančnost meritev s ThetaProbe ML2X v primerjavi s kapacitivnostnimi EC-10 (Decagon Devices). Tudi rezultati Sharma in sod. (2017), ki so primerjali delovanje impedančnih HydaProbe merilnikov in 5TM (Deacagon devices), ki delujejo po metodi kapacitivnosti, govorijo v prid impedančnim merilnikom.

Merilniki, ki delujejo po principu meritev v frekvenčnem prostoru ter kompleksne upornosti, so v primerjavi s TDR merilnimi sistemi cenejši zaradi uporabe nižje frekvenčnega standardnega vezja. Lahko jih povežemo s konvencionalnimi shranjevalniki podatkov (DC izhodni signal) in jih uporabljamo v slanih tleh z veliko električno prevodnostjo. Slabost FD metod je večja občutljivost na temperaturo in spremenljive lastnosti tal, zaradi česar je potrebna talno specifična kalibracija. Tudi pri uporabi ADR impedančnih merilnikov je za zanesljive meritve talno specifična kalibracija priporočljiva (Muñoz-Carpena, 2004; Rowlandson in sod., 2013). V primerjavi s TDR merilnim sistemom, Evett in sod. (2006) poročajo veliko temperaturno občutljivost pri proučevanih kapacitivnostnih merilnikih, ki je večja pri večjih vsebnostih vode.

2.3.3.4 Časovni odziv (Time Domain Transmission - TDT)

Metoda meri čas širjenja elektromagnetnega impulza, enosmerno po transmisijski liniji. Kljub temu, da je metoda podobna TDR, potrebuje električno povezavo na začetku in na koncu transmisijske linije. Ne glede na to je vezje enostavnejše od tistega, ki je uporabljen pri TDR merilnih sistemih, zato so TDT merilniki cenejši (Muñoz-Carpena, 2004; Blonquist in sod., 2005).

2.3.3.5 Fazni prenos

Po potovanju po fiksni razdalji sinusni val izkaže fazni zamik, ki je relativen fazi izvora. Fazni zamik je odvisen od dolžine potovanja po transmisijski liniji, frekvence in hitrosti širjenja, ki je odvisna od vsebnosti vode v tleh. Zato lahko vsebnost vode določimo s faznim zamikom za dano frekvenco in razdaljo potovanja (Muñoz-Carpena, 2004).

2.3.3.6 Zemeljski radar (Ground Penetrating Radar - GPR)

Zemeljski radar je geofizikalna metoda, ki omogoča visoko ločljive, tridimenzionalne posnetke pod površjem (Knight, 2001). Kljub temu, da so znanstveniki že na začetku prejšnjega stoletja začeli proučevati širjenje radijskih valov nad in vzdolž površine Zemlje, sta Waite & Schmidt (1962) prva poročala o ponovljivem radiofrekvenčnem prodoru v podzemno površino skozi naravni material, ledeno ploskev na Grenlandiji (Annan, 2002). Zemeljski radar generira in oddaja radijske frekvence iz širokega nabora kotov iz antene v tla. Ločena antena prejema tako prenešene, kot tudi odbite signale. Za meritve iz površine tal, signali prihajajo do sprejemne antene po treh primarnih poteh: 1) neposredno preko zraka med oddajno in sprejemno anteno, 2) neposredno skozi bližnjo površino tal in anteno ter 3) posredno po oddajanju iz objektov ali meja plasti pod površjem tal. Iz zaznanih signalov je mogoče določiti tako hitrost signala, ki potuje skozi tla, kot tudi slabitev signala. Nato lahko z analizo, podobno kot se uporablja pri TDR, sklepamo na vsebnost vode in električno prevodnost tal, po katerih je radijska frekvenca potovala do antene (Topp & Ferré,

2002). Za razliko od TDR merilnih naprav, GPR antene ne potrebujejo direktnega kontakta s tlemi in so lahko premične. Prednost metode je v ne invazivni sposobnosti prostorske zaznave vsebnosti vode. Merilno območje je na vmesni skali med točkovnimi meritvami in daljinskim zaznavanjem vsebnosti vode pod površjem. Slabost GPR je v količini zahtevanega znanja, ki ga upravljalec potrebuje za pridobitev kakovostnih podatkov in ustrezno interpretacijo (Huisman in sod., 2001; Davis & Annan, 2002; Robinson in sod., 2008). Zemeljski radar je ob primerni kalibraciji z gravimetričnim standardom primeren za zaznavo vsebnosti vode v koreninskem območju tudi za namene upravljanja namakanja (Shamir in sod., 2018).

2.3.4 Meritve matričnega potenciala vode v tleh

Merilne naprave, ki merijo matrični potencial vode v tleh, nam podajo informacijo o sili, s katero je voda vezana na talne delce. Vsi t. i. tenziometrični instrumenti vsebujejo porozni material, ki je v stiku s tlemi in skozi katerega voda lahko prehaja. Med njih uvrščamo tenziometre, avtomatske merilnike za meritve vodnega potenciala in metodo upornostnih blokov (mavčni bloki ali granulirani matrični merilniki). Tenziometri natančno merijo potencial vode v tleh v območju rastlinam dostopne vode, kar je zelo uporabo v hortikulturi. Temperatura in raztopljene soli v talni raztopini na meritve nimajo vpliva. Glavna slabost tenziometričnih merilnikov je pojav kavitacije v suhih tleh. Navadni tenziometri delujejo le do tenzije 80 do 85 kPa, zato je za nemoteno delovanje potrebno vzdrževanje ustrezne tenzije oziroma vsebnosti vode v tleh (Muñoz-Carpena, 2004; Zupanc & Pintar, 2007; Heng & Evett, 2008; Pardossi in sod., 2009).

2.4 NEGOTOVOSTI IN POMANJKLJIVOSTI ANALIZE METOD

V prispevku uporabljena znanstvena literatura je bila, za vsak vsebinski sklop, izbrana na podlagi prebranih izvlečkov. Pomanjkljivost analize je v tem, da smo količino literature morali omejiti in preglednega prispevka nismo izvedli na podlagi vse obstoječe literature. Zato je negotovost analize lahko povzročena zaradi subjektivnosti izbire obravnavanih znanstvenih prispevkov.

3 REZULTATI IN RAZPRAVA

3.1 PRIMERJAVA IN UPORABA METOD MERITEV

Za večjo preglednost obravnavanih metod smo pripravili preglednico 1, ki prikazuje najpogosteje uporabljene metode za določevanje vsebnosti vode v tleh. Metode meritev smo primerjali glede na merjene parametre, način vzorčenja, obliko merilnika, prostorsko skalo zaznave, frekvenco meritev, kakovost podatkov in zahtevnost obdelave podatkov. Pri elektromagnetnih metodah smo dodali še ceno posameznega merilnega instrumenta, ki ne vključuje shranjevalnika podatkov. Določeni merilniki, poleg meritev vsebnosti vode v tleh, omogočajo sočasne meritve temperature in električne prevodnosti. Z daljinskim zaznavanjem lahko dobimo zgolj informacijo o vsebnosti vode neposredno pod površjem. Glavne značilnosti, ki vplivajo na izbiro posamezne merilne metode, vsekakor vključujejo časovno frekvenco meritev, težavnost obdelave podatkov in ceno. Zvezno časovno serijo meritev omogočajo le merilniki, ki merijo dielektričnost tal, a hkrati omogočajo le točkovne meritve. Z zemeljskim radarjem ali daljinskim zaznavanjem lahko z istim merilnim instrumentom meritve izvedemo na večjih površinah, a za interpretacijo podatkov potrebujemo usposobljeno osebje s specifičnim znanjem, medtem ko pri ostalih metodah uporabnik ne potrebuje predhodnega znanja. Podatki o vsebnosti vode v tleh so običajno izraženi z volumskimi odstotki.

Preglednica 1: Pregled najpogosteje uporabljenih metod za določevanje vsebnosti vode v tleh (povzeto po Teixeira in sod., 2003; Muñoz-Carpena, 2004; Robinson in sod., 2008; Pardossi in sod., 2009; Petropoulos in sod., 2015)

Table 1: An overview of the most commonly used methods for determination of soil water content (summarized by Teixeira et al., 2003; Muñoz-Carpena, 2004; Robinson et al., 2008; Pardossi et al., 2009; Petropoulos et al., 2015)

		Merjeni parametri	Način vzorčenja	Oblika merilnika	Prostorska skala	Frekvenca meritev	Kakovost podatkov	Obdelava podatkov	Cena (€)
Gravimetrično		θ (vol. %)	vzorčenje tal znanega volumna	/	točkovna (običajno V = 100 cm^3)	kampanjska, ni zvezna	visoka	preprosta	
Elektromagnetne metode	TDR	θ (vol. %), EC (dS m^{-1})	vgradnja v posamezno plast talnega profila	2 ali 3 vzporedne kovinske vilice TRIME (vilice prevlečene s PVC)	točkovna, po dolžini vilic (10 - 30 cm) in 1 cm okoli vilic na vse strani	zvezna, izmerjeni podatki se samodejno vpisujejo v shranjevalnik podatkov, v željenih časovnih intervalih (npr. urnih)	visoka	preprosta	1500 - 5000
	TDT	θ (vol. %), EC (dS m^{-1}), T (°C)	vgradnja v posamezno plast talnega profila	zunanja kovinska elektroda je ukrivljena in sklenjena			po talno specifični kalibraciji, visoka	preprosta	150 - 500
	FD	θ (vol. %), EC (dS m^{-1}), T (°C)	vgradnja v posamezno plast talnega profila več globinske vstavimo v dostopno cev	v mnogo različnih oblikah, 2 ali 3 vzporedne kovinske vilice več globinski imajo elektrode nameščene koncentrično vzdolž plastične cevi	točkovna, običajno 90 % zaznave pride od 2 cm v stran od merilnih elektrod, vilice so običajno dolge 5 - 10 cm več globinski merijo po profilu v dostopni cevi, z radijem 5 - 10 cm			preprosta	50 - 1000
	ADR: Impedanca		vgradnja v posamezno plast talnega profila	centralna kovinska vilica, s tremi kovinskimi vilicami, ki jo obkrožajo	točkovna, volumen zaznave znotraj zunanjih vilic (dolge 5 - 10 cm)			preprosta	450 - 500
	GPR	θ (vol. %)	premična zaznava po površini	običajno na premični platformi v obliki vozička	med točkovnimi meritvam in daljinsko zaznavo	kampanjska, ni zvezna	visoka	zahtevna	12000 - 15000
Daljinsko zaznavanje		θ pod površjem	snemanje	na premični platformi (letalo, satelit)	lokalna, globalna	kampanjska ali zvezna v več dnevnih intervalih	nizka	zahtevna	

3.2 VPLIV LASTNOSTI TAL NA MERITVE Z ELEKTROMAGNETNIMI METODAMI

Zaradi kompleksnih interakcij med elektromagnetnimi valovi in talnimi komponentami ni mogoče vzpostaviti edinstvenega kalibracijskega razmerja za določanje vsebnosti vode v tleh (Topp & Ferré, 2002). Dejavniki, ki vplivajo na dielektričnost tal, so tekstura in mineralna sestava tal (Gong in sod., 2003; Vaz in sod., 2013; Provenzano in sod., 2015; Iwata in sod., 2017; Hajdu in sod., 2019), delež in vrsta organske snovi (Fares in sod., 2016; Kassaye in sod., 2019), gostota (Zettl in sod., 2015; Matula in sod., 2016; Parvin & Degre, 2016), električna prevodnost tal (Varble & Chávez, 2011; Sevostianova in sod., 2015; Matula in sod., 2016) in temperatura (Bogena in sod., 2007; Chanzy in sod., 2012; Mittelbach in sod., 2012; Bogena in sod., 2017; Walthert & Schleppi, 2018; González-Teruel in sod., 2019). Interakcija elektromagnetnih valov in glinenih mineralov z veliko kationsko izmenjevalno kapaciteto in površino delcev še ni popolnoma jasna (Evett & Parkin, 2005). Gostejša kot so tla, večje je volumsko razmerje trdnih delcev v primerjavi z zrakom in večja je dielektričnost suhih tal. Točnost meritev se izboljša z naraščajočo gostoto substrata, kar poudarja pomembnost dobrega stika med merilnikom in substratom (Gong in sod., 2003; Matula in sod., 2016). V splošnem merilniki izkazujejo velike razlike med organskimi tlemi ter tlemi z veliko prevodnostjo, v primerjavi z mineralnimi. Pri organskih tleh so surovi izhodni podatki merilnika v splošnem manjši kot pri mineralnih tleh, kar je pričakovano zaradi manjše gostote in večje poroznosti organskih materialov (Vaz in sod., 2013).

3.3 TALNO SPECIFIČNA KALIBRACIJA MERILNIKOV

Napake pri meritvah vsebnosti vode v tleh imajo lahko močne negativne posledice pri upravljanju namakanja (Soulis in sod., 2015). Zato je za ustrezno delovanje merilnikov potrebna točnost meritev, ki ob uporabi zgolj proizvajalčeve kalibracijske funkcije ni vedno zagotovljena (Seyfried in sod., 2005; Spelman in sod., 2013; Matula in sod., 2016; Parvin & Degre, 2016; Lima in sod., 2018; Roberti in sod., 2018; Domínguez-Niño in sod., 2019).

Standardna referenčna metoda kalibracije elektromagnetnih merilnikov je gravimetrična. Poznamo laboratorijsko in terensko kalibracijo merilnikov. Laboratorijsko kalibracijo lahko izvedemo na porušenem in neporušenem vzorcu tal. Pri laboratorijski kalibraciji na porušenem vzorcu pridobimo vzorec tal, ga posušimo na zraku, presejemo skozi 2 ali 5 mm sito, zmešamo z znano količino vode ter ga v primerno veliki posodi, zgostimo na naravno gostoto tal. Z merilnikom, vstavljenim v tako pripravljen vzorec, naredimo odčitek in takoj vzorčimo z manjšim cilindrom za gravimetrično določitev vode. Z vsako meritvijo dobimo le eno kalibracijsko točko, zato moramo postopek ponavljati pri različnih dodanih količinah vode (Starr & Paltineanu, 2002). V primerjavi s tradicionalno presejanimi tlemi je kalibracija v neporušenem vzorcu tal po mnenju mnogih avtorjev, naprednejša zaradi ohranitve naravne strukture tal in skeleta (Weitz in sod., 1997; Provenzano in sod., 2015; Holzman in sod., 2017). Neporušen vzorec tal nasičimo z vodo, vanj vstavimo merilnik ter ob sušenju vzorca na zraku tehtamo celoten vzorec znanega in ustreznega volumna za kasnejšo gravimetrično določitev vsebnosti vode ter sočasno odčitamo surove izhodne podatke merilnika. Ob prisotnosti skeleta, čigar dielektrične lastnosti se od fine frakcije značilno razlikujejo, lahko prihaja do občutnega odklona v primerjavi s kalibracijskim razmerjem fine frakcije (Coppola in sod., 2013). Weitz in sod. (1997) so med prvimi laboratorijsko kalibracijo TDR merilnega sistema izvedli v neporušenih talnih monolitih tropskih tal vulkanskega izvora. TDR je ob uporabi Topp-ove enačbe podcenjeval dejansko vsebnost vode. Holzman in sod. (2017) so impedančne merilnike ThetaProbe ML2X (Delta-T Devices), ki so v merilnem omrežju za validacijo satelitskih ocen vsebnosti vode, kalibrirali v neporušenem vzorcu tal. Prav tako so Roberti in sod. (2018) iz 33 lokacij v ZDA kalibrirali kapacitivnostne merilnike EnviroSCAN (Sentek Environmental Technologies, Kent Town, Australia), na preko 150 neporušenih vzorcev tal. Ugotovili so, da se proizvajalčeve kalibracijske enačbe slabo prilegajo podatkom pri skoraj vseh tipih tal. Poudarili so pomembnost kalibracije merilnikov, še posebej, če se vsebnost vode meri na različnih talnih tipih.

V primeru terenske kalibracije merilnike vgradimo v tla ter ob različnih okoljskih razmerah (suho, vlažno, mokro) vzorčimo tla v bližini merilnika za gravimetrično določitev vsebnosti vode ter meritve povežemo z odčitki merilnika (Geesing in sod., 2004). Terenska kalibracija je bolj delovno in časovno zahtevna od laboratorijske, vzorčne točke so lahko zelo blizu in pokrivajo majhen razpon vsebnosti vode v primerjavi z laboratorijsko kalibracijo (Kinzli in sod., 2012). Kassaye in sod. (2019) navajajo, da je kljub izboljšani točnosti meritev po laboratorijski kalibraciji pridobljena kalibracijska funkcija še vedno podcenjevala vsebnost vode v tleh. Preprost postopek kalibracije na terenu pri naravni gostoti tal je zagotovil najbolj točno oceno volumske vsebnosti vode. Visconti in sod. (2014) so ocenjevali laboratorijsko in terensko kalibracijo kapacitivnostnih merilnikov 10HS in 5TE (Decagon Devices). Opisali so določene dejavnike, ki v laboratorijskih razmerah niso prisotni: 1) variabilnost med merilniki, 2) lastnosti tal, kot so raztezanje in

krčenje med namakalnimi cikli, prisotnosti skeleta, korenin, talne favne, temperature spremembe in 3) dejanska razlika med različnimi vsebnostmi vode vzorčnih mest, ki je posledica prostorske variabilnosti. Zato priporočajo metodološki pristop, ki temelji na kalibraciji v laboratoriju in kasnejši validaciji na terenu.

3.4 UPORABNOST MERITEV

Merjenje vsebnosti vode v tleh uporabljamo za izračun vodne bilance v hidrološkem krogu, npr. za merjenje bogatenja podzemnih voda (Šerjak in sod., 2019; Zupanc in sod., 2020). Sicer so tehnološke inovacije, ki lahko izboljšajo trajnost namakanja v kmetijstvu, pomembne za optimalno izrabo vodnih virov in energije (Adeyemi in sod., 2017; Cvejić in sod., 2020). Učinkovitejšo izrabo vode v kmetijstvu lahko dosežemo z dodajanjem vode v točno določenih količinah, na določeno mesto ob določenem času (Lozoya in sod., 2016). Svetovno najbolj razširjena strategija upravljanja namakanja temelji na modelih vodne bilance (Zupanc in sod., 2012), vendar je zaradi naglega tehnološkega napredka povečano zanimanje za upravljanje namakanja, ki temelji na meritvah vsebnosti vode v tleh (Millan in sod., 2019). Zvezne meritve vsebnosti vode v tleh omogočajo delovanje avtomatiziranih namakalnih sistemov (Raine in sod., 2007). Napake v podatkih lahko sprožijo napačne odločitve, kar vodi do neučinkovite izrabe virov, povečanja stroškov in okoljskih tveganj. Zato mora biti pri avtomatiziranih sistemih z majhno intervencijo človeka posebna skrb namenjena kakovosti podatkov (Thessler in sod., 2011).

Zotarelli in sod. (2011) poročajo znatno zmanjšanje porabljene vode za namakanje zelene paprike ob namakanju na podlagi meritev, v primerjavi z namakanjem ob fiksnem času. Papanikolaou & Sakellariou-Makrantonaki (2013) sta preučevala dva pristopa namakanja sirka: 1) količina dodane vode do 100 % dnevne evapotranspiracije, dodane na dva dni in 2) avtomatsko namakanje do 100 % dnevne evapotranspiracije, na podlagi meritev vsebnosti vode v tleh. V drugem primeru sta ugotovila manjšo porabo vode, večjo učinkovitost izrabe vode in prihranke energije. Blonquist in sod. (2006) poročajo za 16 % manjšo porabo vode ob namakanju trate na podlagi meritev vsebnosti vode v tleh s TDT merilniki, v primerjavi z namakanjem na ocenah evpotranspiracije, pridobljenih iz vremenske postaje. Millan in sod. (2019) so testirali avtomatski namakalni sistem, ki deluje na podlagi meritev kapacitivnih merilnikov in omogoča vzpostavitev reguliranega deficitnega namakanja. Avtomatizirano namakanje je uspešno vzpostavilo strategijo deficitnega namakanja, ki ni povzročilo sušnega stresa med občutljivejšimi razvojnimi fazami japonske slive. Natančno namakanje na podlagi meritev vsebnosti vode v tleh se pogosto uporablja pri vzgoji sadik, kjer rastline rastejo v loncih omejenega volumna, zaradi česar je privzem vode omejen in so rastline hitreje izpostavljene sušnem stresu (Bayer in sod., 2013; Incrocci in sod., 2019; Kaptein in sod., 2019).

Skeletna tla lahko ovirajo nameščanje merilnikov (Spittlehouse, 2000). Prisotnost talnih por oziroma zračnih prostorov med merilnikom in tlemi rezultira v merilnih napakah (Cosh in sod., 2005), zato je potrebna posebna pozornost ob nameščanju merilnikov. V praksi na meritve na dolgi rok vplivajo obdelava tal, gnojenje in namakanje - pri majhnih vsebnostih vode se v določenih tleh lahko pojavijo razpoke, ki vodijo do merilnih napak zaradi prisotnosti zraka. Vse omenjene učinke je potrebno ustrezno nasloviti in primerno vzdrževati tla okoli merilnika (Chen in sod., 2013). Za avtomatizacijo in izboljšanje namakalnih praks so trenutno najbolj v uporabi merilni sistemi, ki uporabljajo avtonomne merilnike, ki podatke prenašajo na bazno postajo brezžično, preko nekakšnega radijskega oddajnika. Privlačnost tovrstnih merilnih sistemov je v možnosti delovanja na velikih površinah, meritvah na mnogih točkah in možnosti namestitve na odročnih lokacijah, kar preferenčno vodi do merilnikov, ki imajo majhno porabo energije, so poceni in imajo primerno tovarniško kalibracijo (Robinson in sod., 2008; Vellidis in sod., 2008).

4 SKLEPI

V preglednem prispevku smo, v slovenskem jeziku, povzeli za kmetijstvo relevantne metode meritev vsebnosti vode v tleh, ki se sicer uporabljajo tudi v drugih okoljskih znanostih. Bralcem smo omogočili možnost vpogleda v ozadje delovanja merilnih sistemov in njihovo primernost za različne načine uporabe. Ugotovili smo, da lahko informacijo o stanju vode v tleh pridobivamo na različne načine. S tenziometričnimi metodami izmerimo matrični potencial vode v tleh. Volumsko vsebnost vode lahko merimo neposredno z gravimetrično metodo ali posredno. Posredne metode temeljijo na meritvah neke druge lastnosti, ki je odvisna od vsebnosti vode v tleh. Med seboj se razlikujejo v prostorski skali meritev, kakovosti podatkov, zahtevnosti obdelave podatkov, zveznosti meritev in ceni. Za različne načine uporabe so primerne različne metode meritev, nobena ni univerzalno primerna za vse namene. Izmed nabora posrednih metod meritev se v kmetijstvu na prostorski skali njive ter v okoljskih znanostih, zaradi uporabniku vedno bolj prijazne rabe in prenosa podatkov, najpogosteje uporabljajo merilniki, ki merijo dielektričnost tal.

Upravljanje namakanja na podlagi meritev vsebnos-

ti vode v tleh postaja v kmetijstvu vedno bolj razširjeno, saj omogoča dovajanje vode le na določenem mestu, kjer vode primanjkuje. V luči podnebnih sprememb in vedno večjih pritiskov na vodne vire je zmanjšana poraba vode za namakanje zelo dobrodošla. Ocenjujemo, da bi bilo koristno spodbujati pridelovalce za privzem namakalnih praks, ki temeljijo na meritvah vsebnosti vode v tleh. Pomembno je tudi zavedanje o merilnih napakah, ki imajo lahko negativne posledice pri upravljanju namakanja, zato vsem uporabnikom svetujemo, da za večjo točnost meritev preverijo, če je potrebna talno specifična kalibracija merilnikov. Poleg tega je posebno skrb potrebno nameniti namestitvi merilnikov, ki mora biti izvedena pravilno in na reprezentativnem mestu.

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