We thank students, staff, and external collaborators
for a productive and successful 2022
and we wish that the coming holidays will be full of human kindness
and 2023 will be an inspiring year full of health, joy, and success!

Biotechnical Faculty

118 • 4
2022

ACTA AGRICULTURAE SLOVENICA

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z zahvalo študentom, zaposlenim in zunanjim sodelavcem
za dobro sodelovanje in uspešno zaključeno leto 2022
in z željo, da bodo prihajajoči prazniki
polni človeške topline in leto 2023 navdušujoče, polno zdravja, veselja in uspehov!

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Field performance of cryopreserved seed-derived tomato plants and post-thaw survival of viral-infected meristems

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Field performance of cryopreserved seed-derived tomato plants and post-thaw survival of viral-infected meristems

Abstract: The effectiveness of different cryopreservation techniques of tomato meristems isolated from viral-infected plants 'Irishka' cultivar was determined. The pieces of stem were protected with dimethyl sulfoxide and propylene glycol and cooled in vapour phase of liquid nitrogen (–170 °C). For the vitrification and droplet-vitrification protocols, the meristems were treated with loading solution and dehydrated with different plant vitrification solutions (PVS1 modified, PVS2, 88 % PVS3, PVSN). The samples were placed to sterilized aluminum foil pieces, in 1.2 ml cryovials or in 50 µl aluminum pans for differential scanning calorimetry and were directly immersed into liquid nitrogen. According to the dehydration technique, the meristems were dehydrated with sterile airflow for 120 min. The post-thaw survival rates of meristems (from 34.2 to 78.5 %) were observerved only for 50 µl aluminum pans and airflow dehydration. We determined the productivity of plants, obtaned from cryopreserved seeds ('Seven', 'Potiron Ecarlate' and 'Druzhba' cultivars). We observed increasing in total and marketable yields for the plants grown from the cryopreserved seeds for all the cultivars. Total number of diseased plants decreased by 33 % for 'Seven', for 'Potiron Ecarlate' it did by 6.7 %, for that of 'Druzhba' the total percentage of sick and healthy plants did not differ after seeds cryopreservation.

Key words: seed cryopreservation; dehydration; meristem cryopreservation; plant vitrification solution; *Solanum lycopersicum* L.; yield

Uspevanje paradižnika v poljskem poskusu, vzgojenega iz zamrznjenih semen in preživetje z virusi okuženih meristemov po odtajanju

Izvleček: V raziskavi je bila določena učinkovitost različnih metod shranjevanja meristemov paradižnika z zamrzovanjem, pridobljenih iz z virusi okužene sorte 'Irishka'. Koščki stebel so bili zaščiteni z dimetil sulfoksidom in propilen glikolom in ohlajeni v parah tekočega dušika (–170 °C). Za vitrifikacijo je bil uporabljen protokol kapljične vitrifikacije, meristemi so bili obdelani s standardno nosilno raztopino in dehidrirani z različnimi vitrifikacijskimi raztopinami za rastlinska tkiva (modificirana PVS1, PVS2, 88 % PVS3, PVSN). Vzorci so bili potem položeni na koščke sterilizirane aluminijeve folije v 1,2 ml epruvetkah za zmrzovanje ali v 50 µl aluminijastih posodicah za diferencialno vrstično kalorimetrijo, nakar so bili neposredno potopljeni v tekoči dušik. Glede na dehidracijske tehnike so bili vzorci dehidrirani s sterilnim zrakom za 120 min. Preživetje meristemov po odtajanju (od 34,2 do 78,5 %) je bilo opazovano samo za tiste v 50 µl aluminijastih posodicah, ki so bili dehidrirani z zračnim tokom. Določena je bila produktivnost obravnavanih sort, pridobljenih iz semen, shranjenih z zmrzovanjem ('Seven', 'Potiron Ecarlate' in 'Druzhba'). Za vse sorte, ki so bile vzgojene iz semen, shranjenih z zmrzovanjem, je bilo ugotovljeno povečanje celokupnega in tržnega pridelka. Število okuženih rastlin, vzgojenih iz semen po shranjevanju z zmrzovanjem, se je za sorto 'Seven' povečalo za 33 % in za sorto 'Potiron Ecarlate' za 6,7 %. Pri sorti 'Druzhba' se celoten odstotek okuženih in zdravih rastlin ni razlikoval po shranjevanju semen z zmrzovanjem.

Ključne besede: shranjevanje semen in meristemov z zmrzovanjem; dehidracija; raztopine za vitrifikacijo rastlinskih tkiv; *Solanum lycopersicum* L.; pridelek

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1 INTRODUCTION

Conservation of plant genetic resources is important not only for biodiversity preservation, but also for supporting the biotechnology and plant breeding programs. In this regard, the cryopreservation is a powerful tool for a long-term conservation of genetic diversity of plant species and crop improvement, as well it can be considered as an extra strategy even for species with orthodox seeds (Coste et al., 2015). Recently, cryopreservation has also been used to eliminate various pathogens in numerous plant species, so this was coined as cryotherapy (Wang and Valkonen, 2008, 2009; Vieira et al., 2015; Shin et al., 2013). However, there are conflicting findings on the effect of cryopreservation on different crops seeds. The field performance of cryopreserved garlic plantlets under *in vivo* conditions showed the superiority of the morphological traits that increased gradually with the growth improved the net photosynthetic rate, bulb diameter, bulb mass and clove number per bulb (Liu et al., 2019). Cejas et al. (2012) did not observe any phenotypic changes during the early germination stages (0-14 days) of the cryopreserved *Phaseolus vulgaris* L. seeds; but several convincing effects of seeds cryopreservation were revealed at the biochemical level. Arguedas et al. (2018) reported that the significant differences between adult plants derived from cryopreserved and control maize seeds by leaf indices, internodes and ears numbers, plant height and mass of seeds were not observed in field performance. The wild *Solanum lycopersicum* Mill. seeds showed that liquid nitrogen (LN) exposure increased the percentage of seed germination on day 5 but on day 7, the conversion into plantlets and the plant fresh mass differed slightly between non-cryopreserved and cryopreserved samples. Several indicative effects of cryopreservation were recorded at the biochemical level on day 7 of tomato seeds germination (Zevallos et al., 2016). Seeds cryostorage enhanced subsequent plant productivity in terms of growth, but it reduced the seeds production in *Teramnus labialis* (L.f.) Spreng (Acosta et al. 2019). For 9 among 11 species of wild plants, germination of seeds was deteriorated after cryopreservation (Ballesteros and Pence, 2017).

Tomato (*Solanum lycopersicum* L.) is one of vegetables having a world economic importance. Accordingly to the report of «Global Tomato Market 2019 – Robust Consumption Growth in China and India Drives the Global Market» the world tomato sales increased by 6.5 % in 2018 compared to the previous year – up to $190.4 billion, tomato production reached 188 million tons (+ 3.5% of 2017). However, a large number of viruses that damage the tomato plants can lead to enormous crop losses. *S. lycopersicum* L. plants may be infected by a wide range of viruses from different families including *Bromoviridae (Cucumber mosaic virus* (CMV), *Tomato aspermy virus* (TAV)); *Virgaviridae (Tomato mosaic virus* (ToMV); *Potyviridae (Potato virus Y, M* (PVY)), *Secoviridae (Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV), *Tomato black ring virus* (TBRV)); *Bunyaviridae (Tomato spotted wilt virus* (TSWV)), *Alphaflexiviridae (Potato virus X* (PVX), *Pepino mosaic virus* (PepMV)) and *Geminiviridae (Tomato yellow leaf curl virus* (TYLCV)). Today, the novel viruses appear and spread rapidly in tomato culture. In Ukraine *Tomato yellow leaf curl viru*s (TYLCV), *Tomato Torrado virus* (ToTV) and PepMV, *Tomato brown rugose fruit virus* (TBRFV), CMV, ToMV, *Tobacco mosaic virus* (TMV), TSWV, PVM and PVY have been detected in tomato plants (AlDalain et al., 2014).

Creation of the effective cryopreservation protocol can be vital condition for the virus destruction in the tomato germplasm. The cryopreservation techniques for tomato germplasm were previously described (Kulus, 2019). The different vitrification-based procedures can be identified: encapsulation-dehydration; vitrification; encapsulation-vitrification; dehydration; pregrowth; pregrowth-dehydration and droplet-vitrification (Engelman, 2004). However, the viability of virus-infected samples after cryopreservation is much lower (Wang et al., 2018.). Therefore, the determination of the influence of various cryopreservation techniques on the survival rates of the meristems obtained from viral infected plants is a very topical issue. The previous research demonstrated the transmission of TYLCV and ToMV via seeds (AlDalain et al., 2014, Kil et al., 2016), so their cryopreservation is likely to be able to eliminate these types of viruses.

Our objectives were to: (a) determine effectiveness of different cryopreservation techniques on post-thaw survival of meristems isolated from viral tomato plants of 'Irishka'; (b) study germination, total and marketable yield, number of fruits per plant, mass of one fruit, plant height, number of internodes of cryopreserved seed-derived tomato plants ('Seven', 'Potiron Ecarlate', 'Druzhba').

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The *in vitro* tomato culture was obtained from the field growing infected by CMV, ToMV, PVM, TMV, TYLCV plants of 'Irishka' cultivar, which formed sterile flowers. The parts of the stems with meristems were surface sterilized with 30 % commertial bleach (5 % active chlorine) for 25 minutes, then washed 5 times with sterile distilled water for initiation of *in vitro* cultures. Then they

were transferred into glas vials with agar nutrient medium Murashige and Skoog (MS) (Murashige and Skoog, 1962), supplemented with 3 % sucrose without phytohormones. The specimens were cultured at 20 ± 2 °C, with 16 hours of light and 8 hours of darkness under 37 $\mu mol \times m^{-2} \times s^{-1}$ light intensity. The explants were propagated by micro-grafting every 30 days.

2.2 CRYOPRESERVATION PROCEDURE IN VAPOUR PHASE OF LIQUID NITROGEN

For cryopreservation of tomato meristems the method described by Grout et al. (1978) was applied for seedlings of *in vitro* grown plants. All the leaves were removed from regenerated plants; the stems were cut and transferred stepwise at 20 min-intervals through increasing the concentrations (5.0, 10.0, 15,0 %) of dimethyl sulfoxide (Me_2SO_4) and propylene glycol (PG). Afterwards the stems were dried with sterile gauze. The stem pieces from the control plants and those treated with cryoprotectants were placed into different types of containers. Containers were made from sealed by heat-pulse welding polyimide-fluoroplastic film PMF-351 («Progress», Russia) of 50 μm, or sterile aluminum foil of 14 μm thickness. Five stems were placed in each container. Cooling was carried out in the vapours phase at a distance of 15 cm above the surface of the LN. The containers were immersed into LN when the temperature in the samples reached –170 °C and held to 30 min. A two-channel sensor measured the temperature change. After that, the containers were thawed by plunging into water at 40 °C for 2 min. Then the stems were three times rinsed in fresh MS without Me_2SO_4 and PG. After rinsing all axillary and apical meristems were dissected from the stems and cultured on filter-paper bridges in glass tubes with liquid MS medium.

2.3 PREPARATION TO VITRIFICATION-BASED CRYOPRESERVATION TECHNIQUES

The apical and axillary meristems up to 1-2 mm with primordias were isolated from three-week *in vitro* cultured plants. The isolated samples were transferred into a liquid MS medium, supplemented with 12 % sucrose and exposed at dark for 24 hours.

2.4 DEHYDRATION

The meristems were sterile airflow-dehydrated (AD) for 120-min and immersed into LN at a needle tip. Afterwards they were warmed in MS medium, supplemented with 10 % sucrose at 25 °C.

2.5 VITRIFICATION

The meristems were treated with loading solution (2 M glycerol and 0.4 M sucrose) for 20 min and then transferred in different plant vitrification solutions (PVS) for 40 min at 22 °C. The dehydrated meristems were put into 1.2 ml cryovials («Corning», USA) or 50 μl hermetic aluminum pans for differential scanning calorimetry (DSC) and were directly immersed into LN for 1 hour. The specimens were warmed in water bath at 40 °C for 2 min. The cryoprotectants were washed out by two consequent transfers of the meristems on filter papers saturated with MS medium, supplemented with 10 % sucrose.

2.6 DROPLET-VITRIFICATION

The meristems were treated with loading solution (2 M glycerol and 0.4 M sucrose) for 20 min and then transferred in different PVS at 22 °C for 40 min. The dehydrated specimens were placed individually into 10 μl droplets of PVS on a pieces of previously sterilized aluminum foil (15 × 20 × 0.15 mm), which were then directly immersed in LN. The cryopreserved meristems were thawed and the cryoprotectants were washed out by immersion in liquid MS medium, enriched with 12 % sucrose at 24 °C.

2.7 COMPOSITION OF PVS

Modified PVS 1 (22 % glycerol + 13 % PG + 13 % ethylene glycol + 6 % Me_2SO_4 and 0.4 M sucrose); PVS 2 (30 % glycerol + 15 % ethylene glycol + 15 % Me_2SO_4 and 0.4 M sucrose) (Coste et.al., 2015); 88 % PVS 3 (44 % glycerol + 44 % sucrose) (Nishizawa et.al., 1993, Vilardo et.al., 2019); PVS N (34 % sucrose + 15 % glycerol + 14 % ethylene glycol) (Vitsenia et.al, 2015).

2.8 POST-THAW CULTURE

The post-thaw meristems were plased in semi-solid MS medium, enriched with 3 % sucrose and stored under dark conditions for a week. They were transferred to the agar MS medium, supplemented with 3 % sucrose, 3 mg l^{-1} gibberellic acid and 0.01 mg l^{-1} indoleacetic acid. Than explants were cultured at 20 ± 2 °C, with 16 hours of light and 8 hours of darkness under 37 $\mu mol \times m^{-2} \times s^{-1}$ light in-

tensity. The number of meristems, having a green color for 30 days, determined the survival rate.

To examine the influence of pretreatment steps on the explants the part of meristems was treated with different ways but excluding low-temperature exposure. Non-cooled and not treated with PVS or AD meristems were assumed as the control.

2.9 SEEDS CRYOPRESERVATION

For cryopreservation the tomatoes seeds ('Seven', 'Potiron Ecarlate' and 'Druzhba') were placed into 1.5 ml polypropylene cryovials (FLMedical, Italy) and were directly immersed into liquid nitrogen for 2 days. To thaw, the tubes transferred to air. Seeds were sowed on day 7 after cryopreservation. Substrate to obtain tomato seedlings was potting soil "Rozsada" (Kisson, Ukraine), supplemented with coco coir (Ceres, Sri Lanka).

2.10 CHARACTERISTICS OF TOMATO CULTIVARS

Three cultivars of tomato were obtained from the Institute of Vegetable and Melon Growing of the National Academy of Agrarian Sciences of Ukraine.

The 'Seven' is a breeding cultivar of the Institute of Vegetables and Melons, Ukraine; it is resistant to major diseases. The growing season lasts 107-112 days and this variety is of late maturity with determinate plant type. Fruits are flattened, bright-red colored. The mass of one fruit can reach 350 g.

The 'Druzhba' cv. is a breeding material sourced of the Tiraspol Institute of Vegetables, Moldova; it is especially resistant to late blight. The growing season lasts up to 115 days; it is medium early maturity variety with determinate type of plant. Fruits are round, yellowish-orange in color. The mass of one fruit can gain up to 100 g.

The 'Potiron Ecarlate' is a France native cultivar. It is mid early maturity variety with indeterminate type of plant. Their slightly flat and ribbed shape is reminiscent of pumpkin; the skin is two-tone, of yellow and red color. Each fruit can reach a mass of 600 g.

2.11 FIELD RESEARCH

Tomato seedlings were grown in a greenhouse without heating. The seeds were planted on April 10, 2019. Mass shoots were received after 10-12 days. Seedlings were planted in open ground in the third decade of May. The area of the accounting site was 20 m^2. The planting scheme was 70 × 35 cm. Caring for the plants consisted of systematic hoeing of the soil and irrigation (norm 300-500 m^3ha^{-1}). During the growing season, morphological description was performed according to the classifier of the *Solanum lycopersicum* species. We recorded the seed germination, total and marketable yield, number of fruits per plant, mass of one fruit, plant height, number of internodes and amount of healthy plants.

2.12 STATISTICAL ANALYSIS

In all experiments, 10-25 meristems and 100 seeds were used per experimental condition and the experiments were replicated 3–5 times.The results were statistically analyzed using Software Past 3. The results are presented as mean and standard deviation. For establishing statistical significance, we used non-parametric Mann-Whitney criterion. The differences were considered significant at $p < 0.05$.

2.13 WEATHER DATA FOR EXPEREMENTAL REGION

Some weather data 2019 year and long-term ones were obtained from the experimental area (Kharkiv region), and were listed in Table 1.

3 RESULTS AND DISCUSSION

3.1 CRYOPESERVATION OF MERISTEMS

It was shown that regeneration rate made 78 % (from 70 to 83.3 %) for the control group. This parameter did not statistically change for the meristems isolated from stems after cryoprotectant treatment. The post-thaw survival rate was 0 % for two types of containers (Tab. 2). Thus, dissected of meristems from rewarmed stems was not good for *in vitro* grown plants cryopreservation.

For the vitrification-based procedure, preculture of shoot apices with sucrose-enriched medium prior to dehydration with different PVSs has been reported to be effective to improve post-thaw survival of tomato (Kulus, 2019). Meristems were precultured for 24 hour in liquid MS medium, enriched with 12 % sucrose. The results indicated the survival rate of meristems after cultivation in this medium did not differ from the control value (Fig. 1).

Since vitrification solutions contain high concentrations of cryoprotectants such as glycerol, ethylene glycol, propylene glycol or dimethyl sulfoxide, an essential step for successful vitrification is to identify the survival rate

Table 1: Climate conditions of the Ukrainian Eastern forest-steppe

Climate conditions	April	May	June	July	August	September
Rainfall, mm	25.5	58.5	14.0	51.0	7.5	32.0
Rainfall, long-term average, mm	40.8	55.5	65.0	73.3	41.9	48.8
Average daily temperature, °C	10.3	17.9	24.0	21.5	21.5	15.7
Average daily temperature long-term average, °C	9.6	16.5	20.2	21.3	19.8	14.1
Maximum temperature, °C	26.0	30.0	34.0	32.0	33.0	29.0
Long-term maximum temperature, °C	30.0	33.0	38.0	36.5	37.5	31.8
Minimum temperature, °C	-4.0	4.0	10.0	12.0	6.0	-5.0
Long-term minimum temperature, °C	-11.0	-6.8	1.0	6.0	1.5	-6.0
Minimum temperature of soil surface, °C	-4.0	4.0	10.0	12.0	6.0	-5.0

of meristems after PVSs treatment. This index was also determined for the airflow dehydrated meristems. After treatment of meristems with 88 % PVS 3 the growth recovery ranged from 35.5 to 48.5 %, such differences were significantly lower in comparison with the control group. In other variants, no significant differences were observed; re-growth percentages were between 66.6 and 83.3 % (Fig. 1). We observed same decrease in regeneration rate of meristems of the control group. It was most likely related with their reduced viability due to viral infection or their damage during isolation. The decrease in the number of viable meristems after 88 % PVS 3 treatment may be associated with a toxic effect of high concentrations of cryoprotectants or with osmotic responses that lead to damage of samples. A reduced exposure time for meristems in this solution is likely capable to obtain a higher regeneration rate.

Complete death rates were recorded in a week for all the PVSs treated meristems, wich were cryopreserved by droplet-vitrification and vitrification in cryovials. The death of the meristems immersed into LN on a piece of aluminium foil can be associated with their damage during the liquid nitrogen boiling or during warming in a nutrient medium through active rehydration. In case of vitrification in cryovials, death was caused by an unbalanced cooling and heating rate, which can lead to the formation of ice crystals.

After cryopreservation in aluminum pans for DSC, the survival rates of meristems from 34.2 to 78.5 % were observed. The post-thaw survival rates of meristems were 30–40 % for 88 % PVS 3, 70–78.5 % for modified PVS 1, 60–78.5 % for PVS 2 and 55.5–70 % for PVS N, so, the differences between non-cooled and cryopreserved explants were not significant (Fig. 1).

In case of AD the growth recovery of meristems ranged from 63 to 83 %. After cooling at the needle tip, the survival rates did not change significantly (Fig. 1).

It should be noted that all survived but non-cooled meristems formed shoots and regenerated in plants within a month. Despite the high level of post-thaw meristems survival, we could not achieve the formation of the plants-regenerants. During two weeks of the experimental study in the post-thaw conditions, we observed the onset of meristem growth, which stopped but shoots were green. We transferred them to a fresh MS medium but no growth was observed. A month later, the samples remained green but we stopped monitoring them. Selection of phytohormones in the reculture medium or light condition may be necessary.

Thus, we have shown that meristems obtained from the virus-infected 'Irishka' plants had a reduced viability. Cryopreservation of dehydrated with different PVS meristems by droplet-vitrification or vitrification in cryovials did not result in any survival rate. Freezing the pieces of stem under the PG and Me_2SO_4 protectionin in vapour phase of LN followed by immersion into liquid nitrogen

Table 2: The regeneration rates of tomato meristems after PG, Me2SO4 pretreatment and cryopreservation in vapour phase of liquid nitrogen

	Regeneration rate, %		
Variants	Control	PG	Me2SO4
Non-frozem	78.31 ± 5.24	76.66 ± 5.47	71.4 ± 4.066
Frozen in aluminium foil containers	0	0	0
Frozen in polyimidofluoroplastic containers	0	0	0

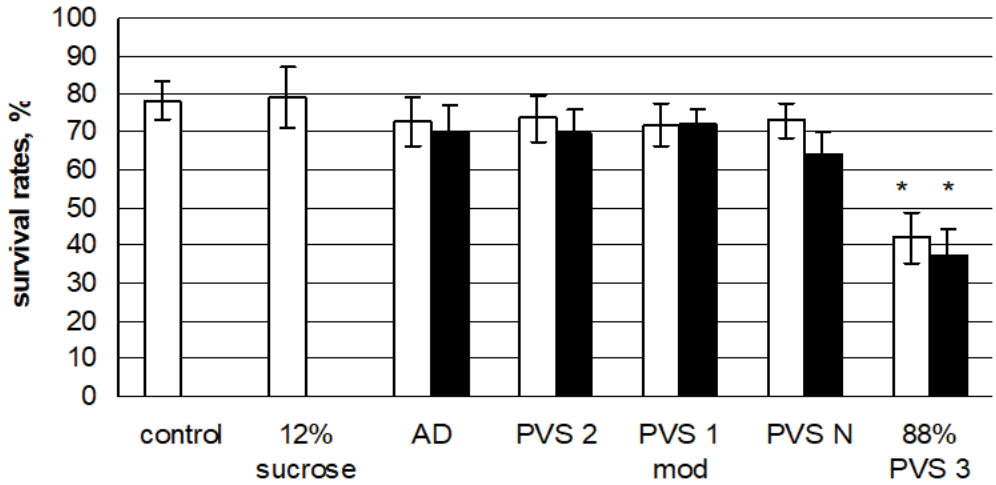

Figure 1: Survival rates of tomato meristems after AD and vitrification in 50 µl aluminum pans for DSC: □ – non-cooled, ▪ – re-warmed. Note: * – differences are significant if compared with the control group, $p < 0.05$

was also ineffective. The use of hermetic aluminum pans for DSC allows us to get a high level of the preserved meristems, independently of PVSs used. Dehydration of the meristems in the airflow and cooling at the needle tip by direct immersion into LN also made it possible to obtain a high survival rate.

3.2 CRYOPRESERVATION OF SEEDS

Cryopreservation of plant materials in LN has been described as a suitable technique to conserve genetic resources of several species. However, the potential effects of LN in subsequent plant growth in field should be studied before large-scale implementation of cryopreserved germplasm banks. In our work, we investigated the effect of LN on the growth and development of plants of three tomato cultivars in field.

For 'Seven' all the studied economically valuable features such as marketable yield and general productivity were significantly higher for the plants grown from the cryopreserved seeds. The increase in total and marketable yields compared with the control group was 351 and 268 % respectively. The productivity from each plant was increased almost in five times, because the mass of one fruit and the number of them were higher (Tab. 3).

For 'Potiron Ecarlate' the marketable yield increased in 220 %, the mass of one fruit was significantly lower for plants grown from cryopreserved seeds; the number of fruits per plant was significantly higher (Tab. 3).

It was shown that for the 'Druzhba' the data of total and marketable yield increased in 27.8 and 71.9 % respectively, number of fruits per plant was significantly higher (Tab. 3).

The height of plants and number of internodes for all the cultivars did not change significantly. However, there was a tendency to an increase in these indices for the plants grown from frozen seeds (Tab. 3).

During the growing season in 2019 in Ukraine, we observed many infected plants with both viral and fungal diseases. It was established that the treatment of seeds with LN led to a decrease in the number of plants infected by viruses. The total number of infected plants grown from the cryopreserved seeds decreased by 33 % for the 'Seven' , for 'Potiron Ecarlate' it did by 6.7 %, for the 'Druzhba' the total percentage of sick and healthy plants did not differ (Tab. 3).

Our results indicate that the germination of tomato seeds after cryopreservation did not change compared to the control. The tomato seeds of relatively small size can be cryopreserved without sophisticated pretreatment, required for more differentiated tissues. They are described as both desiccation and liquid nitrogen tolerant. Up-to-date, seeds of several tomato cultivars were successfully stored in LN. Storage periods ranging from 180 to 1,095 days resulted in germination rates of 99 % when water content of 6–7 % fresh mass. If the moisture content was at 8.7 %, the germination rate of rewarmed seeds fell to 84 % (Grout and Crisp 1995). On the other hand, Montoya et al. (2000) found that 69–88 % of cryopreserved seeds remained viable, but did not germinate after storage in LN.

Cryopreservation of tomato meristems in germetic aluminum pans for DSC, allowed us to obtain a high level of survival rate, but no further recovery was observed. Al-Abdallat et al. (2017) reported the same problem of the divergence between survival and recovery rates. Despite the survival rate of the cryopreserved transgenic

Table 3: Field performance of cryopreserved tomato seeds, 2019

	'Seven'		'Potiron Ecarlate'		'Druzhba'	
	-LN	+LN	-LN	+LN	-LN	+LN
seed germination, %	100	100	100	100	100	100
total yield, kg m^{-2}	1.10	4.97*	3.28	3.49	2.95	3.77*
marketable yield, kg m^{-2}	0.00	2.68*	0.51	1.57*	1.53	2.63*
number of fruits per plant, pcs	5.5	7.0	2.3	6.5*	12.9	18.0*
mass of one fruit, g	50.00	115.65*	181.00	117.11*	50.49	41.15
plant height, cm	66.7	68.3	109.5	114.7	74.7	78.8
number of internodes, pcs	10.2	11.4	16.8	17.7	10.7	12.4
number of healthy plants, %	0	33.33*	13.33	20.0*	6.67	6.67

Note: * – differences were significant compared with non-cryopreserved seeds: -LN – control seeds, +LN – cryopreserved seeds

tomato shoot tips reached even 70 %, however, no further recovery was possible. The authors considered that meticulously plant growth regulators selection, and their concentration optimisation, is required. Additionally, hormonal regulation of tomato explants growth can be altered by the cryopreservation procedure. For example, Grout et al. (1978) reported that viable *S. lycopersicum* explants (cryopreserved shoot tips) produced shoots directly by typical meristem growth when cultured in the presence of gibberellic acid after rewarming. Without gibberellic acid, the surviving explants produced callus and, subsequently, adventitious shoots. On the other hand, non-cryopreserved plant material produced shoots directly without the requirement for addition any plant growth regulators. In our study, we added 3 mg\l gibberellic acid and 0.01 mg/l indoleacetic acid to the reculture medium, but we still did not observe the regrowth of the cryopreserved meristems. Perhaps in order to enhance the uptake of phytohormones, a semi-solid medium, with reduced by half agar concentration, can be applied at the beginning of the recovery culture (Coste et al., 2015).

4 CONCLUSIONS

It was shown that the meristems obtained from virus-infected plants 'Irishka' had a 78 % viability. The effect of 20 % PG, 20 % Me_2SO_4, different PVSs and dehydration by airflow on the regeneration potential of meristems was determined. The significant decrease in the regeneration rate was obtained for meristems treated with 88 % PVS3 (42 % *vs* 78 %), other variants of pretreatment did not strongly change the meristems regeneration rate.

We determined the possibility of tomato meristems cryopreservation by freezing in vapour phase of LN, droplet-vitrification, as well as vitrification in cryovials and in aluminum pans for DSC. Freezing of stems pieces *in vitro* grown plants after PG and Me_2SO_4 treatment in vapour phase of LN was ineffective. Cryopreservation of meristems by droplet-vitrification or vitrification into cryovials did not allow receiving any survival rate. Vitrification in aluminum pans for DSC did not change the meristems survival rate if compared with treated but non-cooled explants. Dehydration of the meristems in the airflow and cooling at the tip of the needle by a direct immersion into LN also made it possible to obtain a high survival rate.

It was shown that all the studied economically valuable features such as marketable and total yield were significantly higher for the plants grown from the cryopreserved seeds of 'Seven', 'Potiron Ecarlate' and 'Druzhba'. The height of plants and number of internodes for all the cultivars did not change significantly; however, there was a tendency to an increase in these indices for the plants grown from the frozen seeds. The total number of infected plants grown from the cryopreserved seeds decreased by 33 % for the 'Seven', for 'Potiron Ecarlate' it did by 6.7 %, for the 'Druzhba'. total percentage of sick and healthy plants did not differ. Thus, the cryotherapy can likely applied for the tomato seeds, but this will demand additional experiments.

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Kazalniki *in vitro* fermentacije in tvorba hlapnih maščobnih kislin iz nestrukturnih ogljikovih hidratov pri kuncih

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Kazalniki *in vitro* fermentacije in tvorba HMK iz nestrukturnih ogljikovih hidratov pri kuncih

Izvleček: Šest čistih nestrukturnih ogljikovih hidratov (glukoza, fruktoza, saharoza, β-glukan iz ječmena, inulin iz cikorije (inulin-C) in inulin nedefiniranega izvora (inulin-N)) smo inkubirali v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev, in spremljali kazalnike kinetike *in vitro* tvorbe plina (skupna potencialna tvorba plina (B), največja hitrost fermentacije (MFR), čas, v katerem je MFR dosežena (TMFR), časovni zamik fermentacije (Lag), tvorba plina (Gas8) in hitrost fermentacije pri 8 urah inkubacije (FR8)) in vsebnosti hlapnih maščobnih kislin (HMK) po 8 urah fermentacije. MFR so bili največji, TMFR pa najkrajši pri fermentaciji sladkorjev: glukoze (MFR 36,0 ml/h; TMFR 8,6 h), fruktoze (MFR 38,6 ml/h; TMFR 9,6 h) in saharoze (MFR 33,2 ml/h; TMFR 9,4 h). Najslabše je fermentiral β-glukan (MFR 12,5 ml/h; TMFR 15,3 h), inulina pa sta fermentirala zelo različno: inulin-N hitreje in intenzivneje (MFR 32,3 ml/h; TMFR 8,3 h), podobno kot sladkorji, inulin-C pa počasi in s slabo intenzivnostjo (MFR 30,5 ml/h; TMFR 11,5 h). Tvorba HMK je bila največja pri sladkorjih in inulinu-N, majhna pri inulinu-C in najmanjša pri β-glukanu ($p < 0,05$). Molarni delež ocetne kisline je bil pri sladkorjih in inulinu-N manjši kot pri inulinu-C in β-glukanu, pri katerih je bil delež maslene kisline najmanjši ($p < 0,05$).

Ključne besede: kunci; prehrana živali; *in vitro* fermentacija; plinski test; nestrukturni ogljikovi hidrati; sladkorji; neškrobni polisaharidi; HMK

***In vitro* fermentation parameters and VFA production of non-structural carbohydrates in rabbits**

Abstract: Six pure non-structural carbohydrates (glucose, fructose, sucrose, β-glucan, chicory inulin (inulin-C) and inulin of undefined source (inulin-N)) were incubated anaerobically in the inoculum prepared from rabbit caecum content and the kinetic parameters of *in vitro* gas production (total potential gas production (B), maximum fermentation rate (MFR), time when MFR was reached (TMFR), lag phase (Lag), the amount of gas (Gas8) and fermentation rate at 8 hours of incubation (FR8)) and volatile fatty acids (VFA) production after 8 hours were determined. MFRs were the greatest and TMFRs the shortest with the fermentation of sugars: glucose (MFR 36.0 ml/h; TMFR 8.6 h), fructose (MFR 38.6 ml/h; TMFR 9.6 h) and sucrose (MFR 33.2 ml/h; TMFR 9.4 h). Fermentation was the lowest in β-glucan (MFR 12.5 ml/h; TMFR 15.3 h), while fermentation of the two inulins was very different: fermentation of inulin-N was intensive and fast and similar to sugars (MFR 32.3 ml/h; TMFR 8.3 h), while inulin-C fermented slowly and with low intensity (MFR 30.5 ml/h; TMFR 11.5 h). VFA production after 8 hours of incubation was the highest for simple sugars and inulin-N, low for inulin-C, and the lowest for β-glucan ($p < 0.05$). The molar proportion of acetic acid was lower in sugars and inulin-N than in inulin-C and β-glucan, which had the lowest molar proportion of butyric acid ($p < 0.05$).

Key words: rabbits; animal nutrition; *in vitro* fermentation; gas test; non-structural carbohydrates; sugars; non-starch polysaccharides; VFA

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1 UVOD

Zaužiti ogljikovi hidrati so za kunce najpomembnejši vir energije. Del teh ogljikovih hidratov se prebavlja z lastnimi prebavnimi encimi do konca tankega črevesa, medtem ko je drugi del sestavljen iz ogljikovih hidratov, za katere žival encimov ne izloča in preidejo v debelo črevo, kjer jih mikrobiota lahko fermentira. Prebavljivi ogljikovi hidrati so mono- in disaharidi (npr. glukoza, fruktoza, saharoza), oligosaharidi in nekateri polisaharidi, npr. škrob. Ti se običajno hitro in učinkovito prebavijo in absorbirajo v prvem delu prebavil in služijo kot pomemben vir hitro dostopne energije. Pri kuncih se mono- in disaharidi učinkovito prebavijo do konca tankega črevesa, a bi se v slepem črevesu lahko pojavili kot produkt kislinske hidrolize celuloze v želodcu, kjer je pri kuncih okolje zelo kislo, pH-vrednost je med 1,5 in 2,0 (Fortun-Lamothe in Gidenne, 2006). Tisti ogljikovi hidrati, ki se ne prebavijo do konca tankega črevesa z lastnimi prebavnimi encimi, pa lahko pri kuncih v precejšnjem obsegu fermentirajo v slepem črevesu. To so predvsem neškrobni polisaharidi (NŠP), rezistentni škrob in nekateri oligosaharidi. Glavni vir teh, v tankem črevesu neprebavljivih ogljikovih hidratov v krmi za kunce so NŠP. Ti so lahko topni (npr. pektini, β-glukani) ali netopni (npr. celuloza). Gidenne in sod. (2020) navajajo, da se pri kuncih pred slepim črevesom lahko prebavi tudi do 37 % vseh NŠP.

Gidenne (1997) navaja, da je stabilna in učinkovita mikrobna fermentacija v slepem črevesu za zdravje kunca nepogrešljiva. Prva stopnja tega fermentacijskega procesa je razgradnja NŠP na oligo-, di- in monosaharide s pomočjo encimov, ki jih izloča mikrobiota v slepem črevesu. Sproščeni sladkorji nato fermentirajo v pline in hlapne maščobne kisline (HMK). Značilnosti fermentacije topnih, torej hitro razgradljivih NŠP (pektinov), pa tudi slabše topnih in netopnih, torej počasneje razgradljivih NŠP (celuloza, ksilan) pri kuncih so opisali že Lavrenčič (2007) in Kermauner in Lavrenčič (2010). V literaturi smo našli tudi nekaj podatkov o *in vitro* fermentaciji krmnih mešanic z različnimi vsebnostmi NŠP (npr. Belenguer in sod., 2011) ali različnih čistih substratov in krmil (npr. Kermauner in Lavrenčič, 2008a, 2008b; Ocasio-Vega in sod., 2018), nekaj raziskav o fermentaciji inulina ter sladkorjev pri kuncih (npr. Marounek in sod., 1997, 2000; Slovakova in sod., 2002; Yang in sod., 2010), medtem ko podatkov o fermentaciji β-glukanov pri kuncih v dostopni literaturi nismo našli.

Inulin je fruktan, sestavljen iz molekul fruktoze, med seboj povezanih z β-2,1 vezmi, zato z lastnimi prebavnimi encimi živali ni prebavljiv. V prehrani neprežvekovalcev in ljudi ga uporabljamo kot prebiotik in ima vrsto ugodnih učinkov, saj ugodno vpliva na črevesno mikrobioto in na imunski sistem, deluje protivnetno, uravnava presnovo maščob in povečuje absorpcijo rudninskih snovi pri ljudeh in živalih (Tawfick in sod., 2022). Volek in sod. (2007) so ugotovili, da dodatek inulina ugodno vpliva na mikrobno aktivnost v slepem črevesu kuncev, saj se je zmanjšalo pojavljanje prebavnih motenj, ki v intenzivni reji kuncev povzročajo največ težav. β-glukane sicer uvrščamo v skupino v vodi topnih polisaharidov, kjer so v linearno verigo z β-1,3 in β-1,4 vezjo povezane molekule glukoze, a so De Arcangelis in sod. (2019) ugotovili, da je pri različnih sortah ječmena lahko delež v vodi netopnih β-glukanov med 16 in 30 %. Tudi β-glukani niso prebavljivi z lastnimi prebavnimi encimi živali, zato jih uporabljamo kot prebiotike, ki povečujejo tvorbo HMK, ugodno vplivajo na prebavo, zmanjšujejo glikemični indeks in holesterol v krvi, imajo antimikrobne in antikancerogene učinke, pomagajo pri kontroli sladkorne bolezni, srčno-žilnih bolezni, pospešujejo imunski odgovor organizma in podobno, nepogrešljivi pa so tudi v živilski industriji (Kaur in sod., 2020).

Fermentacijsko aktivnost mikrobiote v slepem črevesu lahko ocenimo z različnimi metodami. Pogosto so s pomočjo štetja mikrobnih celic ocenjevali aktivnost posameznih bakterijskih vrst v vsebini slepega črevesa (Williams in sod., 2001). Z metodami, ki ocenjujejo tvorbo mikrobnih beljakovin (tvorba ATP; Venkateswaran in sod. (2003)) in/ali produktov fermentacije, predvsem HMK (Kermauner in sod., 1996; Marounek in sod., 1997; Gidenne in sod., 2002), pa lahko ocenimo aktivnost celotne mikrobne populacije v slepem črevesu. Tudi tehnika plinskega testa v kombinaciji z določanjem HMK je zelo uporabna metoda za oceno mikrobne aktivnosti (Calabro in sod., 1999; Lavrenčič, 2007; Villamide in sod., 2009; Kermauner in Lavrenčič, 2010), Carabaño in sod. (2006) pa podajajo podroben pregled tehnik za ugotavljanje mikrobne aktivnosti v slepem črevesu pri kuncih.

S pričujočo študijo smo želeli ugotoviti razlike v kazalnikih kinetike produkcije plina in tvorbi HMK iz različnih nestrukturnih ogljikovih hidratov, ki smo jih inkubirali v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev.

2 MATERIAL IN METODE

2.1 SUBSTRATI

Kot substrat smo uporabili šest čistih ogljikovih hidratov: dva monosaharida (glukoza, Sigma G8270, in fruktoza, Sigma F0127), en disaharid (saharoza, Sigma S9378) in tri NŠP: inulin iz cikorije, Sigma I2250 (inulin-C), inulin nedefiniranega izvora, ki smo ga dobili z Oddelka za živilstvo Biotehniške fakultete Univerze v Ljubljani (inulin-N), in β-glukan iz ječmena, Sigma G6513.

2.2 IN VITRO FERMENTACIJA

Za pripravo inokuluma smo odvzeli vsebino slepega črevesa dveh 78 dni starih kuncev slovenske mesne linije SIKA. Oskrba živali in postopki priprave so potekali po protokolu, ki ga je opisal Lavrenčič (2007). Tvorbo plina v *in vitro* plinskem testu smo ugotavljali po proceduri, ki sta jo opisala Menke in Steingass (1988). Dvesto miligramov substrata smo anaerobno inkubirali pri 39 °C v 100 ml steklenih brizgah, ki smo jim dodali 30 ml inokuluma. Za vsak substrat smo pripravili 4 brizge, poskus smo izvajali v dveh ponovitvah. Meritve smo prvih 12 ur izvajali vsaki 2 uri, nato pa še po 24, 36, 48, 72 in 96 urah fermentacije.

2.3 DOLOČANJE HLAPNIH MAŠČOBNIH KISLIN (HMK)

Dve od štirih steklenih brizg s posameznim vzorcem iz vsake ponovitve smo po 8 urah fermentacije vzeli iz vodne kopeli, njihovo vsebino prelili v 50-ml centrifugirne epruvete in jih zamrznili pri −20 °C do analize vsebnosti HMK. Ekstrakte za določanje vsebnosti HMK smo pripravili po modificirani metodi, ki so jo opisali Holderman in sod. (1977). Uporabili smo plinski kromatograf Hewlett Packard 5890 A (Hewlett Packard, ZDA), opremljen s split/splitless injektorjem in FID detektorjem. Za ločevanje posameznih HMK smo uporabili 30 m NUKOL TM, FUSED SILICA kapilarno kolono (SUPELCO, ZDA).

2.4 IZRAČUNI IN STATISTIČNA OBDELAVA

Meritve *in vitro* tvorbe plina smo korigirali na vsebnost suhe snovi v substratu in na tvorbo plina iz slepih vzorcev. Z Gompertzovim modelom (Lavrenčič in sod., 1997) smo ocenili kazalnike *in vitro* produkcije plina: skupno potencialno tvorbo plina (parameter »B«; ml/g SS), specifično hitrost fermentacije (parameter »C«) in faktor mikrobne (ne)učinkovitosti (parameter »A«). Pri tem smo uporabili nelinearno regresijo (Proc NLIN; SAS Institute Inc., 2015). Iz dobljenih kazalnikov Gompertzovega modela smo izračunali največjo hitrost fermentacije (MFR; ml/h), čas, ko je bila ta dosežena (TMFR; h), prostornino sproščenega plina po 8 urah fermentacije (Gas8; ml/g SS), hitrost fermentacije po 8 urah (FR8; ml/h) in časovni zamik začetka fermentacije (lag phase, Lag; h).

Pri izračunu količine skupnih in posameznih HMK po 8 urah fermentacije smo upoštevali količino HMK v slepih vzorcih in vsebnost suhe snovi v posameznem substratu.

Statistično analizo smo opravili s proceduro GLM v statističnem paketu SAS (SAS Institute Inc., 2015), kjer smo v model vključili substrat kot fiksni vpliv, razlike v kazalnikih fermentacije in vsebnostih HMK med posameznimi substrati pa smo testirali z Duncanovim Multiple Range testom.

3 REZULTATI IN DISKUSIJA

Parametre plinskega testa prikazujemo v preglednici 1, na sliki 1a pa vidimo potek krivulje tvorbe plina iz posameznih substratov v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev. Skupna potencialna količina plina (parameter B) je bila pri vseh substratih večja od 330 ml/g SS, največja pri fermentaciji saharoze (386 ml/g SS), najmanjša pa pri fermentaciji inulina iz cikorije (inulin-C, 338 ml/g SS). Podobne vrednosti smo izmerili v plinskem testu pri različnih nestrukturnih polisaharidih pri kuncih, npr. pri škrobu (Lavrenčič, 2007; Kermauner in Lavrenčič, 2012) ali pektinu (Lavrenčič, 2007), kjer izvor (jabolčni, pesni in citrusovi) ni vplival na potencialno tvorbo plina (Kermauner in Lavrenčič, 2010).

V dostopni literaturi so zabeležene zelo velike razlike v količini proizvedenega plina pri kuncih za različne substrate. Belenguer in sod. (2011) so po 6 urah fermentacije pri kuncih izmerili samo med 21 in 25 ml plina/g SS

Preglednica 1: Ocenjeni kazalniki kinetike *in vitro* tvorbe plina iz nestrukturnih polisaharidov v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev
Table 1: Estimated *in vitro* gas production kinetic parameters of non-structural carbohydrates, incubated in inoculum prepared from rabbit caecum content

Substrat	B (ml/g SS)	C	A
Glukoza	353^{bc}	11,8^{bc}	0,277^{ab}
Fruktoza	345^{c}	25,3^{a}	0,306^{a}
Saharoza	386^{a}	9,4^{bc}	0,234^{b}
Inulin-C	338^{c}	17,3^{ab}	0,246^{b}
Inulin-N	367^{b}	7,5^{bc}	0,240^{b}
β-glukan	342^{c}	4,5^{c}	0,099^{c}
RMSE	13,1	7,31	0,0383
R^2	0,976	0,532	0,762

$^{a, b, c, d}$ = vrednosti v posameznih stolpcih, označene z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p < 0,05$
RMSE = standardni odklon ostanka; R^2 = koeficient determinacije
B = skupna potencialna tvorba plina; C = specifična hitrost fermentacije; A = faktor mikrobne (ne)učinkovitosti

Preglednica 2: Izračunani kazalniki *in vitro* tvorbe plina iz nestrukturnih ogljikovih hidratov v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev
Table 2: Calculated parameters of the *in vitro* gas production of non-structural carbohydrates, incubated in inoculum prepared from rabbit caecum content

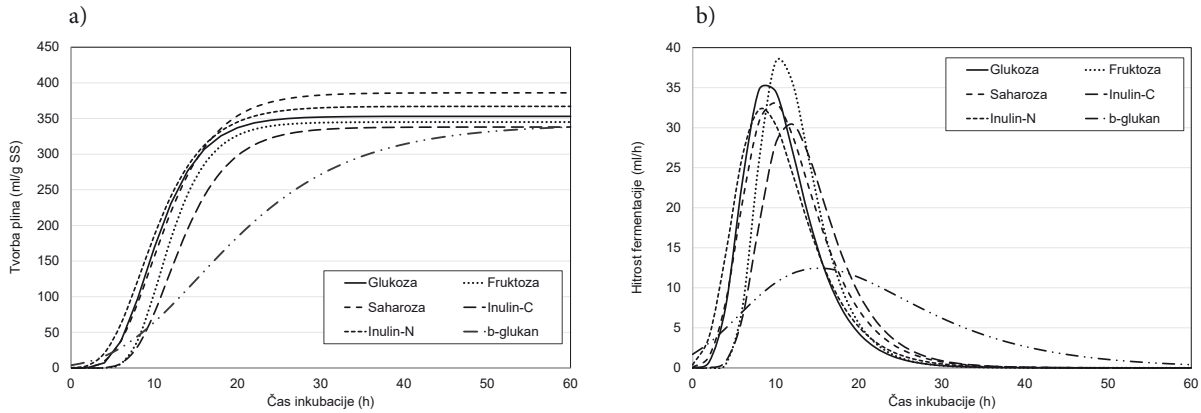
Substrat	MFR (ml/h)	TMFR (h)	Gas8 (ml/g SS)	FR8 (ml/h)	Lag (h)
Glukoza	36,0^{ab}	8,6^{c}	106^{ab}	35,1^{a}	5,0^{bc}
Fruktoza	38,6^{a}	9,6^{c}	66^{c}	31,9^{a}	6,2^{ab}
Saharoza	33,2^{ab}	9,4^{c}	99^{b}	31,5^{a}	4,9^{bc}
Inulin-C	30,5^{b}	11,5^{b}	33^{d}	18,3^{b}	7,4^{a}
Inulin-N	32,3^{ab}	8,3^{c}	125^{a}	31,9^{a}	4,1^{c}
β-glukan	12,5^{c}	15,3^{a}	46^{cd}	9,1^{c}	5,1^{bc}
RMSE	4,97	0,97	15,6	4,24	0,93
R^2	0,850	0,905	0,846	0,883	0,878

$^{a, b, c, d}$ = vrednosti v posameznih stolpcih, označene z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p < 0,05$; RMSE = standardni odklon ostanka; R^2 = koeficient determinacije;
MFR = največja hitrost fermentacije; TMFR = čas, v katerem je MFR dosežena; Gas8 = količina plina po 8 urah fermentacije; FR8 = hitrost fermentacije pri 8 urah inkubacije; Lag = časovni zaostanek začetka fermentacije

krmne mešanice z različno sestavo, Ocasio-Vega in sod. (2018) pa po 24 urah fermentacije med 15 (pšenična slama) in 94 ml/g SS (celobioza). Ferreira in sod. (2019) so izmerili 318 ml/g SS pri koruznem zrnju, 256 ml/g SS pri pšeničnih otrobih in 138 ml plina/g SS pri lucerninem senu, Marounek in sod. (1997) pa po 8 urah fermentacije pri kuncih okrog 200 ml plina/g inulina, kar je bistveno več kot v našem poskusu.

Če naše rezultate primerjamo z *in vitro* fermentacijo pri drugih živalskih vrstah ali pri človeku, ugotovimo, da je bila fermentacija inulina pri kuncih v našem poskusu podobna kot pri človeku (Karppinen in sod., 2000) in pri sesnih pujskih (Williams in sod., 2005), pri odstavljenih pujskih pa je bila tvorba plina iz inulina nekoliko manjša (Pellikaan in sod., 2007).

Raziskav o fermentabilnosti β-glukana pri kuncih v literaturi nismo zasledili. Količina plina, nastalega pri fermentaciji ječmenovega β-glukana pri kuncih v našem poskusu, je bila večja od tiste, ki so jo izmerili pri fermentaciji ovsenih otrobov (Karppinen in sod., 2000) in β-glukana iz ovsa (Lu in sod., 2021) v človeškem blatu, pa tudi pri fermentaciji β-glukana v blatu prašičev (Lu in sod., 2020). Lu in sod. (2021) menijo, da na obseg fermentacije β-glukana vplivajo tako izvor in sestava celičnih sten posameznih žit kot tudi vrsta uporabljenega inokuluma.



Slika 1: Rezultati *in vitro* plinskega testa nestrukturnih ogljikovih hidratov v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev a) krivulja poteka tvorbe plina in b) krivulja hitrosti fermentacije
Figure 1: Results of *in vitro* gas test of non-structural carbohydrates in inoculum, prepared from rabbit caecum content a) gas production kinetics and b) fermentation rates kinetics

Precej večje razlike kot pri parametru B smo zasledili pri specifični hitrosti fermentacije (parameter C), ki je variirala od 4,5 pri β-glukanu do 25,3 pri fruktozi, kjer se je statistično značilno ($p < 0,05$) razlikovala od ostalih substratov (razen od inulina-C). Razlike v parametru C med ostalimi substrati so bile velike, a večinoma neznačilne, podobno kot je za škrob, pektin in ksilan ugotovil Lavrenčič (2007), medtem ko sta pri pektinih različnega izvora Kermauner in Lavrenčič (2010) ugotovila veliko manjše razlike (od 2,6 do 5,5), podobno kot Ferreira in sod. (2019) za različna krmila (od 2,7 do 4,2). Kazalnik A, ki označuje mikrobno (ne)učinkovitost, je bil za večino substratov zelo podoben (med 0,234 in 0,277), nekoliko višji je bil le pri fruktozi (0,306), a značilno nižji pri β-glukanu (0,099). Podobne vrednosti sta dobila pri pektinih različnih izvorov tudi Kermauner in Lavrenčič (2010), medtem ko je Lavrenčič (2007) izmeril precej nižji A (od 0,12 do 0,19) pri škrobu, pektinu in ksilanu. Do podobno nižjih vrednosti so prišli tudi Ferreira in sod. (2019) pri lucerninem senu (0,18), pšeničnih otrobih (0,12) in koruznem zrnju (0,09).

Izračunani kazalniki tvorbe plina in hitrosti so prikazani v preglednici 2, potek hitrosti fermentacije pa na sliki 1b. Največja hitrost fermentacije (MFR) je bila podobna pri večini substratov, znašala je od 30,5 ml/h pri inulinu-C do 38,6 ml/h pri fruktozi, razlika med tema dvema vrednostma je bila tudi statistično značilna. Veliko slabše pa je fermentiral β-glukan (MFR 12,5 ml/h, $p < 0,05$). Tudi čas, v katerem je bila MFR dosežena (TMFR), se je pri večini substratov malo razlikoval, od 8,3 pri inulinu-N do 9,6 ur pri fruktozi, z veliko večjim TMFR pa sta močno odstopala inulin-C (11,5 ur) in β-glukan (15,3 ur).

Pellikaan in sod. (2007) so izmerili precej manjšo MFR in krajši TMFR pri fermentaciji inulina v človeškem blatu, rezultati fermentacije cikorijinih inulinov različnih proizvajalcev v blatu sesnih pujskov pa so bili bolj podobni našim, čeprav z nekoliko nižjimi MFR (Williams in sod., 2005). Karppinen in sod. (2000) so ugotovili, da je bil inulin v *in vitro* pogojih pri človeku popolnoma porabljen v 4 urah fermentacije. Naši rezultati tega ne kažejo, saj je pri inulinu-N fermentacija dosegla vrh šele po dobrih 8 urah, pri inulinu-C pa celo po več kot 11 urah (preglednica 2).

Marounek in sod. (1997) so v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev, izmerili obsežnejšo fermentacijo inulina v primerjavi s škrobom in hemicelulozami, a nekoliko manjšo od pektina. Tudi Karppinen in sod. (2000) so v inokulumu, pripravljenem iz blata človeka, ugotovili hitrejšo in obsežnejšo fermentacijo inulina v primerjavi z drugimi substrati (ovseni, pšenični in rženi otrobi), podobno so Pellikaan in sod. (2007) izmerili obsežnejšo in hitrejšo fermentacijo pri substratu, ki je vseboval 40 % inulina in 60 % oligofruktanov, ne glede na krmo, ki so jo pujski dobivali pred odvzemom vsebine debelega črevesa za pripravo inokuluma. Williams in sod. (2005) so sicer ugotovili, da so razlike v fermentaciji cikorijinih inulinov različnih proizvajalcev velike, a so v povprečju bolje in hitreje fermentirali kot drugi substrati (inulin iz artičoke, različne vrste škrobov, netopne vlaknine) v inokulumu, pripravljenem iz blata sesnih pujskov. Očitno bi morali pri inulinu poznati več lastnosti, npr. stopnjo polimerizacije, in ne le izvora.

Ker je bila fermentacija inulina vedno hitrejša kot fermentacija β-glukana, lahko potrdimo ugotovitev, da tipi vezi med posameznimi molekulami sladkorjev v ogljikovih hidratih določajo tako obseg njihove fermentacije kot tudi hitrost fermentacije (Salvador in sod., 1993).

Največja hitrost fermentacije (MFR) ječmenovega β-glukana pri kuncih v našem poskusu je bila veliko manjša od hitrosti fermentacije β-glukana iz ovsa v blatu prašičev (Lu in sod., 2020), še posebej pa od fermentacije β-glukana v človeškem blatu (Lu in sod., 2021). Lu in sod. (2021) menijo, da je hitrost fermentacije najbolj odvisna od uporabljenega inokuluma, predvsem pa od tega, ali je bila mikrobiota inokuluma že navajena na določen substrat, kar potrjuje rezultate, ki smo jih dobili v naših raziskavah pri fermentaciji škroba pri različno starih kuncih (Lavrenčič, 2007).

Parameter Gas8 opisuje količino plina, ki se iz določenega substrata sprosti v 8 urah fermentacije, kar odgovarja povprečnemu času zadrževanja krme v slepem črevesu kuncev (Gidennne, 1997). Razlike med substrati so bile velike in večinoma statistično značilne, največ plina je v 8 urah nastalo iz inulina-N (125 ml/g SS) in glukoze (106 ml/g SS), najmanj pa iz β-glukana (46 ml/g SS) in inulina-C (33 ml/g SS) (preglednica 2). Največja hitrost fermentacije pri 8 urah (FR8) je bila zelo podobna pri večini substratov (med 31,5 in 35,1 ml/h), odstopala sta le inulin-C (18,3 ml/h) in β-glukan (9,1 ml/h), kjer je bila FR8 najnižja. Kljub tem razlikam v fermentaciji pa so bile vrednosti za Lag precej bolj podobne, čeprav sta se najkrajši (pri inulinu-N 4,1 h) in najdaljši Lag (pri inulinu-C 7,4 h) značilno razlikovala ($p < 0,05$). Lag je definiran kot čas, ki je potreben za mikrobno kolonizacijo in za navlažitev substrata. Ker je v *in vivo* pogojih substrat že navlažen, je Lag odvisen le od hitrosti kolonizacije. Zato menimo, da je v *in vivo* pogojih Lag bistveno krajši kot v *in vitro* pogojih.

V preglednici 3 prikazujemo tvorbo HMK ter molarne deleže posameznih najpomembnejših HMK. Koncentracije HMK, nastalih ob fermentaciji sladkorjev in inulina-N, so bile zelo podobne (od 2,09 mmol/g SS pri fruktozi do 2,60 mmol/g SS pri inulinu-N), medtem ko je ob fermentaciji inulina-C in β-glukana nastalo veliko

Preglednica 3: Tvorba hlapnih maščobnih kislin (HMK) in molarni deleži ocetne, propionske in maslene kisline, nastalih ob fermentaciji nestrukturnih ogljikovih hidratov v 8 urah fermentacije v inokulumu, pripravljenem iz vsebine slepega črevesa kuncev
Table 3: Synthesis of volatile fatty acids (VFA) and molar proportions of acetate, propionate and butyrate produced from non-structural carbohydrates after 8 hours of fermentation in inoculum, prepared from rabbit caecum content

Substrat	HMK (mmol/g SS)	Ocetna kislina (mmol/mmol HMK)	Propionska kislina (mmol/ mmol HMK)	Maslena kislina (mmol/ mmol HMK)
Glukoza	2,25^{a}	0,558^{c}	0,129^{b}	0,313^{b}
Fruktoza	2,09^{ab}	0,564^{c}	0,119^{b}	0,318^{b}
Saharoza	2,26^{a}	0,573^{c}	0,086^{c}	0,341^{a}
Inulin-C	1,50^{bc}	0,623^{a}	0,132^{a}	0,244^{c}
Inulin-N	2,60^{a}	0,566^{c}	0,134^{a}	0,300^{b}
β-glukan	1,39^{c}	0,590^{b}	0,098^{c}	0,234^{c}
RMSE	0,428	0,0192	0,0127	0,0155
R^2	0,649	0,832	0,935	0,920

$^{a, b, c, d}$ = vrednosti v posameznih stolpcih, označene z različnimi črkami, se med seboj statistično značilno razlikujejo pri $p < 0,05$ / means in columns with different superscripts are significantly different at the level $p < 0.05$
RMSE = standardni odklon ostanka; R^2 = koeficient determinacije / determination coefficient

manj HMK (1,50 in 1,39 mmol/g SS). Ti rezultati so podobni rezultatom poskusa, ki sta ga opravila Kermauner in Lavrenčič (2011), kjer so pri različnih krmilih z večjo vsebnostjo ogljikovih hidratov izmerili med 2,86 mmol HMK/g SS pri dehidrirani lucerni in 1,50 mmol/g SS pri ječmenovem zrnju. Sta pa Kermauner in Lavrenčič (2011) ugotovila, da pri fermentaciji čistega škroba in koruznega zrnja nastane veliko manj HMK (0,38 in 0,80 mmol/g SS), kar je verjetno posledica omejene amilolitične aktivnosti mikroorganizmov v slepem črevesu kuncev (Kermauner in Lavrenčič, 2012).

Razmerja med posameznimi HMK se pri kuncih močno razlikujejo od drugih živalskih vrst. V vsebini slepega črevesa zdravih kuncev prevladuje ocetna kislina (60 do 80 %), sledi ji maslena kislina (8 do 20 %), najmanj pa je propionske kisline (3 do 10 %) (Carabaño in sod., 2006). Običajno razmerje med propionsko in masleno kislino je med 0,5 in 0,8 (Gidenne in sod., 2020). Ti deleži in razmerja so odvisni od vrste substrata in zdravstvenega stanja kuncev.

V našem poskusu se deleži posameznih HMK nekoliko razlikujejo od običajnih vrednosti. Izmerili smo manjši delež ocetne kisline (med 55 in 62 mol%) ter večja deleža propionske (od 8 do 13 mol%) in maslene kisline (od 23 do 34 mol%), pri čemer pa je razmerje med propionsko in masleno kislino (med 0,25 in 0,54) ostalo v običajnih mejah. Objave o tvorbi HMK pri fermentaciji sladkorjev so za kunce zelo redke: pri fermentaciji glukoze je nastala enaka količina HMK kot pri fermentaciji ksiloze (Marounek in sod., 2000) ali celobioze (Yang in sod., 2010), medtem ko so se deleži posameznih HMK med substrati močno razlikovali. Zanimivo pa je, da smo v našem poskusu ugotovili največje razlike med obema inulinoma. Iz inulina-N je nastalo največ HMK (2,60 mmol/g SS) z najmanjšim deležem ocetne kisline (56,6 mol%), iz cikorijinega inulina (inulin-C) pa najmanj HMK (1,50 mmol/g SS) z največjim deležem ocetne kisline (62,3 mol%) in najmanjšim deležem maslene kisline (24,4 mol%).

Marounek in sod. (1997) so v *in vitro* poskusu pri kuncih ugotovili manjši delež ocetne kisline, znatno večji delež propionske in rahlo povečan delež maslene kisline pri fermentaciji inulina v primerjavi s škrobom, hemicelulozami ali pektini, pa tudi nekoliko večjo tvorbo skupnih HMK. Da ob fermentaciji inulina nastaja več HMK, so ugotovili tudi Castellini in sod. (2007), ki so obroku dodajali sveže liste cikorije, in Volek in sod. (2007), ki so krmi za kunce dodajali cikorijin inulin, a je bil delež ocetne kisline večji, delež propionske kisline pa manjši. Manjši delež propionske kisline sta izmerila tudi Volek in Marounek (2011) pri dodatku 10 % cikorijine korenine, kar se ne sklada z našimi rezultati, na HMK pa delež inulina ni vplival.

Maertens in sod. (2004) menijo, da fruktani z daljšo verigo (inulin) intenzivneje fermentirajo in tako močneje vplivajo na tvorbo in deleže HMK. Na osnovi tega lahko sklepamo, da je bila molekulska masa (dolžina verig) v inulinu-N večja kot v inulinu-C, saj je iz slednjega nastalo manj HMK z večjim deležem ocetne kisline. Pellikaan in sod. (2007) k temu dodajajo še, da na tvorbo HMK in deleže posameznih HMK močno vpliva navajenost mikrobiote na substrat, saj sta se pri vseh uporabljenih inokulumih ob fermentaciji substrata z inulinom povečala deleža ocetne in propionske kisline, zmanjšal pa delež maslene kisline.

Iz β-glukana se je v našem poskusu tvorilo najmanj

HMK (1,39 mmol/g SS), delež ocetne kisline je bil med večjimi (59,0 mol%), deleža propionske (9,8 mol%) in maslene kisline (23,4 mol%) pa najmanjša. Podatkov o fermentaciji β-glukana pri kuncih v dostopni literaturi nismo našli. Bai in sod. (2021a) so v *in vitro* poskusih na miših ugotovili, da se je iz β-glukana tvorilo več HMK kot iz fruktooligosaharidov (FOS) ali osnovne krme (Bai in sod., 2021b). Tudi Jha in sod. (2010) so ugotovili določene razlike v tvorbi in deležih HMK ob krmljenju različnih količin β-glukanov odstavljenim pujskom, a teh razlik niso mogli povezati z vsebnostjo β-glukanov v krmi. Tvorba HMK iz različnih vrst β-glukanov (iz ječmena ali ovsa) z različnimi lastnostmi (dolžina verig, viskoznost) pri človeku je bila prav tako različna, v povprečju pa manjša v primerjavi z inulinom (Hughes in sod., 2008), nasprotno pa so Kaur in sod. (2011) izmerili večjo vsebnost HMK ob fermentaciji β-glukana v inokulumu, pripravljenem iz človeškega blata, v primerjavi s fermentacijo inulina. Navedene primerjave pa moramo jemati previdno, saj so pri kuncih deleži posameznih HMK drugačni kot pri drugih živalskih vrstah, s katerimi najpogosteje primerjamo fermentacijo v slepem črevesu kuncev (podgane, prašiči ali človek). Tvorba in deleži HMK namreč niso odvisni le od substrata, ampak predvsem od mikrobiote v debelem črevesu, ki pa se razlikuje med vrstami živali, pa tudi med posamezniki znotraj vrste (Karppinen in sod., 2000; Hughes in sod., 2008).

4 SKLEPI

Znano je, da pri neprežvekovalcih veliko ogljikovih hidratov fermentira v debelem črevesu s pomočjo črevesne mikrobiote, vendar pa ni veliko študij, ki bi sistematično ugotavljale obseg in potek fermentacije ter tvorbo hlapnih maščobnih kislin pri kuncih. Enostavni sladkorji so sicer zelo dobro prebavljivi z lastnimi prebavnimi encimi v tankem črevesu, a se lahko pojavijo v debelem črevesu zaradi velike količine vlaknine v krmi za kunce ter kot produkt obsežne kislinske hidrolize v želodcu. Mikrobiota v slepem črevesu kuncev zelo intenzivno fermentira neškrobne polisaharide (NŠP), od obsega in intenzivnosti te fermentacije pa je odvisno zdravstveno stanje in pogin kuncev v intenzivni reji. Intenzivnost fermentacije v slepem črevesu kuncev je odvisna od sestave mikrobiote in njene prilagojenosti na določen substrat, pa tudi od samega substrata, predvsem od tipa vezi med posameznimi monosaharidi, pa tudi od stopnje polimerizacije, dolžine in razvejanosti verige in drugih lastnosti ogljikovih hidratov. Pričakovano so glukoza, fruktoza in saharoza fermentirale najhitreje in v največjem obsegu. Ječmenov β-glukan je fermentiral najpočasneje in najmanj intenzivno, medtem ko sta oba tipa inulina fermentirala zelo različno: inulin-N je fermentiral podobno kot sladkorji, inulin-C pa zelo počasi in z nizko intenzivnostjo. Tako velikih razlik med vrstama inulina nismo pričakovali. Ker v dostopni literaturi nismo našli veliko podatkov o fermentaciji nestrukturnih ogljikovih hidratov pri kuncih, smo dobljene rezultate lahko primerjali samo s fermentacijo le-teh pri drugih živalskih vrstah, pri tem pa se moramo zavedati, da obstajajo zelo velike razlike v sestavi same mikrobiote, kar ima za posledico tudi razlike v končnih produktih fermentacije. Glede na dobljene rezultate bi težko ocenili uporabnost preiskovanih nestrukturnih ogljikovih hidratov za zmanjševanje prebavnih motenj pri kuncih, saj bi bil najverjetneje najučinkovitejši inulin-N, za katerega pa nimamo podatkov o njegovih lastnostih. Zato bi bilo potrebno poskus razširiti in vanj vključiti ogljikove hidrate z dobro definiranimi lastnostmi.

5 ZAHVALA

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6 VIRI

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Genetic diversity in - chilli (*Capsicum annuum* L.) based on microsatellite markers: An evaluation of Bangladeshi germplasm

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Genetic diversity in - chilli (*Capsicum annuum* L.) based on microsatellite markers: An evaluation of Bangladeshi germplasm

Abstract: Genetic diversity analysis is a pre-requisite to develop improve variety of any crop. Hence, 39 SSR markers were used to analyze the genetic diversity of local chilli cultivars. PCR-amplified microsatellite loci were shown to be polymorphic in all investigated cultivars. The locus, CAMS-647 produced the highest number of alleles (8) ranging in size from 188 to 279 bp. PIC values for 39 primers ranged from 0.099 for the locus Hpms 1-165 to 0.806 for the locus CAMS-679. All of the SSRs examined were informative in characterizing the genotypic variance of the samples while 12 were more informative with higher PIC values (> 0.6). There was a wide range of genetic diversity varied from 0.117 (HpmsE075) to 0.806 (CAMS-647), whereas the highest (1.713) and the lowest (0.205) value of Shannon's Information Index was registered in the locus CAMS-679 and Hpms 1-165, respectively. There was a higher degree of genetic differentiation (0.927) and a lower amount of gene flow (0.010). Nei's genetic distance (GD) varied from 0.100 to 0.990. Among 96 cultivars, 55 had distinct status in the dendrogram with higher GD values (> 0.6), while 41 cultivars showed a close relationship and yielded lower GD values.

Key words: chilli; genetic diversity; microsatellite (SSR) markers; polymorphism information content

Določanje genetske raznolikosti čilija (*Capsicum annuum* L.) z mikrosateliti: Ovrednotenje genetskega materiala v Bangladešu

Izvleček: Analiza genetske raznolikosti je predpogoj za vzgojo izboljšanih sort katerekoli kulturne rastline. Zatradi tega je bilo uporabljenih 39 SSR lokusov za analizo genetske raznolikosti genotipov čilija. S PCR pomnoženi mikrosatelitski lokusi so bili polimorfni pri vseh preučenih genotipih. Pri lokusu CAMS-647 smo zaznali največje število alelov (8), ki so obsegali dolžine od 188 do 279 bp. PIC vrednosti so za 39 začetnih oligonukleotidov (primerjev) znašale od 0,099 za lokus Hpms 1-165 do 0,806 za lokus CAMS-679. Vsi analizirani mikrosateliti (SSR) so bili za vrednotenje genenotipske variabilnosti vzorcev informativni, med njimi jih je bilo 12 z večjimi PIC vrednostmi (> 0,6) najprimernejših. Genetska raznolikost je bila velika in je variirala od 0,117 (HpmsE075) do 0,806 (CAMS-647), največja (1,713) in najmanjša (0,205) vrednost Shannonovega informacijskega indeksa sta bili ugotovljeni za lokusa CAMS-679 in Hpms 1-165. Ugotovljena je bila visoka stopnja genetske diferenciacije (0,927) in majhen pretok genov (0,010). Neijeva genetska distanca je variirala med 0,100 in 0,990. Med 96 genotipi jih je imelo 55 jasen položaj v dendrogramu z večjimi vrednostmi genske distance (> 0,6) medtem, ko je 41 genotipov pokazalo ožjo sorodnost z manjšimi v rednostmi genske distance.

Ključne besede: čili; genetska raznolikost; microsatelistki markerji (SSR); informacijska vrednost polimorfizma

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1 INTRODUCTION

Chilli (*Capsicum* spp.) belongs to the Solanaceae family, having chromosome number 2n = 2x = 24 (Sharmin et al., 2018)with a mean of 2.00 alleles per primer. Gene diversity ranged from 0.333 to 1.00 with an average of 0.567. Polymorphic Information Content (PIC. The genus is native to Central and South America (Pickersgill, 1991) which includes five species viz., *Capsicum chinense* Jacq., *Capsicum baccatum* L., *Capsicum frutescens* L., *Capsicum pubescens* Ruiz & Pav. and *Capsicum annuum*. Of these, *C. annuum* is the most important one because of its versatile use and is cultivated in both tropical and temperate areas in the world. It is used as vegetables, spice, colorant, and for some medical applications (Hernández-Pérez et al., 2020). Chilli is a valuable spice and one of the most important cash crops grown in Bangladesh. It is available and used in human food preparation in the forms of green, dried and powdered. It has become an essential ingredient in Bangladeshi dietary patterns. A number of cultivars are grown in Bangladesh showing differences in growth habit, size, shape, color, pungency, and yield indicating the presence of wider genetic variation (Farhad et al., 2010). The area and production of Kharif (April to September) chilli was 19,000 ha and 36,000 mt, respectively; while 83,000 ha and 105,000 mt were recorded in Rabi (October to March) chilli, respectively. The average yield was around 1.68 mt ha^{-1} (BBS, 2021). Bangladesh has a high diversity of cultivars belonging to various cultivated chilli varieties. Due to the long history of cultivation, selection and popularity of crops, sufficient genetic variability has been generated. The analysis of genetic diversity and relatedness between or within different species, populations and individuals is a prerequisite towards effective utilization and protection of plant genetic resources (van Zonneveld et al., 2012). As the country has vast chilli resources, and the demand is using those for further development of new materials that can provide a high economic return. Hence, it is necessary to study some basic traits of similarity and/or parent progeny relationship that can indicate their adoption in the country. Selection of useful diversity from the available genetic resources will be an enormous challenge. Collection and maintenance of the genetic diversity in chilli are important to avoid genetic erosion. Besides the identification of species, the characterization and evaluation of cultivars maintained in gene banks are of fundamental importance (van Zonneveld et al., 2012). Traditionally, morphological markers known as descriptors have been utilized in plants for varietal identification and genetic diversity study, which is expensive, time-consuming, requiring huge areas of land, and specialized staff, and is subject to variation owing to environmental factors (Molla et al., 2017). However, in elite germplasm, the level of polymorphism for morphological traits is sometimes too low and insufficient to allow for variety/genotype discrimination (Dhaliwal et al., 2014). The DNA marker provides a one-stop solution in this case. Different molecular markers for pepper have been established in the previous decade or so. Microsatellite SSR (simple sequence repeat) is a DNA-based marker that based on PCR, multi-allelic, highly polymorphic, commonly co-dominant, highly repeatable, randomly and extensively distributed across the genome (Jain et al., 2014). Furthermore, SSRs are the most extensively used marker system for identifying plant varieties and analyzing diversity, particularly in cultivated species with low levels of polymorphism (Anumalla et al., 2015). Although some research has been conducted regarding chilli diversity in Bangladesh, inadequate information was generated because of the limited number of cultivars assessed with a limited number of primers. For instance, 20 local chilli cultivars were evaluated using 11 SSR makers by Sharmin et al. (2018)with a mean of 2.00 alleles per primer. Gene diversity ranged from 0.333 to 1.00 with an average of 0.567. Polymorphic Information Content (PIC and 22 cultivars using four microsatellite markers by Hossain et al. (2014). The present study was, therefore, undertaken to estimate genetic diversity of 96 winter chilli cultivars collected from diverse locations of Bangladesh by means of 39 microsatellite markers to guide genetic improvement and to promote increased utilization.

2 MATERIALS AND METHODS

2.1 COLLECTION AND EXTRACTION OF GENOMIC DNA FROM A PLANT SAMPLE

A total of 96 local cultivars (Table 1) of winter growing chilli (*Capsicum annuum* L. representing different geographical distributions were nominated and collected at Plant Genetic Resources Centre (PGRC), Bangladesh Agricultural Research Institute (BARI) for the current study to investigate molecular diversity by using SSR marker. Seeds of collected cultivars were sown on small plastic pots to grow seedlings. For DNA extraction, we used young, fresh, disease- and insect-free leaves. SDS (Sodium dodecyl sulfate), phenol: chloroform: IAA followed by alcoholic precipitation were used to isolate genomic DNA from the leaf tissue of three-week-old seedlings described by Saghai-Maroof et al. (1984) with some modifications. Excluding the usage of liquid nitrogen, the modified protocol included digestion with homogenization buffer [Solution: Tris-50 mM, ethylene di-

Table 1: List of winter growing local chilli cultivars used in molecular characterization with their collection sites in Bangladesh

Sl. No.	Cultivars	Location of collecting site (Upazila and District)	Latitude (N)	Longitude (E)
01	BD-10878	Kazipur, Sirajganj	24° 41.516′	89° 42.83′
02	BD-10879	Galachipa, Patuakhali	22° 9.8′	90° 25.8′
03	BD-10880	Kazipur, Sirajganj	24° 41.711′	89° 43.059′
04	BD-10881	Kazipur, Sirajganj	24° 41.711′	89° 43.059′
05	BD-10882	Kazipur, Sirajganj	24° 41.711′	89° 43.059′
06	BD-10883	Kazipur, Sirajganj	24° 41.925′	89° 42.978′
07	BD-10884	Sadar, Sirajganj	24° 31.511′	89° 40.982′
08	BD-10885	Sadar, Sirajganj	24° 32.671′	89° 40.560′
09	BD-10886	Sadar, Sirajganj	24° 32.671′	89° 40.560′
10	BD-10887	Kalapara, Patuakhali	21° 58.918′	90° 13.60′
11	BD-10888	Kalapara, Patuakhali	21° 58.918′	90° 13.60′
12	BD-10892	Galachipa, Patuakhali	22° 9.48′	90° 25.48′
13	BD-10894	Galachipa, Patuakhali	22° 9.48′	90° 25.48′
14	BD-10895	Galachipa, Patuakhali	22° 9.48′	90° 25.48′
15	BD-10896	Galachipa, Patuakhali	22° 9.48′	90° 25.48′
16	BD-10897	Galachipa, Patuakhali	22° 9.48′	90° 25.48′
17	BD-10898	Galachipa, Patuakhali	22° 9.48′	90° 25.48′
18	BD-10938	Muksudpur, Gopalganj	23° 19.0′	89° 52.0′
19	BD-10934	Dohazari, Chittagong	22° 9.46′	92° 4.22′
20	BD-10935	Dohazari, Chittagong	22° 9.46′	92° 4.22′
21	BD-10936	Dohazari, Chittagong	22° 9.46′	92° 4.22′
22	BD-10913	Kotalipara, Gopalganj	22° 59.0′	89° 59.30′
23	KASI-49	Kotalipara, Gopalganj	22° 59.0′	89° 59.30′
24	BD-10916	Kashiani, Gopalganj	23° 17.618′	89° 47.259′
25	BD-10917	Daulatkhan, Bhola	22° 36.24′	90° 44.60′
26	BD-10918	Daulatkhan, Bhola	22° 36.24′	90° 44.60′
27	BD-10919	Sadar, Bhola	22°37.517′	90° 38.062′
28	BD-10920	Sadar, Bhola	22° 37.517′	90° 38.062′
29	RISA-23	Sadar, Bhola	22° 37.517′	90° 38.062′
30	BD-10921	Sadar, Bhola	22° 37.517′	90° 38.062′
31	BD-10922	Sadar, Bhola	22° 37.517′	90° 38.062′
32	BD-10923	Charfashion, Bhola	22° 11.60′	90° 45.48′
33	BD-10924	Charfashion, Bhola	22° 11.60′	90° 45.48′
34	BD-10925	Charfashion, Bhola	22° 11.60′	90° 45.48′
35	BD-10927	Charfashion, Bhola	22° 11.60′	90° 45.48′
36	BD-10928	Charfashion, Bhola	22° 11.60′	90° 45.48′
37	BD-10929	Charfashion, Bhola	22° 11.60′	90° 45.48′
38	BD-10930	Daulatkhan, Bhola	22° 36.24′	90° 44.60′
39	BD-10931	Daulatkhan, Bhola	22° 36.24′	90° 44.60′

Continued on the next page

40	BD-10932	Borhanuddin, Bhola	22° 30′	90° 43.3′
41	BD-10933	Borhanuddin, Bhola	22° 30′	90° 43.3′
42	BD-10900	Madarganj, Jamalpur	24° 54.490′	89° 43.075′
43	BD-10903	Melando, Jamalpur	24° 56.962′	89° 52.622′
44	BD-10904	Melando, Jamalpur	24° 56.962′	89° 52.622′
45	BD-10905	Melando, Jamalpur	24° 56.962′	89° 52.622′
46	BD-10906	Melando, Jamalpur	24° 56.962′	89° 52.622′
47	RT-09	Melando, Jamalpur	24° 56.962′	89° 52.622′
48	RT-14	Sadar, Jamalpur	24° 56.170′	89° 55.721′
49	BD-10908	Sharishabari, Jamalpur	24° 45.103′	89° 49.012′
50	BD-10909	Sharishabari, Jamalpur	24° 45.918′	89° 49.108′
51	BD-10910	Sharishabari, Jamalpur	24° 45.440′	89° 49.828′
52	BD-10911	Sharishabari, Jamalpur	24° 45.192′	89° 49.415′
53	BD-10912	Sharishabari, Jamalpur	24° 45.142′	89° 49.914′
54	AM-29	Kazipur, Sirajganj	24° 41.925′	89° 42.978′
55	BD-10899	Galachipa, Patuakhali	22° 14.62′	90° 23.39′
56	BD-10914	Kotalipara, Gopalganj	22° 59.0′	89° 59.30′
57	BD-10926	Charfashion, Bhola	22° 11.283′	90° 47.124′
58	BD-10901	Madarganj, Jamalpur	24° 53.026′	89° 42.296′
59	BD-10902	Madarganj, Jamalpur	24° 53.026′	89° 42.296′
60	BD-10907	Sharishabari, Jamalpur	24° 45.662′	89° 49.828′
61	BD-10939	Khetlal, Joypurhat	25° 1.5′	89° 8′
62	KASI-115	Muksudpur, Gopalganj	23° 19′	89° 52′
63	BD-10940	Sadar, Gazipur	24° 0′	90° 25.30′
64	RI-02	Ramgarh, Khagrachori	22°.59.97′	91° 42.79′
65	RI-12	Ramgarh, Khagrachori	22°.59.58′	90° 41.83′
66	BD-10889	Kalapara, Patuakhali	21°.58.918′	90° 13.60′
67	BD-10890	Amtali, Barguna	22°.05.115′	90° 14.178′
68	AMS-08	Amtali, Barguna	22°.05.115′	90° 14.178′
69	AMS-10	Kalapara, Patuakhali	22°.02.056′	90° 17.005′
70	AMS-21	Galachipa, Patuakhali	22°.10.413′	90° 23.885′
71	AMS-26	Sadar, Patuakhali	22°.16.437′	90° 19.355′
72	AMS-39	Nalsity, Jhalokati	22°.38.203′	90° 20.966′
73	AMS-42	Babuganj, Barisal	22°.46.966′	90° 18.834′
74	AMS-45	Babuganj, Barisal	22°.47.167′	90° 19.888′
75	AHM-46	Babuganj, Barisal	22°.39.03′	90° 00.51′
76	AHM-46(1)	Wajirpur, Barisal	22° 48.42′	90° 14.42′
77	BD-10941	Sadar, Barisal	22°.44.170′	90° 11.124′
78	AHM-142	Jajira, Shariatpur	23°.19.259′	90° 08.421′
79	AHM-143	Jajira, Shariatpur	23°.15.838′	90° 12.381′
80	IA-52	Tongibari, Munshiganj	23°.30.762′	90° 29.715′

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81	BD-10891	Aamtali, Barguna	22° 12.889′	90° 17.853′
82	BD-10893	Galachipa, Patuakhali	22° 10.413′	90° 23.855′
83	AMS-30	Sadar, Patuakhali	22° 21.90′	90° 23.90′
84	AMS-31	Sadar, Patuakhali	22° 21.90′	90° 23.90′
85	AMS-12	Aamtali, Barguna	22° 08.008′	90° 23.831′
86	AMS-32	Dumki, Patuakhali	22° 27.495′	90° 21.298′
87	AMS-33	Bakerganj, Barisal	22° 33.512′	90° 19.893′
88	RT-12	Sadar, Jamalpur	24° 56.167′	89° 55.892′
89	RT-20	Sadar, Jamalpur	24° 50.909′	89° 53.465′
90	RT-22	Sharishabari, Jamalpur	24° 45.312′	89° 49.112′
91	RT-11	Sadar, Jamalpur	24° 56.167′	89° 55.892′
92	RT-13	Sadar, Jamalpur	24° 56.167′	89° 55.892′
93	RT-18	Sadar, Jamalpur	24° 56.909′	89° 53.465′
94	RISA-33	Sadar, Bhola	22° 47.582′	90° 37.837′
95	RM-01	Akkelpur, Joypurhat	25° 01.958′	89° 01.610′
96	KASI-20(1)	Kotalipara, Gopalganj	22° 59.0′	89° 59.30′

amine tetra acetic acid (EDTA) 25 mM, NaCl 300 mM, SDS 1 % and deionized water] at a temperature of 65 °C for about 30 min, extraction by phenol (25): chloroform (24): IAA (1), precipitation with ice-cold and extra pure isopropyl alcohol and purification with absolute ethanol, sodium acetate (3M) and 70 % ethanol chronologically was used. Finally, DNA sample was added in 50 µl of Tris-EDTA (TE) buffer to a 1.5 ml micro centrifuge tube to dissolve. After completely dissolve the DNA pellet, 4 µl RNase @ 10 mg ml^{-1} was added to isolate DNA and incubated for 1.5 hours at 37 °C. Finally, DNA sample was kept in freezer at -20 °C.

2.2 DNA CONCENTRATION MEASUREMENT AND OPTIMIZATION

The occurrence of quality genomic DNA was confirmed on a 1 % agarose gel which was photographed utilizing a photo documentation technique after being visualized under UV light in UV Transilluminator (Uvitec, UK). In this investigation, DNA samples of all cultivars were confirmed to be of good quality. The amount of genomic DNA was quantified through UV spectrophotometer (Spectronic® GENESYS™ 10 Bio) at 260 nm wavelength. Using the spectrophotometer absorbance; the original DNA concentrations were determined according to the following equation:

$$\text{DNA conc. (ng } \mu l^{-1}) = \text{Absorbance} \times \frac{\text{Volume of distilled water (µl)}}{\text{Amount of DNA sample (µl)}} \times \text{CF } (0.05) \times 1000$$

Before PCR amplification of DNA, the DNA concentrations were adjusted to 25 ng μl^{-1} using the following formula: $S_1 \times V_1 = S_2 \times V_2$ Where, S_1: Initial strength (ng μl^{-1}), V_1: Initial volume (µl), S_2: Final strength (ng μl^{-1}) and V_2: Final volume (µl)

2.3 IDENTIFICATION AND SELECTION OF MICROSATELLITE OR SSR PRIMERS

Preliminarily, 50 microsatellite primer pairs were tested to identify discriminating alleles those are located in 12 chromosomes of chilli from different publications. Among them, 39 were selected for their better responsiveness with clear and desired amplified product size, and they were used in the present investigation for microsatellite analysis (Table 2).

2.4 STANDARDIZATION OF PCR AND ITS AMPLIFICATION

The PCR was started with 10 µl volumes comprising 50 ng template DNA, 5X Green GoTaq® Reaction Buffer included 7.5 mM $MgSO_4$, 1.25 U μl^{-1} Taq DNA polymerase, 0.4 mM of the deoxyribonucleotide triphosphate (dNTPs), 10 µM of primer, 0.5 % DMSO (dimethyl sulfoxide) and required amount of deionized water. This reaction was carried out in an oil-free Eppendorf Mastercycler® nexus Gradient thermal cycler. The following

Table 2: List of microsatellite primers used in this study. Ann. T.: Annealing Temperature, Chr. no.: Chromosome number

Sl.	Locus	Primer sequence (5'-3')	Repeat motif	Ann. T.	Chr. no.	Expected Size (bp)	Reference
1	CAMS-336	F: ggtggaaacttgcttggaga R: cccagaaccatccacctact	$(tc)_{16}$	53°C	3	157	(Minamiyama et al., 2006)
2	CAMS-351	F: cgcatgaagcaaatgtacca R: acctgcagtttgttgttgga	$(tg)_3$…$(ag)_{26}$	51°C	4	240	
3	CAMS-405	F: ttcttgggtcccacactttc R: aggttgaaaggagggcaata	$(tc)_{18}$	53°C	8, 11	241	
4	CAMS-460	F: cctttcacttcagcccacat R: accatccgctaagacgagaa	$(tc)_{20}$	54°C	7	215	
5	CAMS-679	F: tttgcatgttttacccattcc R: atgtgaaacacataggtagcactga	$(tat)_{16}$	53°C	1	200	
6	CAMS-864	F:ctgttgtggaagaagaggaca R: gcttctttttcaacctcctcct	$(aga)_{32}$	54°C	7	222	
7	CAMS-072	F: cccgcgaaatcaaggtaat R: aaagctattgctactgggttcg	$(ac)_{13}$	53°C	5	153	
8	CAMS-117	F: ttgtggaggaaacaagcaaa R: cctcagcccaggagacataa	$(tg)_{21}(ta)_3$	52°C	11	223	
9	CAMS-806	F: tgtcacaagtgtcaaggtaggag R: ccccaaaaattttccctcat	$(aga)_{19}$	54°C	10	227	
10	CAMS-844	F: gcaaagaaaaagaaaagcctga R: ctgcaactgctgcttcattc	$(gaa)_6$	53°C	1	223	
11	CAMS-015	F: tcatgttgattatgcttttgttca R: ccatgtattgtatgatacctgagaaa	$(ac)_7$ at$(ac)_8$a $(ta)_7$	53°C	2	112	
12	CAMS-065	F: ccagtctcatccagcagaca R: catatgctgctcctgcattc	$(ac)_{12}$	52°C		213	
13	CAMS-075	F: actaattacacattctgcattttctc R: aggctcgagtaccacgaaga	$(tg)_{10}$	54°C	5	190	
14	CAMS-478	F: gagtgccatgctgattaagga R: cacgactgtcttgcctgaac	(ag)12	52°C	3	248	
15	CAMS-838	F: ccaggatggtgttaagggttt R: gtcgcatcaatgagcatagg	(aga)19	59°C	6	229	
16	CAMS-861	F: gcatgcaagcttagccaac R: tgagattgaagctagaattttgga	(aga)11	52°C	2	183	

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17	CAMS-880	F: gagccaagaaaaaggtggaa R: caactcatcgttcaacaacaca	(gaa)12	53°C	6	237	(Minamiyama et al., 2006)
18	CAMS-236	F: ttgtagtttgcgtaccatttga R: atgaatccagggttccacaa	(ac)14a (ta)10	54°C	2	191	
19	CAMS-885	F: aacgaaaaacaaacccaatca R: ttgaaattgctgaaactctgaa	(gaa)28	53°C	2	248	
20	CAMS-647	F: cggattcggttgagtcgata R: gtgctttggttcggtctttc	(tat)6tg (tta)3… (tat)21	54°C	3	221	
21	CAMS-173	F: caaccgccagtagacaggtt R: gtgcgtgtgcgtgtgtgtat	(cata)7…(ac)4	52°C	4	169	
22	CAMS-163	F: tccatatagcccgtgtgtga R:gcgtgggaatacaatgctaga	(at)7 (gt)14	53°C	5	250	
23	CAMS-826	F: cttgatctcaagaaccagctacaa R: tgtacattgaagacacggaagaa	(gaa)6 ga (gga)9.. (gaa)3 ga (gaa)3	53°C	8	244	
24	CAMS-855	F: aagtgtcaaggaaggggaca R: cctaaccacccccaaaagtt	(agt)14a (gaa)9	54°C	8	243	
25	CAMS-493	F: tcgatgacgaaaaagtgtgaa R: agggcaaaagacccattctt	(ag)6	53°C	8	225	(Mimura et al., 2012)
26	CAMS-454	F: gagcctcttaatgtatctgaaaaca R: aattttggtgaatcgcacct	(ct)3.. (tc)4c (ct)3.. (tc)5.. (tc)5cc (tc)4	54°C	9	243	
27	CAMS-340	F: tttatgcccattcacaaaataa R: ggacgaattcaccgagtgc	(ta)3… (ag)13	53°C	10	250	
28	CAMS-156	F: ccctatgctttcacaactcct R: acgtggttatgacgataggc	(ac)14a (ta)6	54°C	10	181	
29	Hpms 1-1	F: tcaacccaatattaaggtcacttcc R: ccaggcggggattgtagatg	(ca)12 (ta)4	55°C	1	283	(Lee et al., 2005)
30	AF244121	F: tacctcctcgccaatccttctg R: ttgaaagttctttccatgacaacc	(tag)4IP (gtt)3	52°C	1, 3	234	

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31	Hpms 1-165	F: ggctatttccgacaaaccctcg R: ccattggtgttttcactgttgtg	(ga)13	53°C	4	213	(Lee et al., 2005)
32	Hpms 2-23	F: ccctcggctcaggataaatacc R: ccccagactcccactttgtg	(ttg)7 (gt)9	54°C	5	127	
33	Hpms 1-5	F: ccaaacgaaccgatgaacactc R: gacaatgttgaaaaaggtggaagac	(at)11 (gt)17	53°C	6	311	
34	Hpms AT2-20	F: tgcactgtcttgtgttaaaatgacg R: aaaattgcacaaatatggctgctg	(aat)18	52°C	6	148	
35	Hpms CaSIG19	F: catgaatttcgtcttgaaggtccc R: aagggtgtatcgtacgcagcctta	(ct)6 (at) (gtat)58	54°C	7	218	
36	Hpms 2-21	F: tttttcaattgatgcatgaccgata R: catgtcattttgtcattgatttgg	$(at)_{11}$ $(ac)_{9}$ $(atac)_{10}$	55°C	10	295	
37	Hpms 1-172	F: gggtttgcatgatctaagcatttt R: cgctggaatgcattgtcaaaga	$(ga)_{15}$	58°C	11	344	
38	Hpms 2-2	F: gcaaggatgcttagttgggtgtc R: tcccaaaattaccttgcagcac	$(gt)_{9}$	55°C	11	146	
39	Hpms E-075	F: gcggctcagcagaaagagagag R: tgccacagctggagaacgtaaa	$(acc)_{6}$	52°C	12	205	(Yi et al., 2006)

"touchdown" PCR settings were used to amplify SSRs: 94 °C 3 min^{-1} denaturation, 11 cycles of 94 °C 0.5 min^{-1}, 58-60 °C for 1 min, decreasing by 1 °C per cycle, and 72 °C for 1 min; 30 cycles of 94 °C for 0.5 min^{-}, 52-55 °C for 1 min and 72 °C for 1 min; finally, extension for 5 min. The PCR products were resolved electrophoretically on 2 % agarose gel in 1X TBE to check amplification. The PCR procedure was regarded correct when the primer showed decent band, decreased smearing, and amplified the template DNA at target genomic region.

2.5 PCR PRODUCTS SEPARATION AND VISUALIZATION USING ELECTROPHORESIS

The products of PCR were separated on 5 % denatured polyacrylamide gel using acrylamide: bis-acrylamide (19:1), 10 % APS, 10X TBE buffer, and ultrapure Temed. Triple Wide Mini-Vertical Electrophoresis System (Model: MGV-202-33, CBS Scientific, USA) was used to perform the electrophoresis. Upon loading of PCR products, run the gel maintaining 20 ^{0}C temperature

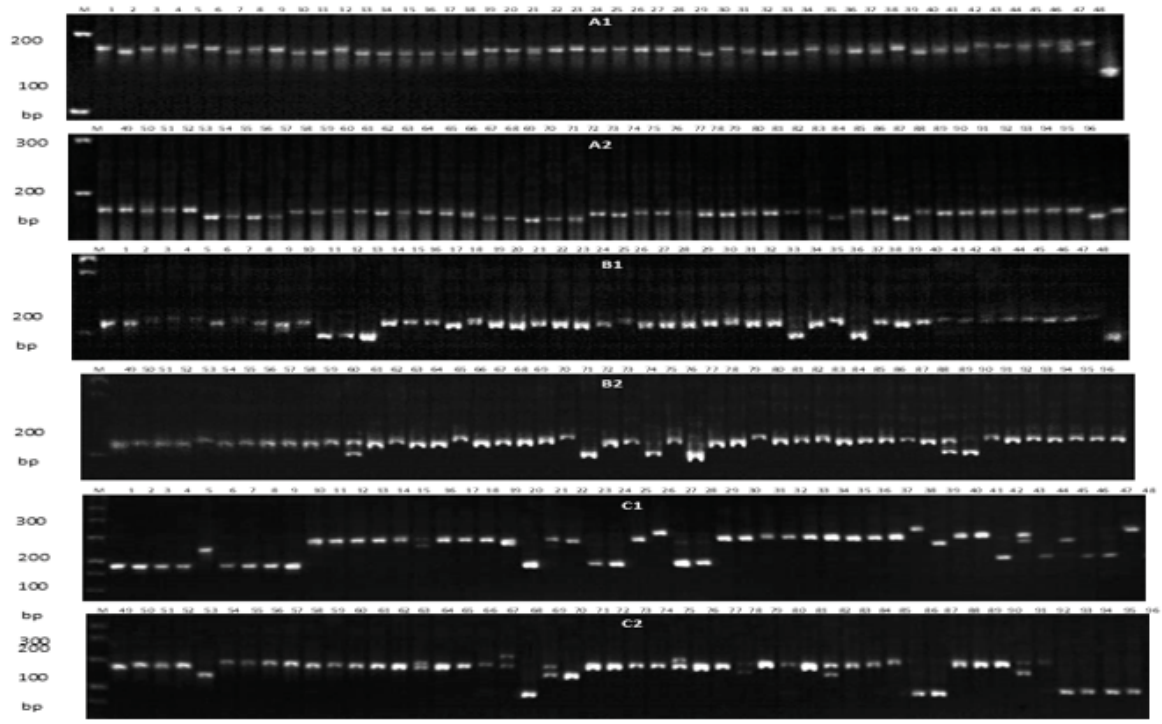

Figure 1: Microsatellite profiles of 96 winter local chilli cultivars at locus CAMS-679 (A1, A2), CAMS-117 (B1, B2) and CAMS-647 (C1, C2); M: Molecular wt. marker (100 bp DNA ladder). Lane 01: BD-10878; Lane 02: BD-10879; Lane 03: BD-10880; Lane 04: BD-10881; Lane 05: BD-10882; Lane 06: BD-10883; Lane 07: BD-10884; Lane 08: BD-10885 Lane 09: BD-10886; Lane 10: BD-10887; Lane 11: BD-10888; Lane 12: BD-10892; Lane 13: BD-10894; Lane 14: BD-10895; Lane 15: BD-10896; Lane 16: BD-10897; Lane 17: BD-10898; Lane 18: BD-10938; Lane 19: BD-10934; Lane 20: BD-10935; Lane 21: BD-10936; Lane 22: BD-10913; Lane 23: KASI-49; Lane 24: BD-10916; Lane 25: BD-10917; Lane 26: BD-10918; Lane 27: BD-10919; Lane 28: BD-10920; Lane 29: RISA-23; Lane 30: BD-10921; Lane 31: BD-10922; Lane 32: BD-10923; Lane 33: BD-10924; Lane 34: BD-10925; Lane 35: BD-10927; Lane 36: BD-10928; Lane 37: BD-10929; Lane 38: BD-10930; Lane 39: BD-10931; Lane 40: BD-10932; Lane 41: BD-10933; Lane 42: BD-10900; Lane 43: BD-10903; Lane44: BD-10903; Lane 45: BD-10905; Lane 46: BD-10906; Lane 47: RT-09; Lane 48: RT-14; Lane 49: BD-10908; Lane 50: BD-10909 Lane 51: BD-10910; Lane 52: BD-10911; Lane 53: BD-10912, Lane 54: AM-29; Lane 55: BD-10899; Lane 56: BD-10914; Lane 57: BD-10926; Lane 58: BD-10901; Lane 59: BD-10902; Lane 60: BD-10907; Lane 61: BD-10939; Lane 62: KASI-115; Lane 63: KASI-115; Lane 64: RI-02; Lane 65: RI-12; Lane 66: BD-10889; Lane 67: BD-10890; Lane 68: AMS-08; Lane 69: AMS-10; Lane 70: AMS-21; Lane 71: AMS-26; Lane 72: AMS-39; Lane 73: AMS-42; Lane 74: AMS-45; Lane 75: AHM-46; Lane 76: AHM-46(1); Lane 77: BD-10941; Lane 78: AHM-142; Lane 79: AHM-143; Lane 80: IA-52; Lane 81: BD-10891; Lane 82: BD-10893; Lane 83: AMS-30; Lane 84: AMS-31; Lane 85: AMS-12; Lane 86: AMS-32; Lane 87: AMS-33; Lane 88: RT-12; Lane 89: RT-20; Lane 90: RT-22; Lane 91: RT-11; Lane 92: RT-13; Lane 93: RT-18; Lane 94: RISA-33; Lane 95: RM-01; Lane 96: KASI-20(1)

at 80-90 V for a set period of time (usually 1 hour for 100 bp) depending on the size of the amplified DNA fragment. Once electrophoresis was completed, the gel was stained with ethidium bromide. For analysis, the individual bands on the glass plate were colored and scored.

2.6 MICROSATELLITE DATA SCORING AND ANALYSIS

Three expert scientists separately assessed the bands representing specific alleles at the microsatellite loci and labelled from the top to the bottom of the gel as A, B & C. Cultivars were hypothetically scored as homozygous (AA, BB, CC) or heterozygous (AB, AC, BC). All loci were combined into a single genotypic data matrix. Allelic frequency estimations were generated to produce statistics of genetic variation (number of observed and effective alleles, Nei's gene diversity, Shannon's information index, heterozygosity, and polymorphism) from genotypic frequency of SSR loci using POPGENE (Version 1.31) (Abouzied et al., 2013). The microsatellite data matrix was deployed to calculate Nei's distance (Nei, 1972), and to produce the corresponding matrix of genetic distance among accessions, while cluster analyses were carried out on the genetic distance matrix by using the UPGMA to determine the relations among accessions (dendrograms) using POPGENE (Version 1.31) (Abouzied et al., 2013). The PIC (polymorphism information content) or gene diversity value of the SSR utilized was computed as PIC= 1- 1- $\Sigma X i^2$; Where, X*i* is the frequency of the *i*-th allele of a particular locus. The software DNA FRAG version 3.03 was used to estimate allelic length (Islam et al., 2012).

3 RESULTS

3.1 MICROSATELLITE POLYMORPHISM

All 39 microsatellite primers employed in this study were confirmed to be polymorphic based on DNA amplification patterns. Figure 1 illustrates three typical SSR profiles. Table 3 shows the results of the variability parameters analysis for the 39 SSRs in the 96 chilli cultivars. With the 39 SSR loci investigated herein, a total of 123 alleles were found among all chilli cultivars, averaging 3.154 alleles per locus. Variation of allele number ranged from 2 to 8. The locus CAMS-647 yielded the most alleles (8) with sizes ranging from 188 to 279 base pairs. Likewise, 6 alleles (142 to 176 bp and 184 to 240 bp) and 5 alleles (223 to 291 bp) were detected at the loci CAMS-679, CAMS-117 and CAMS-855, respectively, in descending order (Table 3). When all cultivars were considered, the expected heterozygosity (H_E, average 0.484) values for each SSR locus were always higher than the observed heterozygosity (H_O), indicating that the population was homozygous.

PIC values for the 39 primers tested in this work ranged from 0.099 for Hpms 1-165 to 0.806 for CAMS-679, with an average value of 0.484 (Table 3). Among the studied markers CAMS-679, CAMS-855, CAMS-117, CAMS-647, CAMS-236, CAMS-351, CAMS-885, CAMS-340, CAMS-864, CAMS-460, CAMS-844, and CAMS-880 showed higher PIC values (> 0.6) followed by HpmsAT2-20 (0.278), CAMS-015 (0.256), CAMS-156 (0.249), Hpms 1-1 (0.249), CAMS-838 (0.170), Hpms 1-172 (0.170), HpmsE075 (0.117), and Hpms 1-165 (0.099) in descending order. Among the studied markers, allele frequency ranged from 0.281 to 0.948 (Table 3).

Effective allele number was also the highest (5.166) for CAMS-679 following 4.046, 3.912, 3.436, 3.364 and 3.322 for CAMS-855, CAMS-647, CAMS-885, CAMS-236 and CAMS-251, respectively (Table 4). Nei's expected heterozygosity (genetic diversity) ranged from 0.117 (HpmsE075) to 0.811 (CAMS-679) with an average value of 0.484.

The mean Shannon's information index (I) was 0.842, and ranged from 0.205 to 1.713 (Table 4). The highest Shannon's information index (1.713) was recorded in the locus CAMS-647 followed by CAMS-679 (1.617), CAMS-117 (1.589), CAMS-855 (1.444) as against the lowest (0.205) in Hpms 1-165. Ranges of genetic differentiation (Fst) values were 0.834 to 1.000 with an average of 0.927 and gene flow (Nm) values ranged from 0.000 to 0.050 with an average of 0.010 (Table 4).

3.2 NEI'S GENETIC DISTANCE BETWEEN THE CULTIVARS

The genetic distance value (GD) of 4560 (1+2+3+...+95) pairs resulting from a permutation combination of 96 winter chilli cultivars ranged from 0.103 to 0.990 on average. While analyzing 96 cultivars, comparatively higher genetic distance values were observed between the pairs of 55 cultivars, while the pairs of 41 cultivars showed lower GD values (Table 5 and Table 6).

The pair BD-10879 vs RT-22 and BD-10926 vs BD-10920 showed the highest (0.990) genetic distance followed by 0.921 and 0.911 in BD-10887 vs RT-12, BD-10931 vs IA-52, BD-10927 vs BD-10879, RT-11 vs BD-10887, RT-11 vs BD-10926, RT-22 vs BD10931 and IA-52 vs BD-10934, respectively (Table 5). The pair between AMS-30 and BD-10893 showed the lowest (0.103) genetic distance followed by BD-10883 with BD-10880

Table 3: Variability of simple sequence repeat marker used for genetic analysis of chilli cultivars

Locus	No. of allele	Allele sizes (bp)	Major allele frequency	Obs Het (H_o)	Exp Het (H_E)	PIC
CAMS-015	3	100, 106, 110	0.854	0.000	0.258	0.256
CAMS-065	4	197, 209, 215, 239	0.479	0.000	0.578	0.575
CAMS-072	3	153, 166, 173	0.677	0.000	0.476	0.474
CAMS-075	4	178, 194, 207, 218	0.615	0.000	0.554	0.551
CAMS-117	6	184, 193, 208, 222, 227, 240	0.500	0.000	0.633	0.750
CAMS-156	2	176, 185	0.854	0.000	0.250	0.249
CAMS-163	2	136, 148	0.802	0.000	0.319	0.317
CAMS-173	3	146, 159, 170	0.688	0.000	0.459	0.457
CAMS-236	4	182, 198, 199, 202	0.385	0.000	0.706	0.703
CAMS-336	3	152, 173, 183	0.750	0.000	0.401	0.398
CAMS-340	4	245, 260, 272, 287	0.432	0.000	0.662	0.658
CAMS-351	4	179, 189, 200, 220	0.427	0.000	0.703	0.699
CAMS-405	3	207, 226, 244	0.552	0.000	0.574	0.571
CAMS-454	2	221, 240	0.583	0.000	0.489	0.486
CAMS-460	3	195, 209, 218	0.490	0.000	0.633	0.630
CAMS-478	2	215, 230	0.646	0.000	0.460	0.457
CAMS-493	3	201, 213, 225	0.510	0.000	0.571	0.568
CAMS-647	8	188, 198, 206, 220, 235, 239, 256, 279	0.406	0.000	0.748	0.744
CAMS-679	6	142, 147, 154, 160, 168, 176	0.281	0.000	0.811	0.806
CAMS-806	3	209, 222, 233	0.667	0.000	0.476	0.474
CAMS-826	3	215, 229, 258	0.823	0.000	0.307	0.306
CAMS-838	2	160, 164	0.906	0.000	0.171	0.170
CAMS-844	3	198, 210, 219	0.490	0.000	0.633	0.630
CAMS-855	5	223, 239, 252, 270, 291	0.292	0.000	0.757	0.753
CAMS-861	3	209, 230, 240	0.563	0.000	0.569	0.566
CAMS-864	4	205, 231, 264, 291	0.427	0.000	0.649	0.645
CAMS-880	3	205, 219, 231	0.521	0.000	0.615	0.612
CAMS-885	4	200, 209, 216, 224	0.354	0.000	0.713	0.683
Hpms 1-1	2	247, 262	0.854	0.000	0.250	0.249
Hpms 1-5	3	266, 285, 312	0.531	0.000	0.559	0.556
Hpms 1-165	2	191, 202	0.948	0.000	0.099	0.099
Hpms 1-172	2	280, 300	0.906	0.000	0.171	0.170
Hpms 2-2	2	156, 167	0.406	0.000	0.485	0.482
Hpms 2-21	2	273, 294	0.625	0.000	0.471	0.469
Hpms 2-23	2	205, 218	0.740	0.000	0.387	0.385
HpmsAT2-20	2	143, 152	0.833	0.000	0.279	0.278
HpmsCaSIG19	2	209, 220	0.760	0.000	0.366	0.364
HpmsE075	2	208, 220	0.938	0.000	0.118	0.117
AF244121	3	93, 111, 120	0.542	0.000	0.526	0.523
Mean	3.154		0.617	0.000	0.484	0.484

Table 4: Summary of genetic variation statistics for all loci used for 96 winter chilli cultivars analysis

Locus	Observed number of alleles (na)	Effective number of alleles (ne)	Genetic diversity	Shannon's Information Index (I)	Genetic differentiation (Fst)	Gene flow (Nm*)
CAMS-336	3	1.662	0.398	0.703	1.000	0.000
CAMS-351	4	3.322	0.699	1.288	1.000	0.000
CAMS-405	3	2.331	0.571	0.942	1.000	0.000
CAMS-460	3	2.700	0.630	1.046	1.000	0.000
CAMS-679	6	5.166	0.753	1.617	1.000	0.000
CAMS-864	4	2.818	0.645	1.159	1.000	0.000
CAMS-072	3	1.900	0.474	0.802	1.000	0.000
CAMS-117	6	2.703	0.744	1.589	1.000	0.000
CAMS-806	3	1.900	0.474	0.781	0.989	0.003
CAMS-844	3	2.700	0.630	1.046	1.000	0.000
CAMS-015	3	1.345	0.256	0.491	0.838	0.093
CAMS-065	4	2.351	0.675	0.968	0.955	0.005
CAMS-075	4	2.227	0.651	0.972	0.978	0.003
CAMS-478	2	1.843	0.458	0.650	1.000	0.000
CAMS-838	2	1.205	0.170	0.311	0.968	0.004
CAMS-861	3	2.305	0.566	0.937	1.000	0.000
CAMS-880	3	2.577	0.612	1.020	1.000	0.000
CAMS-236	4	3.364	0.703	1.287	1.000	0.000
CAMS-885	4	3.436	0.709	1.292	0.863	0.040
CAMS-647	8	3.912	0.806	1.713	1.000	0.000
CAMS-173	3	1.841	0.457	0.762	0.951	0.004
CAMS-163	2	1.465	0.318	0.498	0.901	0.005
CAMS-826	3	1.441	0.306	0.582	0.891	0.031
CAMS-855	5	4.046	0.734	1.444	0.972	0.007
CAMS-493	3	2.312	0.568	0.916	1.000	0.000
CAMS-454	2	1.946	0.486	0.679	1.000	0.000
CAMS-340	4	2.922	0.658	1.145	0.834	0.050
CAMS-156	2	1.332	0.249	0.415	0.962	0.004
Hpms 1-1	2	1.332	0.249	0.415	0.932	0.007
AF244121	3	2.097	0.523	0.804	0.911	0.008
Hpms 1-165	2	1.110	0.099	0.205	0.874	0.034
Hpms 2-23	2	1.627	0.385	0.574	0.901	0.009
Hpms 1-5	3	2.251	0.556	0.894	0.911	0.008
HpmsAT2-20	2	1.385	0.278	0.451	0.904	0.009
HpmsCaSIG19	2	1.573	0.364	0.551	0.952	0.004
Hpms 2-21	2	1.882	0.469	0.662	0.899	0.023
Hpms 1-172	2	1.205	0.170	0.311	0.879	0.032
Hpms 2-2	2	1.932	0.482	0.676	0.918	0.008
HpmsE075	2	1.133	0.117	0.234	0.895	0.022
Mean	3.154	2.220	0.490	0.842	0.927	0.010

Nm* = Gene flow estimated from Fst = 0.25 (1 - Fst)/Fst, Fst = Genetic differentiation

Table 5: List of genotype pairs of winter chilli showed higher values of Nei's (1972) genetic distance

Genotype pair				Genetic Distance	Genotype pair				Genetic Distance
1	BD-10879	vs	RT-22	0.990	29	RT-20	vs	BD-10896	0.799
2	BD-10926	vs	BD-10920	0.990	30	IA-52	vs	BD-10900	0.799
3	BD-10887	vs	RT-12	0.921	31	BD-10940	vs	BD-10909	0.799
4	BD-10931	vs	IA-52	0.921	32	BD-10931	vs	BD-10913	0.799
5	BD-10927	vs	BD-10879	0.921	33	RT-11	vs	BD-10914	0.799
6	RT-11	vs	BD-10887	0.921	34	RT-22	vs	BD-10916	0.799
7	RT-11	vs	BD-10926	0.921	35	RT-22	vs	BD-10930	0.799
8	RT-22	vs	BD-10931	0.921	36	AHM-46	vs	BD-10906	0.790
9	IA-52	vs	BD-10934	0.911	37	RT-20	vs	BD-10938	0.790
10	BD-10878	vs	BD-10918	0.857	38	BD-10917	vs	BD-10922	0.745
11	BD-10879	vs	RT-20	0.857	39	BD-10918	vs	AM-29	0.745
12	BD-10917	vs	BD-10902	0.857	40	BD-10920	vs	BD-10911	0.745
13	BD-10926	vs	BD-10941	0.857	41	BD-10903	vs	BD-10891	0.745
14	BD-10926	vs	RT-18	0.857	42	IA-52	vs	BD-10925	0.745
15	BD-10931	vs	RT-13	0.857	43	BD-10887	vs	BD-10939	0.745
16	BD-10917	vs	BD-10884	0.857	44	BD-10934	vs	BD-10929	0.736
17	BD-10902	vs	BD-10917	0.857	45	BD-10920	vs	BD-10901	0.695
18	RT-13	vs	BD-10935	0.857	46	BD-10920	vs	BD-10912	0.695
19	BD-10926	vs	AHM-46	0.848	47	BD-10926	vs	RM-01	0.695
20	BD-10878	vs	RI-02	0.799	48	RT-22	vs	BD-10886	0.695
21	BD-10884	vs	RT-11	0.799	49	IA-52	vs	BD-10888	0.695
22	BD-10909	vs	BD-10940	0.799	50	IA-52	vs	BD-10933	0.695
23	BD-10917	vs	BD-10908	0.799	51	BD-10884	vs	BD-10893	0.686
24	BD-10927	vs	IA-52	0.799	52	RT-20	vs	BD-10885	0.648
25	BD-10935	vs	BD-10903	0.799	53	RT-22	vs	BD-10890	0.648
26	BD-10916	vs	BD-10878	0.799	54	BD-10908	vs	BD-10921	0.648
27	BD-10926	vs	BD-10881	0.799	55	BD-10939	vs	BD-10936	0.648
28	BD-10879	vs	BD-10889	0.799					

and BD-10882 (0.122), BD-10923 vs BD-10932 (0.144), RISA-33 vs KASI-20(1) (0.167), BD-10897 vs BD-10898 (0.181) and so on (Table 6).

3.3 PHYLOGENETIC DENDROGRAM

The UPGMA cluster analysis generated a dendrogram that divided 96 chilli cultivars into two main group "A" and "B" where only one cultivar i.e. BD-10917 congregated in a separate group "B" and others (95 cultivars) belong to group "A" (Figure 2). However, Group "A" divided in two sub-group "A1" and "A2". Sub-group "A1" was split into two more sub-group ("A1.a" and "A1.b"), where sub-clusters "A1.a1" gathered eight cultivars (BD-10878, BD-10879, BD-10880, BD-10883, BD-10885, BD-10881, BD-10882 and BD-10884). Sub-cluster "A1.a2" grouped 17 cultivars forming, A1.a2.a3.a5, A1.a2.a3.a6 and A1.a2.a4 sub-clusters contained five (BD-10900, BD-10903, BD-10904, BD-10905, BD-10906), nine (BD-10908, BD-10909, BD-10911, BD-10910, BD-10901, BD-10902, BD-10912, BD-10907 and BD-10939) and three (RT-09, RT-14 and AHM-142) cultivars, respectively (Figure 2 and Table 7).

A total of 29 cultivars were clustered into sub-cluster A1.b, which was further divided into four sub-clusters viz., A1.b1, A1.b2.b3.b5, A1.b2.b3.b6 and A1.b2.b4. Similarly, sub-cluster A2 divided into another two sub-

clusters ("A2.a", "A2.b"), where sub-clusters "A2.a1" assembled seven cultivars (BD-10886, KASI-49, BD-10916, KAI-115, RI-12, BD-10934 and BD-10913) whereas only four cultivars viz., BD-10935, BD-10918, BD-10919 and BD-10920 accumulated in sub-cluster "A2.a2" (Figure 4 and Table 7). However, 30 cultivars separated into seven sub-clusters of A2.b in where maximum five cultivars accumulated in four sub-clusters (A2.b1.b4, A2.b2.b5, A2.b2.b6.b8.b11 and A2.b2.b6.b8.b12), four cultivars gathered in 2 sub-clusters (A2.b2.b6.b7.b9 and A2.b2.b6.b7.b10) and sub-cluster A2.b1.b3 accumulated only two cultivars viz., BD-10929 and AMS-39 (Figure 2 and Table 7).

4 DISCUSSION

Among 50 primers screened, only 39 produced polymorphism and were used for final analysis of 96 winter chilli cultivars on the basis of easily scorable amplified bands (Table 3). All markers were observed to be polymorphic, expressing a total of 123 alleles with an average value of 3.15 alleles per locus in the analysis of 96 winter chilli cultivars. The majority of the primers (25) amplified 3-8 alleles per locus (Table 3), where the highest number of alleles (8) were amplified by the locus CAMS-647. However, Di Dato et al. (2015) observed 10 alleles in *Capsicum annuum* while analyzing with CAMS-647 marker. In another experiment carried out by Dhaliwal et al. (2014) identified the most divergent genotype among the six elite lines of chilli pepper by employing 58 SSR markers. Thirty produced polymorphic bands, revealing a total of 83 alleles with an average of 2.67 alleles per locus. Hossain et al. (2014) evaluated the genetic diversity within 22 chilli germplasm by using four microsatellite markers. All the microsatellite markers were found polymorphic in all studied germplasm. A total of 27 alleles were detected and the number of alleles per marker ranged from 4 to 13 (size range was 153-315 bp). The average numbers of allele (3.15) showed substantial variations compared with those of previous studies might be due to the high number of diverse chilli cultivars used in present study. The observed differences in allelic length for each locus indicated the presence of broad genetic base amongst the chilli cultivars. The wide genetic base might due to the high yield of polymorphic markers as reported by Molla et al. (2015)but there is possible uncertainty of linkage with the important genes. In contrast, there are better possibilities of linkage detection with important genes if SSRs are developed from candidate genes. To the best of our knowledge, there is no such report on SSR markers development from candidate gene sequences in rice. So the present study was aimed to identify and analyse SSRs from salt responsive candidate genes of rice. Results: In the present study, based on the comprehensive literature survey, we selected 220 different salt responsive genes of rice. Out of them, 106 genes were found to contain 180 microsatellite loci with, tri-nucleotide motifs (56%.

The PIC values, the reflection of allele diversity, offer an estimate of the discriminating power of a marker by taking into account not only the number of alleles at a locus, but also relative frequencies of these alleles. The genetic diversity of the cultivars chosen determines the PIC values, and this study featured a large number of traditional varieties, which would increase the PIC values. It is important to point out that the selection by breeders have increased the frequency of the alleles or allelic combination with favorable effects at the expense of the others, eventually eliminating many of them (Cao et al., 1998).

All of the SSRs were found to be polymorphic and useful for defining genotypic variation (i.e., PIC values different from zero) (Table 3). Twelve of these SSRs were very informative with higher PIC values (> 0.6) which was in accordance with the previous findings reported by Lee et al. (2005), Mimura et al. (2012) and Minamiyama et al. (2006). Lower PIC values indicate the presence of closely related cultivars; while higher PIC values indicate the presence of diverse cultivars. The observed high PIC values could be related to the utilization of di-nucleotide repeats as well as genotypic variations as reported by Islam et al. (2018). The present investigation had a high proportion of traditional varieties which would have the effect of increasing the PIC values. It is important to indicate that the selection by the breeders had increased the frequency of alleles or allelic combination with favorable effects at the expense of the others, eventually eliminating many of them (Roychowdhury et al., 2014). The number of alleles amplified by a primer and its PIC values also depends upon the repeat number and the repeat sequence of the microsatellite (Rahman et al., 2010). The results of present investigation are in agreement with those of Minamiyama et al. (2006) who showed that (tat), (tg), (ta) and (gaa) repeats yield higher number of alleles and higher PIC values. CAMS-647, CAMS-679, CAMS-117, CAMS-855, CAMS-885 and CAMS-236 having (tat)n, (tg)n, (gaa)n and (ac)n repeat were the most informative microsatellite markers for this set of cultivars, as they yielded five to eight alleles. For CAMS-647 [$(tat)_6tg(tta)_3$…$(tat)_{21}$], CAMS-117 [$(tg)_{21}(ta)_3$] and CAMS-679 [$(tat)_{16}$], showed eight, six and six alleles and average PIC values 0.744, 0.808, 0.806 and 0.750, respectively in analysis of 96 winter chilli cultivars that were not uncommon in terms of the number of repeats and the repeat motif (Table 2 and Table 3). Indeed, the incredibly beneficial markers are extremely valuable for genetic

Table 6: List of cultivars pairs of winter chilli showed lower values of Nei's (1972) genetic distance

Genotype pair				Genetic Distance	Genotype pair				Genetic Distance
1	AMS-30	vs	BD-10893	0.103	22	RT-09	vs	BD-10910	0.267
2	BD-10883	vs	BD-10880	0.122	23	AMS-21	vs	AMS-08	0.267
3	BD-10883	vs	BD-10882	0.122	24	BD-10895	vs	AMS-10	0.267
4	BD-10932	vs	BD-10923	0.144	25	AMS-21	vs	AMS-39	0.267
5	KASI-20(1)	vs	RISA-33	0.167	26	AHM-143	vs	AHM-142	0.267
6	BD-10898	vs	BD-10897	0.181	27	AMS-32	vs	AMS-31	0.267
7	BD-10894	vs	BD-10892	0.191	28	AHM-46(1)	vs	AMS-45	0.276
8	BD-10892	vs	BD-10895	0.191	29	AHM-143	vs	AHM-46(1)	0.286
9	RT-09	vs	BD-10904	0.191	30	AHM-46(1)	vs	KASI-20(1)	0.286
10	RT-14	vs	RT-09	0.191	31	BD-10924	vs	BD-10932	0.295
11	BD-10892	vs	AMS-26	0.191	32	BD-10910	vs	BD-10907	0.295
12	BD-10895	vs	BD-10894	0.216	33	AMS-39	vs	AMS-42	0.295
13	BD-10892	vs	BD-10928	0.216	34	RISA-33	vs	AHM-143	0.295
14	AMS-26	vs	AMS-21	0.216	35	AMS-33	vs	RT-12	0.295
15	AMS-33	vs	AMS-12	0.216	36	BD-10894	vs	BD-10898	0.314
16	RISA-33	vs	AMS-33	0.216	37	BD-10894	vs	RISA-23	0.323
17	BD-10895	vs	BD-10924	0.241	38	AMS-08	vs	BD-10919	0.353
18	RT-12	vs	KAI-115	0.241	39	AMS-10	vs	BD-10899	0.353
19	BD-10928	vs	AMS-32	0.241	40	AMS-08	vs	RI-12	0.353
20	AMS-31	vs	AMS-30	0.258	41	BD-10880	vs	RT-14	0.416
21	AHM-143	vs	KASI-49	0.267	-	-	-	-	-

investigations and determining the level of variation on a certain marker locus (Minamiyama et al., 2006; Sundaram et al., 2008).

According to Nei (1972), higher level of gene diversity values were observed in loci CAMS-679, CAMS-855, CAMS-647 and CAMS-117 and the lower level of gene diversity value was observed in loci HpmsE075, Hpms 1-172, Hpms 1-165 and Hpms 1-1 in analysis of 96 winter chilli cultivars (Table 4). It was observed that marker which detected the highest/higher number of alleles showed higher gene diversity than those detected lower number of alleles which revealed lowest/lower gene diversity. The maximum number of repeats within the SSRs was also positively correlated with the genetic diversity. This result is consistent with previous work done by Chen et al. (2012) and Hossain et al. (2014), who observed that the gene diversity at each SSR locus was significantly correlated with the number of alleles detected, number of repeat motif and with the allele size range. The higher genetic diversity as observed in the current study has also been reported in rice (Rahman et al., 2010), mung bean (Molla et al., 2016) and musk melon (Molla et al., 2017). The current study's findings are similar to those of previous could be owing to higher diversity of cultivars used in this analysis.

Study results also demonstrated higher level of genetic differentiation and low level of gene flow values in 96 chilli cultivars were indicative of diversity among the cultivars due to local origin/cultivars (Table 4). Higher genetic distance between genotype pair indicates that genetically they are diverse compare to lower genetic distance value. Basically, this value is an indication of their genetic dissimilarity. Genotype pair with higher value is more dissimilar than a pair with a lower value. The analysis of molecular data revealed different levels of gene diversity among 96 winter chilli cultivars as determined based on the Nei (1972) genetic distance. According to the results of genetic distance, higher values generated in participation of 55 out of 96 cultivars while rest 41 cultivars yielded lower values (Table 5 and Table 6). Hence, 55 chilli cultivars having higher GD values were selected for further evaluation. From the difference between the highest and the lowest genetic distance values, it was revealed that there was wide variability among

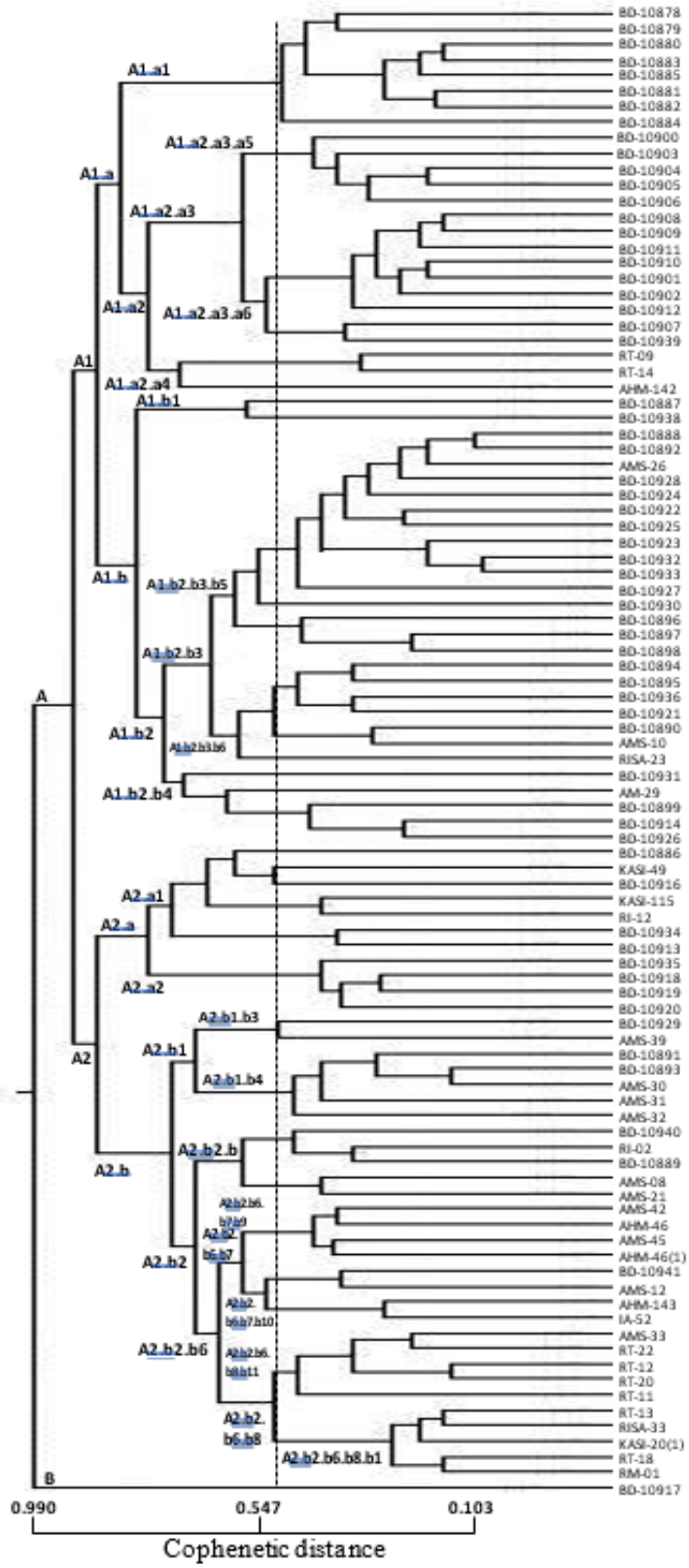

Figure 2: Dendrogram based on Nei's genetic distance, which summarizes the data on the variation between 96 winter local chilli cultivars according to microsatellite analysis

Table 7: Distribution of 96 cultivars according to cluster analysis and selection of diverse cultivars from this cluster

Sl. no.	Genotype	Cluster position	Sl. no.	Selected cultivars	Sl. no.	Genotype	Cluster position	Sl. no.	Selected cultivars
1	BD-10878	A1a1	1	BD-10878	50	BD-10931	A1.b2.b4	28	BD-10931
2	BD-10879		2	BD-10879	51	AM-29		29	AM-29
3	BD-10880		3	BD-10885	52	BD-10899		30	BD-10914
4	BD-10883		4	BD-10881	53	BD-10914		31	BD-10926
5	BD-10885		5	BD-10884	54	BD-10926			
6	BD-10881				55	BD-10886	A2.a1	32	BD-10886
7	BD-10882				56	KASI-49		33	BD-10916
8	BD-10884				57	BD-10916		34	BD-10934
9	BD-10900	A1.a2.a3.a5	6	BD-10900	58	KAI-115		35	BD-10913
10	BD-10903		7	BD-10903	59	RI-12			
11	BD-10904		8	BD-10906	60	BD-10934			
12	BD-10905				61	BD-10913			
					62	BD-10935	A2.a2	36	BD-10935
13	BD-10906				63	BD-10918		37	BD-10918
14	BD-10908	A1.a2.a3.a6	9	BD-10908	64	BD-10919		38	BD-10920
15	BD-10909		10	BD-10909	65	BD-10920			
16	BD-10911		11	BD-10911	66	BD-10929	A2.b1.b3	39	BD-10929
					67	AMS-39			
					68	BD-10891	A2.b1.b4	40	BD-10891
17	BD-10910		12	BD-10901	69	BD-10893		41	BD-10893
18	BD-10901		13	BD-10902	70	AMS-30			
19	BD-10902		14	BD-10912	71	AMS-31			
20	BD-10912		15	BD-10939	72	AMS-32			
21	BD-10907				73	BD-10940	A2.b2.b5	42	BD-10940
22	BD-10939				74	RI-02		43	RI-02
23	RT-09	A1.a2.a4			75	BD-10889		44	BD-10889
24	RT-14				76	AMS-08			
25	AHM-142		-	-	77	AMS-21			
26	BD-10887	A1.b1	16	BD-10887	78	AMS-42	A2.b2.b6.b7.b9	45	AHM-46
27	BD-10938		17	BD-10938	79	AHM-46			
28	BD-10888	A1.b2.b3.b5	18	BD-10888	80	AMS-45			
29	BD-10892		19	BD-10922	81	AHM-46(1)			
30	AMS-26		20	BD-10925	82	BD-10941	A2.b2.b6.b7.b10	46	BD-10941
31	BD-10928		21	BD-10933	83	AMS-12		47	IA-52
32	BD-10924		22	BD-10927	84	AHM-143			
33	BD-10922		23	BD-10930	85	IA-52			

Continued on the next page

34	BD-10925		24	BD-10896	86	AMS-33	A2.b2.b6.b8.b11	48 RT-22
35	BD-10923				87	RT-22		49 RT-12
36	BD-10932				88	RT-12		50 RT-20
37	BD-10933				89	RT-20		51 RT-11
38	BD-10927				90	RT-11		
39	BD-10930				91	RT-13	A2.b2.b6.b8.b12	52 RT-13
40	BD-10896				92	RISA-33		
41	BD-10897				93	KASI-20(1)		
42	BD-10898				94	RT-18		53 RT-18
43	BD-10894	A1.b2.b3.b6	25	BD-10936	95	RM-01		54 RM-01
44	BD-10895		26	BD-10921	96	BD-10917	B	55 BD-10917
45	BD-10936		27	BD-10890				
46	BD-10921							
47	BD-10890							
48	AMS-10							
49	RISA-23							

studied chilli cultivars. However, closeness may be possible in the genetic makeup of the locus for which the primers were responsible to distinguish along with low variation also in the morphological traits and geographical sources. The highest genetic distance may be elucidated by the fact that local cultivars or land races collected from different location have been included in the study. The existing distance can further be used to add gene sources from the traditional varieties to HYVs, using genetic fingerprinting and correlating the values with that of the morpho-physiological features to find out the best performing varieties through appropriate breeding programs. Information on variability expression rate through genetic distance based on morphological traits and geographical origin was also reported in previous investigations conducted by Rahman et al. (2010), Hossain et al. (2014), Molla et al. (2016) and Molla et al. (2017).

Dendrogram portrayed winter chilli cultivars based on Nei (1972) genetic distance UPGMA cluster analysis broadly placed 96 chilli cultivars into two major groups "A" and "B" in which only one genotype namely BD-10917 congregated in a distinct group "B", and other 95 cultivars clustered in group "A" (Figure 2). The genotype BD-10917 had a distinct status in the dendrogram, because there might have effect of higher genetic distance (Table 5) which might be designated through geographical sources and morphological traits. This genotype was collected from Daulatkhan upazila of Bhola district which is island district of Bangladesh. Moreover, distinct morphological features like hypocotyl color (Purple), stem color before transplanting [Mixture (Green+Purple)], leaf pubescence density (Intermediate), fruit shape (Triangular), blossom end (Sunken and pointed) was observed in this genotype (Molla et al., 2021). Locus CAMS-117 generated 227 bp fragments which was distinguishing band pattern for the cultivars BD-10917, BD-10889 and AHM-46. Among the representation of 96 cultivars, BD-10879 and RT-22 scattered in different sub-cluster (A1a1 and A2.b2.b6.b8.b11) exhibiting the highest genetic distance (0.990) (Table 5 and Figure 2). These two cultivars varied in respect of 14 morphological descriptors in which notable were stem color before transplanting, pedicel position at anthesis, calyx margin shape, anther color, fruit shape at peduncle attachment, fruit shape at blossom end (Molla et al., 2021). Moreover, two cultivars, BD-10879 collected from Galachipa, Patuakhali, and RT-22 collected from Sharishabari, Jamalpur (Table 1) are two widely distanced locations of Bangladesh. However, BD-10880 and BD-10883 were grouped together in same sub-cluster (A1a1) and those cultivars showed similar states in respect of 19 morphological traits such as stem color before and after transplanting, leaf shape, leaf color, pigmentation at node, calyx margin shape, filament color, fruit shape at peduncle attachment, fruit shape, fruit shape at blossom end were remarkable (Molla et al., 2021). In addition, similar geographical sources viz. Sirajganj was observed in case of both cultivars (Table 1). Results of the present study and those reported by Rahman et al. (2010), Hossain et al. (2014), Molla et al. (2016) and Molla et al. (2017) suggested that genetic distance value separated the cultivars in different sub-clusters where such values depend on their morphological characters as got selected in different geographical locations.

5 CONCLUSIONS

From this study, it can be concluded that a comparative assessment of the reproducibility of molecular markers has been made for determination of genetic variability among winter growing chilli cultivars in Bangladesh. Higher genetic variability within populations and significant genetic differentiation between populations indicate rich genetic resources of a species. The study also indicated that 55 cultivars derived from different origin were identified as diversified and could be utilized in breeding program for traits of interest. SSR markers have proved to be powerful tools for molecular genetic analysis of chilli cultivars for plant breeding program to assess genetic diversity available. This would allow for the development of new varieties aiming at the improvement of crop productivity withstanding biotic and abiotic stresses.

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Effects of high vitamin and micro-mineral supplementation on growth performance and pork quality of finishing pigs under heat stress

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Effects of high vitamin and micro-mineral supplementation on growth performance and pork quality of finishing pigs under heat stress

Abstract: The objective of this study was to determine the effects of heat stress (HS) on the production performance of fattening pigs and whether the supplementation of vitamins (C and E) and micro-minerals (Se and Zn) at increased concentrations can mitigate HS adverse effects. Thirty six Danbred hybrid barrows (65.1 ± 2.81 kg) were randomly distributed into four treatments 1) HS (28.9 ± 0.9 °C, RH- 60.4 ± 4.3 %) + control diet (HC), 2) HS + diet 1 (HT1), 3) HS + diet 2 (HT2), and 4) thermo-neutral conditions (19.5 ± 0.9 °C, RH- 85.9 ± 7.3 %) + control diet (TC). Bodyweight and feed intake were measured weekly for four weeks. After the experiment, six pigs from each treatment were slaughtered, and the *longissimus lumborum* muscle was sampled to evaluate meat quality. At week four, HS significantly affected pig body weight ($p < 0.05$). However, the other parameters were not significantly affected by HS,while slight improvements in these parameters were observed by supplementing vitamins and micro-minerals in the diet of the pigs despite exposure to HS. Therefore, the pigs used in the study showed resilience to adverse effects of HS on growth and meat quality parameters. The content of vitamins C and E and micro-minerals Se and Zn in the diet seems to play an important role in resilience to HS, therefore their requirement and supplementation should be carefully evaluated.

Key words: pigs; animal nutrition; heat stress; feed additives; vitamins; micro-minerals; growth performance; meat quality

Vpliv dodatka vitaminov in mikrorudninskih snovi v krmo za prašiče pitance na prirast in kakovost mesa v pogojih vročinskega stresa

Izvleček: Cilj raziskave je bil ugotoviti učinke vročinskega stresa (HS) na prirast prašičev pitancev in ali lahko z dodajanjem vitaminov (C in E) in mikrorudninskih snovi (Se in Zn) v povečanih koncentracijah ublažimo škodljive učinke HS. Šestintrideset kastratov Danbred križancev (65,1 ± 2,81 kg) je bilo naključno razdeljenih v štiri skupine 1) HS (28,9 ± 0,9 °C, RV- 60,4 ± 4,3 %) + kontrolna krmna mešanica (HC), 2) HS + krmna mešanica 1 (HT1), 3) HS + krmna mešanica 2 (HT2) in 4) kontrolni pogoji (19,5 ± 0,9 °C, RV- 85,9 ± 7,3 %) + kontrolna krmna mešanica (TC). Telesno maso in zauživanje krme smo merili štiri tedne. Po koncu poskusa smo žrtvovali šest živali iz vsake skupine in vzorčili mišico *longissimus lumborum* za oceno kakovosti mesa. Ugotovili smo, da je v četrtem tednu HS negativno vplival na telesno maso prašičev ($p < 0,05$). HS ni statistično značilno vplival na druge parametre, pri čemer smo opazili trend izboljšanja teh parametrov ob dodajanju vitaminov in mikrorudninskih snovi v krmo prašičev, ki so bili izpostavljeni HS. Prašiči, uporabljeni v študiji, so pokazali odpornost na škodljive učinke HS na rast in parametre kakovosti mesa. Zdi se, da ima vsebnost vitaminov C in E ter mikrorudninskih snovi Se in Zn v prehrani prašičev pomembno vlogo pri odpornosti na HS, zato je potrebno skrbno oceniti potrebe po dodajanju omenjenih dodatkov v krmo prašičev.

Ključne besede: prašiči; prehrana živali; vročinski stres; krmni dodatki; vitamini; mikrominerali; rast; kakovost mesa

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1 INTRODUCTION

The ongoing rise in global temperature caused by climate change and the ensuing heat stress (HS) intensification is a severe threat to food security that is expected to persist throughout the 21st century (FAO, 2016; Wang & Zhang, 2019). Pigs are negatively affected by HS, and it was suggested that modern genotypes are more sensitive to its negative effects than traditional genotypes (Brown-Brandl et al., 2001; Brown-Brandl et al., 2004; Renaudeau et al., 2011). Pigs under HS focus more on reducing metabolic heat production by reducing feed intake by up to 23 % per day since they have a low capacity for thermoregulation; as a result, production performance and product quality are compromised (Renaudeau et al., 2011; Song et al., 2011; Yang et al., 2014a). Regardless of the duration of exposure to high ambient temperature (AT), pig production parameters such as average daily gain (ADG), feed conversion ratio (FCR), and pork quality parameters are negatively affected (Song et al., 2011; Pearce et al., 2013; Morales et al., 2014; Yang et al., 2014a). Pigs exposed to HS at 30 °C have decreased pH value and increased crude fat, drip loss percentage, and toughness (Shear Force) of their meat (Yang et al., 2014a; Mun et al., 2022). Influence of HS on pigs' meat pH is critical as low pH can increase protein denaturation, resulting in decreased water holding capacity, lighter color, and subsequently poor eating quality (Kim et al., 2016; Jankowiak et al., 2021).

Such performance depression can also be attributed to oxidative stress (OS) induced by HS. The balance between the reactive oxygen species (ROS) and the endogenous antioxidant is disturbed by HS. HS causes excessive generation of reactive oxygen species (ROS) and reduction of endogenous antioxidants, which causes the accumulation of dysfunctional proteins, lipid peroxidation products, and impaired mitochondrial DNA (Slimen et al., 2014; Cui et al., 2019). ROS can induce protein modifications upon direct reaction with proteins leading to protein oxidation, which leads to higher drip loss and toughening of the meat (Huff Lonergan et al., 2010; Traore et al., 2012). Moreover, OS can reduce collagen synthesis, leading to decreased collagen solubility and greater meat toughness (Archile-Contreras and Purslow, 2011). Therefore, there is a need for mitigation strategies to combat detrimental effects of HS. Nutritional intervention strategies using nutritional tools have shown progress in abating adverse effects of HS on pig performance (Rhoads et al., 2013; Babinszky et al., 2019). Exogenous antioxidants, such as vitamins C and E, and minerals selenium (Se) and zinc (Zn) can respond to such stressors (Traber & Stevens, 2011; Jarosz et al., 2017; González de Vega et al., 2018; Kiełczykowska et al., 2018). Individual use of vitamins and micro-minerals as supplements in heat-stressed pigs has been studied in the following concentrations: Se (0.5 ppm), vitamin E (100 IU/kg), Zn (60 mg/kg Zn sulfate + 60 mg/kg Zn amino acid complex) (Liu et al., 2016; Mayorga et al., 2018). However, their effectiveness against negative effects of HS on production performance remains unclear. Therefore, the present study aims to examine the impact of increased concentrations of combination of Zn, Se, vitamin E, and vitamin C on growth performance and meat quality of pigs under HS conditions.

2 MATERIALS AND METHODS

2.1 ANIMALS, DIET, AND MANAGEMENT

All experimental procedures were reviewed and approved by the University of Debrecen Animal Care Committee (Debrecen, Hungary – 9/2019/DEMÁB). At the University of Debrecen's Institute of Agricultural Research and Educational Farm's Animal Husbandry Experimental Station (Kismacs, Hungary), a total of 36 Danbred hybrid barrows (65.1 ± 2.81 kg) were allocated to one of the following environmental and dietary treatments (Tables 1, 2 and 3): 1) HS (28.9 ± 0.9 °C, RH-60.4 ± 4.3 %) + basal diet (treatment code: HC), 2) HS + diet 1 (treatment code: HT1), 3) HS + diet 2 (treatment code: HT2), and 4) thermo-neutral environment (19.5 ± 0.9 °C, RH- 85.9 ± 7.3 %) + control diet (treatment code: TC). All pigs were allowed a 7 day adaptation period to their pens (3 pigs per pen with a total of 12 pens), fed *ad libitum* (with control feed) in a thermoneutral (TN) environment (average 19.5 ± 1.5 °C). Afterwards, the temperature of the HS room was gradually raised to 30 °C (heat increment, HI), and pigs in this period started receiving different dietary treatments. A week after the heat increment, the main period of the experiment commenced, which lasted for two weeks. Three diets were formulated: the basal or control feed (B), formulated on a corn-soybean basis according to the (NRC, 2012) recommendation for 75–100 kg live weight pigs having 155 g mean protein deposition per day (Table 1), and with the nutrient content of the premixture added (Table 2). The control diet contained levels of vitamins C and E and micro-minerals Zn and Se in accordance to the NRC recommendation (Table 3). Nutrient recommendation tables do not recommend vitamin C supplementation for growing and finishing pigs, but in DSM Optimum Vitamin Nutrition (OVN®) guidelines (a standard for maximised performance) for breeders the recommendation is set at 315 mg/kg of maximum supplementation. We choose to add 300 mg/kg for diet

Table 1: Composition and calculated nutrient content of basal feeda [a]

Ingredients	Inclusion rate (%)	Energy and Nutrients	Calculated value
Corn	78.68	Digestible energy, MJ/kg	14.24
Soybean meal	16.33	Crude protein, %	12.81
Plant oil	2.11	SID [b] Lys, %	0.78
Limestone	0.92	SID Met+Cys, %	0.45
MCP	0.80	SID Thr, %	0.49
L-Lysine	0.30	SID Trp, %	0.14
DL-Methionine	0.01	Ca, %	0.59
L-Tryptophan	0.03	digestible P, %	0.23
L-Threonine	0.06	Na, %	0.10
Salt	0.26		
Vitamin and mineral premixture	0.50		

[a] NRC (2012) recommendation for 75–100 kg live weight pigs having 155 g mean protein deposition per day, [b] standardized ileal digestible

2 and 150 mg/kg for diet 1. Regarding vitamin E the OVN guidelines recommend 64–105 mg/kg, therefore we decided for a 30 mg/kg two-step increase of the basal diet concentration, resulting in 41 mg/kg in diet 1 and 71 mg/kg in diet 2. In case of Zn, the maximum allowed supplementation in the EU is 150 mg/kg, therefore that was chosen as the maximum value in diet 2, whereas concentration in diet 1 was 100 mg/kg, and in basal diet 50 mg/kg. Due to toxicity problems complete pig feeds should not contain more than 0.5 mg/kg Se in total. Therefore, usually not more than 0.2–0.35 mg/kg is added to the feed. We decided to increase the NRC supplementation of the basal diet (0.16 mg/kg) for 0.05 mg/kg for two times, resulting in 0.21 mg/kg (diet 1) and 0.26 mg/kg (diet 2).

Table 2: Nutrient content of the premixture (in 1 kg of premixture) *

Nutrient	Inclusion rate	Concentration
Zn	mg/kg	9999
Cu	mg/kg	1454
Fe	mg/kg	7281
Mn	mg/kg	9999
I	mg/kg	136
Se	mg/kg	32
Vitamin A	IU/kg	410000
Vitamin D-3	IU/kg	82000
Vitamin E	mg/kg	2205
Vitamin K-3	mg/kg	82
Vitamin B-1	mg/kg	62
Vitamin B-2	mg/kg	205
Ca-d-pantothenate	mg/kg	492
Vitamin B-6	mg/kg	164
Vitamin B-12	mg/kg	1
Biotin	mg/kg	5
Niacin	mg/kg	1026
Folate	mg/kg	25
Choline chloride	mg/kg	60000

* At or above NRC (2012)

2.2 PRODUCTION PARAMETERS

Each pen had a designated plastic container, of which tare-weight was measured and written on the container. Weekly feed intake was measured as feed disappearance and feed residues (both from the feeder and the container) were measured and recorded. Body weights (BW) were obtained weekly from the start of the adaptation period to the end of the experiment. Average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) were calculated weekly, and all represented the mean value per pen.

Table 3: Dietary treatments (supplementation mg/kg)

Nutrient	Basal feed*	Diet 1	Diet 2
Vitamin C	0	150	300
Vitamin E	11	41	71
Zn**	50	100	150
Se**	0.16	0.21	0.26

* NRC (2012); ** organic source

2.3 SLAUGHTER AND PORK QUALITY MEASUREMENT

At the end of the trial, six pigs from each treatment (24 pigs in total) were slaughtered. The slaughtering comprised of two days where three pigs from each treatment (12 pigs in total per day) were slaughtered in similar manner after electrical stunning. The pigs for slaughter were selected randomly. About 500 g of *longissimus lumborum* muscle was removed between the 12th rib and 5th lumbar vertebrae of the pig carcass for meat quality measurements, as described by Rezar et al. (2017).

2.3.1 Physical meat quality assessment

The meat pH was measured at 45 minutes and 24 hours after slaughter in the loin by Testo AG Germany 205 pH value gauge (immersed in a buffer solution before measurement). The meat color was measured using Konica Minolta CR-410 Chroma Meter (Konica Minolta Corporation, Japan) 24 hours after slaughter with a 21-minute blooming time. The Chroma Meter was calibrated with the use of a white calibration plate before the analysis, setting the Y, x, and y illuminant coordinates (Y = 93.7, x = 0.3144, y = 0.3204). Regarding meat color, L* (the degree of lightness, on a scale between 0 (black) and 100 (white), a* (is red-green), and b* (stands for the yellow-blue color characteristic) values represent a color space defined by CIE. In the CIELAB system by using the measured a*, b*, L* features (a* = red, b* = yellow, L* = paleness).

For drip loss (%) determination, one cm thick meat pieces of 50 ± 5 g were cut. Pieces were packed in an inflated nylon bag, then hung up in the fridge at 4 °C for 48 hours and weighed again. For freeze loss (%) determination, from the frozen meat samples (–20 °C), meat of 100 ± 5 g and 1 cm thick pieces was cut, stored at 4 °C for 24 hours, and weighed. The same samples used for thawing loss were cooked. For the evaluation of cooking loss, pieces were packed in nylon bags and cooked for half an hour until reaching the 75 °C core temperature and weighed. After cooking, meat pieces were chilled at 4 °C overnight then sliced in cuboids and measured. For the firmness, shear force measurement (N) was conducted on cooked samples, with shear blade set using 25 kg load cell, with 1.5 mm/s test speed from 40 mm distance, using a Warner-Bratzler shear machine (TA.XT + Texture Analyzer 6.1.18.0 version (Texture exponential, Stable Microsystems Ltd., Vienna Court, Lammas Road, Godalming, Surrey GU7 1YL, United Kingdom)). Once the trigger force is attained, the blade proceeds to shear through the sample. The maximum force denotes the point at which the sample completely fills the triangular cavity of the blade and cuts through the sample surface. After this point shearing continues throughout the whole sample until the blade has passed through the base plate slot. The blade then returns to its starting position. Curves were evaluated to get shear force data.

2.3.2 Chemical analysis of the meat samples

Total nitrogen was analyzed using Kjeldahl-Method and protein content was calculated using the factor of 6.25. Fat was measured according to MSZ ISO 1443:2002 standard. For the elemental analysis (vitamin C and minerals) ground meat samples (1.000 g) were loaded into digester tubes. To all samples ten ml of distilled conc. HNO_3 was added and heated at 60 °C for 30 min, then 3 ml 30%(v/v) H_2O_2 (Scharlab, Magyarország Kft., Debrecen, Hungary) was added, and the samples were digested further at 120 °C for 90 mins. After the digestion, all samples were washed into 50 ml volumetric flasks with distilled water, homogenized, and filtered (MN 640 W paper; Macherey-Nagel). ICP-OES technique was applied on the iCAP 7000 spectrophotometer (Thermo Scientific). For the calibration, a multielement standard solution was applied.

2.4 STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 7.05 software (GraphPad Software Incorporated, San Diego, USA). One-way ANOVA was used to evaluate the production and pork quality parameters. Data were expressed as mean, and group means differences were separated by the Tukey test. Differences among the treatments were considered significant when $p < 0.05$.

3 RESULTS

3.1 GROWTH PERFORMANCE

After two weeks of exposure to HS, pigs in HC and HT2 groups were significantly lighter ($p < 0.05$) compared to those in the TC group. Interestingly, pigs in HT1 had comparable weights to TC (Figure 1e). Duration of HS and supplementation of elevated levels of vitamins (C and E) and micro-minerals (Zn and Se) did not significantly affect the rest of the growth performance parameters (Table 4).

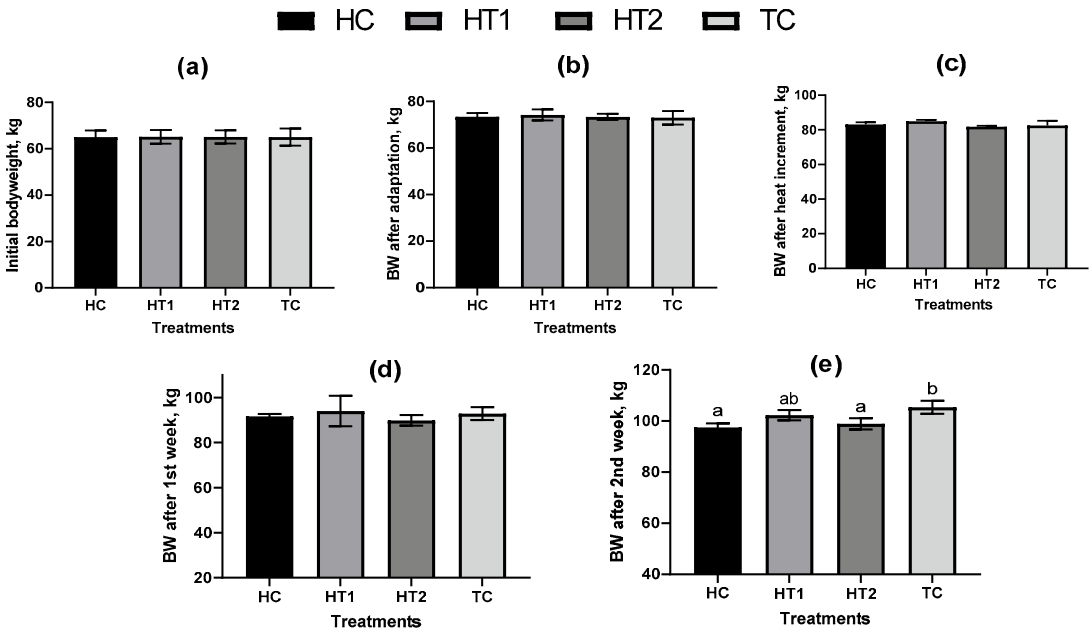

Figure 1: Initial body weight (a), body weight after adaptation period (b), body weight after heat increment (c), body weight after 1st week of the experiment (d), and body weight after 2nd week of the experiment (e). Values are means, with their standard deviation represented by vertical bars; [a, b] means with common letter are not significantly different ($p > 0.05$). HC = heat stress + basal diet; HT1 = heat stress + diet 1; HT2 = heat stress + diet 2; TC = thermal comfort + basal diet.

3.2 MEAT QUALITY

As shown in Table 5, chemical and physical analysis of meat samples obtained from pigs kept in TN and HC environment fed their respective dietary treatments (basal, diet 1, and diet 2) have similar results among all experimental treatments ($p > 0.05$). HS, vitamin C, E, and micro-minerals Zn and Se supplementation did not significantly affect the meat quality parameters of pigs ($p > 0.05$).

4 DISCUSSION

4.1 GROWTH PERFORMANCE

Several studies reported that high ambient temperature induced detrimental effects on growing parameters of pigs (Kellner et al., 2016; Cervantes et al., 2016; da Fonseca de Oliveira et al., 2019). Slow growth, decreased feed efficiency, and carcass lean are affected by pigs under such stressors, which are significant contributors to economic losses in pig production (Gonzalez-Rivas et al., 2020). Mitigation of such adverse effects through vitamins and micro-minerals has been reviewed and documented (Cotrell et al., 2015); however, information regarding the mitigation capacity of their combinations is limited. In our study, 14 days of HS caused a significant decrease ($p < 0.05$) in the body weight (BW) of pigs fed with basal feed (HC) and diets supplemented with higher concentrations of vitamins and micro-minerals (HT2). Interestingly, pigs fed diet 1 (HT1) had a comparable body weight with those in TN conditions (TC). A decline in BW is commonly observed in some animals as a response to HS, which can be attributed to the lower feed and nutrient intake of such animals exposed to the stressor (Xin et al., 2018; Goo et al., 2019). However, the reduction in the BW observed in HT2 pigs was unexpected. This observation might be due to the decreased feed consumption as supported by lower feed intake by pigs in HT2 regardless of the supplementation. A similar observation was reported by Sanz Fernandez et al. (2013), where pigs exposed to HS-fed diets containing inorganic and organic zinc supplemented at a level of 120, 100 + 120, and 120 + 200 mg/kg, respectively, had comparable performance in terms of feed intake and BW. Contrastingly, Romu-Valdez et al. (2019), and Lv et al. (2015), reported that high levels of antioxidants (organic zinc 360 mg/kg, and selenium-enriched probiotics 0.46 mg/kg, respectively) in the diet have positive effects on production performance of pigs under HS, as supported by improvements in BW, FI, ADG, and FCR. Statistically, comparable performance was exhibited by pigs in all the treatment groups in terms of ADG, FI, and FCR under all periods of observation in our experiment. However, pigs under HS fed basal diet (HC) had

Table 4: Growth performance of pigs kept in thermo-neutral and heat-stress environment fed basal diet and diets containing increased concentrations of vitamins C and E and microminerals Zn and Se

Parameter	Treatment				SEM [e]	*p* value
	HC [a]	HT1 [b]	HT2 [c]	TC [d]		
	Average daily gain, g/d					
HI [f]	1536	1626	1383	1375	59.10	0.4031
HS [g], 1st week	1598	1721	1483	1850	86.18	0.5311
HS, 2nd week	1119	1421	1357	1700	100.89	0.2537
HS, 2 weeks	1358	1571	1420	1775	65.38	0.0814
	Average daily feed intake, g/d					
HI	3498	3557	3265	3423	45.44	0.0970
HS, 1st week	3544	3665	3495	3806	86.43	0.6500
HS, 2nd week	3898	3501	4227	3915	114.57	0.1573
HS, 2 weeks	3730	3781	3498	4017	106.83	0.1018
	Feed conversion ratio					
HI	2.30	2.20	2.41	2.51	0.08	0.5896
HS, 1st week	2.23	2.21	2.42	2.06	0.09	0.7117
HS, 2nd week	3.53	2.95	2.61	2.50	0.17	0.1369
HS, 2 weeks	2.73	2.41	2.51	2.26	0.07	0.0955

[a] heat stress + basal diet; [b] heat stress + diet 1; [c] heat stress + diet 2; [d] thermal comfort + basal diet; [e] standard error of mean; [f] heat increment; [g] heat stress

the lowest observed values of all the studied parameters. At the same time, supplementation of vitamins and micro-minerals at increased level in the diet 1 (vitamins C –150 mg/kg, E – 41 mg/kg, and minerals Se – 0.21 mg/kg and Zn – 100 mg/kg) mitigated negative effects of HS. The proposed level of supplementation might be effective enough as there are also studies that reported improvements in the growth of pigs under HS-fed diets containing only a slight increase in dietary antioxidant (Zn – 75 mg/kg) supplementation (Mani et al., 2019).

4.2 MEAT QUALITY

Chronic HS can influence the chemical composition and physical characteristics of meat, which has been observed in meat obtained and evaluated from various species of animals such as broilers, beef cattle, goats, sheep, and pigs (Gregory et al., 2010; Weglarz, 2010; Zhang et al., 2012; Cruzen et al., 2015; Gonzales-Rivas et al., 2020). Pig productivity is affected by HS, and the stressor highly affects pork quality attributes. Decrease in lean tissue, increase in carcass fatness and impaired pork quality, such as pale soft exudative meat (PSE), manifested by increased lightness and yellowness, and decreased redness of meat are common adverse effects of HS on pigs' productive and meat quality performance (Sanz Fernandez et al., 2015; Kellner et al., 2016; Liu et al., 2021). Moreover, HS induced OS can promote protein modifications due to the increase of ROS production. This can lead to protein oxidation that can decrease the water holding capacity (WHC) of the meat and causes its toughness (Huff Lonergan et al., 2010; Traore et al., 2012). As reported by Yang et al. (2014b), exposure of pigs to long term HS at 30 °C increased the drip loss and shearing force of longissimus muscle. These attributes are also correlated to HS effect on pork pH (significant decrease - 5.43, which is below the optimal pH range of 5.7–6.1) signifying its importance (Klont, 2005; Yang et al., 2014b; Jankowiak et al., 2021). Nevertheless, as observed in different poultry and livestock animals (broilers, lambs, and pigs), vitamins and micro-minerals supplementation to the diet can be an effective tool to mitigate detrimental effects of HS on meat quality (Shakeri et al., 2019; Silva et al., 2019; Chauhan et al., 2020; Liu et al., 2021). Interestingly, the 14-day chronic HS exposure of Danbred hybrid pigs in our study did not cause any significant changes in the quality of their meat. Supplementation of vitamins and micro-minerals did not significantly influence the meat quality of pigs exposed to HS. Although our results are in contrast to the observations of Yang et al. (2014b) (pigs exposed to chronic HS (30 °C)); Shi et al. (2016) (pigs exposed to chronic HS (35 °C)); Ma et al. (2019) pigs exposed to chronic HS (35 °C)); and Liu et al. (2021) (pigs exposed to chronic HS (35 °C)). But it is in agreement with

Table 5: Meat quality of thermo-neutral and heat-stressed pigs fed basal diet and diets containing increased content of vitamins C and E and microminerals Zn and Se

	Treatment					
Parameter	HC [a]	HT1 [b]	HT2 [c]	TC [d]	SEM [e]	*p* value
Moisture, %	67.87	67.68	68.17	67.45	0.29	0.8636
Protein, % [f]	22.12	22.85	22.25	22.38	0.22	0.7095
Fat, % [f]	8.46	7.93	8.04	8.62	0.13	0.1937
Vitamin C, mg/100g [f]	9.60	8.31	8.40	9.26	0.64	0.8807
Zn, mg/kg [f]	12.48	11.74	12.30	11.70	0.33	0.8075
Se, mg/kg [f]	0.3910	0.3385	0.3342	0.3172	0.02	0.6864
pH 45 minutes	6.38	6.44	6.44	6.46	0.04	0.9118
pH 24 hours	5.53	5.53	5.22	5.47	0.01	0.3297
L* (lightness)	51.09	50.87	51.52	49.84	0.38	0.4764
a* (redness)	15.95	16.47	15.88	15.09	0.22	0.1824
b* (yellowness)	4.22	4.17	4.62	3.60	0.15	0.1126
Drip loss, %	2.68	2.96	2.41	2.54	0.15	0.6453
Freeze loss, %	9.58	11.51	12.48	11.25	0.40	0.0764
Cook loss, %	25.10	24.86	24.31	23.60	0.42	0.6370
Firmness, N	53.58	62.83	58.98	55.48	1.83	0.3089
Shear force, N	528.70	587.80	554.30	499.50	16.64	0.2933

[a] heat stress + basal diet; [b] heat stress + diet 1; [c] heat stress + diet 2; [d] thermal comfort + basal diet; [e] standard error of mean; [f] in dry matter basis

the observations of Lehotayová et al. (2012) in pigs exposed to constant HS (30 °C) throughout the growing and finishing period. They concluded that several meat quality parameters, such as shear force, drip loss, and meat colour, were not significantly affected by HS. The resilience of pigs to HS, as observed by the quality of their meat evaluated in this study, might be due to their ability to tolerate such stressors over time (Campos et al., 2017). Several studies reported that a longer duration of HS exposure could result in gradual performance improvement in pigs. Such observation may result from the adaptive changes, such as a decrease in heat production during the acclimation stage upon chronic exposure to HS (Renaudeau et al., 2008; Renaudeau et al., 2010, Renaudeau et al., 2013). Moreover, as concluded in the growth performance evaluation no decisive effect of either temperature or diet could be seen, it is therefore expected that the meat quality evaluation would have similar results.

5 CONCLUSIONS

The pigs used in the study were not severely affected by chronic HS. They responded with slightly lower growth performance (ADG, ADFI, and FCR) than pigs in TN environment and have comparable meat quality characteristics. Supplementation of the diet with vitamins and micro-minerals (vitamin C (150 mg/kg) E (41 mg/kg), Zn (100 mg/kg) and Se (0.21 mg/kg)) contributed to the slight improvement in the growth performance of pigs under chronic HS; however, such supplementation did not significantly affect the meat quality of the pigs. Therefore, we can conclude that the high ambient temperature challenge of 14 days has no significant effect on the growth performance and meat quality of the pigs used in this study. Careful evaluation of vitamin and micro-mineral supplementation levels seems important in pigs reared under chronic HS conditions since the pigs in the study responded with improved growth performance in HT1 group, but not in HT2 group.

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Biological and biochemical effects of lufenuron on *Xanthogaleruca luteola* (Muller, 1766) (Coleoptera: Chrysomelidae)

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Biological and biochemical effects of lufenuron on *Xanthogaleruca luteola* (Muller, 1766) (Coleoptera: Chrysomelidae)

Abstract: *Xanthogaleruca luteola* (Mull., 1766) is the major defoliator pest of elm trees in urban area. In this study the effect of lufenuron on some biochemical and biological characteristics was investigated on *X. luteola*. The LC_{30} and LC_{50} of lufenuron were determined on the second instar larvae as 20.22 and 36.65 mg l^{-1}, respectively. Effects of LC_{30} and LC_{50} concentrations of lufenuron on some biological parameters showed that lufenuron caused an increase in larval, pre-pupal and pupal developmental periods. Also, none of the female insects that emerged from the treated larvae did not spawn during their life. The LC_{50} concentration of lufenuron decreased carbohydrate, lipid and protein content and increased glycogen content. But there was not a significant difference in glycogen, and protein contents following the exposure to LC_{30} concentration. However, glutathione-s-transferase (GST) and esterase activities were significantly increased at LC_{50}. In conclusion, due to lethal and sublethal effect of lufenuron on biochemical and biological traits of *X. luteola*, it can be recommended for control this pest in IPM program.

Key words: *Xanthogaleruca luteola*; lufenuron; developmental periods; sublethal effects; biochemical parameters

Biološki in biokemični učinki lufenurona na hrošča *Xanthogaleruca luteola* (Muller, 1766) (Coleoptera: Chrysomelidae)

Izvleček: Hrošč *Xanthogaleruca luteola* Mull je najpomembnejši defoliator brestov v urbanem okolju. V raziskavi so bili preučevani učinki lufenurona na nekatere biokemične in biološke lastnosti tega hrošča. LC_{30} in LC_{50} lufenurona sta bili določeni na drugem razvojnem štadiju ličink in sicer 20,22 in 36,65 mg l^{-1}. Učinki LC_{30} in LC_{50} koncentracij lufenurona na nekatere biološke parametre so pokazali, da je lufenuron povzročil povečanje razvojnih obdobij ličinke, obdobja pred zabubljenjem in obdobja bube. Nobena od samic, ki so se izlegle iz obravnavanih ličink v celotnem življenskem obdobju ni odlegla jajčec. Koncentracija LC_{50} je zmanjšala vsebnost ogljikovih hidratov, maščob in beljakovin ter povečala vsebnost glikogena, ni pa bilo značilnih razlik v vsebnosti glikogena in beljakovin pri izpostavitvi. LC_{30} koncentraciji. Aktivnosti glutation-s-transferaze (GST) in esterase sta se pri izpostavitvi LC_{50} značilno povečali. Zaključujemo, da bi zaradi letalnih in subletalnih učinkov lufenurona na biokemične in biološke lastnosti tega hrošča to sredstvo lahko priporočili za uravnavanje škodljivcev in v programih integriranega uravnavanja škodljivcev.

Ključne besede: *Xanthogaleruca luteola*; lufenuron; razvojna obdobja; subletalni učinki; biokemični parametri

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1 INTRODUCTION

The elm leaf beetle, *Xanthogaleruca luteola* (Muller, 1766) (Coleoptera: Chrysomelidae), is one the most destructive pests of elm trees in Iran. This beetle in both larval and adult stages by feeding on the elm leaves (**Ulmus** spp.) causes severe injurious to trees. In addition to defoliation and morphological changes, this pest causes physiological stress which increases the elm susceptibility to secondary pests and diseases. Additionally, *X. luteola* transmits the fungi spores of Dutch elm disease, *Ophiostoma* (*Ceratocystis*) *novo-ulmi* Brasier, that assumption the serious menace to these trees (Huerta et al., 2010). Due to the widespread plantation of elm trees in urban areas, the application of pesticides against *X. luteola* poses some problematic side-effects on human societies. Therefore, the application of pesticides with high selectivity to this pest and low toxicity to humans and environment is highly appreciated (Defagó et al., 2006).

The estimation of insecticide effects are accessed by lethal and sublethal studies through mortality assays and observation of biology, physiology, behavior and demographic aspects of insect pests and natural enemies(De França et al., 2017). Among the insecticides, insect growth regulators (IGRs) seem to have most adverse effects on insect pests. IGRs may affect the development of insect pests by the interruption of the molting process and cuticle formation, as well as disruption in the endocrine system of insects (Desneux et al., 2007). IGR compounds play a crucial role in control of insect pests, especially pests associated in urban area. Because of specificity in their mode of action and safety to humans, wild life and the environment, these compounds are suitable for pest control than other synthetic insecticides (Tunaz & Uygun, 2004).

Chitin synthesis inhibitors is categorized as IGR which have been detected for controlling of wide variety of immature insect pests (Tunaz and Uygun, 2004). Lufenuron (IRAC group 15) is a benzoylureas that classified as an inhibitors of chitin biosynthesis affecting chitin synthase 1 on insects (Dhadialla et al., 2009; IRAC, 2020) which has been successfully effective against pest species from Lepidoptera, Coleoptera, Hemiptera, Diptera and Thysanoptera (FAO, 2008) due to larvicidal effect along with transovarial–ovicidal and ovicidal effects (Yasir et al., 2019; Abdel Rahman, 2017). Based on results of Arruda et al. (2020), resistance inheritance to lufenuron was incompletely recessive, autosomal, and monogenic in *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae). Due to autosomal and recessive nature, resistance to this compound spreads at low rate lufenuron was registered against lepidopteran and psylla in Iran (Nourbakhsh, 2019).

The increasing in larval, pre-pupal and pupal developmental periods were reported in lufenuron affection in sublethal treatment of *Glyphodes pyloalis* Walker, 1859; which was associated reduction in fecundity and fertility of female adults (Piri Aliabadi et al., 2016). The reduction of glucose, protein and carbohydrate contents has been reported in IGRS treatments of *Pectinophora gossypiella* (Saunders, 1844) (Lepidoptera: Gelechiidae) (Kandi et al., 2012).

Toxicity and sublethal effects of lufenuron on elm leaf beetle has not been studied; besides, based on the mode of action of this pesticide, it seems that this compound is appropriate for control of this pest. The objective of this research was evaluation of the toxicity of lufenuron on *X. luteola*. Subsequently, some biological and biochemical parameters on 2th instar larvae were directed at LC_{30} and LC_{50} levels.

2 MATERIAL AND METHODS

2.1 CHEMICALS

Lufenuron (Match®, EC 50) was prepared from Syngenta Crop Protection (Iran, https://www.syngenta.ir/product/crop-protection/insecticide/match). Other chemical materials were purchased from Merck (Darmstadt, Germany), Wako (Tokyo, Japan) and Fluka (Buchs, Switzerland).

2.2 LABORATORY MASS CULTURE OF *X. LUTEOLA*

The elm leaf beetle adults were collected from University of Guilan campus (Rasht, Guilan province, Iran) without history of pesticide application. Insects were reared under laboratory conditions on the elm leaves at 25 ± 2 °C, 16:8 photoperiod (L:D) and 75 % relative humidity (RH). Transparent plastic jars (10 cm × 7 cm × 5 cm) containing holes in the lids were used for the rearing of larvae and adults. In order to obtain larvae in the same age for bioassay tests, each pair of male and female adults was kept in similar plastic jars and the laid eggs were put in a new container (14 cm × 12 cm × 5 cm) in laboratory condition as mentioned above, daily.

2.3 BIOASSAY

Bioassay tests were carried out on 2th instar larvae based on leaf-dip method (Memarizadeh et al., 2011). Five concentrations (10, 17.78, 31.62, 56.23 and 100 mg

l^{-1}) of lufenuron were used for determination of LC_{30} and LC_{50} which were diluted in distillated water. Elm leaf discs (3 cm × 3 cm) were dipped in the desired concentrations for 30 seconds and dried at room temperature for 30 min before being offered to *X. luteola* larvae. The distillated water was used as control. Ten 2th instars were transferred to each plastic container containing treated leaf. Five replications were used for each treatment. Mortality was recorded 72 h after treatment. The LC_{30} and LC_{50} values were calculated using the Polo-PC software (Software, 1987).

2.3.1 Sublethal and lethal assays

The evolution of sublethal and lethal effects of lufenuron in LC_{30} (20.22 mg l^{-1}) and LC_{50} concentrations (36.65 mg l^{-1}) were studied on 2th instar larvae with leaf-dip method. Larvae were fed on treated leaves for 48 h. Then, alive larvae were transferred to plastic jars which were nourished with fresh leaves up to adult emergence. During this test, mortality, larvae duration, pre-pupal duration, pupal duration, and fecundity of female adults were recorded.

2.3.2 Biochemical assay

2.3.2.1 Amounts of carbohydrate, lipid, and glycogen

Biochemical assays were carried out on treated 2th instars with LC_{30} or LC_{50} doses of lufenuron. After 48 h, the whole body of surviving larvae were homogenized in sodium sulphate buffer solution (Na_2SO_4 2 %) for determination of carbohydrate (Singh & Sinha, 1977), lipid and glycogen contents (Yuval et al., 1998).

2.3.2.2 Esterase activity measurement

Esterase activity (Van Asperen, 1962) and glutathione-s-transferase (GST) activity (Habig et al., 1974) were measured based on using α- and β-naphthyl acetate (NA) and 1-choloro-2,4-dinitrobenzene (CDNB) as substrates, respectively. Three to four replicates were conducted for all previously mentioned enzyme assays.

Protein concentration was measured according to Bradford (1976) method with bovine serum albumin as standard.

Table 1: Determination of sublethal and lethal concentrations of lufenuron on 2th instar larvae of *Xanthogaleruca luteola*

	Concentration (mg l^{-1})	CL*
LC_{30}	20.22	14,71-25,93
LC_{50}	36.65	29,45-46,38

*CL (confidence limits) which has been calculated with 95 % confidence

2.4 DATA ANALYSIS

All statistical analyses were performed using SAS software ($p \leq 0.05$). Tukey's test statistic was used as comparison means (Rodenhouse et al., 2004).

3 RESULTS

The LC_{30} and LC_{50} values were determined as 20.22 and 36.65 mg l^{-1}, 72 h after treatment, respectively which presented in Table 1. These concentrations were used as lethal and sublethal concentrations for the following experiments.

3.1 SUBLETHAL EFFECTS OF LUFENURON ON BIOLOGICAL PARAMETERS

3.1.1 Developmental periods

The larval developmental duration was increased by 13.38 % and 27.06 %, when the larvae treated with LC_{30} and LC_{50} concentrations, respectively. Also, LC_{30} and LC_{50} concentrations were increased the pre-pupal by 13.2 % and 17.5 % and pupal by 16.65 % and 26.74 %, respectively. Totally developmental periods were significantly increased at LC_{30} and LC_{50} concentrations in comparison to the control which have been reported in Table 2. The investigation on female fecundity showed that emerged females from the treated 2th instar larvae did not oviposit any eggs during their lifetime have been lasted 10 days.

3.2 SUBLETHAL EFFECTS OF LUFENURON ON ENERGY RESERVES

Significant differences were observed in carbohydrate and lipid contents of the larvae in LC_{30} concentration of lufenuron in comparison to the control which was significantly decreased 29.1 % and 45.44 %, respectively. However, protein and glycogen contents in this sublethal concentration showed non-significant differences in comparison controls. The protein content was decreased by 12.38 % and glycogen content was increased significantly by 17.79 % (Table 3).

Table 2: Life stages duration of *Xanthogaleruca luteola* after treatment with lufenuron

Treatment	larval developmental duration (3^{th} instar larvae) (day) ± SE*	Pre-pupal duration (day) ± SE**	Pupal duration (day) ± SE	Fecundity (%)
Control	6.93 ± 0.06[c]	2.17 ± 0.06[c]	6.96 ± 0.08[c]	41.01
LC_{30}	8± 0,08[b]	2.5 ± 0.12[a]	8.35 ± 0.19[b]	0
LC_{50}	9.5 ± 0.12[a]	2.63 ± 0.15[a]	9.5 ± 0.18[a]	0

*Means followed by the same letter do not differ significantly ($p \leq 0.05$).
**SE: Standard Error
Larval developmental duration (F = 421.86, df = 2, 66, p value < 0.0001)
Pre-pupal duration (F = 9.88, df = 2,53, p value = 0.0002)
Pupal duration (F = 62.54, df = 2,45, p value < 0.0001)

The LC_{50} treatment was associated with significantly decreasing carbohydrate, lipid, and protein contents in comparison with untreated larvae, 27.8 %, 60.37 %, and 24.9 %, respectively, while glycogen content was significantly increased by 45.56 % (Table 3).

3.3 SUBLETHAL EFFECTS ON DETOXIFICATION ENZYME

3.3.1 Total esterase activity

The esterase activity was increased by LC_{50} concentration 52.16 % and 62.75 %, respectively; when α-NA and β-NA used as substrates. Whereas, there were no significant differences between LC_{30} concentration and control (Table 4)

3.3.2 GST activity

The GST activity were increased by 14.64 % and 69.71 %, when treated by LC_{30} and LC_{50} concentrations, respectively, which was significant at LC_{50} (Table 4).

4 DISCUSSION

Investigation on sublethal effects of insecticides might be associated with variations in life history characteristics as growth developmental stages, fecundity, fertility (Stark & Banks, 2003; Saber et al., 2013; Rehan & Freed, 2015; Su et al., 2022), in addition to behavioral and physiological disturbances (Desneux et al., 2007). In this study, bioassay results showed that lufenuron is effective against *X. luteola* and LC_{50} was determined as 36.6 mg l^{-1}. The results of present study showed that LC_{50} and LC_{30} concentrations had the considerable effects on the developmental stages and fecundity of emerged female adults of *X. luteola*. Sublethal concentrations of lufenuron increased developmental stages in larvae, pre-pupal and pupal after the 2^{th} instar larvae treated with LC_{30} and LC_{50} concentrations which were longer in LC_{50} concentration.

This result is in consistent with the results of Kandi et al. (2012) which showed that lufenuron in LC_{50} concentration increased the larval and pupal durations in *Pectinophora gossypiella*. Besides, the reducing in the adult longevity, fertility and pupal weight of *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) was reported in the sublethal treatment of lufenuron, methoxyfenozide, spinosad, endosulfan, novaluron and

Table 3: Effects of lufenuron on energy reserve of 2^{th} instar larvae resulting from treated second instar larvae of *Xanthogaleruca luteola*

Treatment	Carbohydrate (mg/Larvae) ± SE*	Protein (mg/Larvae) ± SE**	Glycogen (mg/Larvae) ± SE	Lipid (mg/Larvae) ± SE
Control	77.7 ±2.2[a]	71.9 ± 2.1[a]	66.1 ± 6.8[b]	603 ± 2.8[a]
LC_{30}	55.1 ± 1.6[b]	63 ± 0.8[ab]	80.4 ± 1.6[b]	329 ± 14.2[b]
LC_{50}	56.1 ±1.6[b]	54 ± 2.4[b]	121.4 ± 3.6[a]	239 ± 12.8[c]

*Means followed by the same letter do not differ significantly ($p \leq 0.05$).
**SE: Standard Error
The carbohydrate contents (F = 19.61, df = 2,6, p value = 0.0023)
The lipid contents (F = 286.11, df = 2, p value = 0.0004)
The protein content (F = 6.93, df = 2,6, 11, p value = 0.028)
The glycogen content (F = 1.24, df = 2,6, p value = 0.354)

Table 4: Effects of lufenuron on enzyme activities of 2th instar larvae resulting from treated second instar larvae of *Xanthogaleruca luteola*

Treatment	α-Esterase (μmol.min^{-1}.mg protein^{-1}) ± SE*	β-Esterase (μmol.min^{-1}.mg protein^{-1}) ± SE**	GST (μmol.min^{-1}.mg protein^{-1}) ± SE
Control	367.537 ± 14.496^{b}	36.703 ± 0.903^{b}	6.876 ± 1.593^{b}
LC_{30}	442.370 ± 84.631^{b}	55.084 ± 3.915^{b}	8.055 ± 1.004^{b}
LC_{50}	768.221 ± 34.693^{a}	98.506 ± 10.387^{a}	22.7 ± 2.9^{a}

*Means followed by the same letter do not differ significantly ($p \leq 0.05$).
**SE: Standard Error
The α-NA activity (F = 15.88, df = 2.6, *p* value = 0.004)
The β-NA activity (F = 26.55, df = 2.6, *p* value = 0.0124)
The GST activity (F = 19.41, df =2 , *p* value = 0.0192)

tebufenozide (Storch et al., 2007). Also, the delay in the developmental duration of *Cotesia flavipes* (Cameron, 1891) (Hymenoptera: Braconidae) in parasitizing of *Diatraea flavipennella* (Box, 1931) (Lepidoptera: Crambidae) was observed in sublethal affection of lufenuron (Fonseca et al., 2015). Evaluation of flufenoxuron on biological parameters was associated with increasing the larval and pupal periods and morphogenic abnormalities in developmental stages of *Spodoptera littoralis* (Boisduval, 1833) (Reda et al., 2010).

In this study, the fecundity and reproduction of *X. luteola* was influenced by lufenuron. Females that emerged from the treated larvae with LC_{30} and LC_{50} doses of lufenuron did not lay any eggs during their lifetime. The effect of lufenuron on fertility has been attributed to morphological changes in the ovipositor, interference with vitellogenesis, testicular size reduction, and sperm transport incapacity (Sáenz-de-Cabezón et al., 2006). Decreased fertility in IGR-treated insects may be associated with IGR intervention in egg protein accumulation, vitellogenesis synthesis, uptake, and ovariole growth (Pineda et al., 2007). The results are accordance with the reduction of oviposition period in treatment with lufenuron (Josan & Singh, 2000), and cantharidin, selective inhibitor of protein phosphatase 2A (Zhang et al., 2003), has been reported on *Plutella xylostella* (L., 1758) (Huang et al., 2015). Hexaflumuron decreased the oviposition period, egg numbers, and adult emergence of *P. xylostella* (Mahmoodvand *et al*., 2012). Embryonic development changes have been reported in azadirachtin, lufenuron and deltamethrin sublethal treatments on *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) (Correia et al., 2013). In addition, fecundity declined in LC_{50} value of lufenuron on *Heticoverpa armigera* (Hubner, (1808)) (Butter et al., 2003). The percentage of egg hatching was reduced as a dose-dependent manner when *S. littoralis* treated with flufenoxuron (Reda et al., 2010).

On the other hand, the exposure to insecticide sublethal concentrations could influence the biological, physiological and biochemical parameters such as carbohydrates, lipids, and proteins content (Klowden, 2013). When an insect is treated with insecticide, high level energy consumption occurs during detoxification of insecticides. This phenomenon may be leads to lower or higher larval duration or a reduction in reproductive performance (Boivin et al., 2001) which is evidence also in present results. Carbohydrates are assumed as the basic source of energy and a starting material in chitin synthetase (Genc et al., 2002; Nation, 2008). Furthermore, lipids have major roles in preparation of energy, metamorphosis, exoskeleton substrates and biosynthesis of pheromones (Nation, 2008). Proteins are involved in structural and enzymatic functions as hormones and enzymes biosynthesis which are able to convert as an energy source (Klowden, 2013; Wigglesworth, 2012). Nutritional deficiencies along with the increase in metabolic activities for detoxification process during the exposure to pesticides are among the main reasons for the reduced energy level (De Coen & Janssen, 1997; Verslycke et al., 2003). Our results showed that a decrease in the level of energy sources, carbohydrate, lipid and protein, of the larvae following their treatment with LC_{30} and LC_{50} values of lufenuron. According to these results, the protein contents of *X. luteola* larvae decreased at both LC_{30} and LC_{50} treatments, however there was only significant difference in LC_{50} concentration compared to the control. This reduction could be due to the breaking the proteins into amino acids and their entry into the tricarboxylic acid (TCA) cycle as keto acid (Schoonhoven, 1982) to compensate for lower energy caused by lufenuron stress. The present results are in agreement with those of Kandi et al. (2012) who reported LC_{50} of lufenuron caused reduction in the total soluble protein. Piri Aliabadi et al. (2016) showed a reduction in the protein content of *Glyphodes pyloalis* Walker, 1859 larvae when treated with LC_{30} and LC_{50} of lufenuron. The exposure to pesticides can affect carbohydrate metabolism in different species of insects by either decreasing or increasing its content

(Mansingh, 1972). In this study, carbohydrate content of the elm leaf beetle larvae dropped when they were treated with lufenuron. Kandi et al. (2012) had observed the same results by using lufenuron against *Pectinophora gossypiella* (Saunders). Results of this study demonstrated a significant decrease in the lipid content of the larvae of the elm leaf beetle following their exposure to LC_{30} and LC_{50} concentrations of lufenuron. According to some studies, the exposure to pesticides affects synthesis and storage of lipids more than their breakdown (Ali et al., 2011; He et al., 2020). A similar result was reported for *G. pyloalis* larvae treated with lufenuron (Piri Aliabadi et al., 2016). Bashari et al. (2014) also showed a decrease in the lipid content of *X. luteola* larvae treated with hexaflumuron. Glycogen is one of the essential nutrient reserves in insect which can also be affected by pesticide treatment (Fahmy & Dahi, 2009). Our results revealed a significant increase in the glycogen level of the *X. luteola* when treated with LC_{50} concentration of lufenuron. Changes in glycogen level may be due to disruption of the homeostasis mechanism in insects (Nath, 2002; Oguri & Steele, 2007). Lufenuron significantly reduced larval and pupal mass and extended duration of both developmental stages of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in LC_{90}, LC_{50} and LC_{10} concentrations (Butter et al., 2003).

The detoxification enzymes including monooxygenases, GST and hydrolases play the important roles in insecticide metabolisms (Yu, 2014). Additionally, improvement of insecticide tolerance in short time and insecticide resistance after a long period of time exposure may even incorporated by increasing in specific activities and much expression of metabolic enzymes (Yin et al., 2008). Results of our study showed an increase in α- and β-esterase activities of the treated larvae which was significant at LC_{50} and not significant at LC_{30} compared to the control. In lufenuron treatment on *H. armigera,* the esterase activity was reduced significantly with correlation to dose- and time-dependent manner compared with control. It has been recommended the modification in esterase enzyme activities could have important role in fecundity and fertility reduction and inhibition of metamorphosis (Reda et al., 2010).

GSTs are another group of detoxifying enzymes which play an important role in the physiology of stress and intracellular transport and biosynthesis pathways of different cycles (Wilce & Parker, 1994). Results of this study showed that GST activity was increased after the treatment of the larvae with LC_{50}. This result demonstrates that GST activity of the elm leaf beetle maybe are involved in the detoxification of lufenuron as detoxification enzyme for conjugating pesticides and their metabolite with glutathione. Bashari et al. (2014) also reported enhanced activities of GST in *X. luteola* after hexaflumuron treatment.

5 CONCLUSION

Lufenuron in lethal and sublethal concentrations (LC_{50} and LC_{30}) caused affections on the developmental, survival and fecundity of the second instar larvae of *X. luteola* that has been significant influences. In practical approach, these impacts could modify the offspring numbers and maintain population under economic threshold level (ETL). Considering the effect of sublethal concentrations of lufenuron on energy reserves, enzyme activities and spawning rate of elm leaf beetle, it can be concluded that this compound has good potential to control this pest.

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Influence of organo-chemical fertilizer mixed with hormones on yield of *Zea mays* (L.) and soil productivity

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Influence of organo-chemical fertilizer mixed with hormones on yield of *Zea mays* (L.) and soil productivity

Abstract: The effect of five fertilizers; NPK (15-15-15), organo-chemical hormone mixed formula 1 (HO-1), Formula 2 (HO-2), Formula 3 (HO-3) and granular organic fertilizer (GOF) were investigated on maize yield and soil properties in a Randomized Complete Block Design with 4 replications. The new hybrid maize (GT 822) was sown at 75 x 25 cm² spacing. The fertilizer rate was 300 kg ha^{-1}. Initial soil analysis showed that the soil had a lower rate of nutrients but after the second season of trial a significant ($p \leq 0.05$) improvement was observed in soil properties, the highest residual NPK of 0.875 %, 0.0275 %, and 0.0267 % were recorded in HO-3 plots. Vegetative data showed that maize height, dry matter, leaf area, and leaf chlorophyll of 270.85 cm, 282.66 g, 156.02 dm², and 56.70, respectively were also highest in HO-3 fertilizer. Plant growth indices; RGR, LAI, and dry matter use efficiency of 0.132 g g^{-1}day^{-1}, 5.90 and 34.4 %, respectively were best in HO-3. Grain yield and crude protein of 8276.68 kg ha^{-1} and 8.99 % were recorded in HO-3, followed by HO-2 and NPK. Lower yields were obtained from the control and GOF. Our finding revealed that the integration of nutrient sources in a balanced ratio produced the greatest yield output, improved soil properties, and is therefore the future approach to planning an effective fertilizer strategy. Reliance on GOF as a sole fertility package for maize production may result in significant yield losses compared to the integrated approach or use of NPK fertilizer.

Key words: fertilizers; grain yield; integrated nutrient management; soil properties; *Zea mays*

Vpliv mešanice organsko kemičnih gnojil in hormonov na pridelek koruze (*Zea mays* (L.)) in rodovitnost tal

Izvleček: V raziskavi je bil na pridelek koruze in lastnosti tal v popolnem bločnem poskusu s štirimi ponovitvami preučevan učinek mešanice petih odmerkov gnojil NPK (15-15-15) pomešanih z organskimi snovmi in hormoni v naslednjih kombinacijah: 1 (HO-1), 2 (HO-2), 3 (HO-3) ter dodatkom granularnega organskega gnojila (GOF). Novi hybrid koruze GT 822 je bil posejan v razmaku 75 x 25 cm². Odmerek gnojila je bil 300 kg ha^{-1}. Izhodiščna anliza tal je pokazala, da so bila tla slabo preskrbljena s hranili, a je bilo že po drugi sezoni trajanja poskusa opazno značilno izboljšanje ($p \leq 0,05$) v lastnostih tal, pri čemer so bile zabeležene največje vsebnosti ostankov NPK gnojil (0,875 %; 0,0275 % in 0,0267 %) na ploskvah HO-3. Tudi meritve lastnosti obravnavanega posevka koruze kot so višina rastlin, vsebnost suhe snovi, velikost listne površine in vsebnost klorofila (270,85 cm; 282,66 g; 156,02 dm² in 56,70) so imele največje vrednosti pri gnojenju HO-3. Rastlinski rastni kazalniki, RGR, LAI, in učinkovitost izrabe suhe snovi (0,132 g g^{-1} dan^{-1}; 5,90 in 34,4%) so imele največje vrednosti pri obravnavanju HO-3. Pridelek zrnja in vsebnost surovih beljakovin (8276,68 kg ha^{-1} in 8,99 %) sta bila največja pri obravnavanju HO-3, nato pri HO-2 in obravnavanju samo z NPK. Manjši pridelki so bili doseženi pri kontroli in obravnavanju z GOF. Rezultati so pokazali, da je integrirana in uravnotežena raba virov hranil dala največji pridelek in izboljšala lastnosti tal in jo zato priporočamo v bodočih programih gnojilne strategije. Vztrajanje na uporabi granuliranih organskih gnojil kot edinega gnojilnega postopka pri pridelavi koruze lahko povzroči znatne izgube pridelka v primerjavi s temi integriranimi postopki gnojenja ali uporabi NPK gnojil.

Ključne besede: gnojila; pridelek zrnja; integrirano upravljanje z gnojili; lastnosti tal; *Zea mays*

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1 INTRODUCTION

Declining soil productivity is a major problem to sustain maize production globally (Chen et al., 2014). A balanced supply of essential plant nutrients is beneficial for maize growth (Chu et al., 2007), however, farmers' fertilization strategies often focus mainly on major nutrients and economic gains rather than a sustainable agronomic impact. This has led to decreased fertility levels and nutrient imbalances worldwide (Chu et al., 2007). The continuous application of sole inorganic fertilizer to intensify crop cultivation has resulted in unsustainable production due to its inability to condition the soil and sustainable micronutrient levels, soil pH, and soil microbial populations (Chen et al., 2014). The effect of inorganic fertilizers on soil fertility is short-term and without appropriate interventions may pose a negative threat to soil health in the long term (Keteku et. al., 2022; Yang et al., 2015; Meena et al., 2014). Organic fertilizers/amendments had long been reported to promote good soil health and fertility. Fang et al. (2021) and Wang et al. (2018) even recommended that organic fertilizers should replace inorganic fertilizers. Other works recommended a blend due to the low nutrient turnout of organic fertilizers (Voltr et al., 2021; Wang et al., 2017; Ng et al., 2016). Hence, intensive cropping systems without organic inputs are assumed to be unsustainable (Voltr et al., 2021; Ng et al., 2016; Li and Han, 2016). According to Rosegrant et al. (2014), the use of agricultural bioproducts is necessary for inorganic fertilizer conservation and the maintenance of soil productivity. Integration of organic and inorganic fertilizers has nowadays gained attention as the best and the most practical method of promoting short-term plant growth and yield while improving soil organic carbon (SOC) sustainability in the long term (Li and Han, 2016). Several previous studies have reported that combining inorganic fertilizer and organic amendments significantly increased crop yield, SOC, residual nutrients, and microbial activity (Wang et al., 2015). Khaliq et al. (2006) also demonstrated that the combined use of NPK + effective microorganisms (EM) + organic manure significantly increased the growth and yield of cotton as well as residual soil NPK compared to their sole applications. In this light, a new organochemical hormone mixed fertilizer (HO), developed by the Faculty of Agriculture, Naresuan University, Thailand by combining inorganic fertilizer, powder of mixed compost, soil amendments, herbal plant extracts liquid, bio-liquid hormone, and bio-liquid fertilizer at the optimum rate for each plant and coated to control nutrient release (Intanon et al., 2011), seems an interesting product. Previous studies have reported on how the various components of this fertilizer affect crop growth and yield. Intanon (2013) studied the effect of pellet compost, compost mixed bio-liquid fertilizer and compost mixed mineral formulation rice yield and concluded that compost mixed mineral formula produced the highest yield of 6996.25 $kgha^{-1}$, about 43.3 % increase over the unfertilized plot. In addition, Intanon et al. (2017) reported that the HO sugarcane formula increases sugarcane yield by 51.3 % and soil properties; nitrogen from 0.582 to 0.86 %, organic matter (OM) from 0.595 to 0.954 %, EC. 25 °C from 56.81 to 148.72 dS m^{-1} and CEC from 0.17 to 0.87cmol kg^{-1} when compared to the unfertilized plot. Therefore, this experiment was designed on the hypothesis that HO fertilizer can increase maize yield and improve soil properties than the basal recommended NPK fertilizer. The soil of Phitsanulok is slightly acidic and the HO fertilizer has not yet been tested in this area. Several previous researchers have reported on the integration of chemical and organic fertilizer (Voltr et al., 2021; Wang et al., 2017; Ng et al., 2016; He et al., 2015; Wang et al., 2015; Wei et al., 2016) but none have worked on a holistic combination as in HO. This formula is unique and may serve as the basis for new integrated fertilizer formulations.

2 MATERIALS AND METHODS

2.1 RESEARCH FERTILIZERS

The HO fertilizers were sourced from the Faculty of Agriculture, Naresuan University, Thailand. The differences between the formulas are the concentration of their ingredient composition (Intanon et al., 2011) as in Table 1. The NPK: 15-15-15 and GOF were procured from the Department of Land Development, Thailand. The GOF was developed from chicken manure. The compositional analysis of the fertilizers is shown in Table 3.

2.2 RESEARCH SITE

The trial was conducted in the Phitsanulok Province located at 16° 55′ 0″ N and 100° 30′ 0″ E in Thailand. The research soil was sandy clay loam and low in soil fertility (Table 2). The average annual rainfall and temperature in the province are 1339 mm and 27.8 °C, respectively and about 85 % of rain occurs between (June-October, 2018-2019). During the trial, the average monthly rainfall for the two seasons was 73.12 mm, while maximum and minimum temperatures were 34.1 °C and 24.6 °C, respectively. The average monthly rainfall recorded during the experimental period is in Figure 1.

Table 1: Material components of HO maize formula

	Materials (by mass, kg)						Total
Fertilizer	A	B	C	D	E	F	
HO-1	25	30	20	5	10	10	100 kg
HO-2	30	25	20	5	10	10	100 kg
HO-3	36	20	15	5	12	12	100 kg

Note: A = inorganic fertilizer (7:2:1 major, secondary and micronutrients), B = compost powder (OM), C = soil amendment, D = herbal plant extract liquid, E = bio-liquid hormone, F = Bio-liquid fertilizer

2.3 EXPERIMENTAL PLAN

The trial was performed in Randomize Complete Block Design (RCBD) with 6 treatments and 4 replications. The treatments were; T1 control (no fertilizer), T2 NPK: 15-15-15, T3 organo-chemical hormone mixed fertilizer formula 1 (HO-1), T4 formula 2 (HO-2), T5 formula 3 (HO-3), T6 granular organic fertilizer (GOF). The new hybrid maize (GT 822) was sown at an inter-row and intra-row spacing of 75 cm × 25 cm in a 6 m x 5 m plot size. A seed rate of 18 kg ha^{-1} was used with one plant maintained per hill. The fertilizer rate was 300 kg ha^{-1} (0.9 kg plot^{-1}) and was applied in two splits, 30 % at 14 days after planting (DAP) and 70 % at 45 DAP by side placement method.

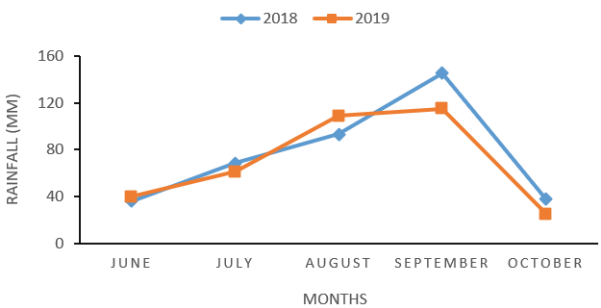

Figure 1: Average monthly rainfall during the experimental period (Source: Phitsanulok weather station)

2.4 RESEARCH DATA

2.4.1 Soil and fertilizer analysis

Soil cores were randomly sampled from the experimental plot before and after the experiment at a depth of (0-20 cm) with a hand auger for soil physical, chemical, and biological properties analysis. The physical and chemical analysis of the fertilizers and soil were determined by the routine methods of (A.O.A.C., 1975). Total N was estimated by the Kjeldahl method, available P by Bray's no. II method and available K, Fe, Zn, Cu, and Mn by the inductively coupled plasma emission spectrometry 4300 Optima DV (PerkinElmer Instruments, Norwalk, CT). Soil pH was recorded at a 1:1 solution ratio with the electrode (H19017 Microprocessor) pH meter. The potassium dichromate oxidation method was adopted to determine organic matter content. Also, cation exchange capacity (CEC) was determined by the ammonium acetate method while electrical conductivity (EC) was measured with the EC meter. The procedures of A.O.A.C. (1975) were again adopted to analyze soil bulk density and porosity. For the fertilizers, total nitrogen was determined by the Kjeldahl analysis, while the determination of other nutrients concentration was done by the inductively coupled plasma emission spectrometry 4300 Optima DV (PerkinElmer Instruments, Norwalk, CT). Also, soil microorganisms (bacteria, fungi, and actinomyces) population in the fertilizers and soil before and after the trial were analyzed by the serial dilution and pour plate method by (Sanders, 2012). The number of microbes was calculated as in Equation 1.

$$\text{No. of microbes g}^{-1}\text{ oven dry sample} = \frac{Average\ plate\ count \times dilution\ factor}{1\ g\ of\ oven\ dry\ sample} \quad \text{(Eqn 1)}$$

The hormones (indole-3-acetic acid (IAA), gibberellic acid (GA_3) and cytokinins) in the HO were estimated by the high-performance liquid chromatography (HPLC) system (Waters 2695 Separations Module,

Table 2: Compositional analysis of the soil before trial

N	P	K	Ca	Mg	S	Fe	Cu	Zn	Mn
	%					mg kg^{-1}			
0.394	0.013	0.015	4.400	1.274	0.216	5.867	0.023	1.355	1.864
OM		CEC	EC. 25°	Bulk density	Porosity	Bacteria	Fungi	Actinomyces	
%	pH 1.1	Cmol kg^{-1}	dS m^{-1}	g cm^{-1}	%	CFU g^{-1} (10^4)		CFU g^{-1} (10^3)	
0.536	5.1	0.183	46.713	1.553	23.093	34.0	39.0	17.0	

Note: sample size (n) = 4

Waters, USA) equipped with a photodiode array detector (Waters 2996 Detector, Waters, USA). The reversed-phase ProntoSil 120-5-C18-ACE-EPS column (150 × 4.6 mm, 5 µm, Bischoff analysis technology, Leonberg, Germany) was used for IAA and GA_3 analysis. The mobile phase for IAA analysis was with A) 0.1 M acetic acid and B) 0.1 M acetic acid in methanol at the flow rate of 1 ml min^{-1}. Conversely, 30 % methanol (adjusted to pH 3 with 0.1 M phosphoric acid) was used for the elution of GA_3 analysis at the flow rate of 0.8 ml min^{-1}. Cytokinin analysis was performed with the reversed-phase C18 ProntoSil HyperSorb ODS (250 × 4.6 mm, 5 µm, Bischoff analysis technology, Leonberg, Germany) column. The mobile phase was with A) 0.1 M acetic acid in ultrapure water (containing 50 ml ACN, pH 3.4 triethanolamine) and B) acetonitrile at the flow rate of 1 ml min^{-1} (Szkop and Bielawski, 2012).

2.4.2 Vegetative growth analysis

Twenty representative sample plants were randomly selected per plot for the assessment of maize height, number of leaves, leaf chlorophyll, and leaf area per plant. Measurements were taken at 10 days interval after 14 DAP until flowering. The SPAD-502 Plus meter was used to measure leaf chlorophyll content (surrogate chlorophyll) on the first five fully open leaves from the plant tip. Leaf area per plant was also measured following the method of (Saxena and Singh 1965) as in Equation 2.

$$Leaf\ area/plant\ (dm^2) = L \times D \times N \times 0.75 \quad \text{(Eqn 2)}$$

L, D and N represents leaf length, leaf diameter and leaves number. 0.75 is leaf area constant for maize.

One representative plant was uprooted at 20 days interval intervals after 14 DAP for dry matter measurement, and after harvesting (120 DAP), the 20 sampled plants were oven-dried at 65 ± 2 °C for 24 h for total dry matter measurement. Plant growth indices; relative growth rate (RGR), leaf area index (LAI), and dry matter use efficiency (DMAE) were calculated by the method of Fisher (1921) shown in (Equation 3, 4, and 5). RGR was calculated at 20 days interval.

$$RGR\ (\mathrm{g\ g^{-1}\ day^{-1}}) = \frac{(Log_e W_2 - Log_e W_1)}{t_2 - t_1} \quad \text{(Eqn 3)}$$

$$LAI = \frac{Leaf\ area\ per\ plant\ (\mathrm{dm^2})}{Ground\ area\ per\ plant\ (\mathrm{dm^2})} \quad \text{(Eqn 4)}$$

$$DMAE\ (\%/\mathrm{day}) = \frac{\text{Grain mass/plant (g)}}{\text{Total dry matter/plant (g)}} \times \frac{100}{\text{Duration of crop}} \quad \text{(Eqn 5)}$$

W_1 and W_2 represents total dry matter $plant^{-1}$ at time t_1 and t_2 respectively. Log_e is the natural logarithm (e = 2.3026).

2.4.3 Yield and yield quality

Grains mass was measured on a plot-wise basis, all the entire grains per plot were taken into consideration. Gain mass measurement was done at 13 % moisture content, measured with a moisture meter (FARMEX model, Delhi, India) and later converted into grain mass ha^{-1}. The average mass of the two seasons is reported here. Afterward, grain NPK contents were determined for quality assessment by the methods; Kjeldahl digestion and its content quantified by an auto-analyzer and vanadomolybdate phosphoric acid digestion methods, respectively (A.O.A.C., 1975). Sriperm et al. (2011) maize convection factor (5.68) was used to convert percent grain nitrogen into crude protein content. Also, the IAA, GA_3, and cytokinins content in the maize shoot were analyzed by the procedure mentioned above.

2.5 DATA ANALYSIS

All growth, yield, and soil analyses results are averages of two season data. Data analysis was conducted with the Analysis of Variance (ANOVA) using the statistical software SPSS version 21 for Windows (SPSS Inc., Chicago, USA). A comparison of treatment means was done by Tukey's test at a 95 % significance level.

3 RESULTS AND DISCUSSION

3.1 COMPOSITIONAL ANALYSIS OF FERTILIZERS

The compositional analysis of the experimental fertilizers is shown in Table 3. The chemical properties of the fertilizers are a major determinate factor of their inherent capacity to supply nutrients. The contents of NPK elements were the highest in NPK: 15-15-15 and HO-3 fertilizers. Secondary nutrients; Ca, Mg, and S were all present in the HO fertilizers and GOF, however, the HO-3 formula contained the highest of 7.97, 1.628,

and 0.055 mg kg^{-1}, respectively. Similarly, the micronutrients Fe, Zn, Cu, and Mn contents followed the same trend. The fertilizer with a balance of major and minor nutrients stands a better chance of promoting optimum crop yield (Sharma et al., 2017). The micronutrients Fe + Mn + Zn were reported by Salem and El-Gizawy, (2012) to be the best combination for maize growth as they enhance the utilization of major nutrient elements. In addition, other properties such as pH, EC, CEC, and OM were well expressed in the HO formulas and GOF. The highest CEC of 21.97 cmol kg^{-1} was noted in the HO-3 and is an indication of available nutrient cations ready to be released. This is due to the presence of high secondary and micronutrient elements. The pH of all the fertilizers was ideal to facilitate the release and uptake of nutrients. The hormone quantification showed that IAA and gibberellic acid (GA_3) were more pronounced in the HO-3 formula with 32.44 mg kg^{-1} and 17.22 mg kg^{-1}, respectively than HO-2 and HO-1, but were absent from NPK fertilizer and GOF. Also, bacteria, fungi, and actinomycetes cell count were similarly present in the HO fertilizers and GOF as well. It is therefore evident from Table 3 that, the NPK fertilizer lacks micronutrients, hormones, and microorganisms, which are important requirements for promoting higher crop yield.

3.2 VEGETATIVE GROWTH

Maize growth was in accordance with the balance nutrient status of the fertilizers (Table 4). Maize height and leaf numbers were comparable between HO-3, NPK, and HO-2. Leaf area/plant was clearly significant ($p \leq 0.05$) with 156.02 dm^2 in HO-3 and was followed closely by NPK and HO-2 (Table 4). Besides the fertilizers having nitrogen which is an important factor for cell division, secondary and micronutrients also significantly affect cell division, chlorophyll construction, and photosynthesis (Kadam et al., 2020). This might have accounted for the higher and comparable maize height and

Table 3: Compositional analysis of fertilizers

			Fertilizer treatments					
Fertilizer properties			T2 (NPK)	T3 (HO-1)	T4 (HO-2)	T5 (HO-3)	T6 (GOF)	CD 5 %
Primary nutrients	N	%	15^{a}	7.061^{d}	8.754^{c}	10.96^{b}	4.92^{e}	0.82
	P	%	15^{a}	6.547^{d}	7.832^{c}	9.302^{b}	4.68^{e}	0.82
	K	%	15^{a}	6.451^{d}	7.795^{c}	9.215^{b}	4.84^{e}	0.15
Secondary nutrients	Ca	Mg kg^{-1}	0	6.54^{b}	6.61^{b}	7.97^{a}	2.35^{c}	0.03
	Mg	Mg kg^{-1}	0	1.526^{c}	1.587^{b}	1.628^{a}	0.960^{d}	0.04
	S	mg kg^{-1}	0	0.050^{a}	0.050^{a}	0.055^{a}	0.021^{b}	0.01
Supplementary nutrients	Fe	mg kg^{-1}	0	9.74^{c}	11.36^{b}	14.24^{a}	2.55^{d}	2.55
	Cu	mg kg^{-1}	0	0.034^{a}	0.035^{a}	0.043^{a}	0.011^{b}	NS
	Zn	mg kg^{-1}	0	1.523^{a}	1.612^{a}	1.679^{a}	0.517^{b}	NS
	Mn	mg kg^{-1}	0	1.325^{c}	1.522^{b}	1.750^{a}	0.761^{d}	0.04
Organic matter (OM) %			0	1.05^{c}	1.13^{c}	1.25^{b}	1.37^{a}	0.07
(pH) = 1:1			6.2^{d}	7.2^{b}	7.5^{a}	7.6^{a}	6.9^{c}	1.18
CEC (cmol kg^{-1})			10.54^{d}	18.62^{b}	21.84^{a}	21.97^{a}	10.78^{c}	0.76
EC. 25° (dS m^{-1})			1.44	1.55	1.57	1.58	1.46	NS
Bacteria CFU g^{-1} ($\times 10^4$)			0	32.36^{a}	29.60^{a}	32.90^{a}	22.33^{b}	3.49
Fungus CFU g^{-1} ($\times 10^4$)			0	48.90^{b}	39.40^{c}	53.93^{a}	40.17^{d}	4.28
Actinomycetes CFU g^{-1} ($\times 10^3$)			0	19.52^{b}	16.68^{c}	23.42^{a}	14.17^{d}	1.93
IAA mg kg^{-1}			0	23.26	24.17	27.11	0	NS
GA_3 mg kg^{-1}			0	11.17^{c}	13.25^{b}	17.22^{a}	0	0.92
Cytokinins mg kg^{-1}			0	8.58	7.05	8.59	0	NS

Note: Mean values with identical superscript letters (a,b,c,d.e) are not significantly different at ($p \leq 0.05$), (n = 4); NS = Non significant, CD = Critical difference

leaf area/plant of HO-3 and HO-2 fertilizers to that of NPK, respectively. HO-3 and HO-2 produced the highest dry matter as well because the sink capacity of a crop is mostly dependent on vigorous vegetative growth (Lima et al., 2017). At maximum leaf area, there was more green area for the interception of active radiation for photosynthesis. In addition, leaf chlorophyll, an important factor in photosynthesis was also the highest (56.7 SPAD units) in the HO-3 fertilizer. As a result, the maximum total dry matter of 282.66 g/plant was recorded in HO-3 (Table 5). A similar finding was reported by Azarpour et al. (2014). Probably plants under this treatment could absorb enough nutrients for better growth leading to the higher RGR of 0.132 g g^{-1} day^{-1}, LAI of 5.90 and DMAE of 34.4 %day^{-1} expressed in HO-3, HO-2, and NPK fertilizers (Table 4 and 5). LAI is an important indicator of the photosynthesis system, it relates to economic yield, and its increment results in high yield (Azarpour et al., 2014). The high growth and growth indices recorded could also be attributed to the IAA, GA_3, and cytokinins contained in HO fertilizers as these hormones are well known to promote crop growth. Our results agree with Timothy and Joe (2003) who reported that nitrogen interacts with GA_3 and cytokinins to increase plant growth. All the fertilized plots outperformed the control plot significantly.

Figure 2: Average cob size under various fertilizers

3.3 YIELD AND GRAIN QUALITY

Maize grain mass varied significantly among the fertilizers (Table 5). The nutrients (N, Fe, Cu, Zn, S, and Mg) are important elements in the synthesis of organic compounds (carbohydrates) in crops (Keteku et al., 2019). Under favourable soil conditions, the production of organic compounds is enhanced by these nutrients. The HO-3 formula contained a higher amount of these nutrients except N, producing the maximum average grain mass over the two seasons (24.83 kg/plot and 8276.68 kg ha^{-1}, respectively). This was followed by HO-2 and NPK fertilizers. The mean cob size of the various fertilizers is depicted in Figure 2. According to Cai et al. (2014), the endosperm makes up about 80 % of the total grain mass, therefore hormones that affect cell proliferation can accelerate greater grain sink capacity and endosperm cell number for greater grain yield. In their study, a significant 6.2 - 40.4 % rise in endosperm cells was promoted by GA_3, which intern accelerated grain filling rate and grain weight by 2.9 - 16.0 % when compared to the control. Our findings support their statement because even though the HO-3 and HO-2 fertilizers contained less NPK nutrients compared to T2, they produced a greater grain mass. Previous investigation by Wei et al. (2016) concluded that organic + inorganic fertilizers treatment significantly increased maize yield by 29 % relative to sole organics and by 8 % compared to inorganic fertilizer alone. Consistent with their result, in our work, HO-3 significantly increased maize yield by 30 % relative to GOF and 12.2 % compared to the NPK fertilizer. Khaliq et al. (2006) also similarly, recorded the

Table 4: Influence of fertilizers on vegetative growth

Treatments	Height $plant^{-1}$ (cm)	Leaves no. $plant^{-1}$	Leaf area $plant^{-1}$ (dm^2) DAP			Leaf area index DAP		
			34	44	54	34	44	54
T1 (control)	165.67^{e}	14.70^{d}	31.12^{d}	55.23^{e}	75.80^{e}	1.04^{e}	1.84^{f}	2.53^{d}
T2 (NPK)	247.31^{ab}	18.13^{a}	71.63^{b}	116.4^{b}	147.41^{b}	2.39^{b}	3.88^{b}	4.91^{b}
T3 (HO-1)	217.95^{cd}	16.90^{b}	57.37^{c}	94.94^{c}	125.65^{c}	1.91^{c}	3.16^{d}	4.19^{b}
T4 (HO-2)	230.64^{bc}	18.10^{a}	70.52^{b}	110.1^{b}	134.57^{bc}	2.35^{b}	3.67^{c}	4.49^{b}
T5 (HO-3)	270.85^{a}	18.50^{a}	76.73^{a}	129.26^{a}	156.02^{a}	2.56^{a}	4.11^{a}	5.90^{a}
T6 (GOF)	193.54^{de}	16.07^{c}	42.17^{d}	74.09^{d}	94.25^{d}	1.41^{d}	2.47^{e}	3.14^{c}
CD @ 5 %	27.98	0.43	4.44	10.76	18.93	0.14	0.10	0.98

Note: Mean values with identical superscript letters (a,b,c,d.e) are not significantly different at ($p \leq 0.05$), (n = 4); CD = Critical difference

Table 5: Influence of fertilizers on growth and yield

	Leaf chlorophyll (SPAD unit)			Relative growth rate (g g^{-1} day^{-1})					
	DAP			DAP					
Treatments	34	44	54	34	54	Total dry matter plant^{-1} (g)	Dry matter accumulation efficiency % day^{-1}	Grain mass plot^{-1} (kg)	Grain mass ha^{-1} (kg)
T1 (control)	18.48^{d}	15.42^{d}	11.76^{d}	0.113	0.051^{c}	134.20^{e}	15.9^{d}	12.67^{d}	4223.31^{d}
T2 (NPK)	33.84^{a}	55.36^{a}	51.95^{a}	0.136	0.068^{b}	248.20^{b}	32.8^{a}	21.80^{b}	7266.69^{b}
T3 (HO-1)	28.81^{b}	52.08^{b}	48.62^{b}	0.130	0.063^{bc}	212.38^{c}	26.9^{b}	19.72^{bc}	6573.31^{bc}
T4 (HO-2)	35.15^{a}	56.04^{a}	52.18^{a}	0.138	0.067^{b}	255.77^{b}	33.7^{a}	22.26^{ab}	7420.00^{ab}
T5 (HO-3)	35.73^{a}	56.70^{a}	52.97^{a}	0.147	0.132^{a}	282.66^{a}	34.4^{a}	24.83^{a}	8276.67^{a}
T6 (GOF)	23.02^{c}	46.22^{c}	39.49^{c}	0.121	0.061^{bc}	159.73^{d}	21.1^{c}	17.39^{c}	5796.67^{c}
CD @ 5 %	4.67	2.35	2.29	NS	0.07	15.34	2.17	2.76	859.69

Note: Mean values with identical superscript letters (a,b,c,d.e) are not significantly different at ($p \leq 0.05$), (n = 4); NS = Non significant, CD = Critical difference

highest seed cotton yield of 2470 kg ha^{-1} under $N_{170}P_{85}K_{60}$ + EM + OM fertilization. With regards to grain quality, nitrogen, phosphorus, potassium and crude protein contents were comparable between HO-3 and NPK with average values of 1.58, 0.54, 0.86, 8.99 % and 1.53, 0.53, 0.85, 8.67 %, respectively as shown in Figure 3. Analysis of the maize shoots also revealed higher concentrations of IAA, GA_3, and cytokinins in the HO-3 treated plot, most particularly IAA (0.03 mg kg^{-1}), which may justify Cai et al. (2014) results in figure 4.

3.4 SOIL IMPROVEMENT

After the second growing season, fertilization caused a significant ($p \leq 0.05$) improvement in soil properties (Table 6). When compared to the initial soil properties in Table 2, all the fertilizers improved soil physical, chemical, and biological properties. The HO-3, HO-2 and NPK fertilizers, significantly increased N levels to 0.875, 0.865 and 0.654 %, respectively compared to the control. This increase represents 57.4 %, 56.9 %, and 42.9 %, respectively. Similarly, secondary and micronutrients were also much improved by the HO fertilizers and GOF, when compared to NPK fertilizer. The best improvement in OM and CEC of 0.785 % and 0.8815 cmol kg^{-1}, respectively were observed in GOF, while EC, bulk density, and porosity of 134.75 dS m^{-1}, 1.445 g cm^{-3}, and 33.32 %, respectively were also found in the plots under HO-3 nourishment. The result in Figure 5 showed a rise and drop pattern in the mean soil pH values in all plots, nevertheless, a significant ($p \leq 0.05$) improvement of 6.0 was observed in HO-1 at the end of the second trial compared to the control. The improvement in soil physicochemical properties might be due to the minerals, compost, and soil amendment (dolomite) contained in these fertilizers (He et al., 2015). The HO-1 contained more soil amendments in its formula than the other HO formulas, there-

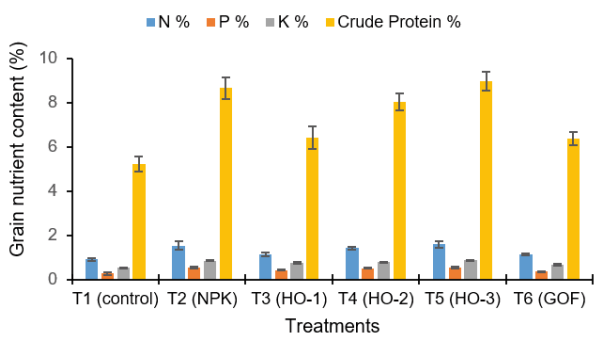

Figure 3: Grain nutrient composition

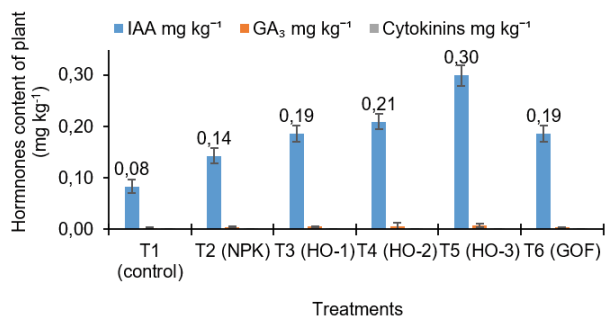

Figure 4: Phytohormones content of maize shoot

fore the calcium might have reacted with water to form CO_3^-, which binds to H^+ ion to adjust soil acidity (Intanon et al., 2011). The effect of the fertilizers on soil pH may also be due to the initial pH of the fertilizers which relates to the materials used in their formulation (Lehmann et al., 2011). The improvement in OM correspondingly decreased soil bulk density and increase porosity (Li and Han, 2016), for good aeration and easy penetration of maize roots. Significant increases in bacteria, fungi, and actinomycetes abundance were also noticed in the HO fertilizers and GOF (Figure 6). An increase in OM due to the addition of organic materials could also increase soil microbial activity (Khaliq et al., 2006). Several previous studies have reported that OM and EM interrelate to improve soil properties (Khaliq et al., 2006; Abujabhah et al., 2016). Abujabhah et al. (2016) reported significant ($p \leq 0.009$) differences in fungi and bacterial abundance in compost amended plots. Our findings support the argument that soil amendments can improve OM and can potentially assist in improving soil physiochemical and biological properties. The significant increase in OM by the HO fertilizers and GOF does indicate that the soil's health and resilience to retain and release nutrients has been improved (Li and Han, 2016) and may intend enhance soil quality through soil aggregation.

4 CONCLUSION

Our findings support the hypothesis that the organo-chemical hormone mixed fertilizer (HO) can increase maize yield and improve soil properties more than NPK fertilizer. Our findings suggest that (i) an optimum combination of inorganic fertilizer, powder of mixed compost, soil amendments, bio-liquid hormone and bio-liquid fertilizer at an optimum rate as in HO-3 can improve soil properties and lead to a maximum maize growth and yield. (ii) the incorporation of growth hormones into fertilization strategies is useful. (iii) grain quality with regards to nitrogen and crude protein contents can be improved by balanced nutrition. This work will serve as a basis for such holistic fertilizer formulation and promote the concept of integrated nutrient management.

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Table 6: Soil properties after the experiment

Soil properties			T1 (Control)	T2 (NPK)	T3 (HO-1)	T4 (HO-2)	T5 (HO-3)	T6 (GOF)	CD @ 5%
Primary nutrients	N	%	0.373^{f}	0.654^{c}	0.707^{b}	0.865^{a}	0.875^{a}	0.597^{d}	0.01
	P	%	0.0126	0.0172	0.0194	0.0204	0.0275	0.01541	NS
	K	%	0.0113	0.0177	0.0188	0.0196	0.0267	0.01552	NS
Secondary nutrients	Ca	mg kg^{-1}	3.452^{d}	3.557^{d}	5.527^{b}	9.257^{a}	9.461^{a}	5.287^{b}	0.35
	Mg	mg kg^{-1}	1.274^{d}	1.576^{c}	3.007^{b}	7.776^{a}	7.785^{a}	2.967^{b}	0.07
	S	mg kg^{-1}	0.193^{d}	1.170^{b}	0.318^{c}	1.707^{a}	1.716^{a}	0.309^{c}	0.01
Supplementary nutrients	Fe	mg kg^{-1}	5.846^{b}	5.972^{b}	6.364^{b}	15.872^{a}	16.200^{a}	6.336^{b}	1.42
	Cu	mg kg^{-1}	0.001^{d}	0.059^{b}	0.057^{b}	0.077^{a}	0.085^{a}	0.050^{b}	0.01
	Zn	mg kg^{-1}	1.343^{d}	1.475^{d}	7.192^{b}	9.777^{a}	9.788^{a}	2.178^{c}	0.68
	Mn	mg kg^{-1}	1.846^{f}	1.787^{g}	3.515^{c}	4.388^{b}	4.401^{a}	2.505^{d}	0.01
Organic Matter (OM) %			0.514^{c}	0.523^{c}	0.616^{b}	0.611^{b}	0.606^{b}	0.785^{a}	0.02
(pH) = 1:1			5.20^{e}	5.25^{e}	6.00^{a}	5.90^{b}	5.80^{c}	5.41^{d}	0.06
EC. 25°(dS m^{-1})			46.843^{e}	115.513^{c}	121.643^{b}	132.44^{a}	134.75^{a}	81.535^{d}	3.97
C.E.C. (cmol kg^{-1})			0.183^{d}	0.373^{c}	0.763^{b}	0.783^{b}	0.800^{a}	0.815^{a}	0.02
Bulk Density (Db) g cm^{-3}			1.573^{a}	1.533^{c}	1.473^{e}	1.453^{f}	1.445^{f}	1.495^{d}	0.01
Porosity (E) %			23.113^{c}	25.613^{c}	29.103^{b}	33.273^{a}	33.32^{a}	26.035^{b}	3.49

Note: Mean values with identical superscript letters (a,b,c,d.e) are not significantly different at ($p \leq 0.05$). (n = 4); NS = Non significant, CD = Critical difference

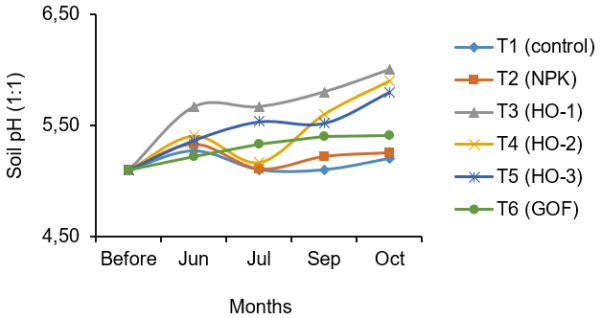

Figure 5: Soil pH during the second cropping season

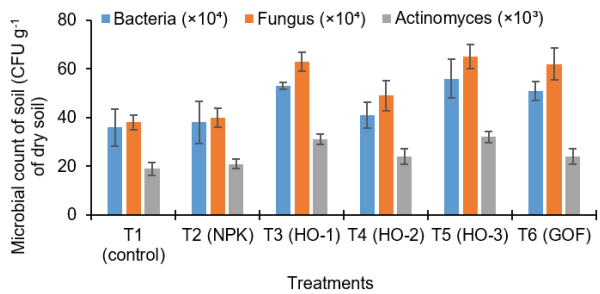

Figure 6: Soil microbial abundance after the trial

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Antioxidant response of *Impatiens walleriana* L. to drought

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Antioxidant response of *Impatiens walleriana* L. to drought

Abstract: Stress caused by drought induces plant morphology, biochemistry, and physiology changes, leading to considerable reductions in plant growth and productivity. This study aimed to evaluate the antioxidant defence system of impatiens seedlings (*Impatiens walleriana* L.) under drought. The antioxidant response of impatiens to drought was evaluated using following parameters: the activity of catalase, guaiacol peroxidase, pyrogallol peroxidase and ascorbate peroxidase, total phenolic and flavonoids contents and total antioxidant capacity. The experiment was conducted during 2020 in a greenhouse under controlled conditions. Half of the impatiens seedlings (20 plants), after the acclimation period in the greenhouse, were exposed to drought for a period of five days, while the second half was not (20 plants were regularly watered). The results of the study showed that the exposure of impatiens seedlings to drought increased the activity of enzymatic components, total phenolics and flavonoids contents and total antioxidant capacity of leaves. Greater exposure of impatiens to drought (in the observed period) implied a higher plant enzymatic and non-enzymatic antioxidant defence system activity. These results confirm that impatiens has evolved both enzymatic and non-enzymatic antioxidant defence mechanisms to adapt and survive the short-term drought exposure.

Key words: defence system; free radicals; leaves; plant growth; stress

Antioksidacijski odziv vodenke (*Impatiens walleriana* L.) na sušo

Izvleček: Stres, ki ga povzroča suša sproži v rastlinah spremembe v morfologiji, biokemični zgradbi in fiziologiji, kar vodi k znatnemu zmanjšanju rasti in produktivnosti rastlin. Namen raziskave je bil ovrednotiti antioksidacijsko obrambo sejank vodenke (*Impatiens walleriana* L.) v sušnem stresu. Antioksidacijski odziv vodenke na sušo je bil ovrednoten z naslednjimi parametri: aktivnostjo katalaze, guajakol peroksidaze, pirogalol peroksidaze in askorbat peroksidaze, vsebnostjo celokupnih fenolov in flavonoidov in celokupne antioksidacijske kapacitete. Poskus je bil izveden v rastni sezoni 2020 v rastlinjaku v nadzorovanih razmerah. Polovica sejank vodenke (20 rastlin), je bila po aklimatizaciji razmeram rastlinjaka izpostavljena sušnemu stresu za pet dni, medtem ko je bila druga polovica (20 rastlin) redno zalivana. Rezultati raziskave so pokazali, da je izpostavitev sejank vodenke sušnemu stresu povečala aktivnosti analiziranih encimov, vsebnosti celokupnih fenolov in flavonoidov ter celokupno antioksidacijsko sposobnost listov. Večja izpostavitev vodenk suši je v opazovanem obdobju povzročila večji encimski in neencimski antioksidacijski obrambni odziv. Rezultati potrjujejo, da ima vodenka sposobnost razvoja encimskega in neencimskega antioksidacijskega obrambnega sistema in lahko preživi krajša obdobja izpostavitve suši.

Ključne besede: obrambni sistem; prosti radikali; listi; rast rastlin; stres

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1 INTRODUCTION

Drought is the most important abiotic factor limiting crop productivity. The lack of water in soil reduces the soil water potential and the ability of plants to take up water, resulting in growth inhibition and reproductive failure (Fahad et al., 2017). In addition, the inevitable consequence of drought is an increase in the production of reactive oxygen species (ROS) in plant cells. ROS include free radicals such as superoxide radical, hydroxyl radical as well as non-radical molecules like hydrogen peroxide (H_2O_2). Increased levels of ROS can cause cellular damage and even cell death (Tola et al., 2021).

Plants, however, have evolved numerous mechanisms to contend with oxidative stress, including the enzymatic and non-enzymatic antioxidant systems. Non-enzymatic defences include compounds with antioxidant properties such as phenolic compounds, vitamin C and carotenoids, while the enzymatic defences include antioxidant enzymes associated with ROS scavenging in plants such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), pyrogallol peroxidase (PPX), ascorbate peroxidase (APX) and catalase (CAT) (Mehla et al., 2017).

SOD protects cells against ROS by catalysing the dismutation of highly toxic superoxide anions to less toxic hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). After dismutation of the superoxide anions by SOD into O_2 and H_2O_2, the CAT decomposes the released H_2O_2 into H_2O and O_2 (Berwal & Ram, 2018). GPX and PPX also protect cells against the damaging effect of H_2O_2 by catalysing their decomposition through oxidation of phenolic substrates (Gill & Tuteja, 2010). APX is also a H_2O_2-scavenging enzyme. APX utilizes ascorbic acid as specific electron donor to reduce H_2O_2 to H_2O (Hasanuzzaman et al., 2019).

The aim of this study was to evaluate the enzymatic and non-enzymatic antioxidant defence system of impatiens seedlings (*Impatiens walleriana* L.) under drought stress. Impatiens was selected as subject of this study primarily because the global production of this flowering plant species is consistently increasing. Therefore any new knowledge about the behaviour of these plants, especially under stress conditions, is of great interest to both producers and scientists.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL CONDITIONS

The experiment was conducted in May 2020 under controlled conditions in the greenhouse of public communal company 'Park' in Sarajevo. The temperature in the greenhouse during the experiment was maintained at 24 °C/21 °C during day/night, while the relative humidity (RH) was maintained between 60 % and 70 %, with combined venting to reduce RH and fogging systems to increase RH.

In the beginning of the experiment, the impatiens seedlings were in the initial stage of flowering. The first part of the study involved transplanting impatiens seedlings into individual pots (20 cm diameter × 13 cm height), containing substrate Florahum-SP. Impatiens seedlings used in the experiment were produced in the nursery near the greenhouse and showed no significant difference in size and appearance.

Ten days after transplanting, half of the impatiens (20 plants) were exposed to drought for next five days (non-watering). However, the second half was not exposed to drought, they served as controls (20 plants were regularly watered). Leaves of impatiens were sampled at the beginning and at the end of experiment (2nd and 5th day after drought treatment). Each leaf sample consisted of three fully expanded and healthy impatiens leaves. Fresh leaves were cut and immediately frozen with liquid nitrogen and then stored in ultra-freezer at -20 °C until further use.

2.2 PROTEIN AND ENZYME ACTIVITY MEASUREMENTS

To obtain the extracts that were used to determine the protein content and activities of catalase and peroxidases, 0.5 g of fresh leaf sample was macerated using a mortar and pestle with liquid nitrogen and 0.015 g polyvinylpyrrolidone (PVP). The powder thus obtained was homogenized in 1.5 ml 50 mM potassium phosphate buffer (pH 7) containing 1 mM dithiothreitol (DTT) and 1mM ethylenediaminetetraacetic acid (EDTA). The homogenized material was centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant was used for protein and enzyme activity measurements.

The extracted proteins were quantified using the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976). The pyrogallol peroxidase (PPX) activity was determined by the oxidation of pyrogallol according to the method of Chance & Maehly (1955) and the results were expressed as μmol purpurogallin per min per mg protein. The guaiacol peroxidase (GPX) activity was determined by the oxidation of guaiacol according to the method of Chance & Maehly (1955) and the results were expressed as μmol tetraguaiacol per min per mg protein. The ascorbate peroxidase (APX) activity was determined by the oxidation of ascorbic acid

according to the method of Nakano & Asada (1981) and the results were expressed as µmol ascorbic acid oxidized per min per mg protein. The catalase (CAT) activity was determined by monitoring the decrease in absorbance at 240 nm at an interval of 5 to 120 sec as a result of H_2O_2 consumption (Aebi, 1984). Results were expressed as µmol of H_2O_2 consumed per min per mg protein.

2.3 EXTRACTION OF PHENOLIC COMPOUNDS FROM LEAVES

The collected fresh leaves of impatiens were oven-dried at 40 °C (3 days) to avoid degradation of their phenolic compounds. After that, dried leaf samples were ground to a fine powder using an electric blender and stored at 4 °C until extraction and analysis. Extraction of phenolic compounds from dried leaf sample was done as follows: 1 g of sample was extracted with 30 ml of 60 % ethanol aqueous solution at room temperature for 24 h. Thereafter, the extract was filtered through Whatman filter paper (11 µm pore size) into 50 ml volumetric flask and diluted to the mark with 60 % ethanol aqueous solution. The extract thus obtained was used to estimate total phenolic content, total flavonoid content and total antioxidant capacity.

2.4 TOTAL PHENOLICS CONTENT

The colorimetric reaction with Folin-Ciocâlteu reagent was performed to determine the content of phenolic compounds in leaf samples of impatiens (Ough & Amerine, 1988). The reaction mixtures consisted of 0.1 ml of extract, 6 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent (before use diluted in distilled water 1:2, v/v) and 1.5 ml of 20 % Na_2CO_3 were mixed thoroughly. Thereafter, the mixture was heated in a water bath at 40 °C for 30 min. After cooling to room temperature, the absorbance of the mixture was read at 765 nm. The results were calculated on the basis of the calibration curve for gallic acid (0-500 mg l^{-1}) and were expressed as mg of gallic acid equivalents per g of dry mass (mg GAE g^{-1} DM).

2.5 TOTAL FLAVONOIDS CONTENT

The aluminum chloride colorimetric assay was performed to determine the total flavonoid contents (Zhishen et al., 1999). The reaction mixtures consisted of 1 ml of extract, 4 ml of distilled water, 0.3 ml of 5 % $NaNO_2$, 0.3 ml of 10 % $AlCl_3$ and 2 ml of 1 M NaOH were mixed thoroughly. The mixture was made up to 10 ml with distilled water and incubated at room temperature for 1 h, and then the absorbance of the mixture was read at 510 nm. The results were calculated on the basis of the calibration curve for catechin (0-100 mg l^{-1}) and were expressed as mg of catechin equivalents per g of dry mass (mg C g^{-1} DM).

2.6 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

Ferric reducing antioxidant power (FRAP) assay was performed to estimate the total antioxidant capacity (Benzie & Strain, 1996). The reaction mixture consisted of 80 µl of extract, 240 µl of distilled and 2080 µl of fresh FRAP reagent were mixed thoroughly. The FRAP reagent was prepared immediately before use by mixing acetate buffer (300 mM, pH = 3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ in a volume ratio of 10:1:1. Thereafter, the mixture was heated at 37 °C for 15 min in a water bath. After cooling to room temperature the absorbance was read at 595 nm. The results were calculated on the basis of the calibration curve for $FeSO_4 \times 7\ H_2O$ (0-2000 µM) and were expressed as µmol of Fe^{2+} per g of dry mass (µmol Fe^{2+} g^{-1} DM). Amersham ultrospec 2100 spectrophotometer (Biochrom, USA) was used for all spectrophotometric measures.

2.7 STATISTICAL ANALYSIS

All experimental measurements were done in triplicates and the results were presented as mean ± standard deviation. Pearson correlation coefficient was used to reflect relationship total phenolic, total flavonoids and total antioxidant activities. One-way analysis of variance (ANOVA) and least-significant-difference test (LSD) at 0.05 level of probability ($p < 0.05$) were performed to evaluate statistical significance between the means using Microsoft Excel 2010 software (Office 2010, Redmond, WA, USA).

3 RESULTS

The activity of all tested enzymes (GPX, PPX, APX and CAT) in the observed period were higher in leaves of impatiens exposed to drought compared to impatiens grown under standard growth conditions (without stress), as shown in Table 1. The results of this study also showed that the activities of all enzymes were increased with the progress of stress.

Table 1: Effect of short-term exposure to drought on antioxidant enzymes of impatiens leaves

	Enzyme activity (μmol min^{-1} mg^{-1} protein)			
Treatment	GPX	PPX	APX	CAT
2nd day of exposure to drought	0.25 ± 0.11[b]	0.41 ± 0.13[b]	0.27 ± 0.26[b]	0.009 ± 0.001[b]
2nd day without stress	0.16 ± 0.07[c]	0.33 ± 0.21[b]	0.13 ± 0.12[c]	0.008 ± 0.002[b]
5th day of exposure to drought	0.31 ± 0.04[a]	1.03 ± 0.13[a]	0.51 ± 0.16[a]	0.033 ± 0.014[a]
5th day without stress	0.18 ± 0.04[c]	0.48 ± 0.07[b]	0.22 ± 0.08[bc]	0.009 ± 0.004[b]
$LSD_{0.05}$	0.054	0.159	0.114	0.006

The activity of GPX and APX were significantly higher in the leaves of impatiens exposed to drought than in the control, regardless of the duration of plant exposure to stress. However, the activity of PPX and CAT in leaves of all stressed impatiens seedlings was significantly higher only at the end of the experiment i.e. on the fifth day of plant exposure to drought. In controls i.e. in variants where impatiens seedlings were not exposed to drought, the activity of all enzymes tested did not change significantly during the experiment.

In this study, the non-enzymatic antioxidant defence system (total phenolic contents (TPC), total flavonoids (TFC) and total antioxidant capacity (TAC) were also affected by growth conditions (Table 2).

As shown in Table 2, TPC, TFC and TAC were higher in the leaves of impatiens exposed to drought than in control. The increases were statistically significant for both the second and the fifth day of plant exposure to drought.

In this study, there was a positive and strong significant relationship between the total phenolic/flavonoids and the total antioxidant capacity of impatiens leaves regardless of growth conditions, indicating that phenolic compounds are mainly responsible for total antioxidant capacity of plants (Table 3).

4 DISCUSSION

A key sign of drought stress at the cellular level is the overproduction of reactive oxygen species (ROS), which is being considered as the most common cause of cellular damage. However, plants have evolved an efficient enzymatic and non-enzymatic antioxidant system to protect themselves against ROS. Within a cell, the SOD constitutes the first line of plant antioxidant defence against ROS. However, H_2O_2, which results from the action of SOD, is toxic to cells. Therefore, the efficient scavenging of H_2O_2 is regarded as a key feature in the cellular antioxidant defence system. Fortunately, plant cells are endowed with H_2O_2-metabolizing enzymes such as peroxidases and catalase. Peroxidases are group of enzymes that catalyse the conversion H_2O_2 into H_2O using a wide variety of substrates as an electron donor (Abedi & Paknyat, 2010).

In this study, generally, stress caused by drought increased the CAT and peroxidase enzymatic activity, and the increase was in line with stress duration; greater exposure of impatiens to drought (in the observed period) implied a higher activity of antioxidant enzymes. However, there was a differential level of activity among enzymes. The activities of GPX and APX enzymes at the early stage of drought stress (2nd day after drought treatment) were significantly higher as compared to CAT, although both peroxidases and CAT act on the same substrate (H_2O_2). Lower CAT activities in plants at the early stage of stress have been reported in many studies (Chugh et al, 2013; Antonić et al., 2016; Wang et al., 2019). Smirnof & Araound (2019) noted that CAT does not have a high affinity for H_2O_2 and this is probably one of the main reasons for its low activity. However, CAT has

Table 2: Effect of short-term exposure to drought on non-enzymatic antioxidants of impatiens leaves

Treatment	TPC (mg GAE g^{-1} DM)	TFC (mg C g^{-1} DM)	TAC (μmol Fe^{2+} g^{-1} DM)
2nd day of exposure to drought	6.92 ± 0.18[b]	2.08 ± 0.24[b]	92.91 ± 5.82[b]
2nd day without stress	5.65 ± 0.22[c]	1.50 ± 0.18[c]	65.23 ± 3.65[c]
5th day of exposure to drought	7.98 ± 0.70[a]	2.68 ± 0.34[a]	103.95 ± 4.17[a]
5th day without stress	6.37 ± 0.75[bc]	2.20 ± 0.20[b]	89.42 ± 12.38[b]
$LSD_{0.05}$	0.83	0.26	10.58

Table 3: Pearson's correlation between total phenolic (TPC), total flavonoids (TFC) and total antioxidant capacity (TAC)

Treatment		TPC	TFC	TAC
2nd day of exposure to drought	TPC	1	0.95	0.93
	TFC		1	0.94
	TAC			1
2nd day without stress	TPC	1	0.92	0.93
	TFC		1	0.91
	TAC			1
5th day of exposure to drought	TPC	1	0.94	0.95
	TFC		1	0.96
	TAC			1
5th day without stress	TPC	1	0.93	0.94
	TFC		1	0.92
	TAC			1

a very high reaction rate (Smejkal & Kakumanu, 2019). The Braunschweig Enzyme Database (BRENDA) reports that one molecule of catalase can convert over 2.8 million molecules of hydrogen peroxide to water and oxygen per second (Schomburg et al., 2017). Therefore, CAT is a sink for H_2O_2 and is indispensable for plant defence system against oxidative stress (Willekens et al., 1997).

Besides enzymatic antioxidants, plants synthesize a wide range of non-enzymatic antioxidants capable of decreasing ROS-induced oxidative damage (Kasote et al., 2015). Non-enzymatic antioxidants include vitamin C, vitamin E, phenolic compounds, carotenoids, etc. Among all non-enzymatic antioxidants, phenolic compounds appear to be the most important since they have a great potential to clear ROS. The antioxidant properties of phenolic compounds are mainly due to their high redox potential, allowing them to act as reducing agents, hydrogen donors or singlet oxygen quenchers (Liang et al., 2010).

In the present study, the accumulation of phenolic compounds was significantly higher in leaves of impatiens exposed to drought than in controls (without stress). Moreover, an increase in phenolics contents was more significant in impatiens exposed to drought for longer duration. These results suggest that plant initiates the intensive synthesis of phenolic compounds as a response to drought, and this hypothesis has been confirmed by many other scientists (Basu et al., 2010; Cramer et al., 2011; Šamec et al., 2021).

Sharma et al. (2019) reported that the considerable accumulation of phenolic compounds in plants is usually a consistent feature of non-enzymatic antioxidant defence mechanisms under stress. However, the capacity of antioxidant defence mechanisms depends on each phenolic compound's chemical structure. Among the phenolic compounds with known antioxidant activity, flavonoids are highlighted (Dibacto et al., 2021). In this study, TFC in leaves of impatiens were progressively influenced by drought. An increase in TFC in leaves of impatiens was already recorded in the 2nd days after drought treatment, and with the progress of stress (5th days after drought treatment), TFC was gradually increased. In this study, an increase of TFC was in line with increase of TPC in impatiens leaves regardless of growth conditions. This was expected since the flavonoids are the biggest group of phenolic compounds.

In the present study, the total antioxidant capacity level estimated with FRAP assay was also significantly higher in leaves of impatiens exposed to drought than in controls. Furthermore, the present study indicates a very strong relationship between the TPC/TFC and TAC in leaves of impatiens, regardless of growth conditions. In short, the antioxidant activity in leaves of impatiens increased by increasing the total phenolic and flavonoid contents. These results were also expected since it is known that phenolic compounds are among the most potent antioxidants from plants.

The levels of enzymatic and non-enzymatic antioxidants in impatiens leaves were very high in the fifth day after drought treatment. Accumulation of these antioxidants suggests a high level of stress convened to the impatiens during this period (Sharma et al., 2012). It can also be assumed that the impatiens during this period continues to defend itself against ROS by producing a high amount of enzymatic and non-enzymatic antioxidants (Kim et al., 2014). However, numerous studies reported a decline in the activity of antioxidant enzymes in various plants in the final stage of stress (three days or more after exposed plant to stress), indicating that antioxidant capacity and thus drought tolerance can vary among plants (Almeselmani et al., 2006; Sabzmeydani et al., 2021). It is evident that plant response to drought depends not only on the extremity and time duration of the stress but also on the plant genetic background.

5 CONCLUSIONS

Exposure of impatiens seedlings to drought increased the activity of enzymatic antioxidants, total phenolic and flavonoid contents and total antioxidant capacity of leaves. Greater exposure of impatiens to drought (in the observed period) implied a higher activity of plant enzymatic and non-enzymatic antioxidant defence systems. These results confirm that impatiens have evolved both enzymatic and non-enzymatic antioxidant

defence mechanisms to adapt and survive the short-term drought exposure.

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Effect of foliar application of glucose and fructose to reduce codling moth (*Cydia pomonella* [L., 1758]) damages on apple orchard

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Effect of foliar application of glucose and fructose to reduce codling moth (*Cydia pomonella* [L., 1758]) damages on apple orchard

Abstract: The apple is a dominant crop in Batna region and codling moth (CM) (*Cydia pomonella*) pressure is constantly very high. In this study, foliar application of single sugars is proposed as a novel control strategy, in an orchard located in Beni Fedhala (province of Batna-Algeria). The effect of spraying fructose (100 ppm), glucose (100 ppm), and insecticide (Deltamethrin) was tested against CM larval damages on the Royal Gala variety. This research showed that CM own four generations in this region. The spraying of glucose alone, fructose alone strongly reduced the percentage of damaged fruits with a very important value of Abbott's efficacy. In addition, fructose and insecticide induced a significant decrease in the percentages of fallen and damaged fruits. Besides, the use of fructose, glucose and the insecticide has significantly reduced the number of diapausing larvae in corrugated cardboard banding. Foliar application of sugars is a completely innovative way in the field of plant protection. These results open new crop management methods.

Key words: *Cydia pomonella,* glucose, fructose*,* Deltamethrin

Učinek foliarnega nanosa glukoze in fruktoze za zmanjšanje škod po jabolčnem zavijaču (*Cydia pomonella* [L., 1758]) v nasadu jablan

Izvleček: Jablana je dominatna sadna vrsta na območju Batne in napad jabolčnega zavijača je stalno zelo velik. V raziskavi je bil uporabljen nanos posameznih sladkorjev kot novi način uravnavanja škodljivca v sadovnjakih na območju Beni Fedhala (provinca Batna-Alžirija). Preiskušeno je bilo škropljenje s fruktozo (100 ppm), glukozo (100 ppm) in insekticidom (Deltametrin) glede na poškodbe, ki so jih ličinke jabolčnega zavijača povzročile na sorti Royal Gala. Raziskava je pokazala, da ima jabolčni zavijač na tem območju štiri generacije. Škropljenje samo z glukozo ali samo s fruktozo je močno zmanjšalo delež poškodovanih plodov s pomembno vrednostjo Abbottove učunkovitosti. Dodatno je obravnava s fruktozo in insekticidom vzpodbudila značilno zmanšanje odstotka odpadlih in poškodovanih plodov. Poleg tega je uporaba fruktoze, glukoze in insekticidov značilno zmanjšala število bub v lovilnih trakovih na deblu jablan. Foliarni nanos sladkorjev je popolnoma nov pristop na področju varstva rastlin. Izsledki te raziskave odpirajo nove metode pri gojenju rastlin.

Ključne besede: *Cydia pomonella*; glukoza; fruktoza; Deltametrin

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1 INTRODUCTION

The codling moth (*Cydia pomonella* [L., 1758]) (CM) is one of the greatest hindrances to apple production in Algeria. The adoption of environmentally acceptable pest control management has increased in the face of dwindling conventional control methods. Sugars not only fuel cellular carbon and energy metabolism but also play pivotal roles as signaling molecules, in plants, different sugar signals are responsible to modulate growth, development, and stress responses (Rolland et al., 2006). The existence of Lepidoptera species is dependent on the site that the female chooses to lay the eggs since the hatching larvae are less mobile (Derridj et al., 2012). The composition of a metabolite blend on the leaf surface consisting of glucose, fructose, sucrose, sorbitol, quebrachitol, and *myo*-inositol is one of the factors that could explain the variation of the intensity of egg-laying by the moth from one cultivar to another (Lombarkia et al., 2013). The studies of Lombarkia (2002) and Lombarkia et al., (2008 and 2013) were tested the link of these six metabolites on *C. pomonella* egg-laying behaviour to reduce the damage. Furthermore, other researchers (Derridj et al., 2011, Arnault et al., [2015, 2016]) have shown an increased interest in the concept of exogenous application of sugars on apple trees to reduce the damage of *C. pomonella* in commercial orchards in several countries (France, Italy, Greece, and Algeria).

As for all agricultural crops, plant protection problems such as pests and diseases are the major factors decreasing apple production. CM cause economical losses in orchards in the Batna region, the interest in strategies in order to keep the pest populations at an economically negligible level is consequently increasing.

In this context, the present research proposed exogenous application of sugars specifically fructose alone and glucose alone on apple trees. A main objective is the assessment in the orchard of the impact of low doses of sugars on fruit damages in comparison to the untreated control, for developing a new environmentally acceptable control method.

2 MATERIAL AND METHODS

This research was conducted at the Batna region, eastern Algeria, (35°21′21,6″ N, 006°01′ 16,5″ E) and in a Royal Gala apple orchard. Four apple plots test treatments of *C. pomonella*. The treated orchard (surface of 2 ha, 9 years old) was managed under common practices of the zone.

One attractive sex pheromone trap type Russell IPM was used to follow the pest dynamics and population of CM during 2019 and 2020, placed at eye level, trapping took place between 17th March to 04th September 2019 and 17th March to 04th September 2020, the observations were carried out every 3 days. The total number of captured moths was counted.

2.1 TREATMENTS

The orchard was divided into four plots adjusted in a randomized Latin square with four repetitions and each plot has three trees, the modalities tested were fructose at 100 ppm (10 g 100 l^{-1}), glucose at 100 ppm (10 g 100 l^{-1}) and insecticide Decis 25 EC (25 g l^{-1} Deltamethrin) at (0.5 l) 1000 l^{-1} dose, in addition to the unsprayed control modality, The treatments were applied using an electrical pressure sprayer (12 V-12 Ah), capacity 16 l. (Fructose and glucose are produced by Fluka Biochemika and Decis by Bayer).

The morning treatments (sugars and insecticide) were carried out every 20 days throughout the season from the flowering end until harvest (Derridj et al., 2012).

2.2 DAMAGE ASSESSMENTS

According to the Abbott's formula, T0 – Tt / T0 × 100 (where T0 is the percentage of infested fruits in the untreated plots and Tt is the percentage of infected fruits in the treated plots), the percentage of fruit damaged at harvest, efficiency of treatments at harvest and percentage of fallen and damaged fruits were measured.

2.3 COUNTING DIAPAUSING LARVAE

The larva at the end of its growth (fifth instar larvae) overwinters in a cocoon (diapausing larvae) in the crevices of the trunk. The sequestration of diapausing larvae by bandaging tree trunks provides a simple and effective means to estimate the CM population, a strip of corrugated cardboard (20 cm wide) was placed around the trunk of all trees, of the four plots and at a height of 20 cm from the ground, installed between mid-April to the end September, the captured diapausing larvae were counted.

2.4 STATISTICAL ANALYSIS

All analyses were performed using SPSS software v 2016. The means of each variable (percentage of fruit damaged at harvest, efficiency Abbott of treatments at

harvest and percentage of fallen and damaged fruits, number of diapausing larvae), were compared by ANOVA on ranks test, followed by post hoc analysis using Fisher's and Tukey's tests or Kruskal-Wallis test. A P-value of 0.05 was used to establish significance in all tests.

3 RESULTS AND DISCUSSION

3.1 SEXUAL TRAPPING OF ADULTS

Two trials were conducted during the 2019 and 2020 seasons, where there are four full codling moth generations (Table 1).

The determination of generations is based on the following principle described by Hmimna and Iraqui (2015), the division a significant and stable increase in catches, followed by a sufficiently long inter-flight (± 30 days) with few catches, indicates a nascent or finishing flight comparable to the start or the end of a generation.

In Algeria, several studies revealed that the CM has two at four generations depending on the climate conditions and the regions. For instance, Soltani et al. (1986), has been stated that CM in quince orchard has four generations while the fourth is partial. Furthermore, Abdesselam (2016) reported two generations of CM in Inoughissen (Batna) and Meradi (2015) found that CM produced three generations in the season. Besides, Tiffrent and Lombarkia (2021) indicated that CM in Batna province, has four generations in the season on the Golden Delicious variety.

3.2 DAMAGE ASSESSMENTS AND TREATMENTS EFFICACY

Foliar sprays of fructose and glucose reduced significantly the percentage of fruit damaged at harvest compared to the control, the analysis of variance (Kruskal-Wallis test), ($p < 0.05$) identifies three groups, control (76.99 ± 1.97 %, a group), glucose and fructose (15.27 ± 1.06 % and 16.45 ± 0.59 % respectively (b group), followed by the spraying of the insecticide with 10.97 ± 0.56 % (c group) (Figure 1).

The Abbott efficiency at harvest of glucose treatments is 80.25 % and fructose generates an average percentage efficiency of 78.58 % compared to the insecticide 85.64 %. The analysis of variance (ANOVA) followed by the Tukey test ($p < 0.05$) identifies two groups (Table 2).

On the other hand, the spraying of fructose alone and the insecticide induced a significant decrease in the percentages of damaged and fallen fruits compared to the untreated control and glucose alone. The analysis of variance (ANOVA) followed by the Tukey test ($p < 0.05$) identifies three groups, control and glucose 32.54 ± 1.25%, 31.93 ± 1.16 % respectively (a group), fructose 19.16 ± 0.91 %, (b group) and insecticide 09.78 ± 0.8 % (c group) (Figure 2).

Moreover, the spraying of glucose, fructose and the insecticide induced a significant decrease in the number of diapausing larvae compared to the untreated control. The analysis of variance (Kruskal-Wallis test), ($p < 0.05$) identifies two groups, control 34.50 ± 2.55 % (a group), glucose 10.08 ± 1.55 %, fructose 06.67 ± 1.67 % and insecticide 08.25 ± 2.22 % respectively (b group) (Figure 3).

By drawing on the concept of the exogenous foliar application of a single sugar can induce plant resistance to the pest, Arnault et al. (2015) indicated that on Granny Smith variety, sprays of fructose at 100 ppm in combination with organo-phosphorus (OP) and insect growth

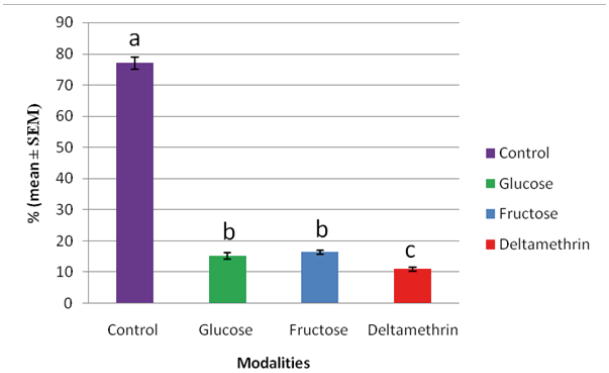

Figure 1: Percentage of damaged fruits at harvest in apple orchard (n = 12) on different modalities (control, fructose, glucose, insecticide). Different letters indicate a significantly different percentages of fruit damaged at harvest at $p < 0.05$

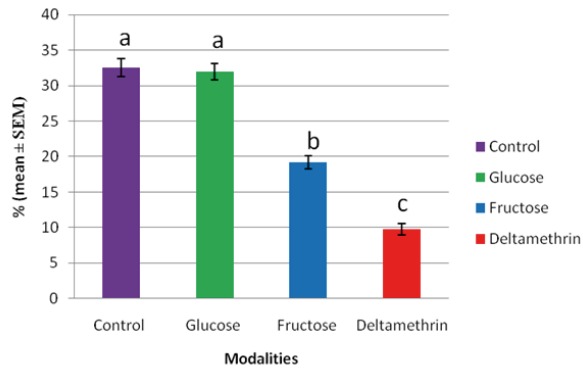

Figure 2: Percentage of fallen and damaged fruits in apple orchard (n = 12) on different modalities (control, fructose, glucose, insecticide). Different letters indicate a significantly different percentage of fallen and damaged fruits at $p < 0.05$

Table 1: The number of catches of *C. pomonella* adult males during the four generations

Dates of trapping	Traps G1		Dates of trapping	Traps G2		Dates of trapping	Traps G3		Dates of trapping	Traps G4	
	2019	2020		2019	2020		2019	2020		2019	2020
20/03	1	1	22/04		10	16/05		10	12/07		06
23/03	0	0	25/04		18	19/05		17	15/07		14
26/03	1	1	28/04		20	22/05		10	18/07		16
29/03	0	0	01/05		40	25/05		10	21/07		10
01/04	1	0	04/05	10	50	28/05	03	11	24/07		08
04/04	1	4	07/05	14	48	31/05	19	10	27/07		10
07/04	2	6	10/05	31	52	03/06	25	11	30/07	5	11
10/04	0	7	13/05	13	27	06/06	09	13	02/08	13	13
13/04	0	23	16/05	13		09/06	26	13	05/08	21	18
16/04	8	40	19/05	10		12/06	16	14	08/08	15	15
19/04	23	18	22/05	38		15/06	11	12	11/08	31	7
22/04	36		25/05	19		18/06	14	12	14/08	10	7
25/04	34					21/06	10	15	17/08	7	6
28/04	35					24/06	14	20	20/08	45	4
01/05	26					27/06	29	22	23/08	3	5
						30/06	80	27	26/08	2	4
						03/07	75	20	29/08	2	4
						06/07	70	14	01/09	20	4
						09/07	42	13	04/09	09	5
						12/07	20				
						15/07	24				
						18/07	24				
						21/07	10				
						24/07	14				
						27/07	17				

regulator (IGR) have significantly reduced the percentage of damaged fruits by CM at harvest compared to the OP and IGR alone (6.5 %, 10 %); and the efficiency is improved by 35 %. In 2013, 2014, at organic orchards study; Arnault et al. (2015) demonstrated that, the foliar applications of fructose to 100 ppm could reduce CM damage by 55 % on the Gala variety. In addition, Arnault et al. (2016) showed that fructose 100 ppm reduced the damage of CM in four commercial apple orchards from 2013 to 2014 in Algeria and France, and the results revealed an efficacy Abbott of 48.9 % and the average percentage of infested fruits was 8.1 % which was significantly lower compared to the untreated modality (23.8 %).

Similarly, previous studies have demonstrated the potential of foliar application of sucrose in micro-doses to control *C. pomonella* performed in the Batna region support our findings.

For instance, Meradi (2015) has demonstrated in her

Table 2: Percentage of Abbott efficiency at harvest in apple orchards (n = 12) on different modalities (fructose, glucose, insecticide)

Treatments	% Abbott efficiency (Mean + SEM*)
Glucose 100 ppm	80.25 % ± 1.18 a
Fructose 100 ppm	78.58 % ± 0.86 a
Insecticide (Deltamethrin)	85.64 % ± 0.85 b

* Values indicated with different letters are significantly different at $p < 0.05$

study conducted on the Starkrimson variety, that fructose 100 ppm has reduced the percentage of fallen and damaged fruits compared to the control (56.0 %and64.0 % respectively), and the Abbott effectiveness at harvest was 31.71 %. While in the study of Abdesselam (2016) on the Golden Delicious variety, sprays of fructose at 100 ppm and glucose at 100 ppm significantly reduced the percentage of fallen and damaged fruits at harvest (92.28 %, 67.47 % respectively) compared to the control (92.28 %); the Abbott effectiveness at harvest were 69.06 % and 8.28 % respectively. In addition, Tiffrent and Lombarkia (2021) have specified that in the study leading on Golden Delicious variety, Abbott's efficiency at harvest obtained for fructose 100 ppm and glucose 100 ppm were 15.54 % and 23.75 % respectively. Whereas in the study of Nasri (2015) on the Royal Gala variety the use of glucose at 10 ppm has significantly reduced the percentage of fallen and damaged fruits compared to the control (24.77 %) and (54.19 %) respectively, and the Abbott effectiveness at harvest was 11.86 %.

This finding, apparent from these studies while preliminary, suggests that the Royal Gala variety was better suited to the concept of the exogenous foliar application at doses of 100 ppm for glucose and fructose in our current study, they have reduced the percentage of fallen and damaged fruits at harvest (31.93 %, 19.16 % respectively) compared to the control (32.54 %); the Abbott effectiveness at harvest was 80.25 % and 78.58 % respectively.

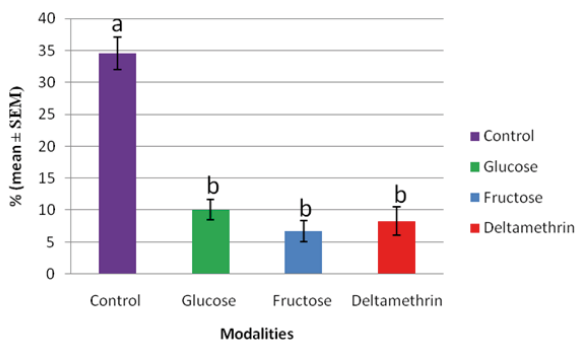

Figure 3: Number of diapausing larvae (n = 12) on different modalities (control, fructose, glucose, insecticide). Different letters indicate significantly different number of diapausing larvae at $p < 0.05$

Walters et al. (2013) explained that induced resistance is a host response; its expression under field conditions is likely to be influenced by a number of factors, including the environment, genotype, crop nutrition and the extent to which plants are already induced.

The overwintering is often a critical part of the insect life-cycle; CM overwinters as a diapausing fifth instar larva. As mentioned in our results, the influence of the spraying of glucose and fructose in diapausing larvae has been demonstrated. These results are in agreement with the findings of other studies. Meradi (2015) reported that treated plots with fructose 100 ppm showed a significantly reduced number of CM diapausing larvae compared to the control, and Nasri (2015) proved that glucose 10 ppm decreased significantly the number of diapausing larvae compared to the control (untreated). Consequently, with this decrease in the number of diapausing larvae, we predict and estimate a low population of adults in the next generation and allow adapted the protection strategy for the following year.

4 CONCLUSION

Based on the obtained results, it can be concluded that the exogenous application of sugars can reduce codling moth damage and small molecules such as glucose and fructose can induce resistance to *C. pomonella* by foliar applications. Possible future studies using the same trials on Royal Gala variety is proposed to confirm these findings for the development of biocontrol strategies.

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Frost hardiness of apple generative buds during dormancy

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Frost hardiness of apple generative buds during dormancy

Abstract: The success of apple production is influenced by frost damages. Occurrence of extreme temperatures is increasing worldwide because of global warming, so the risk of frost damages is also increasing in apple orchards during dormancy and blooming time. In our work the frost hardiness of flower buds of eight apple cultivars was observed with artificial freezing tests during four subsequent dormancy periods in Hungary. The studied cultivar assortment contained two standard commercial cultivars ('Gala', 'Idared'), two scab-resistant cultivars from abroad breeding programmes ('Florina', 'Prima') and four new Hungarian multi-resistant (mainly scab-resistant) cultivars ('Artemisz', 'Cordelia', 'Hesztia', 'Rosmerta'). There were remarkable differences between cultivars and years from the aspect of frost hardiness of generative overwintering organs. At the end of hardening period, in January, the LT_{50} values of flower buds were between -22.4 °C and -30.4 °C according to cultivar and year. LT_{50} means the temperature causing 50 % frost damage in the flower buds of the certain cultivar in the certain time. 'Gala' and 'Florina' were the most frost hardy, while 'Prima', 'Cordelia' and 'Idared' the most sensitive to frost. Cold hardiness values of flower buds of 'Artemisz', 'Rosmerta' and 'Hesztia' cultivars were regularly between the values of two extreme groups. In winters with inappropriate weather the generative overwintering organs were unable to reach the genetically possible frost hardiness of them.

Key words: *Malus x domestica*; artificial freezing tests; LT_{50} values; dormancy; generative buds

Odpornost cvetnih brstov jablane na mraz med mirovanjem

Izvleček: Na uspešno pridelavo jabolk vplivajo poškodbe zaradi mraza. Pojavljanje ekstremnih temperatur se povečuje po vsem svetu zaradi globalnega segrevanja, zato se tudi povečuje tveganje za pozebe v nasadih jablan med mirovanjem ali v času cvetenja. V raziskavi je bila preučevana odpornost na mraz cvetnih brstov osmih sort jablane z umetnim zmrzovanjem v štirih zaporednih rastnih sezonah na Madžarsekm. Preučevani izbor sort je obsegal dve standardni komercialni sorti ('Gala', 'Idared'), dve na škrlup odporni sorti iz tujih žlahniteljskih programov ('Florina', 'Prima') in štiri madžarske multirezistentne sorte (v glavnem odporne na škrlup) ('Artemisz', 'Cordelia', 'Hesztia', 'Rosmerta'). Med sortami in leti so bile opazne razlike glede na mrazno odpornost njihovih prezimnih generativnih organov. Na koncu obdobja odpornosti na mraz, v januarju, so bile LT_{50} vrednosti cvetnih brstov med -22,4 °C in -30,4 °C, odvisno od sorte in leta. LT_{50} pomeni, da temperatura povzroči 50 % mraznih poškodb v cvetnih brstih pri določeni sorti v določenem času. 'Gala' and 'Florina' sta bili na mraz najbolj odporni medtem, ko so bile 'Prima', 'Cordelia' in 'Idared' na mraz najbolj občutljive. Odpornost cvetnih brstov na mraz je bila pri 'Artemisz', 'Rosmerta' in 'Hesztia' vedno med vrednostmi obeh prej omenjenih ekstremnih skupin. V zimah z netipičnim vremenom cvetni brsti niso bili sposobni doseči genetsko pogojene odpornosti na mraz.

Ključne besede: *Malus x domestica*; preiskusi z umetnim zmrzovanje; LT_{50} vrednosti; dormanca; cvetni brsti

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1 INTRODUCTION

Apple is the most important temperate zone fruit, with high economic importance worldwide. The success of apple production is influenced by many factors, one of them is the risk of frost damages. Attention was drawn to this fact in early literature (Modlibowska, 1946; Childers, 1949; Chandler, 1954). There are growing districts, especially in the colder parts of temperate zone, where low temperatures often cause damages to the overwintering organs of apple trees during the dormancy period, hence the knowledge of the winter frost tolerance of the cultivars is especially important in those regions. For example, in the northern parts of the United States millions of apple trees died off in November 1955 and December 1964 due to severe temperature drops. Significant apple tree decay occurred in Poland in the winter of 1986/87 because of persistent frost. Low temperatures have caused significant tree destruction on several occasions in apple orchards of Canada as well (Coleman, 1992; Palmer et al., 2003; Tóth, 2013). The forecasting models of global warming predict more frequent occurrence of extreme climatic conditions, including extraordinary temperatures, which may affect the phenological processes and tolerance of our plants against frost and other environmental factors. It should be stated that frost damage is becoming more common in apple orchards (Eccel et al., 2009; Kaukoranta et al., 2010; Vitasse et al., 2018).

Frost hardiness of generative organs can be studied by field surveys, artificial freezing tests, and indirect laboratory methods (Palonen & Buszard, 1997; Lindén et al., 1999; Palmer et al., 2003). The differences between cultivars and frost tolerance at the given developmental stage could be observed with filed assessments after severe cooling. The frost hardiness of overwintering organs is changing gradually. The process of this changing could be studied by frequent freezing tests. Generative buds are the most frost-sensitive parts of apple trees in winter. Flower buds are the most frost resistant at the end of their endodormancy period, sometimes they tolerate temperatures even below -30 °C, while in the flowering period, temperature only a few degrees below freezing point may be critical. The pistils are the most frost sensitive parts of the flower buds, but other organs are often damaged in low temperatures as well (Holubowicz, 1982; Warner, 1982; Nybom, 1992; Lindén et al., 1999; Lindén, 2002; Rodrigo, 2000; Palmer et al., 2003; Tóth, 1982, 2004; 2013; Soltész, 1988; Lysiak et al., 2016; Tudela & Santibanez 2016).

There are results of frost hardiness of vegetative organs of apple cultivars measuring by field observations and artificial freezing tests as well, the vegetative organs usually survive lower temperature than generative organs (Quamme et al., 1973; Ashworth et al., 1988; Lindén et al., 1996; Palmer et al., 2003; Cline et al., 2012; Pramsohler et al., 2012; Ozherelieva & Sedov 2017).

Differential thermal analysis, electrical impedance spectroscopy, infrared spectroscopy, electrolyte leakage measurement as indirect laboratory methods can provide information about the differences between genotypes from the aspect of frost hardiness of them (Quamme, 1976, 1991; Quamme et al., 1972, 1982; Ceccardi et al., 1995, Kang et al., 1998; Pearce, 2001; Salazar-Gutiérrez et al., 2016; Wu et al., 2019; Yu & Lee 2020; Kaya et al., 2020).

In our study the frost hardiness of flower buds of eight apple cultivars was observed with artificial freezing tests during four subsequent dormancy periods in Hungary. The studied cultivar assortment contained standard commercial cultivars, Hungarian and foreign resistant cultivars as well.

2 MATERIALS AND METHODS

The research work was carried out in the experimental orchard of the Department of Pomology HUALS (predecessor SZIE), which is located on the outskirts of Budapest, in Soroksár. The studies were conducted during four consecutive dormancy periods, from September 2016 to April 2020. The cultivars included in the study were as follows: 'Artemisz', 'Cordelia', 'Hesztia' and 'Rosmerta', four multi-resistant (mainly scab-resistant) cultivars from the breeding programme of our Department (Tóth et al., 2012). 'Prima' and 'Florina', two scab-resistant cultivars from earlier foreign breeding programmes. 'Idared' and 'Gala', two standard commercial cultivars. Thus, a total of 8 cultivars were examined in 4 consecutive test seasons. The studies were performed in the middle of each month from September to March. The last test date for each cultivar was the onset of flowering, which fell to April. In the experimental plantation, the trees were planted with a row and tree spacing of 4 x 2 m in 2007. The growing system is slender spindle. The plantation incorporates integrated cultivation technology, with regular maintenance pruning, manual fruit thinning, nutrient replenishment, drip irrigation and integrated plant protection every year.

The artificial freezing experiments were performed in the climate chamber Rumed 3301 (Rubarth Apparate GmbH, Laatzen, Germany), according to the method and protocol developed on the Department of Pomology (Szalay et al., 2018, 2019). Four or five appropriate freezing temperatures were used at each testing date, according to the rate of dormancy. Both cooling and warming were performed at a rate of 2 °C/h and shoots were kept

at the given freezing temperature for four hours. Five branches were used for all of treatments, each of them with 20-25 flower buds. Every branch was one repetition for statistical analysis. After freezing, all branches were kept at room temperature until analysis. The flower buds were cut longitudinally, and the frost damage was determined based on the rate of discoloration of tissues (green – unharmed, brown – frost damaged) on a numerical scale from 0 % to 100 %, respectively. Based on the results of artificial freezing treatments the LT_{50} values (mean value of frost hardiness) were determined in each sampling dates. LT_{50} means the temperature causing 50 % frost damage in the flower buds of the certain cultivar in the certain time. The LT_{50} was calculated using a linear regression model, assuming that the section of the hardiness sigmoid curve between 20 % and 80 % can be regarded as linear (Gu, 1999). Mean and standard deviation values were calculated during the statistical analysis, and two-way analysis of variance with replicates was performed with Microsoft Excel 365 programme.

3 RESULTS

The frost hardiness of flower buds of observed cultivars is characterized by LT_{50} values. The results of four years experiment are demonstrated on the Figure 1. The process of the changing of frost tolerance can be divided into two periods. Until January the frost hardiness of flower buds increased gradually, it was the hardening period. After it, the generative buds lost their frost hardiness gradually, it was the dehardening part of this process. The rate of hardening and dehardening was not the same in different years, because of different climatic conditions, but the frost hardiness profile of the observed cultivars during dormancy, and the sequence of cultivars from the aspect of frost tolerance in different sampling dates, both were very similar. It was not consistent the changing of LT_{50} values during the hardening period. After the initial fast decreasing, the changing slowed down until a certain point, after it this process was accelerated again, until the lowest value of LT_{50}. So, the hardening period can be divided into two phases as well. The first stage took place at temperatures above freezing. The start of the second, accelerated phase was when the ambient temperature was continuously below freezing point. In the second half of the winter, dehardening also took place at different rates each year due to different climatic conditions. Based on our results, it can be stated that the survival of the flower buds of the studied apple varieties was ensured by the current frost tolerance formed during their hardening and hardening processes in the four test seasons. During the winter, even the most sensitive cultivars did not experience frost damage. There was an intensive cooling in early April of 2020 with -9 °C, this arrived at the end of the deherdening period, just before

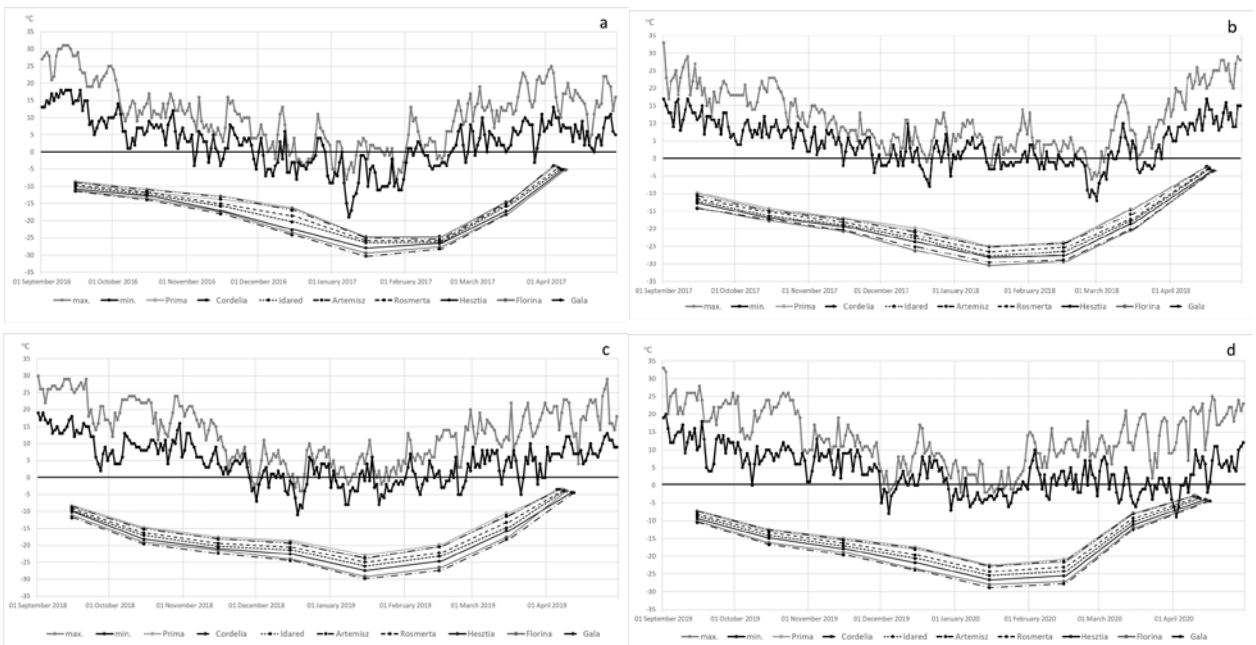

Figure 1: The LT_{50} values of the flower buds of the studied apple cultivars determined by artificial freezing tests (below) and the daily maximum and minimum ambient temperatures in the experimental orchard (above) in 2016/17 (a), 2017/18 (b), 2018/19 (c) and 2019/20 (d) test season

the blooming time of apple trees. It caused a severe frost damages in the generative organs.

The differences between the cultivars were smaller in the autumn and spring months, and higher during the three winter months (December, January and February). Therefore, differences between cultivars are evaluated based on the test results of these three months of four years (Fig. 2.).

Based on the statistical analysis, the studied varieties can be divided into three groups. The most frost sensitive group includes 'Prima', 'Cordelia' and 'Idared'. The most frost tolerant were 'Florina' and 'Gala' according to our study, while 'Artemisz', 'Rosmerta' and 'Hesztia' belonged to the group of medium frost tolerant. In the four-year studies, the frost resistance of the flower buds of the cultivars did not reach the genetically possible maximum value in each year. This is analyzed based on the January measurement results, as this month was the most frost tolerant period in each year. At this geographical location, during the four-year study, the flower buds of the studied varieties reached the most frost-resistant values in 2018 and partly in 2017 (Fig. 3.).

Further studies are needed to determine whether these values are genetically encoded maximum values. During the study period, flower buds were least hardened in 2020. There was a difference of 1.5-2.6 °C between the LT_{50} values of the years with the best and the weakest frost resistance, depending on the cultivar. In 2019 the frost resistance of flower buds was between the extreme values.

4 DISCUSSION AND CONCLUSIONS

There is a certain amount of information in the literature about frost hardiness of apple cultivars. The lowest critical temperatures were determined on the basis of the frost damages of vegetative organs and the cultivars were classified into frost tolerance groups based on these results. Usually four groups were created from tender to very hardy (Forsline, 1983; Friedrich & Fischer 2000; Palmer et al., 2003; Tóth, 2013). Generative organs are more sensitive to frost than vegetative organs, so their examination is also very important. Flower buds and flowers are vital organs for fruit trees, they will produce the fruit, it is also very important to know their frost tolerance. In addition, the frost resistance of vegetative and generative organs is often not closely related (Westwood, 1993; Palonen & Buszard 1997; Palmer et al., 2003; Tóth, 2013). In many places of production, strong cooling also occurs during the flowering period, so it is worthwhile to study the frost tolerance of the cultivars during this period as well. The more advanced the flowering, the more sensitive the flower organs (Palmer et al., 2003; Aygün & San 2005; Szalay et al., 2019).

Photoperiod and temperature are key environmental factors in cold acclimation of apple trees (Howell & Weiser 1969; Faust, 1989; Tromp, 2005; Heide & Prestrud 2005; Wu et al., 2019). During the dehardening period the temperature is the most important environmental factor affecting the frost hardiness of overwintering organs (Tromp, 2005; Quinones et al., 2020). Frost tolerance of trees is also influenced by numerous other factors, such as the cultivar, the rootstock, the cultivation system, the cropping technology, the health status of the trees, the characteristics of the geographical location (Westwood, 1993; Faust, 1989; Janick & Moore 1996; Palmer et al., 2003; Tóth, 2013). Due to all these, there are large differences in the development of frost tolerance between cultivars, production sites and years.

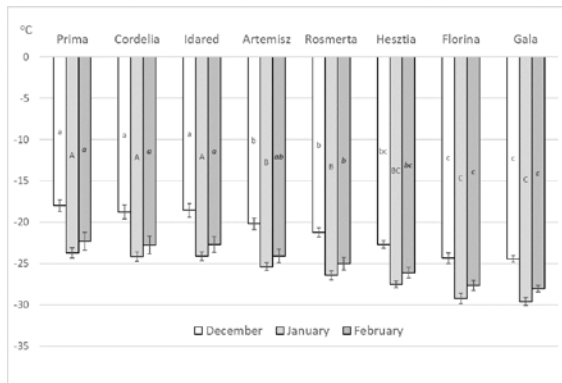

Figure 2: LT_{50} values of flower buds of the studied apple cultivars in December, January, and February, averaged over the four study years; The columns show the mean values, the lines the standard deviation, and the letters the homogeneous groups, the different letters indicate significantly ($p \leq 0.05$) different values

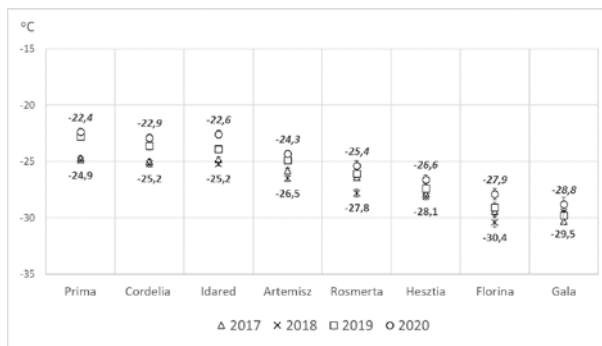

Figure 3: LT_{50} values of flower buds of the studied apple cultivars in mid-January in the four study years; the values of 2018 (normal numbers) and 2020 (italics) are shown; the lines represent the standard deviation

In the present experimental work, the frost tolerance of flower buds of eight apple cultivars has been investigated by artificial freezing method for four consecutive years in our experimental plantation, in Central Hungary. The trees stand on the same rootstock and have received the same cultivation technology. Thus, we were mostly able to establish the differences between the cultivars. In addition, we were able to describe the course of the change in frost resistance as the tests were performed monthly during the winter dormancy periods. The four years offered only a limited opportunity to determine the impact of environmental factors and years. However, restricted conclusions can be drawn from the differences between the years, based on our results.

We have data on the frost tolerance of the vegetative and generative organs of the most important apple cultivars grown in Hungary based on field studies (Zatykó, 1986; Tóth, 1982, 2004, 2013; Soltész, 1988; Soltész et al., 2010; Dremák, 2011). However, resistant cultivars recently introduced into cultivation were not included in these studies. The international literature contains data about frost resistance of different apple cultivars. The lowest survival temperatures were determined with the observation of the frost hardiness of vegetative organs, trunk, branches, twigs (Ashworth et al., 1988; Lindén et al., 1996; Palmer et al., 2003; Cline et al., 2012; Pramsohler et al., 2012; Ozherelieva & Sedov 2017). For example, the lowest survival temperature of 'Gala' was -35.6 °C, in the case of 'Fuji' it was -37.7 °C, while vegetative tissues of 'Jonagold' survived just -31.2 °C. There is limited information about the frost hardiness of generative organs, but general conclusion is, the lowest survival temperature of them is higher (frost hardiness is weaker) than vegetative organs (Lindén et al., 1999; Lindén 2002; Pramsohler & Neuner 2013; Lysiak et al., 2016; Salasar-Gutiérrez et al., 2016; Tuleda & Santibanez 2016; Ozherelieva & Sedov 2017).

In our present work the frost hardiness of flower buds of eight cultivars was determined with artificial freezing tests. From the studied assortment 'Florina' and 'Gala' belonged to frost hardy category, 'Artemisz', Rosmerta' and 'Hesztia' belonged to the middle frost-tolerant category, while 'Prima' 'Cordelia' and 'Idared' represented frost sensitive cultivars.

In our previous work the frost hardiness of flowers of some apple cultivars was determined during blooming time (Szalay et al., 2019).

The frost resistance of the vegetative organs of apple trees develops in two stages in autumn, so the hardening period of them can be divided into two stages. The first stage takes place at temperatures above freezing, but the second stage requires permanently low temperatures. It has been experimentally demonstrated that in the absence of low temperatures vegetative overwintering organs cannot harden properly (Howell & Weiser 1970; Palmer et al., 2003; Wu et al., 2019). The functioning of the generative organs is similar. In our earlier work, the role of low temperature in the hardening of peach flower buds was experimentally confirmed (Szalay et al., 2010). Our present experimental results suggest this for apple cultivars as well. In the first part of frost tolerance profile of flower buds of apple cultivars a breaking point is observed, after which hardening continues at persistently low temperatures. The role of temperature in hardening is also indicated by the fact that the LT_{50} values of the flower buds of the studied apple cultivars were different from year to year in January. In case of unfavorable weather, the genetically possible level was not reached. Dehardening of flower buds also took place at different rates in the study years, which suggests the role of temperature in this process as well. Further studies are planned to better understand the role of environmental factors in hardening and dehardening of apple overwintering organs.

It is difficult to compare our results with previous research results, as such a systematic study of the frost resistance of flower buds has not yet been performed. Research results suggest that there is no strong correlation between frost resistance of vegetative and generative organs. For this reason, it is worth examining the vegetative and generative organs separately. Frost tolerance of vegetative organs of 'Idared' is good, but the flower buds and flowers of it are very sensitive to frost during dormancy (Mittelstadt & Murawski 1975; Soltész et al., 2010; Tóth, 2013; Szalay et al., 2019). Frost sensitivity of 'Idared' flower buds is confirmed in our present work as well. Vegetative organs of 'Gala' is moderately tender (Palmer et al., 2003; Tóth, 2013), the flower buds of this cultivar have good frost tolerance during dormancy according to our experimental results.

Knowledge of frost hardiness of cultivated apple varieties in different phenological stages is important for estimation of suitability of them for growing sites. In this area of research, field studies and artificial freezing experiments together can give good results. Our experimental work provides new information about the second one.

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Assessing agricultural commercialization and rural infrastructure development in rural Southwestern Nigeria: evidence from smallholder cassava farmers

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Assessing agricultural commercialization and rural infrastructure development in rural Southwestern Nigeria: evidence from smallholder cassava farmers

Abstract: This study assessed agricultural commercialization and rural infrastructure development of smallholder cassava farmers in rural Southwestern Nigeria. The study was conducted in Nigeria with cross-sectional data collected from 352 smallholder cassava farmers. Crop commercialization index (CCI) was used to compute each farmer's CCI and categorized into four levels while ordered logit model was employed to analyze the determinants of agricultural commercialization of cassava farmers in the study areas. Availability of some important rural infrastructures were assessed across cassava farmers' commercialization levels. The results revealed that 13.1 % of cassava farmers did not participate in the sale of cassava roots while 86.9 % of them participated actively in the output market. The mean and maximum CCI in the study areas was 59.1 and 95.5 respectively. The results also showed that less than 40 % and 20 % of cassava farmers in all commercialization levels had access to electricity and piped water respectively. The ordered logit regression analysis indicated that age, transport cost, cassava marketing experience, and distance to market were among the determinants of agricultural commercialization. Therefore, stakeholders should expedite policy actions capable of promoting rural infrastructure development that will enhance agricultural production, marketing and improve the quality of life of rural farming communities.

Key words: Crop Commercialization Index (CCI); cassava farmers; subsistence agriculture; rural infrastructure; ordered logit model

Ocena tržne usmerjenosti in razvoja infrastrukture na podeželju na kmetijskih območjih jugozahodne Nigerije: primer manjših pridelovalcev manioke

Izvleček: V raziskavi je bil ocenjen razvoj podeželske infrastrukture in razvoj tržne usmerjenosti pri manjših pridelovalcih manioke na podeželju jugo-zahodne Nigerije. Raziskava je bila izvedena z zbiranjem različnih podatkov pri 352 manjših kmetih, ki pridelujejo manioko. Za vsakega kmeta je bil izračunan indeks tržne usmerjenosti (CCI) vseh poljščin, njegove vrednosti so bile nato razvrščene v štiri nivoje, za analizo glavnih determinant tržne usmerjenosti pridelovalcev manioke na območju je bil uporabljen model hierarhične logistične regresije. Dostopnost nekaterih pomembnih podeželskih infrastruktur je bila med pridelovalci manioke ocenjena glede na raven tržne usmerjenosti. Rezultati raziskave so pokazali, da 13,1 % pridelovalcev manioke ne sodeluje pri prodaji pridelka, 86,9 % pa jih aktivno sodeluje na trgu. Vrednosti poprečnega in maksimalnega indeksa (CCI) sta bili v preučevanih območjih 59,1 in 95,5. Rezultati so še pokazali, da ima manj kot 40 % in 20 % pridelovalcev manioke na vseh ravneh razvoja tržne usmerjenosti dostop do elektrike in vodovoda. Model hierarhične logistične regresijske analize je pokazal, da so imele danosti kot so starost, stroški transporta, izkušnje s prodajo manioke in oddaljenost do trga največji vpliv na razvoj tržne usmerjenosti. Zaradi tega bi morali odločevalci razviti aktivnosti, ki bi vzpodbujale razvoj infrastrukture na podeželju, kar bi pospešilo kmetijsko proizvodnjo, razvoj trga in izboljšalo kvaliteto življenja v ruralnih kmečkih skupnostih.

Ključne besede: indeks tržne usmerjenosti (CCI); pridelovalci manioke; samooskrbno kmetijstvo; infrastruktura na podeželju; hierarhični logit model

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1 INTRODUCTION

In recent time, access to food by over 7 billion people has become one of the most challenging issues in our contemporary world (Otekunrin and Otekunrin 2021a; Ayinde et al. 2020). The number of people affected by hunger has increased globally by the emergence of COVID-19 pandemic in early 2020, bringing the achievement of the decline in the prevalence of undernourished (PoU) from 2005 to 2014 to an abrupt end (FAO et al. 2021). From 2019-2020, the PoU witnessed a surge from 8.4 % (650.3 million) to about 9.9 % (768.0 million), making the achievement of Sustainable Development Goal 2 (SDG 2) by 2030 close to becoming a mirage especially in the developing countries (FAO et al. 2021). In 2022, the PoU further increased with estimated value between 702 and 828 million people in the world with an unprecedented impact occassioned by COVID-19 (FAO et al. 2022).

As world food demand escalates with attendant global population increase especially in low- and middle-income countries (LMICs), the subsistence agriculture (small-scale farming) practiced by most farmers in the developing countries can no longer meet the food demand of the people and thereby, in dire need of real transformation. The rejigging of subsistence agriculture in Africa is pivotal to the economic prosperity of the region especially those that depend mainly on agriculture (von Braun, 1994, 1995; Pingali and Rosegrant, 1995; Timmer, 1997; World Bank, 2008; Gebremedhin and Jaleta, 2010; Otekunrin et al. 2019). Transforming the small-scale agricultural practice will lead to the promotion of agricultural commercialization that enhances commerce and optimum productivity at both national and household levels. The increased income gain by the household will equally promote food consumption and nutritional outcomes of both rural and urban households (Carletto et al. 2017)

According to Agricultural Policy Research in Africa (APRA), agricultural commercialization emerges when agricultural enterprises depend mainly on the market for the sale of produce and for the purchase of production inputs (APRA, 2018). In another word, agricultural commercialization implies increased market transactions (that is, market participation) for capturing the gains from specialization (Carletto et al. 2017). Commercialization process may occur on the output-side of production usually with sales of farm produce or on the input-side mainly through increased use of purchased inputs. The estimation of the level of commercialization of subsistence agriculture from the output-side of production gives the opportunity to gain the marketing behavior of each household (APRA, 2018; Carletto et al. 2017; Otekunrin et al. 2019).

In past decades, agricultural commercialization in Africa is synonymous to large-scale farming involving cash crops (Martey et al. 2012). Moreover, this is no longer the same in recent time as these cash crops which are usually rain-fed and are negatively impacted by the unfavorable weather conditions. These lead to a reduction in annual harvest of the crops and hence, calls for urgent crop diversification (Martey et al. 2012; Obisesan, 2012; Opondo et al. 2017). Recently, food crops such as cassava and sorghum are being supported for their drought-resistance and other attributes which make them suitable as food security crops in the Africa (Martey et al. 2012; Obisesan, 2012; Opondo et al. 2017).

It is evident that infrastructural development especially in the rural settings in Africa (like Nigeria) will promote agricultural commercialization. Moreover, infrastructure development in the region is crucial to advancing economic growth and promoting quality of life of the people (AfDB, 2020). With the recent population increase in Africa coupled with the United Nations (UN) projection of the continent's population increase from 1.3 billion in 2019 to 2.4 billion in 2050. Noting that the majority of the growth is projected to come from Sub-Saharan Africa (SSA) (UNDESA, 2019; OECD/ACET, 2020). In order to meet up with the growing demand for food, there is need for the African countries (Nigeria inclusive) to scale up infrastructure development especially in the rural areas to match the demands of ever-increasing population in the region mainly in the aspects of production capacity, labour participation and food security (OECD/ACET, 2020).Where rural infrastructural facilities like good rood network, reliable information and communication technology, uninterrupted power supply, healthcare facilities and access to improved water and sanitation are available and functioning properly, will create an enabling environment for smallholder farmers and enhance the production and processing of agricultural produce that would lead to increased income for farmers, improve quality of life of the rural households.

Cassava (*Manihot esculenta* Crantz) is regarded as one of the most cultivated root crops in the tropics and unarguably the six most important crop in the world after wheat, rice, maize, potato, and barley (Saranraj et al. 2019; Otekunrin & Sawicka, 2019). Cassava is commonly referred to as "drought-tolerant crop" (Otekunrin & Sawicka, 2019). Global production of cassava reached 303.6 million tonnes with countries like Nigeria, Democratic Republic of Congo (Congo, DR), Thailand and Ghana were among the top 5 producers globally in 2019. The cassava production in Africa reached 192 million and is recognized as the largest cassava growing region while Nigeria maintained the top position as the highest producer of the crop both in Africa and globally with an

estimated value of 59 million tonnes and 19.5 % share of total global production in 2019 (FAOSTAT, 2021).

Cassava in Nigeria, is regarded as the most important crop by production and second most important by consumption (SAHEL, 2016; Otekunrin & Sawicka, 2019). Majority (90 %) of the fresh cassava roots are consumed locally as food, about 10 percent is used for industrial purposes while Nigeria is yet to tap the enormous trade potential of the crop because less than 1 percent of cassava produced in the country entered the international market (Otekunrin & Sawicka, 2019).

This study assesses agricultural commercialization and rural infrastructure development among smallholder cassava farmers in South West Nigeria. This study contributes to the body of knowledge on the importance of rural infrastructure development in Africa especially in Nigeria which is capable of boosting agricultural productivity, marketing of agricultural produce and, enhancing the quality of life of the rural households in South-West Nigeria.

This study stated five hypotheses and are given as follow;

H_0: Gender of the cassava farmers does not have any relationship with commercialization levels of the farmers.

H_1: Gender of the cassava farmers have relationship with commercialization levels of the farmers.

H_0: Farmers' educational qualification does not have any relationship with commercialization levels of the farmers.

H_1: Farmers' educational qualification have relationship with commercialization levels of the farmers.

H_0: Farmers' transport cost does not have any relationship with commercialization levels of the farmers.

H_1: Farmers' transport cost have relationship with commercialization levels of the farmers.

H_0: Farmers' cassava marketing experience does not have any relationship with commercialization levels of the farmers.

H_1: Farmers' cassava marketing experience have relationship with commercialization levels of the farmers.

H_0: Farmers' distance from farm to nearest market does not have any relationship with commercialization levels of the farmers.

H_1: Farmers' distance from farm to nearest market have relationship with commercialization levels of the farmers.

The study therefore, contributes to the existing body of knowledge by analyzing factors affecting agricultural commercialization and the challenges confronting smallholder cassava farmers in South-West Nigeria.

2 MATERIALS AND METHODS

2.1 STUDY AREA

Nigeria is the seventh most populous nation in the world. Based on Worldometer elaboration of the latest United Nations data, the current population of Nigeria (at September 4, 2021) is 212,108,984 people representing 2.64 % of total world population (Worldometer, 2021). South-West is one of the six geopolitical zones of Nigeria and is located in Western region of Africa with total land mass of 923,768 square kilometer (Maps of World 2021). Nigeria is a multi-ethnic country having Hausa, Igbo and Yoruba as the three predominant ethnic groups and national languages. The six states in South-West are; Lagos, Ekiti, Ogun, Ondo, Osun and Oyo. The region lies between latitude 9° 4.9199ˡ N and longitude 8° 4.9199ˡ E (Find Lattitude and Longitude, 2021). It is largely a Yoruba speaking region of the country with diversity of dialects within and across the states in the zone. There are two distinct seasons in the zone i.e. rainy and the dry seasons. Agriculture remained the most common means of livelihood of about 70 percent of the rural population (Lawal & Samuel, 2010; Otekunrin & Otekunrin 2021b). The main cash crops mostly grown in the zone include cocoa, citrus and timber, while the food crops are cassava, yam, maize, cowpea, melon, and millet. Livestock production include pigs, rabbits, sheep, goats, poultry and snails (Lawal & Samuel, 2010; Otekunrin & Otekunrin 2021b).

2.2 DATA COLLECTION AND SAMPLING PROCEDURE

The study employed multi-stage sampling procedure. In the first stage, random sampling of two (Ogun and Oyo) from six cassava producing States in the South western region of the country was done. The 2nd stage involved random selection of five Local Government area (LGAs) (Egbeda, Ona-Ara, Ido, Afijio and Oyo East) from Oyo State and three LGAs (Odeda, Ewekoro and Odogboolu) from Ogun State. In stage 3, 24 villages (Badeku, Akintayo, Ajoda, Bodunde, Ajoda-Ajobo, Kupalo, Jago, Akinwaare, Morakinyo, Akinmoorin, Abujakan, Bodija-Omikiti, Bodija-Tekun; Olodo, Adao-Alabata, Ogbere, Oluwaji, Imodi-Ijebu, Surulere, Omu-Ijebu, Oke-Ola, Sabo-Imodi, Ita-Ale Imodi, Eyiwa) were selected from the eight LGAs. The Stage 4 involved a random selection of 16 cassava farming households resulting in a

total of 384 farming households. The data were collected through structured, interviewer-administered questionnaire which include; the household socioeconomic characteristics, food consumption and expenditure pattern, rural infrastructure related factors and other salient information. These questionnaires were answered by the smallholder cassava farmers in the study areas. After data cleaning, 32 out of 384 (resulting to a total of 352 respondents) of the questionnaires were discarded due to incomplete information resulting in 91.7 % total responses from the survey.

2.3 ANALYTICAL FRAMEWORK

2.3.1 Estimating agricultural commercialization

The agricultural commercialization levels of cassava farmers was estimated using Crop commercialization Index (CCI) by Strasberg et al. 1999; Carletto et al. 2017; Otekunrin et al. 2019 defined as :

$$CCI_i = \frac{Gross\ value\ of\ crop\ sale_{hhi,\ \ yearj}}{Gross\ value\ of\ all\ crop\ production_{hhi,yearj}} \times 100 \qquad (1)$$

We have hh_i is the ith household in year *j*.

The commercialization levels of the cassava farmers in the study areas can be represented by a scale from absolute subsistence farmer (*CCI* = 0) to perfectly commercialized (*CCI* = 100) (Carletto et al. 2017; Otekunrin et al. 2019). This method allows for more than just the usual dicotomy of sellers and non-sellers, or between staple and cash crop producers (Carletto et al. 2017; Otekunrin et al. 2019). This also informs about how much of the harvested produce farmers decided to offer for sale in the output market. The crop sold ratio is the ratio of gross value of crop sold and gross value of all crop production (Shively & Sununtnasuk, 2015).

Cassava farmers were categorized based on their cassava commercialization levels. Farmers that did not participate (non-sellers) in the sale of the cassava roots were categorized as zero commercialization level (CCI 1 = 0 %) while those that participated actively (sellers) are grouped into; low commercialization level (CCI 2 = 1.00-49.9 %), Medium-High commercialization level (CCI 3 = 50.0-75.9 %) and Very High commercialization level (CCI 4 = 76.0-100.0 %) levels (Otekunrin and Otekunrin, 2021b).

2.4 MODELLING THE DETERMINANTS OF COMMERCIALISATION LEVELS AMONG CASSAVA FARMERS

2.4.1 Ordered Logit Model (OLM)

The multivariate ordered Logit model is used to determine factors influencing commercialisation levels of smallholder cassava farming households in South West Nigeria. This analysis is adopted when the dependent variable has more than two categories and the values of each category have an ordered sequential structure where a value is indeed "higher" than the previous one (Torres-Reyna, 2014).

The logit coefficients are in log-odds unit and they are not read as OLS coefficients as such in interpreting, we need to estimate predicted probabilities of Y = 1 or the marginal effects which measures changes in the probability of commercialisation outcome with respect to a change in the regressors. The probabilities of the respondents of being in any of the identified levels are estimated using natural log of the cumulative distribution (Booroah, 2002; Obayelu, 2012). A positive marginal effect estimate for a category indicates that an increase in that variable will increase the probability of being in that category while a negative estimate implies a decrease in probability of being in that category.

In the ordered logit model, there is an observed ordinal variable Y which is a function of another variable y^* that is not measured. The latent variable y^* has various threshold points.

In this study, following Oluwatayo & Rachoene (2017); Ogutu et al. (2020) and Hussayn et al. (2020), this model specification was used:

$$y_i^* = x_i'\beta + \varepsilon_i \qquad (2)$$

where y_i^* is the latent variable of the commercialization levels of cassava farmer *i*, x'_i is a vector of explanatory variables describing farmer *i*, β is a vector of parameters to be estimated and is a random error term which follows a standard normal distribution.

Choice rule:

$$y_i = \begin{cases} 1 & if\ y_i^* \le \mu_1\ (Zero\ level\ (0.0\%)) \\ 2 & if\ \mu_1 \le y_i^* \le \mu_2\ (Low\ level\ (1.0 - 49.9\%)) \\ 3 & if\ \mu_2 \le y_i^* \le \mu_3\ (Medium - High\ level\ (50.0 - 75.9\%)) \\ 4 & if\ y_i^* > \mu_3\ (Very\ High\ level\ (76.0 - 100.0\%)) \end{cases} \qquad (3)$$

μ_1 to μ_3 are the cut-off values for the ordered logit model.

Hence, the dependent variable is the commercialisation levels; CCI 1, 2, 3 and 4 are the various categories (Zero, Low, Medium-High and Very High levels). As the ordered classes increase, the parameter set (β) is interpreted as: positive signs (+) indicate higher commercialisation level as the value of the variables increase, while negative signs (-) suggest the opposite (Hussayn et al. 2020). These interplays will be compared to the ranges between the various thresholds, μ_1, so as to establish the appropriate commercialisation level for a particular farmer.

The description and definition of the selected explanatory variables indicating the mean, standard deviation, minimum and maximum of each of the selected variables are shown in

Table 1.

3 RESULTS AND DISCUSSION

3.1 DESCRIPTION OF SOCIOECONOMIC CHARACTERISTICS OF CASSAVA FARMERS

The socioeconomic description of cassava farming households are presented in Table 1 and 2. The results indicated that about 40 percent of the farmers were between the age of 41 and 50 years while the mean age was 51 years revealing that cassava farmers are in their advanced age. This result was similar to findings from Adeyemo et al. (2019) and Adepoju et al. (2019). About 79 percent of the farmers were men indicating that cassava production is male dominated in the study areas. This result agrees with Otekunrin (2011), Awoyemi et al. (2015) and Adepoju et al. (2019) that cassava production are male dominated in South West Nigeria.

About 69 percent of cassava farmers in Ogun State were male while only 14.7 % were female in Oyo State. Majority (86.6 %) of the cassava farmers in the study areas were married while those that are still single were less than 5 percent. The large percent of married respondents indicated that more members of farm family were possibly going to be available for cassava production in the study areas. According to Awoyemi et al. (2015) and Kolapo et al. (2020) who also corroborated that large percent of married respondents in cassava production and processing could imply that cassava farmers in the study areas were matured and ready to take vital farming decisions jointly with their spouses.

About 46 percent of the farmers in the study areas had family size that is less than 5 persons. The mean household size in the study areas was 6 persons, implying that the farmers had relatively large family size which could possibly be available as family labour against short fall of hired labour. This results corroborates the findings of Effiong (2005); Adepoju et al. (2019) and Kolapo et al. (2020) that a relatively large household size enhances the availability of family labour which reduces constraint on labour demand in cassava production, processing and marketing.

Table 2 also revealed that 53.4 % of the farmers in the two states had only primary education qualification while 15.9 % were with no formal education. The results

Table 1: Description and definition of study explanatory variables

Variable	Description	Mean	Std. Dev	Minimum	Maximum
AGE	Age of farmers (years)	51.29	11.31	27	89
HHS	Number of Household members	6.18	2.82	20	1
EDUCATION	Number of years spent in school	7.05	4.28	0	16
FSIZE	Size of the farm used for cassava production (hectare)	1.50	1.05	0.20	4.86
FEXP	Cassava Farming experience of the farmers (years)	15.30	10.61	1	50
FINCOME	Farm income of the farmers (Naira)	129,420.82	113,164.30	0	950,000
NFINCOME	Non-farm income of the farmers (Naira)	58,616.48	71,380.35	0	500,000
MKTEXP	Cassava marketing experience of the farmers (years)	11.46	9.31	0	45
TRANSCOST	Cost of transport incurred by farmers (Naira)	3,576.70	1,334.45	0	10,000
DISTMKT	Distance from farm to closest market (km)	8.86	3.93	1	10,000
FOODEXP	Farmers' household Food expenditure (Naira)	21,974.43	9,668.99	20,000	60,000

Computed from field survey data, 2020

Table 2: Socioeconomic characteristics of cassava farmers

	Ogun State (n = 141)	Oyo State (n = 211)	Pooled (n = 352)
Variable	Frequency (%)	Frequency (%)	Frequency (%)
Age (years)			
≤ 40	11 (7.8)	40 (19.0)	51 (14.5)
41-50	52 (36.9)	88 (41.7)	140 (39.8)
51-60	46 (32.6)	49 (23.2)	95 (27.0)
> 60	32 (22.7)	34 (16.1)	66 (18.8)
Gender			
Male	97 (68.8)	180 (85.3)	277 (78.7)
Female	44 (31.2)	31 (14.7)	75 (21.3)
Marital Status			
Married	118 (83.7)	187 (88.6)	305 (86.6)
Single	5 (3.5)	10 (4.7)	15 (4.3)
Divorced	9 (6.4)	5 (2.4)	14 (4.0)
Widowed	9 (6.4)	9 (4.3)	18 (5.1)
Household size (Persons)			
≤ 5	65 (46.1)	95 (45.0)	160 (45.5)
6-10	75 (53.2)	97 (46.0)	172 (48.9)
> 10	1 (0.7)	19 (9.0)	20 (5.6)
Education background			
No formal education	5 (3.5)	51 (24.2)	56 (15.9)
Primary	100 (70.9)	88 (41.7)	188 (53.4)
Secondary	35 (24.8)	59 (28.0)	94 (26.7)
Tertiary	1 (0.7)	13 (6.2)	14 (4.0)
Farm size (hectare)			
≤ 1.00	63 (44.7)	85 (40.3)	148 (42.0)
1.01-2.00	53 (37.6)	69 (32.7)	122 (34.7)
2.01-3.00	22 (15.6)	33 (15.6)	55 (15.6)
> 3.00	3 (2.1)	24 (11.4)	27 (7.7)
Farm Experience (years)			
≤ 10	57 (40.4)	94 (44.5)	151 (42.9)
11-20	52 (36.9)	74 (35.1)	126 (35.8)
21-30	22 (15.6)	28 (13.3)	50 (14.2)
> 30	10 (7.1)	15 (7.1)	25 (7.1)
Cassava marketing Experience (years)			
≤ 10	73 (51.8)	132 (62.6)	205 (58.2)
11-20	47 (33.3)	51 (24.2)	98 (27.8)
21-30	17 (12.1)	19 (9.0)	36 (10.2)
> 30	4 (2.8)	9 (4.2)	13 (3.7)

Continued on next page

Farm Income (Naira)			
≤ 50,000	27 (19.1)	46 (21.8)	73 (20.7)
51,000-100,000	48 (34.0)	52 (24.6)	100 (28.4)
101,000-200,000	53 (37.6)	76 (36.1)	129 (36.6)
> 200,000	13 (9.2)	37 (17.5)	50 (14.2)
Food Expenditure (Naira)			
≤ 10,000	6 (4.3)	25 (11.8)	31 (8.8)
11,000-20,000	62 (44.0)	117 (55.5)	179 (50.9)
> 20,000	73 (51.8)	69 (32.7)	142 (40.3)
Transport cost (Naira)			
≤ 2,000	24 (17.0)	33 (15.6)	57 (16.2)
2,100-5,000	115 (81.6)	166 (78.7)	281 (79.8)
> 5,000	2 (1.4)	12 (5.7)	14 (4.0)
Distance from farm to market (km)			
≤ 5	12 (8.5)	69 (32.7)	81 (23.0)
6-10	98 (69.5)	92 (43.6)	190 (54.0)
> 10	31 (22.0)	50 (23.7)	81 (23.1)

Computed from field survey data, 2020

also indicated that only 4.0 % had tertiary education qualification while farmers' mean year spent in school was 7.05 (Table 1) years in the study areas. This results indicated relatively low level of education among the cassava farmers in the study areas. This means that higher formal education may not be a prerequisite to engaging in smallholder crop production and marketing but rather hands-on (on-farm) experience may be more crucial (Huffman 2001; Awotide et al. 2012; Adepoju et al. 2019).

Table 2 also revealed that 44.7 % and 40.3 % of the farmers had less than 1.0 hectare of cassava farm land in Ogun and Oyo respectively. The mean size of the farmland used for cassava production in the last cropping season was 1.50 hectare, indicating that most of the farmers in the study areas are largely smallholder farmers cultivating less than 5.00 hectare farmland. These findings are supported by the works of Sebatta et al. 2014; Rapsomanikis, 2015; Otekunrin et al. 2019; Otekunrin & Sawicka, 2019; Ikuemonisan et al. 2020.

The distribution of cassava farmers by their experience in farming activities (Table 2) indicated that about 36 percent of them had years of farming spanning from 10-20 years in both Oyo and Ogun State. The mean cassava farming experience in the two states was 15.30 years. This revealed that cassava farmers had considerably high years of farming experience which may possibly translate to increased productivity. This results agreed with Okoye et al. (2016) in the study of smallholder cassava farmers in Madagascar with 15 years farming experience while Kolapo et al. (2020) in the study of cassava bio-fortified Vitamin-A processors with 17 years.

Furthermore, the income distribution of cassava farmers in the study areas revealed that 20.7 % of the farmers in the two states earned less than ₦50 000 annually while 36.6 % of the farmers' income in both Ogun and Oyo States ranged between ₦101 000 to ₦200 000 annually. The mean farm income of cassava farmers was ₦129, 420.82 (Table 1).

Meanwhile, nearness to closest market centers promotes higher income, provides employment opportunities especially in the rural communities and enhances seamless access to farm inputs especially for smallholder farmers. The results (Table 2) indicated that about 70 percent and 44 percent of cassava farmers were 6-10 km far away from closest market centers in Ogun and Oyo States respectively. The mean distance from farm to market of cassava farmers was 8.86 km (Table 1). The farther the farmers to the closest market centers, the lower the likelihood of the farmers' market participation and this may lead to reduced household income (Renkkow et al. 2004; Otekunrin et al. 2019).

3.2 CASSAVA FARMERS' AGRICULTURAL COMMERCIALIZATION LEVELS

This section presents the agricultural commercialization levels of cassava farmers in Ogun and Oyo States

(Table 3). The results were computed through crop commercialization index (CCI) of each cassava farmers as specified above. The results showed that about 13 percent of the cassava farmers in the study areas (Ogun, 8.5 %; Oyo, 16.1 %) did not participate in sale of their cassava produce (non-sellers) in the last cropping season and are categorized as zero commercialization level (CCI 1). About 30 percent of the cassava farmers in the two states were categorized as medium-high commercialized farmers while the highest percentage (40.1 %) of the farmers sell above 75 % of their cassava roots categorizing them as "very high commercialization level" (CCI 4). The mean crop commercialization index in the study areas was 59.08 (Ogun, 67.24; Oyo, 53.64), belonging to medium-high commercialization level (CCI 3). These results are similar to that of Hussayn et al. (2020) and Kolapo et al. (2020) who reported higher level of market participation by cassava farmers and processors in South-West, Nigeria.

The results of the Chi-Square test to show if there exist any significant relationships between cassava farmers' commercialization levels and some selected explanatory variables is presented in Table 4. The results indicated that there were no significant relationships between cassava farmers' commercialization levels and farmers educational qualification, gender and distance from farm to market. This revealed that the educational levels, gender of the farmer and farmers' distance from farm to market may not possibly determine the extent of commercialization of cassava produce by the cassava farmers in both Ogun and Oyo states, Southwest Nigeria. In terms of education attainment, this result was in line with Huffman (2001); Awotide et al. (2012); Adepoju et al. (2019) who posited that educational background of the farmers may not be a necessary condition for smallholder households' decision to increase investment in the cassava value chain.

Furthermore, the results (Table 4) also revealed that transport cost incurred ($p < 0.01$) and cassava farmers' marketing experience ($p < 0.01$) had significant association with the cassava farmers' commercialization levels in the study areas. This is in line with *a priori* expectation that the cost of transporting farmers' produce to the market (as determined by the distance from farm to nearest market) may determine the extent of their output market participation. This agrees with the findings of Renkkow et al. (2004); Okoye et al. (2016); Otekunrin et al. (2019a) that the farther the farmers to the closest market centers, the lower the likelihood of their market participation and also their commercialization levels. However, the marketing experience of the cassava farmers also had a significant association with the extent of

Table 3: Smallholder cassava farmers' commercialization levels

	Ogun State	Oyo State	Pooled
Crop commercialization index (CCI) levels	Frequency (%)	Frequency (%)	Frequency (%)
Zero Level (Non-sellers)	12 (8.5)	34 (16.1)	46 (13.1)
< 50.0% (Low Level)	15 (10.6)	45 (21.3)	60 (17.0)
50.0-75.9% (Medium-High Level)	44 (31.2)	61 (28.9)	105 (29.8)
76.0-100.0% (Very High Level)	70 (49.6)	71 (33.6)	141 (40.1)
Mean CCI	67.24	53.64	59.08
Minimum CCI	18.23	7.62	7.62
Maximum CCI	95.45	95.45	95.45
N	141	211	352

Source: computed from field survey data, 2020. N means number of cassava farmers

Table 4: Hypotheses testing

Hypothesis	Pearson Chi-Square statistic (χ^2)	Asymp. Sig (2-sided)	Decision
a	7.949	0.242	Do not reject H_0
b	3.748	0.290	Do not reject H_0
c	6.237	0.101	Do not reject H_0
d	16.105	0.001	Reject H_0
e	42.901	0.000	Reject H_0

cassava commercialization of farmers in the study areas. As the farmers gain more experience in the sale of their cassava produce in the output market, it tend to improve the extent of their cassava commercialization and leading to increased farm income (Okoye et al., 2016; Otekunrin et al., 2022a).

3.3 RURAL INFRASTRUCTURE-RELATED FACTORS ACROSS CASSAVA FARMERS' COMMERCIALIZATION HOUSEHOLD LEVELS

Tables 5-8 present the distribution of cassava farmers' commercialization levels according to availability of infrastructure-related factors. Among the factors considered are; (i) access to electricity (ii) access to improved toilet, (iii) access to healthcare service, (iv) access to piped water in the study areas of Ogun and Oyo State, Nigeria. Table 5 revealed the level of access to electricity of smallholder cassava farmers across their four commercialization levels in the study areas. The result indicated that in the two states, above 50 percent of farmers in all the four commercialization levels opined that they did not access to electricity while the highest percent (69.5 %) of farmers in this category belonged to medium-high commercialization level (Ogun, 47.7 % and Oyo, 85.2 %). This result is lower than the national average (38.9 %) of rural households who have access to electricity as reported in 2018 Nigeria Demographic Household Survey (NDHS) (NCP & ICF, 2019; Otekunrin et al. 2022b; Otekunrin 2022). Similarly, in Table 5, the relationship between access to electricity and cassava farmers' CCI levels in the two states are not statistically significant (Ogun, $p = 0.16$; Oyo, $p = 0.45$).

However, according to Africa Infrastructure Development Index (AIDI) 2020 where Africa's Electricity index (one of the AIDI components) revealed that Nigeria's electricity index score fluctuate between 2.56 in 2010 to 2.72 on the scale of 100 in 2020, revealing critical challenge in the country's power sector. It is worthy of note that many rural areas in Nigeria are not connected to the national grid. Electricity is pivotal to the farmers' increased production and processing of the agricultural produce (AfDB, 2018; 2020).

Table 6 indicated the level of access to improved toilet among cassava farmers' households as categorized by their commercialization levels in the study areas. The results showed that in all the four commercialization levels, only "very high commercialization level" (CCI 4) farming households had 39.0 % access to improved toilet in the study areas. Less than 30 percent of other commercialization levels (CCI 1-3) had access to improved toilet

Table 5: Percent distribution of access to electricity among cassava farmers' commercialization household levels

		State	
		Ogun (n = 141)	Oyo (n = 211)
CCI levels	Access to Electricity	n (%)	n (%)
Zero Level (Non Seller)	Have access to electricity	9 (75.0)	6 (17.6)
	No access to electricity	3 (25.0)	28 (82.4)
	Total	12 (100)	34 (100)
Low Level	Have access to electricity	11 (73.3)	12 (26.7)
	No access to electricity	4 (26.7)	33 (73.3)
	Total	15 (100)	45 (100)
Medium-High Level	Have access to electricity	23 (52.3)	9 (14.8)
	No access to electricity	21 (47.7)	52 (85.2)
	Total	44 (100)	61 (100)
Very High Level	Have access to electricity	50 (71.4)	16 (22.5)
	No access to electricity	20 (28.6)	55 (77.5)
	Total	70 (100)	71 (100)
Total	Have access to electricity	93 (66.0)	43 (20.4)
	No access to electricity	48 (34.0)	168 (79.6)
	Total	141 (100)	211 (100)
Pearson Chi-Square (χ^2) or Fisher's exact, p-value		–, 0.16	2.65, 0.45

Computed from field survey data, 2020

in both Ogun and Oyo states. Cassava farming households belonging to Zero commercialization level had the lowest percentage (17.4 %) access to electricity among the four commercialization levels. Generally, only 29.3 % of the cassava farming households (Ogun, 46.1 % and Oyo 18.0 %) had access to improved toilet. This result is lower than the national average of 39.1 %. The NDHS 2018 revealed that only 39.1 % of rural households had access to improved toilet and about 33 percent of rural households use open defecation (NPC & ICF 2019). Similarly, in Table 6, the relationship between access to toilet facilities and cassava farmers' CCI levels in the two states are not statistically significant (Ogun, $p = 0.05$; Oyo, $p = 0.52$). This is not unconnected to the fact that many of the cassava farm households did not have access to toilet facilities in the study areas rural Ogun and Oyo states.

Likewise, Nigeria was ranked 24th (out of 54 countries in Africa) in 2020 composite AIDI with 23.26 (23.26/100). Nigeria was ranked 30th (among 54 African countries) with index score of 65.62 in the 2020 Africa water supply and sanitation index (WSS). This result indicated that Nigeria is not among the top 10 countries with best WSS index in the region (AfDB, 2020). According to United Nations Children's Fund (UNICEF), Nigeria is ranked second globally with 38 million people practicing open defecation while West and Central Africa accounted for about 24 percent of global open defecation (UNICEF 2021). This portends high risk of malnutrition and diarrheal disease incidence especially in young children in Nigeria (UNICEF 2021, Omotayo et al. 2021).

When considering rural infrastructure, healthcare service (rural social infrastructure) is categorized as one of the three main classes of rural infrastructures in Nigeria (Idachaba, 1985). Table 7 indicated the level of access to healthcare service across the four categories of cassava commercialization farm household levels in the study areas of Ogun and Oyo States, Nigeria. The results showed that more than 50 percent of all the four commercialization household levels had access to healthcare in the study areas. The result indicated that low level commercialization households (CCI 2) had the highest level (61.7 %) of access to healthcare service in the two states (Ogun, 73.3 % and Oyo, 57.8 %). Comparing access to healthcare among the cassava commercialization households in the study areas, the result revealed that cassava farming households in Ogun State had higher access to healthcare service (80.9 %) than that of Oyo State (41.7 %). Similarly, in Table 7, the relationship between access to healthcare service and cassava farmers' CCI levels was found to be statistically significant in Ogun state ($p < 0.01$) while it was not statistically significant in Oyo state ($p = 0.23$). This results reflected the fact that more

Table 6: Percent distribution of access to improved toilet among cassava farmers' commercialization household levels

		State	
		Ogun (n = 141)	Oyo (n = 211)
CCI levels	Access to Toilet	n (%)	n (%)
Zero Level (Non Seller)	Have access to toilet	3 (25.0)	5 (14.7)
	No access to toilet	9 (75.0)	29 (85.3)
	Total	12 (100)	34 (100)
Low Level	Have access to toilet	7 (46.7)	10 (22.2)
	No access to toilet	8 (53.3)	35 (77.8)
	Total	15 (100)	45 (100)
Medium-High Level	Have access to toilet	15 (34.1)	8 (13.1)
	No access to toilet	29 (65.9)	53 (86.9)
	Total	44 (100)	61 (100)
Very High Level	Have access to toilet	40 (57.1)	15 (21.1)
	No access to toilet	30 (42.9)	56 (78.9)
	Total	70 (100)	71 (100)
Total	Have access to toilet	65 (46.1)	38 (18.0)
	No access to toilet	76 (53.9)	173 (82.0)
	Total	141 (100)	211 (100)
Pearson Chi-Square (χ^2) or Fisher's exact, *p*-value		–, 0.05	2.25, 0.52

Computed from field survey data, 2020

than half of the population of smallholder cassava farmers in Ogun state had access to healthcare service.

The results in Table 8 showed the distribution of access to piped water among CCI levels farm households in the study areas. However, access to piped water is another aspect of WSS index (fourth component of the indicators used to compute AIDI). As indicated above, access to improved water and sanitation is crucial to the nutrition and health status of members of households both in rural and urban settings in Nigeria (AfDB, 2020). The recent report of NDHS revealed that 74 % of households in urban area have access to improved source of drinking water while 42 % of the rural households in Nigeria did not have access to improved source of drinking water (NPC & ICF, 2019). Table 8 revealed the level of access to piped water among cassava commercialization households in Ogun and Oyo States. The results showed that in all the four categories of cassava commercialization households, less than 20 percent (14.5 %) had access to piped water in the study areas of Ogun and Oyo States. This result is lower than the national average of 42 % access to improved sources of water in rural households in Nigeria (NPC & ICF, 2019; Otekunrin et al. 2022b; Otekunrin 2022). Similarly, in Table 8, the relationship between access to piped water and cassava farmers' CCI levels was not statistically significant in the two states (Ogun, p = 0.96; Oyo, 0.67). Additionally, this result portends grave challenge on the unavailability of safe sources of drinking water in the rural settings of Ogun and Oyo states.

Zero commercialization households had the least percentage (10.9 %) of cassava farmers' households' access to piped water. Access to piped water among the cassava commercialization households in the study areas indicated that cassava farming households in Ogun State had higher access to piped water (30.5 %) compared to those in Oyo State (3.5 %). Both of these results are still below the national average of 42 % water access by rural households in Nigeria. This revealed the rural infrastructure gaps in the study areas which has the potential of posing serious health and environmental concern in the rural settings of Ogun and Oyo states (NPC & ICF, 2019; Otekunrin et al. 2022b; Otekunrin 2022).

3.4 DETERMINANTS OF AGRICULTURAL COMMERCIALIZATION AMONG CASSAVA FARMERS

Table 9 presents the factors influencing agricultural commercialization in the study area. This analysis was carried out to assess the drivers of agricultural commercialization among smallholder cassava farmers in the

Table 7: Percent distribution of access to healthcare service among cassava farmers' commercialization household levels

		State	
		Ogun (n = 141)	Oyo (n = 211)
CCI levels	Access to Healthcare service	n (%)	n (%)
Zero Level (Non Seller)	Have access to healthcare	11 (91.7)	12 (35.3)
	No access to healthcare	1 (8.3)	22 (64.7)
	Total	12 (100)	34 (100)
Low Level	Have access to healthcare	11 (73.3)	26 (57.8)
	No access to healthcare	4 (26.7)	19 (42.2)
	Total	15 (100)	45 (100)
Medium-High Level	Have access to healthcare	32 (72.7)	30 (49.2)
	No access to healthcare	12 (27.3)	31 (50.8)
	Total	44 (100)	61 (100)
Very High Level	Have access to healthcare	60 (85.7)	20 (28.2)
	No access to healthcare	10 (14.3)	51 (71.8)
	Total	70 (100)	71 (100)
Total	Have access to healthcare	114 (80.9)	88 (41.7)
	No access to healthcare	27 (19.1)	123 (58.3)
	Total	141 (100)	211 (100)
Pearson Chi-Square (χ^2) or Fisher's exact, p-value		–, 0.23	12.11, <0.01

Computed from field survey data, 2020

Table 8: Percent distribution of access to piped water among cassava farmers' commercialization household levels

		State	
		Ogun (n = 141)	Oyo (n = 211)
CCI Household levels	Access to Piped water	n (%)	n (%)
Zero Level (Non Seller)	Have access to piped water	3 (25.0)	2 (5.9)
	No access to piped water	9 (75.0)	32 (94.1)
	Total	12 (100)	34 (100)
Low Level	Have access to piped water	5 (33.3)	2 (4.4)
	No access to piped water	10 (66.7)	43 (95.6)
	Total	15 (100)	45 (100)
Medium-High Level	Have access to piped water	13 (29.5)	1 (1.6)
	No access to piped water	31 (70.5)	60 (98.4)
	Total	44 (100)	61 (100)
Very High Level	Have access to piped water	22 (31.4)	3 (4.2)
	No access to piped water	48 (68.6)	68 (95.8)
	Total	70 (100)	71 (100)
Total	Have access to piped water	43 (30.5)	8 (3.8)
	No access to piped water	98 (69.5)	203 (96.2)
	Total	141 (100)	211 (100)
Fisher's exact, *p*-value		–, 0.96	–, 0.67

Computed from field survey data, 2020

study areas. The cassava commercialization categories were ordered and the commercialization levels were significant ($p < .001$) (Table 9). The threshold value indicating the commercialization levels; (cut1, cut2 and cut3) indicated that a value of the latent variable with -0.4382 or less represented zero commercialization (CCI 1), between -0.4382 and 1.080 was low commercialization (CCI 2), between 1.080 and 2.7352 represented medium-high commercialization (CCI 3) while a value ≥ 2.7352 was very high commercialization (CCI 4). The dependent variable is the agricultural commercialization levels (crop share ratio) categorized into four outcomes (1= Zero Level, 2 = Low Level, 3 = Medium-High Level and 4 = Very High Level). The predicted probabilities of Y = 1 or the marginal effects was estimated which measured changes in the probability of agricultural commercialization outcome with respect to a change in explanatory variables.

Tables 9 revealed the results of the ordered logistic regression and the marginal effects of each of the explanatory variable on the probability of agricultural commercialization levels.

The marginal effects provide insights into how the explanatory variables shift the probability of cassava farmers' CCI between the four ordinal levels. The statistical significance of the coefficients and the marginal effects as discussed as follows. Age, marital status, farm experience, farm income, distance to market, transport cost, cassava marketing experience, access to toilet and motorcycle ownership were the explanatory variables that had significant influence on agricultural commercialization of cassava farmers (Table 9).

The estimated results indicated that age of cassava farmers are significant at 1% level of probability, and has a negative relationship with the probability of being in the "very high commercialization level (CCI 4). The results of the marginal effects showed a unit increase in age is expected to lead to 0.0097 decrease in the probability of attaining very high commercialization level. This result agrees with *a priori* expectation that the younger the farmer, the higher the productivity which may equally lead to increasing the extent of agricultural commercialization. The result is similar to other works that opined that the older the farmer becomes, the lower the likelihood of market participate and more less likely the farmer increases the extent of his commercialization (Martey et al. 2012; Olwande & Mathenge 2011; Okoye et al. 2016). This result is contrary to the work of Enete et al. 2009 who posited that older farmers are most likely to increase the extent of cassava sales.

The results indicated that a unit increase in married respondents is expected to lead to 0.07 and 0.10 decrease

in the probability of falling in the categories of zero (CCI 1) and low commercialization (CCI 2) levels respectively. But a unit increase in married farmer is expected to lead to 0.16 increase in the probability of extending cassava commercialization to very high level (CCI 4).

This implies that the married farmer will have more household size as well as opportunity of available family labour to work on the farm in case of short fall in hired labour which can result in the increase in commercialization levels of the farmers. This results is similar to that of Effiong (2005); Adepoju et al. (2019) and Kolapo et al. (2020) who opined that larger household size enhances the availability of family labour which reduces constraint on labour demand in cassava production, processing and marketing. A unit increase in farmer's farm experience will increase the probability of the farmer extending cassava commercialization from zero, low and medium-high levels by 0.0064, 0.0121 and 0.0034 respectively. Consequently, a unit increase in farm experience is expected to lead to a decrease in farmer attaining the highest level of commercialization (CCI 4) by 0.0220 assuming other factors are held constant.

Moreover, as the distance from farm to market decreases by a kilometer, the probability of the cassava farmer falling in the categories of zero, low and medium-high commercialization levels is expected to increase by 0.81 %, 1.53 % and 0.44 % respectively. Meanwhile, as the distance from farm to market increases by a kilometer, the probability of farmers engaging in very high level of commercialization (CCI 4) increases by 2.77 %. This implies that farmers may not be willing to participate in very high commercialization level if they are very far from market centers usually because of higher transaction costs. This is in line with the studies of Omiti et al. (2009), Gebremedhin & Jeleta (2010), Agwu (2012), Opondo et al. (2017) and Otekunrin & Sawicka (2019) who found that distance to market centers inhibits access to the market and market participation of smallholder farmers.

The results also indicated that as farmers increase their marketing experience, they are able to extent their level of commercialization up to the highest level (CCI 4). This is in line with *a priori* expectation because farmers with increased marketing experience tend to have good bargain power (for prices of farm produce) at the market than those with little or no experience. This corroborates the findings of Okoye et al. (2016) who concluded that farmers' higher cassava farming experience has significant influence on the probability of households participating in markets and attaining increased commercialization level than selling at the farm gate in Central Madagascar.

Moreover, a unit increase in farmers' motorcycle ownership is expected to increases the probability of farmers extending their cassava commercialization by 0.0445, 0.0854 and 0.0305 from zero, low and medium-high commercialization levels respectively. This indicated that motorcycle ownership enhances agricultural commercialization in the study area.

3.5 STUDY LIMITATIONS AND AREAS FOR FURTHER RESEARCH

The study employed cross-sectional survey data from only rural cassava farmers in Ogun and Oyo states, South-West Nigeria while the findings from this study may not be generalized for all cassava farmers in rural settings and in all geo-political zones of the country. However, all rural cassava farmers involved in this study were smallholder farming households with not more than 5 hectares of cassava farm land while those > 5 ha of farmland are excluded in this study which may give an entirely different result outlook.

Additionally, other studies that investigate agricultural commercialization and infrastructure development in other geo-political zones (e.g. North-East, North-West, North-Central, South-South among others) of Nigeria should be carried out to capture the findings that may stem out of geo-political zone differences among smallholder cassava farmers. Also, further studies may capture the urban commercial farmers cultivating on more than 5 ha farmland

4 CONCLUSIONS

Transition from subsistence to commercial agriculture is a crucial pathway to the growth and development of most developing countries especially those that depend mainly on agriculture. It is equally important to assess the role of rural infrastructure development in promoting agricultural commercialization in Ogun and Oyo States, Nigeria. In this study, we assessed agricultural commercialization and rural infrastructure development among smallholder cassava farmers in Southwestern Nigeria. Crop commercialization index (CCI) was used to categorize cassava farming households into levels and OLM was employed to analyze the drivers of agricultural commercialization of cassava farmers in the study areas. The CCI was computed for each farm household while we explored level of rural infrastructure development across the four cassava farmers' commercialization levels in the study areas. The study found that about 87 percent of cassava farmers participated in the marketing of their cassava produce with mean CCI of 59.1 %.

Table 9: Determinants of agricultural commercialization

Variable (X)	Estimated values	Marginal effects of zero level	Marginal Effect of low level	Marginal Effect of medium-high level	Marginal Effect of very high Level
Age	-0.0424***	0.0028***	0.0053***	0.0015	-0.0097***
	(0.0127)	(0.0009)	(0.0017)	(0.0010)	(0.0029)
+Gender	-0.3680	0.0235	0.0456	0.0160	-0.0850
	(0.2450)	(0.0148)	(0.0303)	(0.0147)	(0.0572)
+Marital Status	0.7850***	-0.0671**	-0.1021***	0.0086	0.1606***
	(0.2903)	(0.0299)	(0.0391)	(0.0215)	(0.0523)
Household Size	0.0556	-0.0037	-0.0070	-0.0020	0.0127
	(0.0508)	(0.0034)	(0.0065)	(0.0021)	(0.0116)
Year of schooling	-0.0030	0.0002	0.0004	0.0001	-0.0007
	(0.0382)	(0.0025)	(0.0048)	(0.0014)	(0.0087)
Farm Size	0.0300	-0.0020	-0.0038	-0.0011	0.0068
	(0.2138)	(0.0142)	(0.0269)	(0.0078)	(0.0488)
Farm Experience	-0.0962***	0.0064***	0.0121***	0.0034*	-0.0220***
	(0.0315)	(0.0023)	(0.0044)	(0.0020)	(0.0070)
Farm Income	2.11e-06**	-1.40e-07**	-2.66e-07**	-7.56e-08	4.82e-07**
	(1.04e-06)	(0.0000)	(0.0000)	(0.0000)	(0.0000)
Nonfarm Income	-9.01e-07	5.98e-08	1.14e-07	3.22e-08	-2.06e-07
	(5.95e-07)	(0.0000)	(0.0000)	(0.0000)	(0.0000)
Distance from farm to Market	-0.1215***	-0.0081***	-0.0153***	-0.0044	0.0277***
	(0.0317)	(0.0023)	(0.0043)	(0.0028)	(0.0072)
Transport Cost	0.0003***	-0.00002***	-0.00003***	-9.71e-06	0.00006***
	(0.00009)	(0.00001)	(0.00001)	(0.00001)	(0.00002)
Food Expenditure	0.00003*	-1.79e-06*	-3.40e-06*	-9.64e-07	6.15e-06*
	(0.00001)	(0.0000)	(0.0000)	(0.0000)	(0.0000)
Cassava marketing Experience	-0.2014***	-0.0134***	-0.0254***	-0.0072*	0.0460***
	(0.0352)	(0.0029)	(0.0053)	(0.0041)	(0.0078)
+Access to credit	-0.6126	0.0521	0.0803	-0.0068	-0.1256
	(0.8666)	(0.0919)	(0.1138)	(0.0521)	(0.1547)
+Access to Extension	0.0552	-0.0036	-0.0069	-0.0021	0.0126
	(0.3040)	(0.0200)	(0.0381)	(0.0117)	(0.0697)
+Access to healthcare services	-0.0490	0.0032	0.0062	0.0018	-0.0112
	(0.2888)	(0.0190)	(0.0363)	(0.0109)	(0.0661)
+Access to toilet	0.7743**	-0.0454***	-0.0916***	-0.0452	0.1822**
	(0.3072)	(0.0164)	(0.0339)	(0.0299)	(0.0738)
Own motorcycle	-0.6966**	0.0445***	0.0854**	0.0305	-0.1604**

Continued on next page

	(0.2858)	(0.0169)	(0.0352)	(0.0210)	(0.0661)
/cut1	-0.4382				
	(0.7125)				
/cut2	1.0801				
	(0.7137)				
/cut3	2.7352				
	(0.7250)				

Note: (+) is dummy variable from 0 to 1, ***Significance at 1 % level **Significance at 5 % level
*Significance at 10% level. Figures in parentheses are robust standard errors
Number of observation = 211, Log Pseudo likelihood = -376.53844, Wald $chi^2(18)$ = 86.00
Probability >chi^2 = 0.0000, Pseudo R^2 = 0.1739

Furthermore, the study revealed many cassava farming households did not have access to some rural infrastructure such as electricity, improved toilet, access to healthcare service and access to piped water especially farming households from Oyo State. The ordered logit regression analysis showed that age, marital status, transport cost, cassava marketing experience, distance to market and ownership of motorcycle were among the significant factors influencing agricultural commercialization in the study areas. Therefore, stakeholders should expedite policy actions capable of promoting rural infrastructure development that will enhance agricultural production, marketing and improve the quality of life of rural households.

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In vitro direct and indirect regeneration of plants from nodal and petiole explants in *Pelargonium odoratissimum* (L.) Herit.

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In vitro direct and indirect regeneration of plants from nodal and petiole explants in _Pelargonium odoratissimum_ (L.) Herit.

Abstract: Nodal and petiole explants were employed to study the direct and indirect regeneration from *Pelargonium odoratissimum in vitro*. Direct shoots regeneration of nodal segments was tried in MS medium containing 1 and 2 mg l^{-1} BAP and 0.5 mg l^{-1} IBA. The highest mean shoots number and the greatest shoots per explant number were obtained in the medium containing 2 mg l^{-1} BAP. Nodal segments were the source of petiole explants and the resulting petioles were cultured in ½ MS medium supplemented by 1, 1.5, 2 and 4.5 mg l^{-1} BAP enriched with 0.1, 1 and 1.5 mg l^{-1} NAA. With the petiole explants, the lowest browning percentage, the highest callus induction and also, the top number of shoots per explant were recorded in 2 mg l^{-1} BAP + 0.1 mg l^{-1} NAA medium. The medium supplemented with 0.2 mg l^{-1} NAA exhibited the desired effect on rooting percentage and mean root number and length. The rooted young plants were transferred to the pots containing peat-moss and perlite (1:1) and the acclimatization was successful since, more than 90 % of plants adapted-well in the greenhouse conditions. This *in-vitro* propagation methodology would be advisable to the plant production systems and to whom wish to produce the clonal homogenous plants for the commercial ideas and for the detailed molecular studies.

Key words: callus induction; MS medium; nodal segments; *Pelargonium odoratissimum*; shoot regeneration

Neposredna in posredna _in vitro_ regeneracija rastlin iz izsečkov nodijev in listnih pecljev pri muškatki (_Pelargonium odoratissimum_ (L.) L Herit)

Izvleček: V raziskavi so bili uporabljeni izsečki nodijev in listnih pecljev za preučevanje neposredne in posredne vzgoje rastlin muškatke (*Pelargonium odoratissimum* (L.) L Hér.) *in vitro*. Neposredna tvorba poganjkov iz izsečkov nodijev je bile preiskušena na MS gojišču, ki je vsebovalo 1 in 2 mg l^{-1} BAP in 0,5 mg l^{-1} IBA. Največje poprečno število poganjkov in največje število poganjkov na izseček je nastalo v gojišču, ki je vsebovalo 2 mg l^{-1} BAP. Nodiji so bili vir izsečkov listnih pecljev, ki so bili gojeni na polovičnem MS gojišču z dodatkom 1, 1.5, 2 in 4,5 mg l^{-1} BAP, obogatenega z 0,1, 1 in 1,5 mg l^{-1} NAA. Z uporabo izsečkov listnih pecljev je bil dosežen najmanjši odstotek porjavitev, najboljša indukcija kalusa in največje število poganjkov pri dodatku 2 mg l^{-1} BAP + 0,1 mg l^{-1} NAA v gojišče. Gojišče, kateremu je bil dodano 0,2 mg l^{-1} NAA je pokazalo zaželjen odstotek ukoreninjenja in največje poprečno število in dolžino korenin. Vkoreninjene mlade rastline so bile posajene v lončke z mešanico šotnega mahu in perlita (1:1). Aklimatizacija je bila uspešna, saj se je več kot 90 % rastlin uspešno prilagodilo razmeram v rastlinjaku. Takšno metodologijo *in vitro* razmnoževanja rastlin bi bilo primerno uvesti v sisteme razmnoževanja, v katerih želimo vzgojiti homogene klonske rastline za komercialne namene in podrobnejše molekularne raziskave.

Ključne besede: indukcija kalusa; MS gojišče; nodijski izsečki; *Pelargonium odoratissimum*; regeneracija poganjkov

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1 INTRODUCTION

Pelargonium odoratissimum is an important aromatic plant belonging to the Geraniaceae family and is in common growth in the tropics and sub-tropics (Ghanem et al. 2008). This is a perennial herbaceous plant and, is propagated by the semi-hard wood stem cuttings. However, the success rate with this propagation method is quite limited. The oil extracted from the above ground parts of the plant is in great need with the cosmetic, fragrance and hygienic industries and preparations from the plant have remarkable antimicrobial, antifungal and insecticidal activities (Ghanem et al. 2008; Gupta et al. 2002).

Plants regeneration and propagation by the *in vitro* culture methods have been defined as reliable ways to the breeding and the economical production of medicinal plants. Shoot regeneration with both the direct and indirect ways were experienced to have enough plant materials with the traits of interest. The need for the disease-free plants besides mass-production have been the main ideas to resort to *in vitro* propagation of *Pelargonium odoratissimum*. However, there is no universal protocol suitable for *Pelargonium* spp *in vitro* propagation (Wojtania et al. 2004). In the *in vitro* conditions, regeneration rate is dependent upon genotype, explants type, media components, plant growth regulators (PGRs), light intensity and quality and, the interaction of these factors (Arshad et al. 2012). *In vitro* direct and indirect regeneration of *Pelargonium graveolens* L'Hér. has been reported by leaf and nodal explants (Satyakala et al. 1995; Saxena et al. 2000) as well as shoots formation from calli samples derived from the leaf sections of 'Bipuli' cultivar (Gupta et al., 2002).

With *Pelargoniun radula* L Herit micropropagation (Zuraida et al. 2013), the utmost shoots regeneration rate was acquired for nodal explants in a medium containing 0.5 mg l^{-1} BAP+1 mg l^{-1} IBA. Saxena et al. (2000) were successful to obtain the highest shoots number per leaf explants in a medium enriched with 5 mg l^{-1} Kinetin+1 mg/L NAA. For the nodal segments, the best results were acquired from the combination of 8 mg l^{-1} Kinetin +1 mg l^{-1} NAA. Krishna Raj et al. (1997) reported that somatic embryogenesis of *P. odoratissimum* 'Frensham' cultivar was successful with the young leaf petioles. Hammerchlag and Bottino (1981) noted that callogenesis from the young seedlings of nodal segments was dependent upon the phenological stage of growth and the younger seedlings produced the highest number of shoots *in vitro*. Adventitious shoots regeneration from the petiole explants of *Pelargonium* × *hederaefolium* 'Bonette' cultivar was stimulated by the high amounts (9-18 μM) of thidiazoron and the related embryogenesis and organogenesis crosstalk was studied in detail.

Rooting behavior *in vitro* is another criterion in plants micropropagation which is mainly dependent on auxin and cytokinin equilibrium with the dominancy of auxin type PGRs (Zuraida et al., 2013). Rooting in *P. graveolens and P. radiattum* (Andrews) Pers was the best in half strength MS medium with 0.1 mg l^{-1} NAA (Zuraida et al., 2013). In spite of great attempts on *Pelargonium* species *in vitro* cultures; there are scattered information on *P. odoratissimum* propagation methods *in vitro*. The improvements in tissue culture method with the *Pelargonium* species would be the basal steps in the enhanced production of high valued secondary metabolites from these species. Moreover, the *in vitro* cultural methods will assist molecular biologists and biotechnologists to manipulate the species for the optimized production of the desired constituents. The present experiment was planned to study the potential direct and indirect regeneration capability of nodal and petiole explants of *P. odoratissimum in vitro*. This methodology would be possibly a reliable procedure for the commercial in vitro production of this high-valued crop for the coming molecular and breeding programs.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND STERILIZATION

Nodal explants were taken from mother plants grown under a reference university research greenhouse in Maragheh, Iran. The homogenous mother pot plants (2 years old) available in our greenhouse were employed for sampling of the uniform nodal section of about 5 to 8 mm in diameter from the middle part of shoots. 30 nodal segments (3 treatments and 10 replications per treatment) were rinsed in running water for 45 minutes. Superficial sterilization of nodal cuttings was completed in a laminar-airflow cabinet as followed: immersion in 70 % ethanol for one minute, followed by sodium hypochlorite, 15 % containing 2 drops of Triton X-100 for 13 minutes. Subsequently, the explants were rinsed three-times in distilled water and treated with 70 % alcohol for 20 seconds. Finally, the samples were placed on sterile paper to get dried. The dried sterile plant material was cut into 0.5-1 cm segments and transferred to MS medium. Petiole explants were acquired from the shoots grown from nodal segments *in vitro*. The sterile petiole samples were divided into 0.5 cm explants.

2.2 REGENERATION

The basal medium was MS supplemented with 3 % sucrose and 0.7 % agar along with 500 mg l^{-1} myoinositol. For some instances, we used also ½ MS medium. Nodal segments were cultured in MS medium supplemented by BAP (1 and 2 mg l^{-1}) and IBA (0.5 mg l^{-1}). Petiole explants were cultured in ½ strength MS medium containing BAP (1, 1.5, 2 and 4.5 mg l^{-1}) and NAA (0.1, 1, 1.5 mg l^{-1}). To prevent tissue browning, 50 mg l^{-1} citric acid was added to the medium. Three explants were placed in every 9 cm petri-dish. The cultures were placed under dark conditions in a growth chamber for two weeks under 16:8 hours photoperiod regime at 23 ± 1 °C and 20 ± 1 °C.

2.3 PROLIFERATION STAGE

Two weeks after the cultures establishment; the nodal segments were subcultured in the MS basal medium with the initial treatment combinations. Three successive subcultures were done at two week intervals. The number and length of shoots were recorded twice per week. In the second phase; and 4 weeks after establishment of the petiole explants, the tender creamy colored calli were developed. Callus formation percentage was measured for each sample. Calli were subcultured on the ½ MS medium with the initial treatment combinations as well. Two weeks after calli were sub-cultured; the shoots were produced. Later, the number and length of the shoots were measured. Petiole explants were taken from the shoots produced from the nodal segments *in vitro*. For the indirect regeneration stage, the derived calli were cultured on the initial ½ MS medium.

2.4 ROOTING

The shoots derived from the proliferation stage were cut from the distal ends and were cultured on ½ MS medium containing 0.1 and 0.2 mg l^{-1} NAA combined with 2 g l^{-1} activated charcoal. Rooting percentage and the number and length of the roots were recorded about ten days after transferring to the rooting medium.

2.5 ACCLIMATIZATION OF THE NEWLY-FORMED SEEDLINGS

Warm (50-60 °C) water was used to remove the agar debris from the young roots and, the plantlets were transferred to the pots containing peat moss: perlite (1:1) and were placed in a growth chamber under 16:8 photoperiod regime and 23±1 °C and 20±1 °C temperature regime during the day and night, respectively.

2.6 STATISTICAL ANALYSIS

Experimental design for the nodal segments was arranged with 3 treatments and 10 replications in a completely randomized design (CRD) and for petiole explants with 6 treatments and 5 replications with 3 experimental units as factorial experiment based on CRD. Each experiment was repeated at least twice and the reported data are the means of two experiments. Data were analyzed by SAS (version 9) and mean comparisons were done by LSD (Least Significant Difference) test at the 0.05 probability level.

3 RESULTS

3.1 DIRECT SHOOT REGENERATION

Two weeks after the explant cultures; the shoots were visible on nodal explants. For the multiplication/proliferation, shoots were cultured with the initial treatments. Treatments and growth regulators concentration and combinations had inevitable effects on the growth and developmental response of plant samples.

Mean comparisons revealed that 2 mg l^{-1} BAP+0.5 mg l^{-1} IBA and 2 mg l^{-1} BAP (alone) had significant effects on reducing the browning percentage in samples (Figure 2). With the nodal section explants, the proximal ends were responded to the media and, we observed the browning disorder.

BAP proved to be the desirable compound for initiating the shoot growth in terms of total shoot number per explant and mean shoot number in nodal segments of *Pelargonium odoratissimum* (Figure 1)

Calli were produced on ½ MS medium containing different combinations of BAP and NAA. Calli were induced from the explants during 25 to 30 days under dark conditions. All treatment combinations (NAA+BAP) had positive effects on the calli production from petiole explants. Mean comparisons revealed that 1.5 and 2 mg l^{-1} BAP+0.1 and 1 mg l^{-1} NAA had the highest callus induction potential (Table 1). In the present experiment, the explant browning was overcome with using 50 mg l^{-1} of citric acid in ½ MS medium. Our results shows that the lowest browning rate in petiole explants was achieved in the medium containing 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA (Table 1).

The results revealed that the survival rate and regeneration potential in petiole explants were related to

Table 1: Mean comparison for the effects of treatment combination on callogenesis, and shoots induction in petiole explants of Pelargonium odoratissimum in vitro

Treatment combination	Mean shoots length (cm)	Mean shoots number	Browning percentage (%)	Callogenesis (%)
1.5 mg l^{-1} BAP +1.5 mg l^{-1} NAA	0.45^{c}	1.78^{bc}	79.36^{ab}	45.66^{de}
2 mg l^{-1} BAP + 1 mg l^{-1} NAA	0.21^{c}	0.66^{c}	92.66^{a}	25.32^{e}
2 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	3.33^{a}	19.14^{a}	15.35^{d}	100^{a}
1mg l^{-1} BAP + 1 mg l^{-1} NAA	1.22^{b}	4.36^{b}	58.96^{bc}	73.03^{bc}
1.5 mg l^{-1}BAP + 1 mg l^{-1} NAA	1.35^{b}	2.78^{bc}	40.66^{c}	93.24^{ab}
4.5 mg l^{-1} BAP + 1 mg l^{-1} NAA	1.12^{b}	2.02^{bc}	56.38^{c}	59.63^{cd}

Different letters in columns are significant *($p \geq 0.01$)* based on LSD test

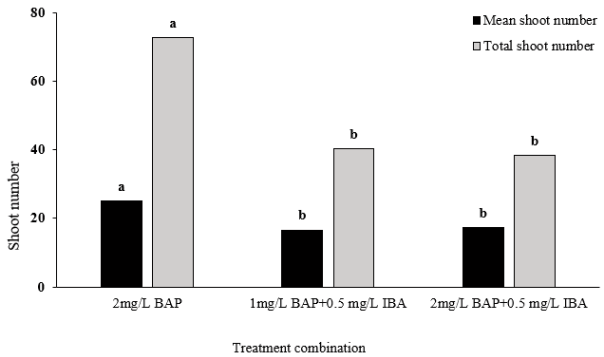

Figure 1: Treatment combination effects on total shoot number per explant and mean shoot number in nodal segments of Pelargonium odoratissimum in vitro (based on LSD test)

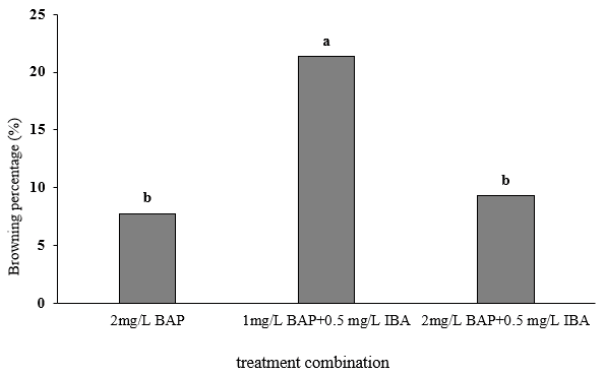

Figure 2: Treatment combination effects on browning rate of nodal segments of Pelargonium odoratissimum in vitro (based on LSD test)

the ratios and amount of plant growth regulators applied. Two weeks after subculture, the shoots were developed. The result showed that the highest number and length of the shoots was obtained in the medium containing 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA (Table 1).

The greatest proliferation rate with nodal explants

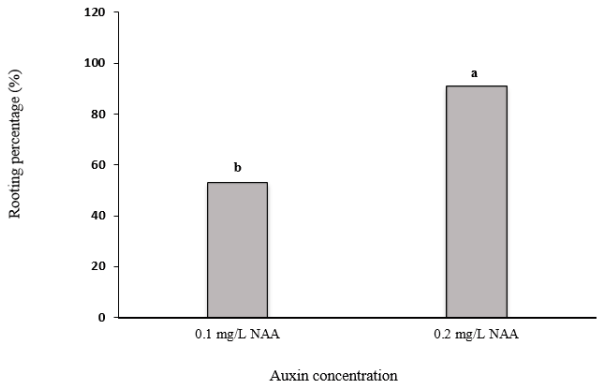

Figure 3: The effect of NAA concentrations on rooting percentage of *Pelargonium odoratissimum* in vitro ($p \geq 0.01$)

was recorded in the medium containing 2 mg/L BAP and with petiole explant in the medium supplemented by 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA (Figure 2 & Table 1)

3.2 ROOTING AND ACCLIMATIZATION

The shoots derived from the nodal and petiole explants were cultured in ½ MS medium enriched with 0.1 and 0.2 mg l^{-1} NAA plus 2 g l^{-1} active charcoal. Figure 3 shows that the highest rooting percentage was observed in 0.2 mg l^{-1} NAA. 0.2 mg l^{-1} NAA was the concentration of choice for both root number and rooting percentage as well.

Furthermore, mean comparisons revealed that, for the root number, there was a difference between NAA concentrations, and explant type. So that, with 0.2 mg l^{-1} NAA, the top root number was belonged to the nodal explants (Figure 4). The results in figure 5 shows that with both nodal and petiole explants; 0.2 mg l^{-1} NAA had more root length.

Eventually, the intact rooted plantlets were selected for the acclimatization stage. The plantlets were trans-

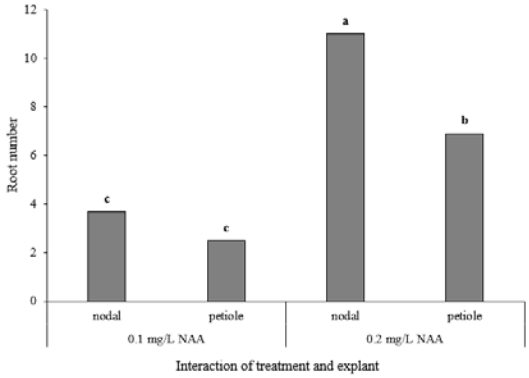

Figure 4: The effect of NAA concentrations on root number of *Pelargonium odoratissimum* in vitro (p ≥ *0.0)*

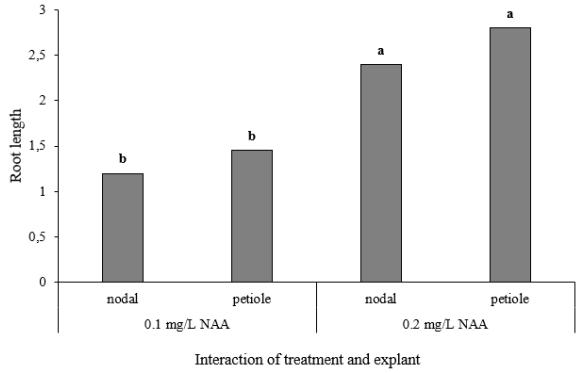

Figure 5: The effect of NAA concentrations on root length of Pelargonium odoratissimum in vitro (p ≥ 0.)

ferred to the pots containing peat moss-perlite (1:1), and were placed in a growth chamber under 16:8 hrs. light: darkness period, and 23±1 ºC and 20±1 ºC day: night temperature regime. After one month, the survived plantlets percentage was recorded and later, the acclimation of the potted plants was quite successful (more than 90 %).

4 DISCUSSION

The results showed that the sole cytokinin compared to the co-application of cytokinin:auxin had more promising effect on mean shoots number. It seems that cytokinins at higher concentrations, weaken the apical dominance and hence, stimulate auxiliary shoot induction.

These findings are concomitant with the reports of Zuraida et al. (2013) with diverse *Pelargonium* species. From the production viewpoint, *in vitro* micropropagation is a crucial step for the optimized proliferation rates, and furthermore, the fast and mass multiplication of the shoots provide the plant material needed for the coming stages of propagation route. The internal hormonal ratios and, especially auxin (IAA) amount were the major factors affecting the multiplication in direct organogenesis from *Jasmine* nodal segments and the MS medium having 3 mg l^{-1} BAP and 1 mg l^{-1} NAA was the best treatment (Farzinebrahimi et al., 2014). In *Pelargonium capitatum* plants incubated under dark conditions for 4 weeks and cultured in the medium containing 0.5 mg l^{-1} NAA+1 mg l^{-1} BAP+1 mg l^{-1} Zeatin; the full (100 %) direct shoot regeneration was achieved (Hassanein and Dorion, 2005). Epinosa et al. (2006) produced the highest indirect organogenesis of *Prunus serotina* Ehrh. plants from the leaf explants incubated under dark conditions for three weeks. Possibly, darkness affects the internal hormonal balance and positively interacts with the auxin:cytokinin exogenous applications in favor of higher organogenesis potential. Furthermore, darkness ameliorates the deteriorative effects of phenolics and hence declines the browning rates in the plant samples *in vitro* (Sukhumpinij et al., 2010).

Our results showed that for the scented geranium, BAP alone was enough for the shoot multiplication. The idea is that for the optimized shoots proliferation in geranium, the high amounts of cytokinins per auxins are functional and essential.

Calli production is dependent upon the morphological characteristics of the plant tissues and the type and concentration of growth regulators employed. Thiruvengadam et al. (2006) reported that MS medium supplemented with 1 mg l^{-1} 2, 4-D attained the maximum callus induction.

Khodadadi et al. (2015) reported the improved calli production and shoot regeneration of *Momordica charantia* L. leaf explants by increasing NAA and kinetin concentrations up to 1 mg l^{-1}. However, high concentrations of the growth regulators mentioned had quite negative effects on callogenesis. Benazir et al. (2013) realized that the greatest callogenesis percentage in *Pelargonium graveolens* was obtained by 20 µM IBA+10 µM KIN and 20 µM IBA+10 µM BAP as well as by 20 µM IAA+10 µM KIN. Overall, the balanced auxin to cytokinin ratio is the essential factor impacts calli production and the further proliferation of leaf explants of scented geranium (Benazir et al., 2013). Furthermore, Charlwood and Charlwood (1991) noted that the callogenesis was improved in scented geranium whenever they used more than 1 mg l^{-1} of auxins and cytokinins. Callogenesis response in different organs and tissues is dependent on the endogenous and exogenous hormonal balance, so, the logical cytokinin:auxin ratios with the dominancy of cytokinins are vital for the petiole explants callogenesis.

The diverse responses of the leaf and petiole explants to the varying concentrations of BA and NAA in the me-

dium possibly are due to the differences in the internal hormonal content or because of the different sensitivity of the organs to the growth regulators employed (Koroch et al., 2002). Arshad et al. (2012) demonstrated that the highest survival rate in *P. capitatum* was obtained in a medium enriched with 2 mg l^{-1} BA+1 or 2 mg l^{-1} NAA. In another study, Saxena et al. (2000) said that using 0.5 mg l^{-1} BAP instead of kinetin along with 0.1 mg l^{-1} NAA drastically increased the survival rate in *P. graveolens*.

Shoots proliferation and growth need suitable rates and amounts of auxin and in this case, NAA. The need for the low concentrations of auxins (0.1 mg l^{-1} NAA) for the formation and elongation of the shoots is maybe due to the near sufficiency of internal auxin. Saxena et al. (2000) reported that cytokinin/auxin rate plays a pivotal role in both direct and indirect organogenesis process of the plants *in vitro*.

The same idea has been reported by Brown and Charlwood (1986). The later scientists noted that organogenesis responses and shoot growth in scented geranium tissue culture was dependent on the exogenous applications of growth regulators and specially was responsive to BAP and NAA. Generally, shoot induction *in vitro* is highly responsive to the cytokinins type and concentration. This bio-molecules stimulate the organogenesis in the potentiate calli cells and hence, encourage the shoots induction and initiation. Cytokinins also increase cell division rates and more specially enhance the adventitious shoots formation via combating the apical dominancy.

Koroch et al. (2002) reported that the medium containing 4.4 μM BAP+0.05 μM NAA achieved the highest regeneration rate and the greatest number of shoots. Moreover, Saxena et al. (2000) demonstrated that the maximum number of shoots in rose-scented geranium was obtained by a medium enriched with 0.5 mg l^{-1} BAP+0.1 mg l^{-1} NAA. Ghanem et al. (2008) wrote that leaf and petiole explants of *P. graveolens* had the suitable regeneration rate at the MS medium filled with 1 mg l^{-1} BAP+0.1 mg l^{-1} NAA. The essentiality of using at least 0.5 to 1 mg l^{-1} BAP for the shoots elongation in the nodal explants of scented geranium has been emphasized by Zuraida et al. (2013).

Satyakala et al. (1995) noted that the maximum number and length of the nodal cutting for the same plant need 1 mg l^{-1} BAP and IAA. The highest regeneration percentage with *P. hortorrum* was obtained in the medium supplemented with 1 mg l^{-1} BAP and/or Zeatin plus 0.2 mg l^{-1} NAA (Hassanein and Dorion, 2005). Generally, the direct and indirect regeneration in rose–scented geranium is responsive to the endogenous and exogenous PGR_S, explant type and the chemical properties of the growing medium.

The most probable idea is that root formation in *Pelargonium* species *in vitro* is most dependent upon the shoots related attributes. Zuraida et al. (2013) noted that 0.2 mg l^{-1} auxin (IAA and IBA) was the best for the highest rooting percentage in *Pelargonium citrosum* and *P. radula* which is in line with our results. Auxins alone or in combination with the low concentration of cytokinin are able to initiate the root primordia (Zuraida et al., 2013). It seems that, deciding on the type and concentration of exogenous auxin application for *in vitro* proliferation and production is quite dependent on the internal hormonal content of the plant tissue. So, owing to the results obtained from the present experiment, 0.2 mg l^{-1} NAA produced the highest root number as well as the longest roots.

5 CONCLUSION

The current experiment was conducted to reach a reliable fast and easy propagation method for the direct and indirect regeneration of *Pelargonium odoratissimum* nodal and petiole explants under *in vitro* conditions. Overall, an established *in vitro* culture regeneration protocol is dependent on several factors such as; plant species, explant type and media composition. In the present study, the best treatment for direct regeneration from the nodal segments was obtained by 2 mg l^{-1} BAP and for indirect regeneration from the petiole explants. The optimized treatment combination was 2 mg l^{-1} BAP+1 mg l^{-1} NAA. Moreover, the results showed that nodal explants compared to petioles had greater regeneration rate and have been considered more suitable for *Pelargonium* regeneration purposes. All in all, difficulty in petioles regeneration rate, along with the highest probability of somatic mutations and the low rate of shoots proliferations, makes this explant not suitable candidate for the *in vitro* proliferation of *Pelargonium* and even makes the indirect regeneration method more time-consuming and expensive. However, since the calli are the initial and easy starting materials for the production of secondary metabolites with suspension cultures, so, the study of callogenesis and calli production and optimization for the accelerated secondary metabolites production will be a motivation for using the petiole explants with *in vitro* commercial insights. Moreover, the results showed that explant type and PGR_S concentration were the main factors influencing regeneration potential and rooting behavior in *Pelargonium in vitro* culture. In such a way, the highest regeneration rate was traced and recorded with nodal segments. The easy protocol experienced with the present experiment could be employed for the clonal propagation and suspension cultures of this high-valued ornamental medicinal species.

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Mulch tillage – principle of preservation of chernozem of the northern steppe of Ukraine

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Mulch tillage – principle of preservation of chernozem of the northern steppe of Ukraine

Abstract: Reclamation and intensive utilization of chernozems of the steppe zone of Ukraine over a long period led to loss of a significant amount of organic matter, agrophysical degradation, and dramatic decrease of soil fertility. Organic products of plant origin – byproducts of field crops (straw, frondiferous residues of arable crops) – play an important role in the renewal of fertility, protection of soils from erosion and accumulation of efficient moisture in the soil. The article presents the results of studying of the agroeconomic efficiency of board, differentiated and shallow (mulching) tillage systems when growing field crops under the conditions of the northern Steppe of Ukraine. There is substantiated the expediency of use of a shallow (mulching) tillage system, which, in terms of crop rotation efficiency against a fertilized background, is highly competitive with board and differentiated systems, as well as has a positive effect on the structural state of the arable layer (the content of agronomically valuable aggregates is 76 %), provides additional (71-85 m^3 ha^{-1}) accumulation of efficient moisture in the autumn-winter period. The most of conditional stubble on the surface remains, of course, in the early fallow (without tillage in autumn) – 630 pcs m^{-2}. A significant amount of it was also after disk processing – 333 pcs m^{-2}. Early fallow is a reliable method to wind erosion (deflation) combat in the spring. Even strong winds with a speed of more than 15 m s^{-1} in early fallow are not able to blow out more than 5-12 g m^{-2} of soil in 5 minutes of exposure, while in case of board tillage these figures increase by 11-26 times and amount to 134 g m^{-2}.

Key words: mulch tillage; agrophysical properties; fertility; crop residues; productivity; humusa

Obdelava tal z mulčenjem kot osnova za ohranjanje črnozema v severnih stepah Ukrajine

Izvleček: Melioracije oz. spremembe naravne stepe v njive in intenzivna raba črnozemov sta v stepah Ukrajine preko daljših obdobij pripeljala do znatne izgube organskih snovi v tleh in sprememb v njihovi zgradbi, kar je povzročilo dramatično zmanjšanje rodovitnosti tal. Organske snovi rastlinskega izvora kot so stranski produkti pridelave poljščin (slama, ostali ostanki poljščin) igrajo pomembno vlogo pri obnavljanju rodovitnosti, ščitijo tla pred erozijo in zagotavljajo zadostno zadrževanje vlage v tleh. Prispevek predstavlja rezultate raziskave agroekonomskega učinka različnih sistemov obdelave tal (oranja in mulčenja) pri pridelavi poljščin v razmerah severnih step v Ukrajini. Uporaba sistemov plitve obdelave tal (mulčenje) se izkaže kot primernejša glede na učinkovitost kolobarja in porabe gnojil. Je bistveno boljša v primerjavi z različnimi sistemi oranja. Ima tudi pozitivni vpliv na zgradbo orne plasti tal (vsebnost agronomsko zaželjenih talnih agregatov je 76 %), dodatno prispeva k zadostni vlažnosti tal v jesensko zimskem obdobju (71-85 m^3 ha^{-1}). Največ primernih rastlinskih ostankov ostaja na površju v začetku prahe (brez obdelave v jeseni; 630 delcev m^{-2}). Znatna količina teh organskih ostankov ostane na površju tudi pri obdelavi z diskasto brano, – 333 delcev m^{-2}. Zgodnja praha je primerna metoda za preprečevanje vetrne erozije spomladi. Celo močni vetrovi, s hitrostjo več kot 15 m s^{-1}, pri zgodnji prahi ne morejo odpihniti več kot 5-12 g m^{-2} tal, pri izpostavitvi za 5 minut, medtem ko se pri obdelavi tal z oranjem ta količina poveča 11-26 krat in znaša 134 g m^{-2}.

Ključne besede: obdelava tal z mulčenjem; agrofizikalne lastnosti; rodovitnost; ostanki poljščin; produktivnost; humus

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1 INTRODUCTION

The strategic goal of Ukraine's agricultural production is an increase of crop yields, significant increase of each hectare productivity to provide sufficient food both for the population and for export abroad, as well as creation of a stable fodder base for livestock. Difficult conditions for the industry economic development require transition to energy and resource-saving agriculture. Herewith, an important task is to preserve and expand the regeneration of potential and effective soil fertility, to maintain environmental safety of agroecosystem. Reformation of the agricultural sector of Ukraine requires improvement of the agricultural system as a whole, therefore, development aimed at forming promising models of agriculture that would simultaneously increase productivity, save energy and resources and preserve soil has strategic perspective (Saiko and Boiko, 2002; Tarariko et al., 2013; Tkalic, 2011).

Therefore, reclamation and intensive utilization of chernozems of the steppe zone of Ukraine over a long period led to loss of a significant amount of organic matter, agrophysical degradation, and dramatic decrease of soil fertility. Over the past 40 years, chernozems have lost 0.5-0.7 % of organic matter. Moldboard tillage is one of the most cost and energy-intensive processes in agriculture, and its implementation in Ukraine consumes about 500 thousand tons of fuel per year. Organic products of plant origin – byproducts of field crops (straw, frondiferous residues of arable crops) – play an important role in the renewal of fertility, protection of soils from erosion and accumulation of efficient moisture in the soil (Adamchuk et al. 2016; Shevchenko et al., 2015; Tebrugge, 2001; Tamames, 2002; Vita, 2007; Cannel, 1985). Crop residues are energy material for arable soil formation and shall be rolled into soil. This makes it possible to close the small biological cycle of substances, opened by the systematic subtraction of most of the biological products of plants. Introduction of crop residues increases humus content, improves soil structure, reduces erosion trend, stimulates nitrogen fixation process. Plant remains are a source of nutrition for soil microorganisms, without which the availability of certain nutrients would be limited. The effectiveness of fertilization with crop residues depends primarily on their harvester grinding, spread over a field and rolled into soil. It is well known that the cutting height of plants during harvest shall not exceed 20 cm, the length of 75 % of crop residues shall not exceed 10 cm, and residues over 15 cm shall not exceed 5 %. Plant remains shall be spread evenly without rolls over a field. Immediately after harvesting, plant remains shall be rolled into soil with disc harrows to a depth of 12 cm, preventing it from drying out. Sufficient humidity ensures efficient activity of microorganisms and rapid decomposition of straw. Before turning the crop residues, it is desirable to apply nitrogen fertilizers, in particular ammonium nitrate at the rate of N_{10} per 1 ton of crop residues to compensate for the utilization of nitrogen by soil microorganisms, since crop residues rolling without nitrogen fertilizers leads to a dramatic decrease in the content of mineral nitrogen in the soil and reduces the yield of subsequent crops in crop rotation. Application of crop residues in the amount of 3.5-4.0 t ha^{-1} with nitrogen compensation (N_{10} per 1 ton of crop residues) is equivalent to the application of 18–20 t ha^{-1} of manure in terms of its effect on increase of soil fertility and crop yield (Holypan et al., 2003; Andrienco, 2011; Crutchfield, 1986; Lebid et al. 2006; Pabat, 2003; Cerepanov, 1991; Troeh and Thompson, 1993).

Mulching also improves the temperature profile, agrophysical condition of the soil, agrochemical and biological indicators. In addition, mulching significantly increases the effectiveness of mineral fertilizers, especially in arid growth environment. Under such extreme conditions crop yields increase by 20-25 % only due to mulching. The correct utilization of post-harvest residues is closely related to mechanical tillage that regulates their distribution on the field surface, which in turn is associated with protection from deflation, with moisture accumulation and the nature of their mineralization, humification (Crutchfield, 1986; Lebid et al., 2006; Pabat, 2003; Cerepanov, 1991; Troeh and Thompson, 1993).

Mulching cultivation is carried out with subsurface cultivators, chisels or diskers. According to the data of different scientists (Pabat, 1992; Lebid et al., 2006; Troeh and Thompson, 1993), each nonmoldboard tool leaves after cultivation different amount of post-harvest residues of the predecessor on the soil surface:

disk tillers and harrows (LDG-15, BDT-7, Great Plains Turbo-Max 3000TM/3500TM/400TM) leave 40-60 % residues on the surface;

subsurface cultivators (KPSh-5, UNIA KOS PREMIUM) – 85-95 %;

subsurface plows (PG-3-5, STAVR, PG-5) – 80-90 %;

anti-erosion cultivators (KPE-3.8, KT-3.9G) – 60-7 5%;

chisel plows (PCh-4.5, Chip, ПЧ-10.01, PCh-6) – 60-70 %;

chisel cultivators (KPCh-5.4, Duolent) – 35-65 %;

soil spikers (BIG-3A, Great Plains UT3030) – 80-85 %;

stubble seeders (SZS-2.1, STS-2, Great Plains NTA3010/ADC1150) – 65-70 %.

2 MATERIALS AND METHODS

Experimental studies were conducted during 2011-2015 in the stationary experiment of the Institute of Grain Crops at the National Academy of Sciences of Ukraine in a short crop rotation: bare fallow – winter wheat – sunflower – spring barley – corn. The effectiveness of the board, differentiated and mulch tillage systems was studied in the crop rotation. Cultivation was carried out with the following implements: 1. Board – with a PLN-4-35 plow to a depth of 20-22 cm for spring barley and sunflower, 23-25 cm for corn, 25-27 cm for black fallow (autumn) 2. Chisel – with a chisel plow to a depth of 14-16 cm for sunflower and spring barley (in autumn); 3. Disk – with a BDT-3 harrow to a depth of 10-12 cm for spring barley and clean fallow (in autumn); 4. Subsoil – with a combined KShN-5.6 or KR-4.5 implement to a depth of 14-16 cm for corn and 12-14 cm for sunflower (autumn) in early fallow (in spring). Black fallow maintenance was based on the principles of minimization and varying of depth of cultivation from 10-12 cm in spring to 6-8 cm before wheat sowing. Clean fallow maintenance after the main cultivation in spring was carried out similar to black fallow maintenance.

All the grinded frondiferous mass of the predecessors was left in the field without subtraction and it was rolled with the above-mentioned implements against the background without fertilizers and with the application of mineral fertilizers together with crop residues. The experimental design included 3 fertilizer backgrounds: 1) without fertilizers + post-harvest residues; 2) $N_{30}P_{30}K_{30}$ + post-harvest residues; 3) $N_{60}P_{30}K_{30}$ + post-harvest residues. Mineral fertilizers were applied in the spring by spreading for preplant cultivation.

Agricultural techniques for growing field crops (winter wheat – Lytanivka variety, corn for grain – 295 SV Belozirskyi hybrid, sunflower – Yason hybrid, spring barley – Ilot variety) are generally accepted for the Steppe zone. For winter wheat and spring barley in the tillering phase the Esteron herbicide 1.2 and 0.8 l ha^{-1} was applied, respectively, for corn and sunflower – Harness soil herbicide at a dose of 2.5 l ha^{-1}. Mineral fertilizers ($N_{30}P_{30}K_{30}$) for winter wheat were applied in autumn before sowing and additionally for plants nutrition – N_{30} in spring at booting stage. In the cultivation of corn, sunflower and spring barley, mineral fertilizers ($N_{30}P_{30}K_{30}$, $N_{60}P_{30}K_{30}$) were applied for presowing cultivation.

To comprehensively study the impact of tillage technologies and fertilizers on agrochemical, water-physical properties, formation of crop productivity, commercial efficiency in a stationary experiment, modern field, measuring-weighing, analytical and mathematical-statistical research methods were applied according to generally accepted methods:

- density of soil structure was studied by the method of "cutting ring" in layers 0-10, 10-20 and 20-30 cm in four repetitions before sowing field crops and at the end of the growing season, in fallow in spring and at the end of fallowing (Vadunina, 1986).

- soil hardness was measured with a Reviakin hardness tester to a depth of 0-30 cm in the spring before sowing and at the end of the growing season (Vadunina, 1986);

- structural and aggregate composition of the soil was determined by the method of "dry" sieving on a column of sieves according to N.I. Savinov. Soil was sampled in the spring from layers 0-10, 10-20, 20-30 cm in ten places before sowing field crops and at the end of the growing season, in fallow in the spring and at the end of fallowing (Revut, 1972);

- anti-deflation assessment of the field surface depending on the tillage technique was determined by the degree of preservation of crop residues on the field surface and lumpiness of the upper layer 0-5 cm in spring before moisture closure and sowing of spring crops (Shiyatuy, 1971);

- availability of a conditional stubble on the field surface (pcs. m^{-2}) was determined with the help of a formula:

$$S = Q/p \bullet d$$

where S – conditional stubble on the field surface (pcs. m^{-2});

Q – mass of air-dried crop residues per 1 m^2, g;

p – mass of one wheat stubble with a straw length of 20 cm (p = 0.26 g);

d – mass equivalent of the conversion of crop residues of different field crops into wheat stubble.

- lumpiness of the upper layer of soil (fractions larger than 1 mm) was determined within the period of accounting for the preservation of crop residues. An average sample weighing 500 g was prepared from 10 individual samples, followed by sieving the soil into fractions through a sieve column (Shiyatuy, 1971);

- the coefficient of wind resistance of the soil surface was determined by the formula:

$$W = A/Q$$

where W – coefficient of wind resistance;

A – maximum allowable wind operations according to E.I. Shiyatuy equal to 120 g (Shiyatuy, 1971);

Q – estimated or actual soil erosion by wind, g/m^2 for 5 minutes.

- depth of tillage was determined using a furrow depth indicator, 50 measurements were performed at each site. After determining the average depth, the co-

efficient of uniformity of cultivation was calculated and evaluated according to a five-point scale (Stebut, 1871);

- soil moisture was determined in a one and a half meter layer of soil by thermostatic-weight method (Vadunina, 1986; Dolgov et al., 1966). Samples were taken every 10 cm in three places of the site and two adjacent repetitions in spring before sowing of spring crops in the phase of earing, flowering, shot and in autumn before sowing of winter wheat, as well as at the end of growing season;

- total crop water consumption was determined by the method of water balance (Vadunina, 1986);

- soil for agrochemical analysis was sampled in the spring to a depth of 0-10, 10-20 and 20-30 cm for all the options of the experiment at five points of the field diagonally in the first and third repetitions. Plants were sampled before harvesting field crops. Sampling and preparation of samples for analysis were performed according to the methodology developed by Yu. K. Kydzin (Kydzin, 1963).

- agrochemical analyzes of soil samples was performed both standard and some specific, namely: general humus according to I.V. Tiurin in the modification of Orlova and Hrindel with spectrophotometric ending; total (gross) nitrogen and phosphorus by the method of K.E. Ginzburg, GM Shchehlova, E.A. Wolfius (Ginzburg, 1975); the content of $N\text{-}NO_3$ in the soil without composting and after 7 days of composting – spectrophotometrically (Kravkov's method) (Borisova, 1968);

- the content of active forms of P_2O_5 and K_2O – with a solution of 0.5 N acetic acid (Vagenin, 1975); the content of readily available forms of phosphorus extracted by solutions of weak hydrochloric acid (Franceson's method) and neutral solution of potassium sulfate (Karpinskii Zamiatin method) (Ginzburg, 1975);

- Keldal nitrogen, phosphorus – on a photoelectric colorimeter, potassium – on a flame photometer were determined in plant samples;

- protein, fat, starch and fiber in grains and seeds – on Infrapid-61;

- accounting for above-ground weediness of crops was accounted by the method of sites (1.0 m^2) in the earing phase of early cereals and before the first inter-row cultivation in row crops, as well as after each cultivation of fallow with simultaneous weeding to determine species and their mass in air dried state. The accounting frame was superimposed on the diagonal of the site in 10 places (Veselovskiy et al., 1998; Ivachenco, 2001);

- yield accounting was carried out separately by the method of direct threshing (winter wheat, barley, peas) with the Sampo-500 combine (sunflower – Niva-Effect combine), corn – manually taking into account the humidity and impurity of products (Bulugin et al., 1999) within phase of full grain maturity. After determining the impurity and moisture content of the grain, yield was reduced to 100 % purity and 14 % moisture. Yield data for all the crops were processed by the method of analysis of variance according to B.A. Dospekhov using computer technology (Dospekhov, 1985);

- assessment of crop rotation productivity depending on tillage and fertilizer systems was given by grain yield, amount of feed, grain units and digestible protein per 1 ha of crop rotation area, as well as the average yield of field crops. The calculation of feed, grain units and digestible protein was determined by multiplying the yield of the product by the normative coefficients (Tome, 1964);

- calculations of the economic efficiency of the studied measures were carried out according to the recommendations of the Institute of Agrarian Economics National Scientific Center and the Institute of Steppe Agriculture (Rubka et al., 2012);

The two-factor stationary experiment is based on the method of splitting plots with their sequential placement in triplicate. The size of the plots of the first order – 1500, the second – 375, the accounting area – 30-100 m^2.

The soil of the experimental field is ordinary low humus medium loam chernozem. The thickness of the humus horizon is 38-43 cm. The humus content in one layer is 0-30 cm – 4.2 %. Absorbed bases are mainly calcium 20.4 and magnesium 7.8 mg/eq per 100 g of soil. The degree of soil saturation with bases is 94.18 %. Due to this fact, the reaction of the soil solution is close to neutral (pH 6.6-6.8). The gross content of nutrients in one layer of soil is in the range of: total nitrogen – 0.15-0.19, phosphorus – 0.11-0.14, potassium – 2.0-2.4 %, active forms of phosphorus (in acetic extract according to Chirikov) – 9-10, exchangeable potassium (according to Maslova) – 14-15 mg per 100 g of soil.

The climate of the study area is temperate-continental with significant fluctuations in weather conditions over the years. The average annual air temperature is +9.6 °C, with a deviation in some years from 8.4 to 10.8 °C. The average annual rainfall is 509 mm and varies from 420.7 to 832.7 mm. Most of them (68 % of the annual amount) fall during the warm period (April-October) and are largely spent on evaporation, as well as runoff due to the predominance of heavy rainfall in the undulating terrain.

In recent decades, the world, and in particular Ukraine, has undergone significant agrometeorological changes in the direction of global warming.

Adverse weather conditions for growing field crops were in 2012 and 2013. The hydrothermal coefficient in the period of the greatest water consumption of plants (May – July) was equal to: 2011 – 0.8, 2012 – 0.6, 2013

– 0.7, 2014 – 0.9, 2015 – 0.8. The SCC index of less than 0.7 indicates the presence of soil and air drought that adversely affects the grain formation and filling.

Mathematical processing of field research data to determine the validity of differences was carried out using computer software and in accordance with the methodology (Dospekhov et al., 1985; Styl et al., 1977).

3 RESULTS AND DISCUSSIONS

According to the research results, the agrophysical indicators of the soil, regardless of its processing, were within the optimal parameters. The density of the soil did not exceed the critical limit – 1.35 g cm^{-3} in the cultivated layer and was 1.18 for plowing, 1.25 for chiselling, 1.26 for subsurface knifing, and 1.26 g cm^{-3} for disk tillage. It should also be noted that in case of shallow disk processing, differentiation of the processed layer was observed in terms of density with its increase in a layer of 10–20 cm to 1.3 g cm^{-3}. This is due to the mechanism of action of a soil tillage implement, as a result of which the above-mentioned layer is compacted.

At the beginning of spring field operations, during the years of research, regardless of the tillage system, favorable density conditions were developed for all studied field crops that were within the range of 1.09-1.32 g cm^{-3} in the arable layer (0-30 cm). With shallow mulching due to the reduction of the depth of loosening to 12-14, 14-16 cm there was some compaction of the layer 0-30 cm by 0.02-0.14 g cm^{-3} that does not exceed the optimal density for crops growing.

Soil hardness during plowing in a layer of 0-30 cm was minimal – 5.0-8.7 kg cm^{-2}, the use of chiselling, subsurface knifing and disking contributed to an increase in indicators up to 11.9, 12.1 and 13.3 kg cm^{-2}, respectively, without exceeding the optimal parameters up to 21 kg cm^{-2} for field crops (Table 1).

The system of differentiated tillage, where the same implements were used as in mulching, but after other crops, occupied an intermediate position in terms of hardness.

The 0-5 cm sowing layer of soil for all the tillage systems was within the range of optimal hardness (3.4-16.2 kg cm^{-2}), and in the layer of 5-15 cm there was an increase in hardness to 12.2-17.1 kg cm^{-2}, as compared with the top one – 1.1-3.5 times.

The soil layer of 15-25 cm was the most compacted both in the board tillage system and at application of disk, subsurface and chisel implements in differentiated and mulching systems.

The change in the hardness of the arable layer of the soil depended not only on the method of tillage, but also on the influence of the root system of a particular crop grown in the field. Thus, against the background of non-

Table 1: Average change in soil hardness indicators depending on tillage systems for 2010-2015, kg/cm^2

Soil tillage system (A factor)	Soil layers, cm	In the spring, at the beginning of field operations (B factor)					At the end of fallowing and crop growing (B factor)				
		complete fallow	winter wheat	sun flower	spring barley	corn	complete fallow	winter wheat	sunflower	spring barley	corn
Board	5	4.2	15.2	4.4	8.1	3.4	15.8	20.5	16.6	21.0	17.0
	15	5.8	15.2	6.5	10.8	5.7	21.1	18.2	20.7	18.4	21.0
	25	14.9	19.8	12.7	19.6	13.5	21.9	17.3	32.5	17.5	31.1
	0-25	7.8	15.9	7.1	11.8	8.3	18.1	18.6	20.8	19.7	20.2
Differentiated	5	14.6	16.3	7.0	8.3	4.1	14.9	22.2	16.2	25.1	17.9
	15	17.1	20.2	11.8	15.7	12.2	24.6	23.0	33.2	20.7	33.3
	25	16.8	24.1	12.9	20.1	16.8	24.1	21.0	33.0	22.3	33.4
	0-25	15.4	18.8	9.8	13.5	11.8	19.7	20.0	24.8	23.2	25.1
Mulching	5	16.2	17.7	6.5	10.4	11.3	19.8	28.4	19.4	29.3	20.1
	15	17.3	17.5	13.6	16.9	13.8	26.5	23.1	33.8	24.4	33.7
	25	22.8	18.9	15.0	18.9	14.9	26.9	22.2	33.6	22.4	33.8
	0-25	17.6	16.8	10.8	14.2	15.6	22.4	25.2	25.8	25.7	26.0
$HIP_{0.95}$, kg cm^{-2}, (0-25 cm layer)											
for A factor		4.5					1.8				
for B factor		4.8					2.3				
for AB interaction		7.3					4.2				

board cultivation, the following year, in the spring the hardness indicators in the 0-25 cm soil layer were distributed in the following order: after winter wheat – 9.8 kg cm^{-2}, spring barley – 15.6, corn – 17.6 kg cm^{-2}; chisel cultivation – after winter wheat – 10.8 kg cm^{-2}, spring barley – 11.8, sunflower – 13.5 kg cm^{-2}.

These data demonstrate that crops with different types of root system have different effects on soil hardness. For example, crops of winter wheat with a highly branched root system and optimal plant density in the area can significantly improve the hardness of the arable layer, while corn, sunflower that have stronger roots but are grown at lower standing densities, have less impact on it.The hardness of the soil in winter wheat crops at the time of resumption of spring vegetation exceeded the areas of fall plowing by 1.08-3.00 times. It was the highest in the field with shallow loosening by fallow disk cultivation – 18.8 kg cm^{-2} and the lowest – by plowing – 15.9 kg cm^{-2}.

The hardness of the arable layer changed in dynamics under the influence of precipitation, air temperature and the development of the root system. From the beginning of spring operations until the harvesting, it increased in winter wheat crops from 15.9-18.8 to 18.6-25.2 kg cm^{-2}, or 1.2-1.3 times, sunflower – from 7.1-10.8 to 20.8-25.8 kg cm^{-2}, or in 2.4-2.9 times, corn – from 8.3-15.6 to 20.2-26.0, or in 1.6-2.4 times, in areas of spring barley – from 11.8-14.2 to 19.7-25.7 kg cm^{-2}, or 1.7-1.8 times. At the end of the growing season of winter wheat and sunflower, as in the spring period, there was a clear tendency to increase the hardness of the soil of the arable layer with depth.

Deterioration of the arable layer in spring barley crops was negatively affected by high temperatures and lack of precipitation for a long time, and as a result of drying of the soil at the end of the growing season that can be judged by hardness, in particular the 0-10 cm upper layer exceeding deeper layers in 1.1-1.3 times, going beyond the optimal values (21.0-29.3 kg cm^{-2}).

The impact of tillage on its hardness, from the moment of autumn, preserved until the end of the growing season of the crops. Thus, in particular, at the end of the growing season the tendency to decrease the hardness of the soil against the background of plowing (18.1-20.8 kg cm^{-2}) is observed for all the crops, compared with the options of differentiated (19.7-25.1 kg cm^{-2}) and mulching (22.4-26.0 kg cm^{-2}) tillage systems, which is primarily due to the reduction of tillage depth and the peculiarity of the action of non-board implements without turning the layer on the soil.

Based on the research, it can be concluded that the use of all the methods of tillage, especially deep board tillage, helps to reduce hardness due to mechanical loosening. Reducing the depth of soil loosening to 12-14, 14-16 cm, when using non-board implements, increases the hardness to 18.8 kg cm^{-2} in the spring before crops sowing (0-30 cm layer) that does not exceed the maximum allowable parameters (21 kg cm^{-2}) for growth and development of field crops. A significant increase in hardness at the end of the growing season to 18.1-26.0 % is primarily due to man-made burden, reduced density and porosity, deterioration of structural and moisture regime in the cultivated layer (0-30 cm) that further is restored to optimal parameters during the autumn-winter period due to increase of moisture and reverse freezing-thawing processes.

Structural analysis of the soil, carried out in the spring in a 0-30 cm layer before pre-sowing cultivation, demonstrated that, regardless of the methods of tillage, the amount of agronomically valuable structural aggregates with a size of 10-0.25 mm did not exceed 73.2-75.9 %. There was a tendency to increase the number of the most valuable structural aggregates with a size of 7-0.25 mm against the background of chisel and disk treatment in the presence of crop residues on the surface.

In general, if we characterize the structural state of the soil depending on the tillage systems, the number of valuable aggregates increases in ascending order: board – differentiated – mulching. The sum of agronomically valuable aggregates of 10-0.25 mm per fall plow in a layer of 0-30 cm after winter wheat before sowing sunflowers and after corn at the beginning of fallowing under the mulching system was 87.7-91.8 % and exceeded the board system option by 4.9-9.6 %.

Methods and systems of basic tillage had less overall impact on the structuring processes than the crops themselves and the plant remains left by them in the field. With shallow tillage, the number of the most valuable aggregates (10-0.25 mm) increased due to the reduction of man-made burden on the soil compared to the board. The mulching system of tillage had a significant advantage in soil structuring with the use of annual shallow tillage, leaving the post-harvest residues of the predecessor on the surface of the field.

The agrophysical properties of the soil, together with the presence of crop residues on its surface, are closely related to soil deflation (wind erosion). As it is known, soil resistance to deflation is determined by soil deflation. Deflation (the amount of soil particles transported by wind) is the most objective indicator of the degree of soil wind resistance. It mainly depends on the properties of the upper soil layer (granulometric composition, cloddiness and cohesion of soil aggregates, amount of stubble, etc.) (Barwicki et al., 2012; Bakker et al., 2008; John, 2013; Novakovsky, 2017; Tarariko, 2017; Baliuk et al., 2016; Rasmussen, 1999; Javůrek et al., 2008). For most soils with the content of clods larger than 1 mm in the

upper layer of 0-5 cm and in an amount above 60 % of the dry mass favorable conditions are created for resistance to wind blowing, and when the amount is less than 50 %, the blowing of soil particles increases (Zayceva, 1970). In our studies, immediately after tillage in autumn, the cloddiness (aggregates > 1 mm) of the upper layer (0-5 cm) of the soil, regardless of fallow treatment, was 61.0-62.9 %, and did not decrease below 60 %, that is, it was wind resistant. During the winter period, as a result of the influence of oppositely directed processes of freezing – thawing, moistening – drying out, soil aggregates were destroyed to erosion-hazardous sizes, the cloddiness of chernozem decreased by 1.3-1.4 times and amounted to only 43-46 %, as a result of which they can deflate on open plains and wind-swept slopes (Table 2).

According to theoretical calculations, according to the method of E.I. Shiyatuy, soil deflation by wind is allowed to the extreme limit 120 g per 5 minutes of exposure. When the erosion is less than or equal to 50 g, the soil surface is considered to be highly wind-resistant, and at values of 50-120 g it is considered to be moderately wind-resistant. In spring conditions (the period of manifestation of maximum deflation), in order to prevent soil blowing out, it is required to have 8-10 pieces/m^2 of conditional stubble 20 cm long in terms of winter wheat for each percent reduction in the top layer cloddiness (Shiyatuy, 1971).

Therefore, during the destruction of erosion-resistant particles (aggregates > 1 mm), plant crop residues of the predecessor left on the soil surface that protect its surface from blowing silt fractions in spring, are of great importance. The most of conditional stubble on the surface remains, of course, in the early fallow (without tillage in autumn) 630 pcs m^{-2}. A significant amount of it was also after disk processing – 333 pcs m^{-2}. Early fallow is a reliable method for wind erosion (deflation) combat in the spring. Even strong winds with a speed of more than 15 m s^{-1} in early fallow are not able to blow out more than 5-12 g m^{-2} of soil in 5 minutes of exposure, while in case of board tillage these figures increase by 11-26 times and amount to 134 g m^{-2} (Figure 1).

The coefficient of wind resistance of the surface (the ratio of the allowable level of deflation of 120 g m^{-2} to its actual value) was the highest in the early fallow – 24, due to the protection of the surface by crop residues. In summer, during fallow care, during cultivation, the risks of soil deflation increase significantly, even for early fallow. But still, the soil is more resistant to blowing out in case of options of non-board tillage compared to the board. The use of board tillage in fallow, as well as for all the crops in the crop rotation, contributed to the emergence of maximum wind erosion (deflation).

Early fallow is not only a radical method of combating wind erosion, but also water erosion. The runoff of melt water in spring does not create significant soil erosion in such a case. This is facilitated by an increase in its density, protection by snow and crop residues. In such a case the water flow breaks up into small streams and

Table 2: Indicators of anti-deflation resistance of soil in spring in a field of complete fallow, depending on the methods of cultivation

Soil anti-deflation resistance indicators	Soil cultivation	Soil anti-deflation resistance value
Quantity of conditional stubble on the soil surface, pieces m^{-2}	board (plowing)	20
	non-board (disk)	333
	non-board (subsurface, early fallow)	630
Cloddiness (aggregates > 1 mm) in 0-5 cm soil layer	board (plowing)	46
	non-board (disk)	43
	non-board (subsurface, early fallow)	45
Clog mechanical resistance, %	board (plowing)	82
	non-board (disk)	67
	non-board (subsurface, early fallow)	75
Soil deflation by wind, g m^{-2} ·5 min.	board (plowing)	134
	non-board (disk)	113
	non-board (subsurface, early fallow)	5
Surface wind resistance coefficient	board (plowing)	0.89
	non-board (disk)	1.06
	non-board (subsurface, early fallow)	24.0

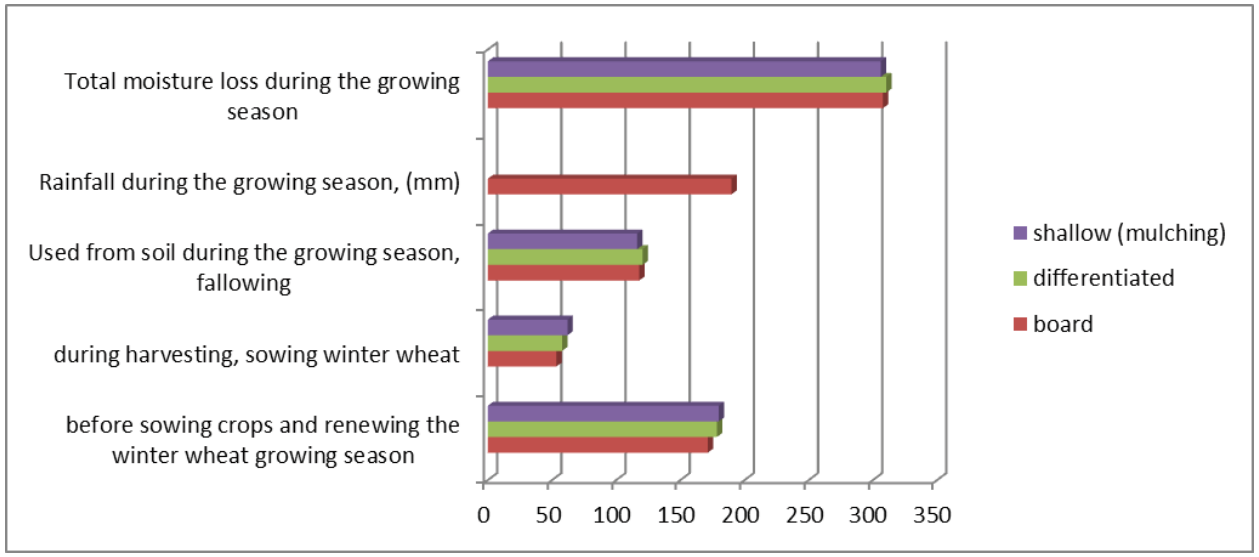

Figure 1: Average moisture balance in short crop rotation against the background of different tillage systems for 2011-2015, mm

loses speed mainly due to mechanical braking. With a high clogging ability of the soil preparation, soil washout outside the field was 1.5-4.3 t ha^{-1}, which is 4-12 times less than in case of plowing (18.6 t ha^{-1}).

The resistance of early fallow to erosion of heavy summer precipitation increases if there is more than 2.5 t ha^{-1} of plant substrate on the surface, postponement of the main cultivation to the period of mass growth of weeds (May), its carrying out with non-board implements to a loosening depth of 12-14 cm in order to preserve the mulching screen and to create a cloddy structure of the topsoil. Under the conditions of artificial sprinkling with an intensity of 3.5 mm min^{-1}. (end of June, slope of 2.5°) in areas of board tillage with the fallow maintenance technology recommended for the zone, the runoff began in 3.2 minutes when supplying 11.2 mm of water, while in the early fallow with spring processing – after 7.6 minutes and supply of 26.6 mm of precipitation. Soil permeability and runoff turbidity in this case were 1.08 mm min^{-1} and 25 g l^{-1}, against 0.65 mm min^{-1} and 39 g l^{-1}, respectively, under control conditions.

The transition from black fallow to early fallow, against the background of mulching the soil surface with crop residues of the predecessor, improves the structure of ordinary chernozem, while reducing the amount of silty fractions (< 0.25 mm) that are most amenable to anthropogenic pressure to a safe indicator of 5.4-5.6 %. The content of agronomically valuable aggregates, 10-0.25 mm in size, at the end of fallow in the arable layer, on the contrary, increases in relation to autumn plowing to 89-90 %. The level of these indicators makes it possible to state that with a positive balance of biogenic compounds, the presence of a sufficient amount of energy material and the absence of erosion, the renewal of the structure in early fallows occurs in a self-regulation mode inherent in natural analogs of fallow or virgin lands (Medvedev, 2007).

According to the level of accumulation of winter precipitation, early fallow had an annual advantage over autumn plowing. This can be explained by the formation of a very dense protective screen, created by standing stubble and grinded crop residues. In the area that has not been cultivated since autumn, there is a significant decrease in wind speed in the aboveground airspace, as well as earlier and more uniform snow deposition, an increase in its viscosity and density. In combination with high buffering and retaining properties of early fallow, this causes less water loss to runoff, evaporation, freezing and blowing, and as a result, it contributes to an increase in the coefficient of precipitation absorption and additional accumulation of moisture in the root-active soil layer (0-150 cm) compared to the board tillage by an average of 130 m^3/ha.

As for the reserves of efficient moisture in the spring in a one and a half meter soil layer, on average over five years of research, they amounted to 171.4 mm for the board tillage system, 178.5 – differentiated, and 179.9 mm -mulching. The advantage in the accumulation of moisture in the autumn-winter period by 7.1-8.5 mm (71-85 t ha^{-1}) was observed against the background of differentiated and mulch tillage systems in comparison with board tillage. This is due to the presence of post-harvest residues on the soil surface, wavy microrelief during chiselling. Ultimately, this contributed to a greater accumulation of snow during December-January with a general shortfall in the standard amount of precipitation

and the virtual absence of significant snow cover during the years of the study (Table 4).

The total consumption of moisture from the soil varied in a narrow range of 306.2-310.4 mm and almost did not change depending on the tillage system. It should be noted more economical moisture consumption by field crops against the background of a shallow mulch tillage system, as evidenced by a decrease in the moisture consumption coefficient by 13.4 mm t^{-1} compared to board tillage.

According to the results of agrochemical analysis, in the mulch tillage system in crops of corn, spring barley, sunflower, with an average and increased supply of N-NO3, there was a tendency to reduce the amount of nitrates by 1.4-3.0 mg kg^{-1} compared to the board system. With an increased and high level of phosphorus and potassium supply, the difference in the range of 11-29 mg kg^{-1} in the content of these elements in the cultivated layer between the options of mulching and board tillage is considered insignificant. The existing reserves of P_2O_5 and K_2O in the 0-30 cm layer before sowing field crops were sufficient to form a high grain yield, regardless of the studied tillage and fertilizer systems.

The use of shallow mulch tillage with crop residues left in crop rotation fields is of great importance for the renewal of soil fertility, especially in the context of a reduction in the use of organic and mineral fertilizers in recent decades. The stubble crop residues of field crops left on the field return a significant amount of previously alienated nutrients, their amount depends mainly on the yield of by-products and the biological characteristics of the crops. The largest number of nutrients is returned with crop residues of winter wheat (N – 57.4-79; P_2O_5 – 13.1-17.3; K_2O – 94.0-140.6 kg ha^{-1}), sunflower stalks (N – 50, 1-70.5; P_2O_5 – 13.2-16.4; K_2O – 148.5-186.5 kg ha^{-1}) and corn (N – 53.3-65.1; P_2O_5 – 29.9-33, 3; K_2O – 90.4-103.6 kg ha^{-1}) that is explained by the high yield of by-products and the significant content of nutrients in them. A significantly smaller amount (by 1.5-2.0 times) of nutrients is returned with by-products of spring barley (N – 32.9-43.2; P_2O_5 – 7.8-10.4; K_2O – 43.5-63 .7 kg ha^{-1}) due to the low yield of straw compared to winter wheat straw, corn and sunflower stalks (Table 3).

A significant amount of nutrients returns to the soil and the root system of field crops. For example, the roots of winter wheat after their mineralization leave in soil: N – 40.2-63.8; P_2O_5 – 6.2-8.9; K_2O – 13.9-19.2 kg ha^{-1} that is somewhat less compared to crop residues, especially in potassium (6-8.5 times), but making up a significant amount in the total amount. The same patterns are inherent in the content of nutrients in the root residues of sunflower, corn and spring barley, the decrease in their number in comparison with the nutrients of above-ground residues in nitrogen was 1.4-3.1; phosphorus 2.4-4.2; potassium 6.2-6.8 times.

In its total mass, crop residues (root + stubble) leave a significant amount of organic matter, which, during humification and mineralization, is partially converted into humus and active nutrients ($N\text{-}NO_3$, P_2O_5, K_2O). The total amount of stubble substances involved in the biological cycle was distributed in cereal crops by individual plant organs in the following ratio: main product – 44 %, by-product – 39-40 %, root system – 16-17 %, in sunflower – 32, 52 and 16 % respectively.

Relative indicators of the possible reuse of macronutrients after the mineralization of the mass of roots and by-products of cultivated crops are 48-53 % for N, P_2O_5 – 30-34 %, K_2O – 72-90 % of the volume of their biological circulation for crop formation, that is, when planning fertilizer systems in the crop rotation there should be taken into account, first of all, the compensation of the used nitrogen and phosphorus.

The use of crop residues as an organic fertilizer provides energy for the culture soil-forming process in agroecosystems, subject to the application of nitrogen fertilizers (nitrogen compensation) of 8-10 kg of active ingredient per ton of crop residues to ensure the vital ac-

Table 3: The content of humus, gross nitrogen and phosphorus under the influence of various tillage systems in 2015, %

Tillage system	Soil layer	Grain-fallow-row crop rotation		
		humus	nitrogen	phosphorus
	Without fertilizers + post-harvest residues			
Board	0-10	4.46	0.22	0.17
	10-20	4.29	0.21	0.15
	20-30	3.90	0.20	0.14
	0-30	4.22	0.21	0.15
Shallow (mulching)	0-10	4.64	0.23	0.15
	10-20	4.25	0.21	0.15
	20-30	3.96	0.19	0.14
	0-30	4.28	0.21	0.15
	Post-harvest residues + $N_{30\text{-}60}P_{30}K_{30}$			
Board	0-10	4.35	0.22	0.16
	10-20	4.29	0.21	0.14
	20-30	4.05	0.20	0.14
	0-30	4.23	0.21	0.15
Shallow (mulching)	0-10	4.66	0.23	0.16
	10-20	4.24	0.21	0.16
	20-30	4.06	0.20	0.15
	0-30	4.32	0.21	0.16
$HIP_{0.95,}$ % (0-30 cm layer)		0.05	-	-

tivity of microorganisms. It is best to use nitrogen in the ammoniac or amide form, i.e. ammonium sulfate, ammonium chloride or urea. At the same time, the use of mineral soil nitrogen is leveled and the process of mineralization of organic substances is inhibited due to the high biogenicity of the soil. During the decomposition of root and stubble residues of crops with a relatively low nitrogen content, mineralization processes prevail over humification processes, since nitrogen-free humus compounds are unstable and quickly mineralize. It has been established that for the root residues of winter wheat, the humification coefficient is 0.15-0.18 (C : N – 35-40 : 1), for straw – approximately 0.10 (C : N – 80 : 1). The coefficient of humification of organic fertilizers (manure) is 0.2-0.3 (C : N – 25-35 : 1). The joint use of crop residues and mineral fertilizers in the recommended doses increases the humification coefficient by 23-25 %.

The use of crop residues (straw of early cereals, sunflower and corn stalks) as an organic fertilizer is also of great environmental importance:

- crop residues evenly spread over the field protect the soil from erosion, drying out and compaction processes;

- large amount of organic matter is utilized, and the half-life elements are completely absorbed by the soil complex;

- organic mass is re-introduced in the circulation of mineral and organic plant nutrition to form a new crop;

- organic matter decomposes in the soil for a long time, does not pollute it with high concentrations of nitrate nitrogen, organic phosphorus and potassium;

- stable balance of input into the soil and losses of plant nutrients from crop residues eliminates the washing out of active elements and their removal with surface runoff to water bodies;

- leaving crop residues on the field contributes to the development of soil fauna that increases the activity of bacteria, worms and other organisms, improves the agrochemical and physical properties of the soil.

The use of post-harvest crop residues in our experiments in combination with mineral fertilizers in moderate doses of $N_{30\text{-}60}P_{30}K_{30}$ caused some changes in the potential and effective soil fertility. Systematic – within 6 years – rolling in the soil (50 % with shallow mulching and almost complete with plowing) of biomass by-products of crop rotation, even without nitrogen to compensate, provided a deficit-free balance of humus. With the initial humus content of the arable layer of 4.2 %, after 6 years, in case of options without mineral fertilizers, but with leaving of crop residues, the content of total humus in the layer of 0-30 cm in the grain-fallow-row crop rotation was 4.22-4.28 % (higher by 0.02-0.08 percentage points (p.p.)), and in combination with the application of mineral fertilizers it increased by 4.23-4.32 %, or 0.03-0.13 p.p. (Table 3). The application of mineral fertilizers in combination with crop residues contributed to the

Table 4: Average influence of basic tillage systems and fertilizers on crop rotation productivity for 2011-2015, t ha^{-1}

	Soil tillage system and fertilizers in crop rotation					
	board		differentiated		mulching	
Sequence of crops in a crop rotation	post-harvest residues	post-harvest residues + $N_{48}P_{18}K_{18}$	post-harvest residues	post-harvest residues + $N_{48}P_{18}K_{18}$	post-harvest residues	post-harvest residues + $N_{48}P_{18}K_{18}$
Complete fallow	-	-	-	-	-	-
Winter wheat	4.51	4.74	4.45	4.88	4.30	4.82
Sunflower	2.38	2.66	2.24	2.68	2.31	2.71
Spring barley	2.51	2.90	2.36	2.88	2.05	2.68
Corn	5.01	5.73	4.94	5.64	4.83	5.59
	Obtained per hectare of crop rotation area, t					
Total grain	2.41	2.67	2.35	2.68	2.23	2.62
Including winter wheat	0.90	0.95	0.89	0.98	0.86	0.96
Feeder grain	1.50	1.73	1.46	1.70	1.38	1.65
Grain yield	4.01	4.46	3.92	4.47	3.73	4.36
Output of feed units	3.57	3.98	2.87	3.99	3.35	3.92
Output of digestible protein	0.40	0.44	0.38	0.44	0.37	0.44
Output of grain units	3.26	3.62	3.15	3.64	3.08	3.59

increase of the humus coefficient and, accordingly, to the greater accumulation of humus not only by shallow mulching, but also by the use of board tillage.

In general, there was a tendency to improve the humus condition of the soil with systematic shallow (mulching) tillage in short crop rotations by reducing mineralization processes and increasing humification processes compared to plowing. The content of gross nitrogen and phosphorus in the soil changed little under the influence of the studied agricultural practices.

In addition to the positive aspects, mulch tillage has a significant drawback that consists in an increase in weed infestation of early grain crops and fallow crops by 1.4-1.6 times, row crops by 1.4-1.8 times, which in some years necessitates the use of soil and postemergent herbicides for reliable control of weed infestation of crop rotation fields, preventing a decrease in their productivity. The application of mineral fertilizers at a dose of N_{30-60} in combination with crop residues of the predecessor increases the competitiveness of cereals to weeds by increasing the optical density of crops. In general, according to the scale of I.V. Veselovskiy, weeds increased in crop rotation in ascending order: board tillage system – weed level is low, differentiated – medium, shallow (mulching) – high (Veselovskiy et al., 1998; Ivachenco, 2001).

The productivity of field crops in a 5-field grain-fallow-row crop rotation was determined mainly by the application of mineral fertilizers, rather than tillage. The main tillage systems on the plots fertilized with mineral fertilizers, together with crop residues, turned out to be equivalent in all productivity indicators: grain yield (2.42-2.68 t ha^{-1}), grain units (3.37-3.64 t ha^{-1}), fodder units (3.65-3.99 t ha^{-1}) and digestible protein (0.41-0.44 t ha^{-1}) per hectare of crop rotation area with a slight downward trend in shallow mulch tillage. In case of option with crop residues without mineral fertilizers, the system of board and differentiated tillage had an advantage in all productivity indicators, due to a slightly better nutritional schedule (Table 4). Grain yield with board tillage system was higher by 0.18 t ha^{-1} (7.5 %), grain units – 0.18 (5.5 %), fodder units – 0.22 (6.2 %), digestible protein – 0.03 t/ha of crop rotation area (7.5 %) compared to shallow mulching.

Mineral fertilizers applied in moderate doses ($N_{48}P_{18}K_{18}$ per 1 ha of crop rotation area), together with crop residues, significantly increased the productivity of the crop rotation as a whole. The maximum increase in grain yield from the use of $N_{48}P_{18}K_{18}$ with the board tillage system was 0.26 (9.7 %), grain units – 0.36 (9.9 %), fodder units – 0.41 (10.3%), digestible protein – 0.02 (5.0%) t ha^{-1} of crop rotation area. The introduction of $N_{48}P_{18}K_{18}$ with a differentiated processing system increased the yield of grain by 0.33 (12.3 %), grain units – 0.49 (13.5 %), fodder units – 1.12 (28.0 %), digestible protein – 0.06 (13.6 %) t ha^{-1} of crop rotation area. The use of $N_{48}P_{18}K_{18}$ in crop rotation with a shallow (mulching) tillage system gave an increase in grain yield by 0.39 (14.9 %), grain units – 0.51 (14.2 %), fodder units – 0.57 (14.5 %), digestible protein – 0.07 (15.9 %) t ha^{-1} of crop rotation area. According to the research results, the highest gains from mineral fertilizers in terms of productivity were inherent in a shallow (mulching) background with characteristic more stringent conditions of the phytosanitary state and nutritional schedule. The introduced mineral fertilizers in moderate doses increase the productivity of the crop rotation by more than 14 % compared with the board tillage system and somewhat better initial conditions of mineral nutrition.

The use of alternative mulching methods for the basic tillage (disking, chiselling, subsurface knifing) for field crops makes it possible to optimize the operating costs of tillage, in particular, to save fuel and energy resources when introducing disking 15.7-17.6 l ha^{-1}, chiselling 7.0-8.3, subsurface knifing 17.4-22.1 l ha^{-1}, which ultimately has a positive effect on conditionally net income and an increase in the level of profitability of agricultural products by 5-14%.

4 CONCLUSIONS

1. Agrophysical indicators of the soil, regardless of its processing, were within the optimal parameters. The density of the soil did not exceed the critical limit – 1.35 g cm^{-3} in the cultivated layer and was 1.18 for plowing, 1.25 for chiselling, 1.26 for subsurface knifing, and 1.26 g cm^{-3} for disk tillage. Soil hardness during plowing in a layer of 0-30 cm was minimal – 5.0-8.7 kg cm^{-2}, the use of chiselling, subsurface knifing and disking contributed to an increase in indicators up to 11.9, 12.1 and 13.3 kg cm^{-2}, respectively, without exceeding the optimal parameters up to 21 kg cm^{-2} for field crops. Structural analysis of the soil, carried out in the spring in a layer of 0-30 cm before pre-sowing cultivation, showed that, regardless of the methods of tillage, the amount of agronomically valuable structural aggregates with a size of 10-0.25 mm did not exceed 73.2-75.9 %. There was a tendency to increase the number of the most valuable structural aggregates with a size of 7-0.25 mm against the background of chisel and disc treatment in the presence of crop residues on the surface. There was a tendency to increase the number of the most valuable structural aggregates with a size of 7-0.25 mm against the background of chisel and disk treatment in the presence of crop residues on the surface.

2. To protect the soil from wind erosion (deflation), the most of conditional stubble on the surface remains, of course, in the early fallow (without tillage in autumn) – 630 pcs m^{-2}. A significant amount of it was also after disk

processing – 333 pcs m^{-2}. Early fallow is a reliable method to wind erosion (deflation) combat in the spring. Even strong winds with a speed of more than 15 m s^{-1} in early fallow are not able to blow out more than 5-12 g m^{-2} of soil in 5 minutes of exposure, while in case of board tillage these figures increase by 11-26 times and amount to 134 g m^{-2}.

3. Application of shallow (mulching) tillage provides additional accumulation of efficient moisture in the autumn-winter period by an average of 71-85 t ha^{-1} compared to board tillage due to the presence of post-harvest residues on the soil surface.

4. Systematic rolling in the soil (50 % with shallow mulching and almost complete with plowing) of biomass by-products of crop rotation, even without nitrogen to compensate, provided a deficit-free balance of humus. With the initial humus content of the arable layer of 4.2 %, after 6 years, in case of options without mineral fertilizers, but with leaving of crop residues, the content of total humus in the layer of 0-30 cm in the grain-fallow-row crop rotation was 4.22-4.28 % (higher by 0.02-0.08 percentage points (p.p.)), and in combination with the application of mineral fertilizers it increased by 4.23-4.32 %, or 0.03-0.13 p.p.

5. In terms of productivity, different tillage systems (board, differentiated, shallow (mulching)) in the 5-field crop rotation were found to be equivalent, except for the options without the application of mineral fertilizers, where the shallow (mulching) system was inferior to the differentiated and board by 5.5- 7.5 %.

The obtained results are of great importance for farms of various forms of land ownership in the steppe zone, as they help to preserve the fertility of chernozem and to protect it from moisture erosion, deflation, etc. The conducted research will be continued in a long-term stationary experiment to identify the dynamics of the balance of humus, erosion processes under climate change.

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The authors declare that they have no competing interests.

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Odstranjevanje potencialno strupenih kovin iz odpadnega blata iz čistilne naprave z uporabo EDTA

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Removal of potentially toxic metals from sewage sludge using EDTA

Abstract: Sewage sludge has the potential to be used as a fertilizer in agriculture because of its high nutritional value, but it is often contaminated with toxic metals (TM). This study investigated whether ReSoil® technology, based on the use of EDTA (50, 70, and 100 mmol l^{-1}), efficiently removes TM from sewage sludge collected after aerobic (blato1) and anaerobic (blato2) treatment. The highest removal efficiency of Pb was achieved in blato1 (up to 60 %) and of Zn and Cu in blato2 (up to 55 and 29 %, respectively). The content of nutrients did not change significantly after remediation, only available phosphorus decreased up to 1.7-times in blato2, but its content remained high (489-510 mg 100^{-1} g^{-1}). After remediation, the concentration of all metals, except Zn, in the leachates was below the limit for non-hazardous substances. To demonstrate the possibility of recycling process solutions and EDTA, blato2 was washed in 5 consecutive batches with 50 mmol l^{-1} washing solution, removing on average 28 % Pb, 48 % Zn, 35 % Cu, 30 % Mn, and 10 % Fe. ReSoil® technology removes metals from sludge and preserves its nutritional value. However, the efficiency of the technology depends on the treatment process used in the wastewater treatment plant.

Key words: EDTA; toxic metals; remediation; sewage sludge; wastewater treatment plants; aerobic biological treatment; anaerobic biological treatmen

Odstranjevanje potencialno strupenih kovin iz odpadnega blata iz čistilne naprave z uporabo EDTA

Izvleček: Odpadno blato iz čistilnih naprav (ČN) ima zaradi velike hranilne vrednosti potencial za uporabo v kmetijstvu, vendar je pogosto onesnaženo s potencialno strupenimi kovinami (PSK). V raziskavi smo preverili ali ReSoil® tehnologija, ki temelji na uporabi EDTA (50, 70 in 100 mmol l^{-1}), omogoča učinkovito odstranjevanje PSK iz odpadnega blata, vzetega po aerobni (blato1) in anaerobni (blato2) biološki obdelavi. Največji delež odstranitve Pb smo dosegli v blatu1 (do 60 %), največji delež odstranitve Zn in Cu pa v blatu2 (do 55 in 29 %). Vsebnost hranil se po remediaciji ni bistveno spremenila, le vsebnost dostopnega fosforja se je v blatu2 po remediaciji do 1,7-krat zmanjšala, vendar je še vedno ostala velika (489-510 mg 100^{-1} g^{-1}). Po remediaciji so bile koncentracije v izpirkih za vse kovine, razen za Zn, pod mejo za nenevarne snovi. Za dokazovanje možnosti recikliranja procesnih raztopin in EDTA smo blato2 oprali s 50 mmol l^{-1} pralno raztopino v seriji 5 zaporednih remediacij in pri tem v povprečju odstranili 28 % Pb, 48 % Zn, 35 % Cu, 30 % Mn in 10 % Fe. ReSoil® tehnologija sicer omogoča odstranjevanje PSK iz blata in pri tem ohrani njegovo hranilno vrednost, vendar je učinkovitost tehnologije odvisna od postopkov obdelave blata na ČN.

Ključne besede: EDTA; potencialno strupene kovine; remediacija; odpadno blato; čistilne naprave; aerobna biološka obdelava; anaerobna biološka obdelava

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1 UVOD

Pri čiščenju odpadnih voda čistilne naprave (ČN) proizvedejo ogromne količine odpadnega blata. Globalna proizvodnja odpadnega blata se ocenjuje na 45 milijonov ton (suhe snovi) letno, od tega je leta 2019 samo na Kitajskem proizvodnja odpadnega blata znašala 8 milijonov ton (suhe snovi) (Wei in sod., 2020). Po podatkih iz leta 2020, je v Evropi proizvodnja odpadnega blata znašala 13 milijonov ton (suhe snovi), kar je za 1,5 milijona tone več kot leta 2010 (Buta in sod., 2021). Naraščajoča proizvodnja blata iz čistilnih naprav vodi v zaskrbljujoče naraščanje razmerja med proizvedeno količino blata in kapaciteto ČN za njegovo obdelavo.

Zaradi velike hranilne vrednosti ima odpadno komunalno blato velik potencial za uporabo v kmetijstvu kot organsko gnojilo, saj je bogato z organsko snovjo in hranili, predvsem z dušikom (N) in fosforjem (P) (Hudcova in sod., 2019). Dodatek odpadnega blata lahko poveča vsebnost N, P in organskega ogljika v tleh, izboljša obstojnost strukturnih agregatov, poroznost, vodno zadrževalne lastnosti in biotske lastnosti tal (Hamdi in sod., 2019; Geng in sod., 2020). Roig in sod. (2012) so v 16-letni raziskavi dokazali, da gnojenje s pregnitim blatom povečuje vsebnost N in organske snovi ter mikrobno in encimsko aktivnost tal. Uporaba odpadnega blata izboljša talne lastnosti, kar lahko spodbudi rast rastlin in s tem poveča količino pridelka ter zmanjša potrebo po dragih sintetičnih gnojilih (Geng in sod., 2020).

Poleg velike vsebnosti hranil, odpadno blato vsebuje tudi strupena organska onesnažila, patogene organizme, mikroplastiko in potencialno strupene kovine (PSK), kar omejuje njegovo uporabo v kmetijstvu (Collivignarelli in sod., 2019). ČN uporabljajo številne tehnološke procese za obdelavo in nevtralizacijo odpadnega blata pred njegovo uporabo v kmetijstvu. Najpogosteje uporabljeni metodi stabilizacije odpadnega blata in mineralizacije organskih komponent sta aerobna in anaerobna razgradnja, ki omogočata zmanjšanje števila patogenih mikroorganizmov, odpravo neprijetnega vonja in preprečujeta nadaljnjo razgradnjo blata (Rorat in sod., 2019). Kljub temu pa PSK ostajajo ena glavnih težav odpadnega blata. PSK niso biorazgradljive in se s časom kopičijo v tleh, od koder zlahka prehajajo v podtalnico in rastline ter se preko prehranjevalne verige prenašajo v človeka (Suanon in sod., 2016). V državah EU je zato uporaba odpadnega blata v kmetijstvu regulirana z EU Direktivo (86/278/EGS), ki določa mejne vrednosti nekaterih kovin. Nekatere kovine so lahko strupene že pri majhnih koncentracijah, imajo kancerogen, mutagen in / ali teratogen potencial, zato predstavljajo nevarnost za okolje in zdravje človeka (Wuana in sod., 2010; Ali in sod., 2019). Med najbolj potencialno strupenimi kovinami in polkovinami so Cd, Cr, Cu, Hg, Ni, Pb, Zn in As (Ali in sod., 2019).

Gnojenje kmetijskih obdelovalnih površin z odpadnim blatom lahko dolgoročno vodi v kopičenje PSK v tleh (Iglesias in sod., 2018) in s tem povečanje onesnaženja. Pred uporabo odpadnega blata v kmetijstvu je zato odstranjevanje PSK nujno potrebno.

Za odstranjevanje kovin iz blata so v uporabi številne tehnike, kot so biološke metode (npr. biološko izpiranje z uporabo mikroorganizmov, vermikompostiranje), kemična ekstrakcija (npr. z organskimi in anorganskimi kislinami, kelatorji, solmi) in fizikalne metode (npr. temperaturna obdelava, elektrokinetična obdelava, ultrazvočna ekstrakcija) (Wen in sod., 2013; Suanon in sod., 2016; Hanay in sod., 2009; Babel in del Mundo Dacera, 2006). Kemična ekstrakcija je zaradi nezahtevnosti postopka, relativno kratkega časa ekstrakcije, velike učinkovitosti odstranitve kovin in nizke cene, najpogosteje uporabljena tehnika. S kovinami onesnažena tla ali blato se pri tem tretira z močnimi anorganskimi kislinami, kot so H_2SO_4, HCl, in HNO_3, organskimi kislinami, kot sta oksalna in citronska kislina ter kelatorji kot so nitrilotriocetna kislina (NTA), N,N-bis(karboksimetil)glutaminska kislina (GLDA) in etilendiamin tetraacetat (EDTA) (Babel in del Mundo Dacera, 2006). V dosedanjih raziskavah je bilo ugotovljeno, da ekstrakcija samo z enim reagentom ni dovolj učinkovita za odstranjevanje kovin. Kou in sod. (2020) so z uporabo EDTA v kombinaciji s citronsko, glutaminsko in asparaginsko kislino, odstranili značilno večji delež PSK kot z uporabi posameznega reagenta, in sicer največ do 76 % Zn, 68 % Ni, 14 % Cu, 16 % Pb in do 27 % Cr. V nekaterih raziskavah so za večjo učinkovitost dekontaminacije blata kemijsko ekstrakcijo združili s fizikalnimi metodami. Na primer, Li in sod. (2019) so z uporabo mikrovalovne pečice in ob dodatku žveplene kisline, ocetne kisline ali EDTA, učinkovito odstrani najmanj 90 % Cu, 70 % Zn in Pb, 45 % Fe in 20 % Ni. Uporaba elektromehanične metode remediacije ob dodatku EDTA učinkovito odstrani 88 % Zn, 78 % Cu in 58 % Pb (Pei in sod., 2016). Uporaba fizikalnih metod je precej draga, zato izvedljivost na večjem, komercialnem navju ni realna. Učinkovita kemijska ekstrakcija pa zahteva veliko količino reagentov (Geng in sod., 2020), zato je za ekonomsko izvedljivost dekontaminacije blata nujno potrebno recikliranje in ponovna uporaba reagentov.

Za remediacijo s kovinami onesnaženih tal smo pred kratkim razvili novo trajnostno tehnologijo ReSoil®, ki temelji na uporabi in recikliranju kelatorja EDTA in procesnih vod v zaprti zanki (Lestan, 2017; Gluhar in sod., 2021; Morales Arteaga in sod., 2022a). V predhodni raziskavi, Morales Arteaga in sod. (2022b) smo uspeli optimizirati novo ReSoil® tehnologijo za učinkovito dekontaminacijo odpadnega blata po anaerobni razgradnji

(> 90 % suhe snovi) v zaprtem procesu, brez proizvodnje odpadne vode. Učinkovitost odstranitve kovin je v veliki meri odvisna od uporabljenih postopkov čiščenja in obdelave blata na posamezni ČN, saj ti postopki vplivajo na kemijske lastnosti obdelanega blata. Blato proizvedeno v različnih ČN ima veliko variabilnost v svoji kemični sestavi (Tytła in sod., 2016), saj ČN uporabljajo različne postopke za obdelavo blata. Kljub porastu uporabe anaerobne biološke obdelave odpadnega blata na ČN v zadnjih letih, predvsem v zahodnoevropskih državah, še vedno ostaja določen odstotek ČN, kjer poteka samo aerobna stopnja obdelave blata (Hanum in sod., 2019). Glavni namen te raziskave je bil preveriti ali je optimizirana tehnologija učinkovita za dekontaminacijo blata neodvisno od kemijskih lastnosti blata, ki so povezane z načinom obdelave blata na ČN. V ta namen smo uporabili odpadno blato po aerobni biološki obdelavi ter blato po anaerobni biološki razgradnji iz ČN, ki uporablja drugačne postopke obdelave blata kot ČN, iz katere smo uporabili blato v predhodni raziskavi, Morales Arteaga in sod. (2022b). Namen te raziskave je bil določiti (i) optimalno koncentracijo reagentov za učinkovito dekontaminacijo blata, (ii) preveriti ali tehnologija omogoča recikliranje procesnih vod in EDTA v seriji 5 zaporednih remediacij in (iii) preveriti vpliv remediacije na lastnosti ter varnost blata za njegovo potencialno uporabo v kmetijstvu.

2 MATERIAL IN METODE

2.1 VZORCI

Vzorce odpadnega blata smo leta 2021 odvzeli na eni izmed čistilnih naprav v Sloveniji. Vzorce smo odvzeli na dveh tehnoloških stopnjah obdelave na ČN, ter ju poimenovali blato1 in blato2. Blato1 je vzorec blata, vzet iz sekundarne sedimentacijske posode po aerobni biološki obdelavi industrijskega in komunalnega blata na ČN po ločevanju vode in zgoščenega blata. Preseženo blato se nato črpa v anaerobna gnilišča z namenom zmanjšanja dostopnosti organskih snovi oziroma stabilizaciji blata. Vzorec blata, vzet po anaerobni razgradnji (digestat oziroma pregnito blato), smo poimenovali blato2. Vsebnost suhe snovi je v blatu1 znašala 1,3 %, v blatu2 pa 25 %. Vzorce blata smo pred uporabo posušili na 60 °C do konstantne suhosti in zmleli v mlinčku (velikost delcev < 2 mm).

2.2 PRANJE BLATA Z RAZTOPINO EDTA IN OBDELAVA PROCESNIH VOD

Za remediacijo odpadnega blata smo kot osnovo uporabili ReSoil® tehnologijo in jo ustrezno modificirali (Lestan, 2017; Gluhar in sod., 2021; Morales Arteaga in sod., 2022a). Odpadno blato smo oprali s pralno raztopino (PR) v razmerju suho blato:pralna raztopina = 1:7. Pralna raztopina je vsebovala 50 mmol l^{-1}, 70 mmol l^{-1} ali 100 mmol l^{-1} Ca-EDTA in H_2SO_4 v razmerju Ca-EDTA:H_2SO_4 = 1:1. Za kontrolo učinkovitosti delovanja kombinacije Ca-EDTA in H_2SO_4 v PR smo blato oprali s 50 mmol l^{-1} Ca-EDTA (kontrola za smiselnost uporabe H_2SO_4) in s 50 mmol l^{-1} H_2SO_4 (kontrola za smiselnost uporabe Ca-EDTA). Koncentracijo reagentov (50 mmol l^{-1}) v kontrolnih vzorcih smo izbrana glede na koncentracije regentov, ki smo jih uporabili za serijo 5 zaporednih remediacij. Ekstrakcija je potekala na krožnem stresalniku 1 h. Po 1 h smo vzorce centrifugirali 10 min na 4000 rpm. Supernatant smo shranili za nadaljnje meritve, blato pa prelili z vodo (izpiralna raztopina 1, IR1), premešali in ponovno centrifugirali pri enakih razmerah. Proces spiranja z vodo smo ponovili še 2x (izpiralna raztopina 2 in 3, IR2 in IR3), da smo iz blata odstranili morebitne ostanke kompleksa EDTA-kovine. Po zadnjem spiranju smo blato posušili na 105 °C do konstantne suhosti. Za nadaljnje analize smo suhe vzorce blata strli v terilnici in presejali skozi 250 µm sito.

Najboljše obravnavanje, glede na učinkovitost odstranitve kovin in porabo reagentov, smo nato izbrali za serijo 5 zaporednih remediacij, pri čemer smo reciklirali vse procesne raztopine in EDTA ter jih uporabili za remediacijo v naslednji seriji. Uporabljene procesne raztopine smo obdelali pri pH gradientu med 12,5 in 2. Za recikliranje EDTA v obliki Ca-EDTA ter za obarjanje in odstranjevanje strupenih kovin v obliki netopnih kovinskih hidroksidov, smo uporabljeno pralno raztopino (uPR) ter uporabljeno prvo in tretjo raztopino za izpiranje (uIR1, uIR3) naalkalili s CaO (pH 12,5, približno 30 min). Oborino in presežek $Ca(OH)_2$, nastal po hidraciji CaO, smo odstranili iz raztopine s centrifugiranjem (10 min na 4000 rpm). Po alkalni fazi smo uIR1, z dodatkom 96 % H_2SO_4, nakisali na pH 2 (120 min reakcija) ter tako oborili in s centrifugiranjem reciklirali preostalo EDTA v kisli obliki kot H_4EDTA. RS1 in RS3 smo po tej obdelavi uporabili v naslednji seriji za izpiranje blata. Druge raztopine za izpiranje (IR2) nismo obdelali in smo jo neposredno uporabili za izpiranje blata v naslednji seriji. Pred vsako naslednjo serijo remediacij smo v obdelano PR iz prejšnje serije dodali H_2SO_4 ter EDTA v obliki H_4EDTA, pridobljeno iz uIR1 in svežo EDTA, v obliki Na-EDTA, za nadomeščanje izgub kelatorja v procesu, predvsem zaradi vezave na trdo fazo blata. Tako pripravljena raztopina je tvorila reciklirano pralno raztopino (PR), ki se je v naslednji seriji uporabila za pranje blata. V naslednji seriji smo blato po pranju s PR izprali z obdelanimi izpiralnimi raztopinami v sledečem vrstnem

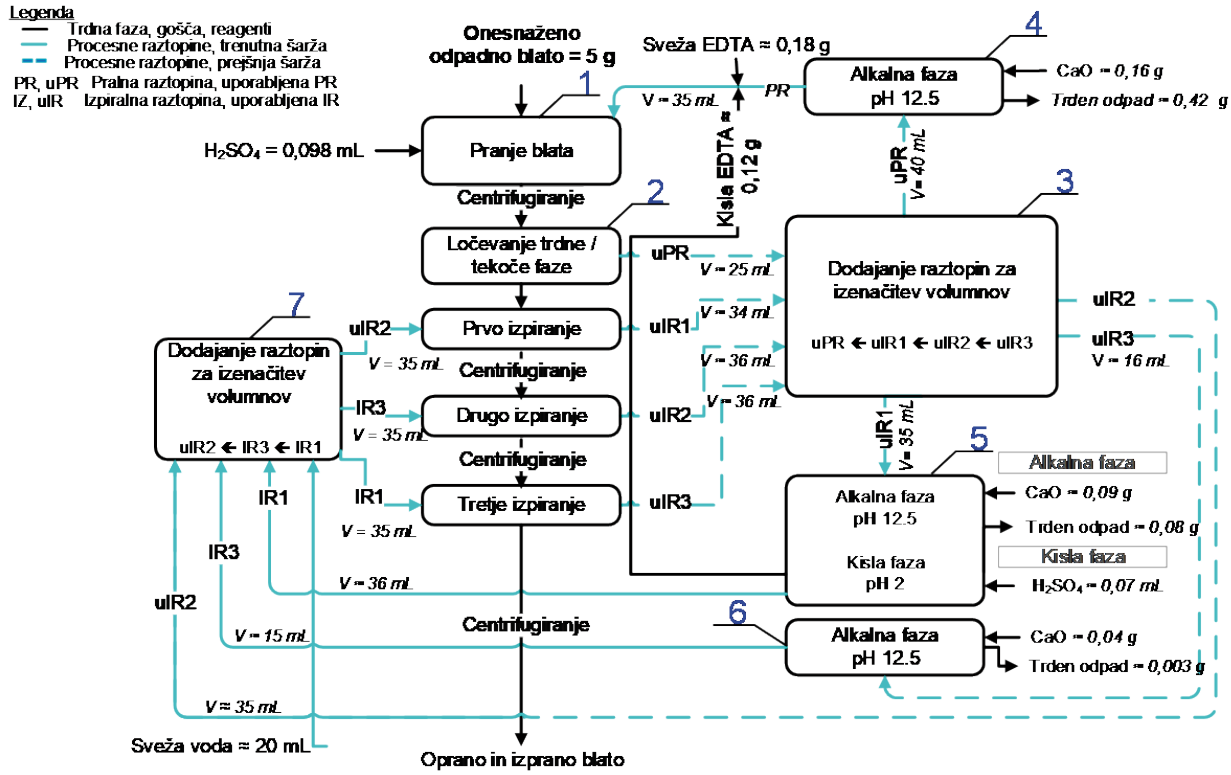
redu: IR2 → IR3 → IR1. V IR1 smo na koncu dodali manjšo količino sveže vode za kompenzacijo izgube procesne vode: zaradi razlik v vlagi vhodnega (suh) in izhodnega (nasičen) vzorca blata ter hidraciji apna. Shema procesa je prikazana na Sliki 1.

2.3 MERITVE EDTA

Za oceno kelatne aktivnosti EDTA v procesnih vodah po remediaciji smo uporabili spektrofotometrično metodo po Wang in sod. (2013), ki temelji na redukciji Fe^{3+} po dodatku Na_2SO_3. Nastali železovi ioni reagirajo z barvilom 1,10-fenantrolin monohidratom pri čemer pride do nastanka feroina. Izmerjena absorbanca feroina predstavlja osnovo za izračun koncentracije EDTA. Meja detekcije je bila 0,15 mmol l^{-1} EDTA.

2.4 KARAKTERIZACIJA ODPADNEGA BLATA

Vzorce blata smo okarakterizirali pred in po remediaciji. Delež suhe snovi oziroma vlažnost blata, smo določili po metodi sušenja na 105 °C do konstantne teže (ISO12880, 2000). Delež suhe snovi smo izračunali na podlagi masnega količnika med vlažnimi in suhimi vzorci. Vsebnost organske snovi, organskega ogljika (C_{org}) in skupnega dušika (TN), smo določili po suhem sežigu na instrumentu vario MAX CNS (Elementar) (ISO15936, 2012 in ISO 16168, 2012). Vsebnosti skupnega (TP) in



Slika 1: Okvirni diagram novega postopka pranja blata onesnaženih s potencialno strupenimi kovinami. (1) Pranje blata. (2) Ločevanje trdne in tekoče faze, spiranje blata. (3) Kompenzacija izgub vode v procesu. (4) Alkalizacija uPR. (5) Alkalizacija in zakisanje uIR1. (6) Alkalizacija uIR3. (7) Dodajanje drugih raztopin in sveže vode vsaki od procesnih raztopin, da se doseže končni volumen. PR in uPR označujeta pralno in uporabljeno pralno raztopino, IR1 in uIR1 predstavljata prvo in uporabljeno prvo raztopino za izpiranje, RS2 in uRS2 predstavljata drugo in uporabljeno drugo raztopino za izpiranje, RS3 in uRS3 predstavljata tretjo in uporabljeno tretjo raztopino za izpiranje. Modre črte označujejo pretok raztopin, črtkane modre črte označujejo pretok raztopin iz prejšnje serije, črne črte označujejo pretok trdnih snovi

Figure 1: The flow chart of the washing process of sewage sludge contaminated with toxic metals. Process steps: (1) sewage sludge washing, (2) solid-liquid separation and sludge rinsing, (3) compensation of water losses, (4) alkalization of uPR, (5) alkalization/acidification of uIR1, (6) alkalization of uIR3. (7) Addition of other solutions and fresh water to each of the process solutions to reach the final volume. PR, uPR denotes washing and used washing solution, IR1 and uIR1 represent first rinsing and used rinsing solution, IR2 and uIR2 represent second rinsing and used rinsing solution, IR3 and uIR3 third rinsing and used rinsing solution. Blue lines denote flow of solutions, dashed blue lines denote flow of solutions from previous batch, black lines denote flow of solids

rastlinam dostopnega fosforja (kot P_2O_5) smo izmerili kolorimetrično (ISO 13346, 2001; ÖNORML 1087, 1993).

2.5 DOLOČANJE IZPIRANJA (MOBILNOST) KOVIN

Varnost remediiranega blata za okolje smo preverili z določanjem kovin v vodnem izpirku. Suh vzorec blata smo v razmerju blato:deionizirana voda = 1:10 ekstrahirali 24 h na krožnem stresalniku. Po 24 h smo vzorce centrifugirali in v supernatantu izmerili pH in koncentracijo kovin v skladu z Uredbo o odlagališčih odpadkov (Ur. l. RS, št. 10) in nemško standardno metodo izpiranja (DIN 38414-S4), ki se v EU pogosto uporablja za ocenjevanje izpiranja kovin v tla in podtalnico ter za primerjavo z zakonsko določenimi mejami za odlaganje nevarnih odpadkov na odlagališča.

2.6 DOLOČANJE KOVIN

Koncentracijo kovin smo v blatu določili po kislinskem razkroju z zlatotopko (mešanica HNO_3 in HCl v razmerju 1:3) v mikrovalovni pečici (Mars Xpress, CEM MDS-2000), razredčili z deionizirano vodo (ISO 54321, 2020) in pomerili na AAS (Varian, AA240FS) oziroma AAS z elektrotermično atomizacijo v grafitni kiveti (GF-AAS, Agilent, 240Z AA). Prav tako smo pomerili kovine v procesnih vodah (pred in po obdelavi z apnom) in vodnih ekstraktih, ki smo jih predhodno filtrirali (45 µm celulozni acetat membranski filter). Meja detekcije za Pb, Zn, Cu, Cr, Mn in Fe, je bila 10, 10, 30, 20, 20 in 60 µg l^{-1}.

3 REZULTATI IN DISKUSIJA

Uporaba odpadnega blata iz čistilnih naprav kot gnojilo v kmetijstvu, je v Evropi urejena z Direktivo Sveta (86/278/EGS), ki je uvedla mejne vrednosti koncentracij za potencialno strupene kovine v obdelanem odpadnem blatu z namenom varovanja zdravja človeka. Primerjava koncentracij kovin v originalnem (ne-remediiranem) odpadnem blatu po aerobni (blato1) in anaerobni (blato2) stopnji obdelave v naši raziskavi z zakonodajo na podlagi Direktive EU in slovenske zakonodaje (Ur. l. RS, št. 62) je pokazala, da je vsebnost Pb (< 250 mg kg^{-1}) in Cr znotraj dovoljenih mejna (< 200 mg kg^{-1}), medtem ko koncentracije Zn (> 1200 mg kg^{-1}) in Cu (> 300 mg kg^{-1}) presegajo dovoljene mejne vrednosti (Preglednica 1). Elementi v sledovih, kot so Cu, Zn, Mo, Mn in Fe, so esencialna mikrohranila, potrebna za rast rastlin, vendar lahko pri velikih koncentracijah postanejo strupena (Parveen in sod., 2015). Poleg kovin, ki imajo v Uredbi določene mejne vrednosti, smo zaznali tudi veliko koncentracijo Mn in Fe. Velika koncentracija Fe je predvsem posledica dodajanja Fe na ČN za lažje ločevanje vode in blata (Wei in sod., 2018). Glede na literaturo lahko koncentracije potencialno strupenih kovin v odpadnem blatu razvrstimo v naslednjem vrstnem redu: Zn > Cu > Cr > Ni > Pb > Cd (Kowalik in sod., 2021), kar je skladno tudi z našimi rezultati (Zn > Cu > Cr > Pb, Preglednica 1). Uporaba blata, ki izpolnjuje zahteve glede dovoljene vsebnosti PSK za kmetijske namene na podlagi direktive EU, lahko predstavlja veliko tveganje za geoakumulacijo v tleh (Kowalik in sod., 2021), zato je za kmetijske namene koncentracijo kovin potrebno čim bolj zmanjšati.

V raziskavi, ki smo jo izvedli pred kratkim (Morales Arteaga in sod., 2022b) smo pokazali, da je tehnologija ReSoil®, sicer razvita za čiščenje onesnaženih tal, dovolj robustna, da omogoča dekontaminacijo peletov dehidriranega blata z > 90 % suhe snovi, vzetega po anaerobni stopnji obdelave. Z novo ReSoil® tehnologijo smo iz dehidriranega blata odstranili 34-43 % Pb, 56-64 % Zn, 57-62 % Cu, 15-25 % Cr, 20-35 % Mn in 0-7 Fe (Morales Arteaga in sod., 2022b).

Lastnosti blata variirajo tako med različnimi ČN, kot znotraj posameznega obrata, zaradi različnih tehnoloških pristopov obdelave in sprememb v sestavi vhodnega vzorca. Ker je učinkovitost odstranitve kovin z ReSoil® tehnologijo v prvi vrsti odvisna od kemijskih lastnosti vzorca, je za preverjanje učinkovitosti tehnologije in njenega potenciala za morebitno komercialno uporabo potrebno raziskati, ali tehnologija omogoča dekontaminacijo blata neodvisno od načina njegove obdelave na ČN. V ta namen samo uporabili blato iz druge ČN kot v predhodni študiji (Morales Arteaga in sod., 2022b) ter vzorce odvzeli po aerobni biološki obdelavi (blato1) in po anaerobni biološki obdelavi (blato2). Lastnosti blata1 in blata2 so prikazane v Preglednici 1.

3.1 UČINKOVITOST ODSTRANITVE KOVIN

Optimalno razmerje med blatom in pralno raztopino, čas ekstrakcije in reagente (EDTA, H_2SO_4, oksalna kislina, ditionit) smo določil v raziskavi Morales Arteaga in sod. (2022b), medtem ko smo koncentracijo reagentov določili v tej raziskavi, saj je koncentracija EDTA odvisna od kemijskih lastnosti blata. Kot je razvidno na Sliki 2, smo z uporabo EDTA in H_2SO_4 iz blata uspešno odstranili Pb, Zn, Cu, Mn in Fe. Za razliko od prve raziskave (Morales Arteaga in sod., 2022b), v kateri smo v 10 zaporednih serijah odstranili 15-25 % Cr, Cr s koncentracijo 71,4 ± 6,9 mg kg^{-1} v blatu1 in 103,4 ± 2,6 mg kg^{-1} v blatu2

Preglednica 1: Kemijske lastnosti originalnega (Orig) in remediiranega (50, 70 in 100 mmol l^{-1}) blata1 (aerobna stopnja obdelave) in blata2 (anaerobna stopnja obdelave). Analiza za pH, organsko snov, C_{org}, TN, TP in P_2O_5 je narejena na združenem vzorcu 3 ponovitev. Koncentracije kovin so prikazane kot povprečne vrednosti s standardno napako (n = 3)
Table 1: The chemical properties of the original (Orig) and remediated (50, 70 in 100 mmol l^{-1}) blato1 (sewage sludge after aerobic treatment) and blato2 (sewage sludge after anaerobic treatment). Analyses of pH, organic matter (organska snov), organic C (C_{org}), TN, TP, and P_2O_5 refer to the combined sample of 3 replicates. Data for metal concentrations are given as average values with standard error (n = 3)

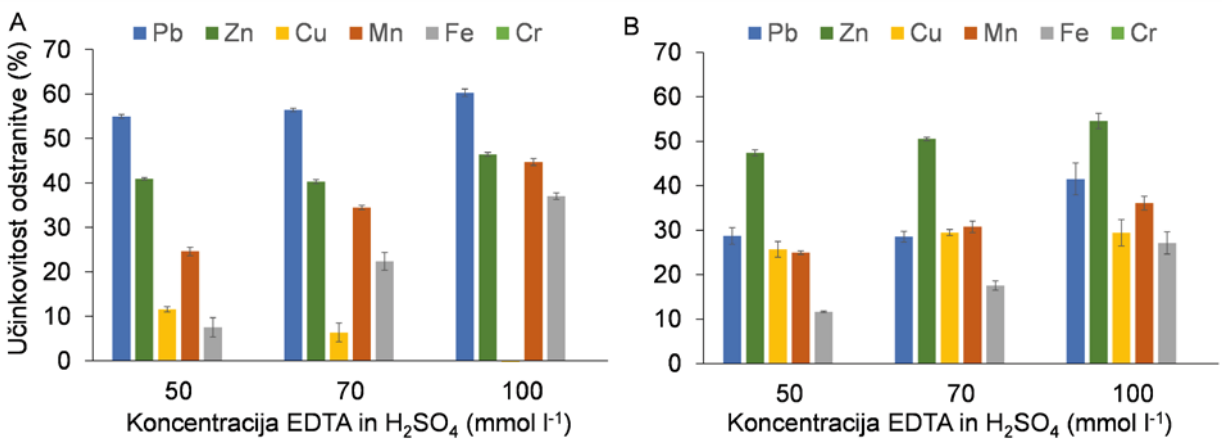
	Blato1				Blato2			
	Orig	50 mmol l^{-1}	70 mmol l^{-1}	100 mmol l^{-1}	Orig	50 mmol l^{-1}	70 mmol l^{-1}	100 mmol l^{-1}
ph	7,2	6,2	6,1	6,0	7,5	7,0	6,7	6,7
Organska snov (%)	59,6	64,6	65,9	67,0	51,4	54,6	54,8	56,2
C_{org} (%)	34,6	37,5	38,2	38,9	29,8	31,7	31,8	32,6
TN (%)	5,4	5,3	5,4	5,5	4,2	4,3	4,3	4,4
TP (g kg^{-1})	27,2	25,3	22,5	20,4	29,0	29,8	27,8	25,6
P_2O_5 (mg $100^{-1}g^{-1}$)	10,0	10,8	11,7	14,8	867,7	510,1	543,1	498,3
Kovine								
Pb (mg kg^{-1})	78,7 ± 0,3	35,5 ± 0,7	34,3 ± 0,3	31,3 ± 0,3	62,6 ± 1,4	44,6 ± 2,2	44,7 ± 0,8	36,6 ± 1,1
Zn (mg kg^{-1})	1258,5 ± 26,5	743,2 ± 5,1	751,4 ± 5,6	674,1 ± 3,1	1450 ± 30,1	762,8 ± 24,6	717,5 ± 5,4	658,9 ± 9,9
Cu (mg kg^{-1})	357,5 ± 2,6	316,0 ± 4,9	334,7 ± 7,6	368,3 ± 2,2	423,5 ± 1,7	300,6 ± 6,5	298,7 ± 2,9	298,9 ± 7,4
Cr (mg kg^{-1})	71,4 ± 6,9	108,1 ± 13,5	113,5 ± 6,1	133 ± 7,0	103,4 ± 2,6	128,6 ± 3,1	130,9 ± 1,4	126,5 ± 5,8
Mn (mg kg^{-1})	347,1 ± 1,5	261,7 ± 2,7	227,4 ± 1,7	191,9 ± 3,2	337,9 ± 1,7	253,6 ± 5,2	233,9 ± 4,4	216,0 ± 1,3
Fe (g kg^{-1})	46,9 ± 0,7	43,4 ± 0,4	36,4 ± 0,9	29,5 ± 1,0	50,0 ± 0,2	44,2 ± 1,3	41,2 ± 0,5	36,4 ± 0,0

v tej raziskavi nismo uspeli odstraniti. V predhodni raziskavi (Kolbl Repinc in sod., 2021) v kateri smo uporabili blato iz iste čistilne naprave, enako blatu1, smo ugotovili, da je 79 % celotnega Cr v blatu vezanega na organsko snov in 20 % vezanega na rezidualni del, kar pomeni, da je le 1 % Cr vezanega na dostopnejše frakcije (Kolbl Repinc in sod., 2021), kar bi lahko razložilo močnejšo vezavo Cr v blatu in s tem neučinkovitost vezave Cr na EDTA.

Največjo stopnjo odstranitve v blatu1 v tej raziskavi, smo dosegli pri Pb (55-60 %), sledili so Zn (40-47 %), Mn (25-45 %), Fe (8-37 %) in Cu (0-12 %) (Slika 2A). V blatu2, smo največjo učinkovitost odstranitve dosegli pri Zn (47-55 %), ki so mu sledili Pb (29-42 %), Mn (25-36 %), Cu (26-30 %) in Fe (12-28 %) (Slika 2B). Remediacija blata samo s H_2SO_4 ni bila uspešna, saj nismo odstranili nobene izmed kovin. Prav tako je bila učinkovitost odstranitve kovin samo s Ca-EDTA majhna in je znašala 12 % za Pb, 32 % za Zn, 19 % za Cu, 13 % za Mn in 0 % za Fe. Ca-EDTA ima v primerjavi z Na-EDTA počasnejšo kinetiko kemijske reakcije, zato je za učinkovito odstranjevanje kovin potrebna več kot 10 h ekstrakcija (Jez in Lestan, 2016; Gluhar in sod., 2021). Dodatek H_2SO_4 v PR aktivira Ca-EDTA preko znižanja pH vrednosti in tvorbe netopnega kompleksa s Ca (v $CaSO_4$), hkrati se zmanjša čas ekstrakcije na 1 h. Kot smo pokazali v nedavni raziskavi (Morales Arteaga in sod., 2022b), s H_2SO_4 aktivirana EDTA za 2,2-krat poveča odstranitev Pb iz tal v primerjavi s samo EDTA.

Primerjava učinkovitost odstranitve PSK v obeh vzorcih blata kaže, da je bila odstranitev Pb v blatu1 med 1,5-2,0-krat večja kot v blatu2 (Slika 2). Odstranitev glavnih onesnažil blata, Zn in Cu, s koncentracijami nad zakonsko določenimi mejami, je bila v blatu2 večja kot v blatu1. Odstranitve Zn je bila v blatu2 v povprečju sicer 1,2-krat večja kot v blatu1, vendar je koncentracija v obeh vzorcih blata (Preglednica 1) značilno padla pod mejno vrednost. Največje razlike med blatom1 in blatom2 v učinkovitosti odstranitve smo opazili pri Cu (Slika 2). Učinkovitost odstranitve Cu iz blata1 je bila med 0 in 12 %, kar je za najmanj 2,2-krat manj kot v blatu2 (Slika 2). Koncentracija Cu v blatu2 je bila po remediaciji v povprečju med 299-301 mg kg^{-1}, kar je ravno na nivoju mejne vrednosti 300 mg kg^{-1} (Ur. l. RS, št. 62), medtem ko je koncentracija Cu v blatu1 (316-368 mg kg^{-1}) presegala dovoljeno mejo tudi po remediaciji (Preglednica 1). Razlike v učinkovitosti odstranitve Cu iz blata1 in blata2 se najverjetneje nahaja v različni moči vezave Cu v obeh vzorcih blata. V zgoraj omenjeni raziskavi (Kolbl Repinc in sod., 2021) smo ugotovili, da je v blatu po aerobni obdelavi iz iste ČN 96 % celotnega Cu vezanega na organsko snov in 2 % na preostanku, kar pomeni, da je le slaba 2 % Cu vezanega na dostopnejše frakcije (1,3 % vezanega na vodotopno fazo, 0,5 % na izmenljivo fazo in 0,2 % vezanega na karbonate). V raziskavi Jenkins in Scheybeler (1981) je bila uspešnost odstranitve Cu s H_2SO_4 iz blata le 1 %, kar so razložili z vezavo večine Cu v blatu na organski kompleks, tako kot v naši raziskavi, ter domnevo, da je Cu v kompleksu močneje vezan z organsko snovjo kot ostale kovine. Pri anaerobni razgradnji kislinske bakterije v odsotnosti kisika razgradnjo organske snovi v nižje organske kisline (Roš, 2001), kar bi lahko povečalo dostopnost Cu v blatu2 in s tem njegovo vezavo z EDTA kompleksom.

Največji odstotek odstranitve kovin smo načeloma opazili pri blatu opranem z največjo koncentracijo EDTA (Slika 2). Da učinkovitost odstranitve kovin narašča z naraščanjem koncentracije EDTA poročajo tudi Ren in sod. (2015). Optimalna koncentracija EDTA je v njihovi razis-



Slika 2: Učinkovitost odstranitve kovin (%) v blatu1 (A) in blatu2 (B)
Figure 2: Removal efficiency of metals (%) in sewage sludge: blato1 (A) and blato2 (B)

kavi znašala 125 mmol l^{-1}, pri čemer so uspeli odstraniti 48 % Pb, 39 % Zn, 43 % Cd, 40 % Cr in 20 % Cu (Ren in sod., 2015). V primerjavi z Ren in sod. (2015) smo z uporabo manjših koncentracije EDTA, manjšega razmerja blato:PR (1:7 vs 1:10) in krajšega časa ekstrakcije (1 h vs 24 h) dosegli boljšo učinkovitost odstranitve Zn, Cu in Pb (Slika 2). Razlike v učinkovitosti odstranitve PSK med najmanjšo in največjo koncentracijo EDTA so bile relativno majhne, zato smo za nadaljnja testiranja možnosti recikliranja procesnih vod izbrali najmanjšo koncentracijo, 50 mmol l^{-1}, saj ravno reagenti predstavljajo glavni strošek ReSoil® tehnologije. Z uporabo 50 mmol l^{-1} PR smo v blatu1 odstranili 55 % Pb, 41 % Zn, 12 % Cu, 25 % Mn in 8 % Fe ter v blatu2 29 % Pb, 47 % Zn, 28 % Cu, 25 % Mn in 12 % Fe (Slika 2).

Rezultati kažejo, da je s H_2SO_4 aktivirana EDTA učinkovita za dekontaminacijo blata tako po aerobni kot anaerobni obdelavi ter da je učinkovitost odstranitve posamezne kovine odvisna od stopnje obdelave blata na ČN.

3.2 VPLIV REMEDIACIJE NA MOBILNOST KOVIN

Z namenom določanja mobilnosti kovin in s tem varnosti remediiranega blata za okolje, smo izmerili koncentracije kovin v vodnih ekstraktih. Mobilnost Pb, Zn, Cu in Cr je bila v obeh originalnih vzorcih blata pod mejnimi vrednostmi za nenevarne snovi določenimi z Ur. l. RS, št. 10 in DIN 38414-S4 (Preglednica 2). Kot je razvidno iz Preglednice 2, je bila mobilnost vseh kovin v blatu2 vsaj za polovico manjša kot v blatu1, kar kaže na stabilizacijo kovin po anaerobni biološki obdelavi.

Po remediaciji se je mobilnost Pb v blatu1 zmanjšala za med 1,9-krat in 5,5-krat v primerjavi z originalnim blatom1, medtem ko je v blatu 2 opranem s 50 in 70 mmol l^{-1} PR mobilnost Pb bila podobna, v blatu2, opranem s 100 mmol l^{-1} PR, pa za 1,8-krat večja kot v originalnem vzorcu (Preglednica 2). Mobilnost Cu in Cr se je po remediaciji povečala v obeh vzorcih blata, in sicer za med 1,3- in 2,1-krat za Cu in med 1,5- in 2,3-krat za Cr v primerjavi z originalnimi vzorci (Preglednica 2). Kljub temu so bile koncentracije Pb, Cu in Cr po remediaciji krepko pod mejnimi vrednostmi izpiranja za nenevarne odpadke v obeh vzorcih blata. Največje povečanje mobilnosti po remediaciji smo opazili pri Zn: koncentracija Zn v remediiranem blatu1 je bila 2,2-4,1-krat večja, v blatu2 pa kar za 10,0-14,5-krat večja od originalnega blata (Preglednica 2). Koncentracije Zn so v vseh vzorcih blata po remediaciji presegale dovoljeno koncentracijo za nenevarne snovi, razen s 50 mmol l^{-1} PR oprano blato2 (Preglednica 2). Mobilnost Mn in Fe, ki sicer nista zakonsko regulirana, se je v izpirkih po remediaciji prav

Preglednica 2: Koncentracije kovin v vodnih ekstraktih (mg kg^{-1}) originalnega (Orig) in remediiranega (50, 70 in 100 mmol l^{-1}) blata1 (aerobna stopnja obdelave) in blata2 (anaerobna stopnja obdelave). Podatki so prikazani kot povprečne vrednosti s standardno napako (n = 3)

Table 2: The concentration of metals in leachate (mg kg^{-1}) in the original (Orig) and remediated (50, 70 in 100 mmol l^{-1}) blato1 (sewage sludge after aerobic treatment) and blato2 (sewage sludge after anaerobic treatment). The data are given as average values with standard error (n = 3)

	Blato1				Blato2			Mejne vrednosti izpiranja mg kg^{-1}
	Orig	50 mmol l^{-1}	70 mmol l^{-1}	100 mmol l^{-1}	Orig	50 mmol l^{-1}	70 mmol l^{-1}	100 mmol l^{-1}	
Pb	1,13 ± 0,01	0,21 ± 0,00	0,41 ± 0,02	0,58 ± 0,01	0,47 ± 0,08	0,44 ± 0,00	0,51 ± 0,00	0,82 ± 0,01	10
Zn	25,25 ± 0,38	51,10 ± 1,50	68,95 ± 1,15	104,25 ± 1,85	4,66 ± 0,09	46,50 ± 0,70	62,17 ± 0,35	67,65 ± 0,81	50
Cu	15,21 ± 0,85	19,51 ± 0,21	19,21 ± 0,09	21,66 ± 0,49	7,93 ± 0,04	17,90 ± 0,61	18,32 ± 0,62	14,62 ± 0,08	50
Cr	0,27 ± 0,00	0,38 ± 0,00	0,41 ± 0,00	0,46 ± 0,00	0,09 ± 0,00	0,21 ± 0,00	0,22 ± 0,00	0,20 ± 0,00	10
Mn	3,12 ± 0,00	1,39 ± 0,00	1,89 ± 0,00	2,60 ± 0,00	1,65 ± 0,00	1,21 ± 0,00	1,21 ± 0,00	1,53 ± 0,00	
Fe	192,3 ± 0,4	139,3 ± 0,7	186,2 ± 1,2	249,4 ± 0,7	40,4 ± 0,8	156,8 ± 1,0	155,8 ± 1,3	162,5 ± 0,9	

[1]Mejne vrednosti izpiranja nenevarnih snovi v skladu z Uredbo o odlagališčih odpadkov (Ur. l. RS, št. 10, 2014) in DIN 38414-S4, ki določata kriterije in postopke za prevzem odpadkov na odlagališčih (L/S = 10 l kg^{-1})

tako spremenila: koncentracija Mn je bila v vseh remediiranih vzorcih manjša, medtem ko je bila koncentracija Fe v blatu1 opranem s 50 in 70 mmol l^{-1} PR manjša, v vseh ostalih vzorcih pa večja kot v originalnem vzorcu (Preglednica 2).

V skladu s pričakovanji je koncentracija kovin v vodnih ekstraktih naraščala z naraščanjem koncentracije reagentov v PR, saj EDTA lahko poveča mobilnost kovin (Chen in Cutright, 2001), kar še dodatno potrjuje izbiro 50 mmol l^{-1} PR za nadaljnje poskuse.

3.3 VPLIV REMEDIACIJE NA KEMIJSKE LASTNOSTI BLATA

Z namenom vrednotenja vpliva remediacije na kakovost odpadnega blata, namenjenega uporabi v kmetijstvu, smo določili vsebnost organske snovi, organskega C (C_{org}), skupnega N (TN) ter predvsem vsebnost skupnega P (TP) in rastlinam dostopnega P (kot P_2O_5). Povprašev anje po P gnojilih se povečuje in pričakovati je, da bodo zaloge ekonomsko upravičenega pridobivanja fosforja z izkopavanjem fosfatne rude oziroma s fosfatom bogatega minerala apatita v naslednjih 50-100 letih izčrpane (Černe, 2017), zato je pridobivanje P iz odpadkov in drugih alternativnih virov ključnega pomena. Blato iz ČN je bogat vir hranil, zato je cilj novega postopka remediacije, poleg odstranitve kovin, ohraniti vsebnost P in ostalih hranil. Znano je namreč, da pranje z EDTA spodbuja razgradnjo organske snovi in povzroča izgube C_{org} (Jez in sod., 2021). Ren in sod. (2015) na primer ugotavljajo, da se po pranju z EDTA v blatu zmanjša vsebnost organske snovi, TN, TP in zniža pH vrednost, koncentracija rastlinam dostopnega P pa je ostala skoraj nespremenjena. Tudi Kou in sod. (2020) poročajo o zmanjšanju vsebnosti TN in TP po pranju blata z EDTA v kombinaciji z organskimi kislinami, medtem je bila vsebnost organske snovi in dostopnega P po remediaciji v nekaterih kombinacijah večja kot v originalnem blatu.

Kot je prikazano v Preglednici 1 se hranilna vrednost blata po remediaciji ni bistveno spremenila. Vsebnost TN je po remediaciji v obeh vzorcih blata ostala skoraj nespremenjena, prav tako nismo zaznali večjih razlik v vsebnosti organske snovi in C_{org} (Preglednica 1). Koncentracija TP je po remediaciji v blatu2 (25,6-29,8 g kg^{-1}) ostala skoraj nespremenjena, medtem ko je bila koncentracija TP v blatu1 po remediaciji (20,4-25,3 g kg^{-1}) za 1,1-1,3-krat manjša kot v originalnem blatu (27,2 g kg^{-1}) (Preglednica 1). Koncentracija rastlinam dostopnega fosforja, izraženega kot P_2O_5, se je po remediaciji v blatu1 povečala (med 1,1- in 1,5-krat), v blatu2 pa zmanjšala (1,6- in 1,7-krat) (Preglednica 1). Kljub temu, je bila koncentracija rastlinam dostopnega P v remediiranem blatu2 za 34-47-krat (498-543 mg 100^{-1} g^{-1}) večja kot v remediiranem blatu1 (11-15 mg 100^{-1} g^{-1}) (Preglednica 1). Razlog za to je precej večja koncentracija dostopnega P v originalnem blatu2 (868 mg 100^{-1} g^{-1}), in sicer 87-krat večja kot v blatu1 (10 mg 100^{-1} g^{-1}) (Preglednica 1). V anaerobnih razmerah fosfat akumulirajoče bakterije za privzem organskih spojin sprostijo fosfat iz svojih celic (Ubukata, 2006), kar bi lahko pojasnilo razlike v dostopnem P med blatoma 1 in 2.

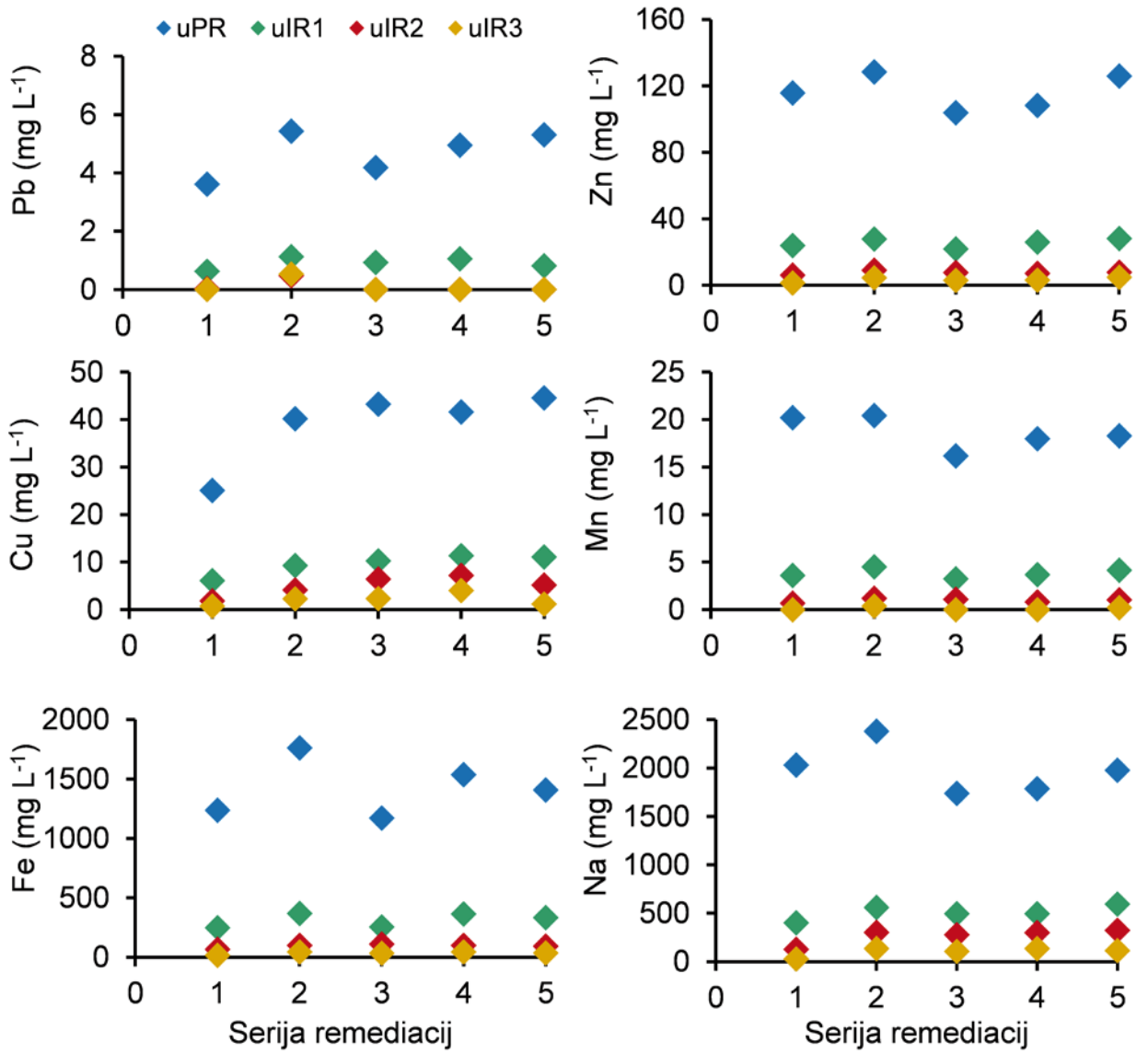
Po remediaciji smo v vseh vzorcih opazili znižanje pH vrednosti (Preglednica 1). Razlog je dodatek H_2SO_4 za aktivacijo Ca-EDTA in recikliranje EDTA iz PR1 (opisano v Poglavju 2.2, Slika 1).

3.4 SERIJA ZAPOREDNIH REMEDIACIJ Z RECIKLIRANJEM RAZTOPIN IN EDTA

Za preverjanje možnosti recikliranja procesnih vod in EDTA, smo uporabili blato2 zaradi: (i) lažje priprave vzorca pred remediacijo – zaradi velikega deleža vode (98,7 %) v blatu1 bi bile potrebne velike količine blata, ki bi ga bilo potrebno centrifugirati; (ii) večje učinkovitost odstranitve Zn in Cu, glavnih onesnažil blata; (iii) majhne mobilnost PSK, koncentracije vseh kovin pod mejnimi vrednostmi za nenevarne snovi; in (iv) večje koncentracije skupnega in rastlinam dostopnega P. Blato2 smo v seriji 5 zaporednih remediacij oprali s 50 mmol l^{-1} PR, kot je razloženo v Poglavju 2.2. Povprečna koncentracija kovin v seriji 5 zaporednih remediacij je znašala 44,7 ± 1,0 mg kg^{-1} Pb, 705,7 ± 13,3 mg kg^{-1} Zn, 308,5 ± 5,0 mg kg^{-1} Cu, 237,7 ± 4,0 mg kg^{-1} Mn in 51,5 ± 4,0 g kg^{-1} Fe. Učinkovitost odstranitve kovin je v seriji 5 zaporednih remediacij bila podobna oziroma nekoliko večja kot v predposkusu in je v povprečju znašala

Preglednica 3: Učinkovitost odstranitve kovin (%) iz blata2 v seriji 5 zaporednih remediacij opranih s 50 mmol l^{-1} pralno raztopino
Table 3: Removal efficiency of metals (%) in blato1 (A) and blato2 (B) over the 5 consecutive remediation batches washed with 50 mmol l^{-1} washing solution

	Učinkovitost odstranitve (%)				
Serija	Pb	Zn	Cu	Mn	Fe
1	30	49	38	32	0
5	30	49	36	31	0
3	22	44	32	26	12
4	29	48	37	31	17
5	32	48	34	32	21

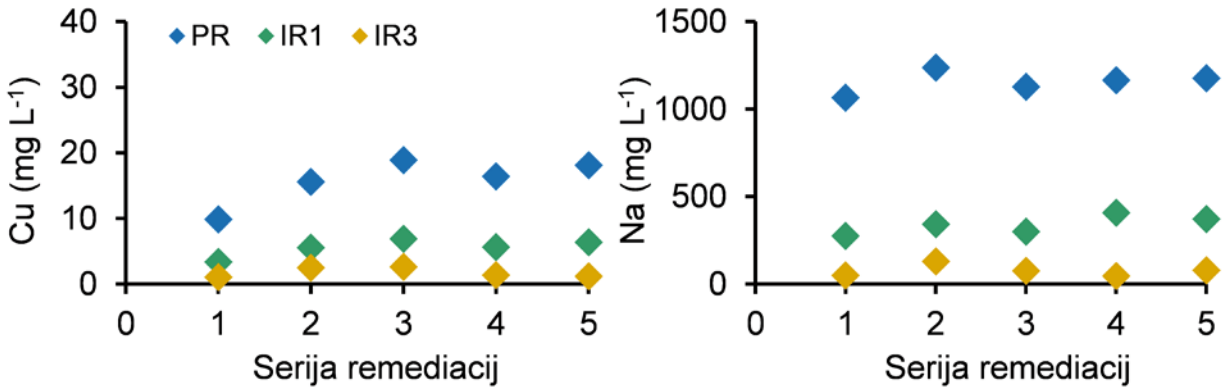


Slika 3: Koncentracija kovin v uporabljeni pralni, prvi izpiralni, drugi izpiralni in tretji izpiralni raztopini (uPR, uIR1, uIR2 in uIR3) v seriji 5 zaporednih remediacij
Figure 3: The concentration of metals in used washing, first, second, and third rinsing solutions (uPR, uIR1, uIR2, and uIR3, respectively) over the 5 consecutive remediation batches

28 % za Pb, 48 % za Zn, 35 % za Cu, 30 % za Mn in 10 % za Fe (Preglednica 3).

Pri ReSoil® se procesne vode vsakič znova uporabljajo v zaprtem procesu, zato je pomembno ohraniti njihovo kakovost. Kot indikatorje kakovosti smo po vsaki seriji v uporabljenih procesnih vodah izmerili koncentracije kovin, Na in EDTA. Koncentracije Pb, Zn, Mn in Fe so v uporabljeni PR (uPR) nihale, vendar trenda naraščanja, ki bi povzročil poslabšanje raztopin ni bilo opaziti (Slika 3). Koncentracija Cu se je po prvi seriji remediacij v uPR sicer za 1,6-krat povečala, vendar se je koncentracija po drugi seriji stabilizirala med 40 in 44 mg l^{-1} (Slika 3). Podobno kot v predhodni raziskavi, Morales Arteaga in sod. (2022b), smo v primerjavi s Pb, Zn, Cu in Mn v uPR opazili visoko koncentracijo Fe (Slika 3), ki smo ga v alkalni fazi, kot je razloženo v nadaljevanju, uspešno odstranili v obliki Fe hidroksidov, saj je koncentracija Fe v obdelani PR, IR1 in IR3 padla pod mejo kvantifikacije.

Koncentracija kovin je z vsakim naslednjim izpiranjem značilno padala in je v uporabljeni izpiralni raztopini 3 (uIR3) bila za večino kovin okrog 0, le pri Fe in Na so bile koncentracije nekoliko večje (16-44 mg l^{-1} za Fe



Slika 4: Koncentracija Cu in Na v pralni, prvi izpiralni in tretji izpiralni raztopini (PR, IR1, IR3) po obdelavi raztopin s CaO v seriji 5 zaporednih remediacij
Figure 4: The concentration of Cu and Na in washing, first, and third rinsing solutions (PR, IR1, and IR3, respectively) after CaO treatment over the 5 consecutive remediation batches

in 27-135 mg l^{-1} za Na) (Slika 3). V ReSoil® tehnologiji remediiran vzorec po pranju z EDTA 3-krat speremo z vodo, da odstranimo večino strupenih kelatov, saj zaradi slabe biološke razgradljivosti in potencialnega izpiranja, lahko EDTA predstavlja težavo za okolje (Bloem in sod., 2017). Kot smo pokazali v predhodnih raziskavah, strupene emisije ne prestavljajo težav v ReSoil® tehnologiji (Kaurin in sod., 2020; Gluhar in sod., 2021).

Uporabljene procesne raztopine (uPR, uIR1, uIR3) smo obdelali in reciklirali v močnem alkalno-kislem pH gradientu. Pri visoko alkalnih razmerah (pH 12,5), ki jih dosežemo z dodatkom CaO, se kovine v EDTA kompleksu zamenjajo s Ca, sproščene kovine pa se oborijo kot hidroksidi, ki jih lahko odstranimo s centrifugiranjem (Slika 1, Lestan, 2017; Gluhar in sod., 2021). Z dodatkom CaO smo iz uPR, uIR1 in uIR3 popolnoma odstranili Pb, Zn, Mn in Fe, saj je koncentracija teh kovin padla pod mejo kvantifikacije. Le v obdelani PR smo izmerili Cu in Na. Koncentracija Cu se je v PR po dodatku apna zmanjšala za v povprečju 2,5-krat in je po drugi seriji remediacij dosegla vrh, saj so koncentracije med drugo in peto serijo nihale med 16 in 19 mg l^{-1} (Slika 4). Do podobnih opažanj smo prišli tudi v naši predhodni raziskavi, kjer je koncentracija Cu dosegla vrh v peti seriji in se do desete ni več bistveno spreminjala (Morales Arteaga in sod., 2022b). Razlog za slabšo odstranitev Cu iz uPR bi lahko bil večja stabilnost Cu-EDTA kelata v alkalnih razmerah v primerjavi s Pb- in Zn-EDTA (Kim in sod., 2003).

Podobno kot pri Cu, je bila koncentracija Na v uporabljeni in obdelani PR velika. Razlog za prisotnost Na je redno dodajanje sveže EDTA v obliki Na-EDTA, saj pride med samim procesom do manjših izgub EDTA. Kljub temu se Na, tako kot Cu, v raztopinah ni kopičil, saj je bila njegova koncentracija precej stabilna skozi vseh 5 serij remediacij (Slika 3,4).

Poleg omenjenih elementov lahko težavo v zaprtem procesu predstavlja tudi kopičenje Ca^{2+} in SO_4^{2-} v procesnih vodah. Presežek Ca^{2+} iz alkalnega in SO_4^{2-} iz kislega dela procesa, se v ReSoil® iz procesnih raztopin odstrani skupaj z opranim blatom kot netopni gips ($CaSO_4$; Lestan, 2017; Gluhar in sod., 2021), ki je bogat vir hranil (Islam in sod., 2021). Tako istočasno povečamo hranilno vrednost remediiranega blata ter preprečimo kopičenje ionov in poslabšanje procesnih raztopin v naslednjih serijah.

Zaradi delnega mešanja procesnih raztopin med spiranjem blata pride do prenosa EDTA iz PR v IR, kar bi v seriji zaporednih remediacij lahko pripeljalo do naraščanja koncentracije EDTA v IR in posledično slabše učinkovitosti izpiranja blata. EDTA smo po vsaki seriji reciklirali v alkalni fazi v obliki Ca-EDTA, ki je ostala raztopljena v PR in IR1 (Slika 1). Za preprečitev poslabšanja kakovosti IR smo iz IR1 v kislih razmerah oborili in odstranili EDTA v obliki H_4EDTA, saj je znano, da se EDTA obarja v močno kislih razmerah. Oborjeno H_4EDTA smo prenesli v obdelano PR. Koncentracija EDTA je bila v obdelani RS1 in RS3 pod mejo kvantifikacije v vseh petih serijah remediacij, medtem ko je v PR v 5 zaporednih serijah znašala: 50, 22, 33, 38 in 36 mmol l^{-1}. Podobno kot v naši prvi raziskavi (Morales Arteaga in sod., 2022b) so bile izgube EDTA v posamezni seriji v povprečju 36 %. Izgube EDTA v procesu smo v vsaki naslednji seriji nadomestili z dodatkom sveže EDTA v obliki Na-EDTA, kot je to opisano v Poglavju 2.2.

V procesu je nastal le trdni odpadek, in sicer 2,55 g v petih serijah. Neposreden prenos vrednosti iz laboratorijskega v večje merilo, tako za količino uporabljenih reagentov in nastanek trdnega odpadka, kot tudi učinkovitost odstranitve kovin iz blata in procesnih raztopin, ni mogoč. Za realnejšo oceno tehnološke in stroškovne

učinkovitosti novega ReSoil® postopka je zato v prihodnje potrebno narediti poskuse v večjem merilu.

4 SKLEPI

V raziskavi smo pokazali, da nova ReSoil® tehnologija v splošnem omogoča odstranjevanje PSK iz odpadnega blata, ne glede na stopnjo njegove obdelave na ČN, vendar je učinkovitost odstranitve posamezne kovine odvisna od stopnje obdelave blata na ČN. Uporaba 50 mmol l^{-1} PR se je izkazala kot dovolj učinkovita za odstranjevanje kovin, kar znatno znižuje stroške nove ReSoil® tehnologije. Nova ReSoil® tehnologija omogoča recikliranje procesnih raztopin in EDTA ter proizvaja le trden odpadek, kar smo dokazali v seriji 5 zaporednih remediacij.

Oba vzorca remediiranega blata sta kljub zmanjšanju koncentracije skupnega in dostopnega P ohranila veliko hranilno vrednost. Skrb je povzročala le povečana koncentracija Zn v izpirkih, ki je presegala dovoljeno mejno vrednost v večini remediiranih vzorcev. Rezultati kažejo, da je blato po anaerobni biološki obdelavi iz ČN, na kateri smo opravili vzorčenje, primernejše za uporabo v kmetijstvu kot blato po aerobni biološki obdelavi, saj je zadovoljilo vse kakovostne in varnostne kriterije. Vendar pa vse ČN ne uporabljajo anaerobne biološke razgradnje za stabilizacijo blata, zato so potrebne nadaljnje raziskave v smeri povečanja učinkovitosti odstranitve vseh PSK, ne glede na stopnjo obdelave blata ter raziskati možnosti za zmanjšanje mobilnosti kovin v remediiranem blatu.

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Diversity of hymenopteran families associated to quinoa crop in Algeria (case of Biskra province)

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Diversity of hymenopteran families associated to quinoa crop in Algeria (case of Biskra province)

Abstract: The quinoa (*Chenopodium quinoa*Willd.) crop is originated from Andean region (South America). Its nutritional values as well as its drought climate and water salinity tolerant character have motivated many countries such as Algeria to adapt such a plant culture. This study aims to assess entomofauna from Hymenoptera order in terms of composition and functional diversity. We aim also to evaluate the consequences of the expansion and uncommon crop on the hymenopteran composition. For the first field trial, the survey was carried out in Biskra province during 2018-2019. The Shannon Wiener index and evenness indices were used to measure family's diversity. The results revealed that 1737 specimens were identified into forty families and 166 species. Formicidae species were the most abundant with 68 % of total number of individuals, followed by Braconidae with 7 % and Crabronidae with 5 %. As well, diversity collected with yellow pan traps was more important in winter and spring seasons. As the functional groups, results indicated the presence of three major groups; parasitoids, pollinators and predators. The parasitoid group is the richest one.

Key words: quinoa crop; hymenoptera order; abundance; Shannon Wiener index; Biskra

Raznolikost družin kožokrilcev v posevkih kvinoje v Alžiriji (primer province Biskra)

Izvleček: Kvinoja ali perujski riž (*Chenopodium quinoa* Willd.) je poljščina, ki izvira iz območja Andov v Južni Ameriki. Njena velika hranilna vrednost kot tudi odpornost na sušo in slanost so motivirale številne države, med njimi tudi Alžirijo, da jo uvedejo v pridelovanje. Name te raziskave je bil oceniti favno žuželk iz reda kožokrilcev v posevkih te poljščine glede na sestavo in funkcionalno raznolikost. Namen raziskave je bil tudi ovrednotiti posledice razširjanja te nenavadne poljščine na sestavo favne kožokrilcev. Prvi poljski pregled je bil izveden v province Biskra, v rastni sezoni 2018-2019. Kot merili raznolikosti družin sta bila uporabljena Shannon Wienerjev indeks in indeks izenačenosti. Rezultati so pokazali, da je bilo določenih 1737 primerkov, ki so pripadali 40 družinam in 166 vrstam. Vrste iz družine Formicidae so bile najbolj pogoste, s 68 % deležem vseh osebkov. Tem so sledile Braconidae s 7 % in Crabronidae s 5 % deležem. Raznolikost, ki je temeljila na ulovu z rumenimi ploščami je bila bolj pomembna pozimi in spomladi. Rezultati so pokazali, da so bili med funkcionalnimi skupinami prisotni parazitoidi, opraševalci in plenilci kot glavne skupine, med njimi je bila skupina parazitoidov najbogatejša.

Ključne besede: kvinoja; redovi kožokrilcev; pogostost; Shannon Wienerjev indeks; Biskra

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1 INTRODUCTION

Insects represent the major component of terrestrial ecosystems (Weisser and Siemann, 2004).In trophic interactions (insect-plant), plants serve as resources for insects. They tend to interact with similar partners and they show a stronger conservatism levels for insect pollinators and herbivorous insects (FontaineandThébault , 2015). However, this relationship between insects and plants is not stable and in several cases, native insects have adapted to the introduced crops(Morrill, 2004).Hymenoptera order is one of the largest orders (Gaston, 1993), it ranks the third after Coleoptera and Lepidoptera (Stork, 1997), important members of hymenopteran species belong to higher trophic levels, they are more sensitive to any change of their habitats. Three main groups, ants, bees and parasitoids play crucial role in preserving diversity (Rot et al., 2021).Quinoa ((L.)Willdenow) crop is a pseudo-cereal with high nutritional value; it can be grown in dry climates and tolerates high levels of irrigation water-salinity. To improve food security, it was introduced in Algeria in 2014.It has been the subject of some field trials in arid regions. In Biskra province, quinoa crop was firstly sown in September 2015 on El-Outaya site. There are various reasons for studying diversity related to an exotic plant species.Firstly, know the taxonomy of local hymenoptera reservoir and understand the influence of quinoa crop on species behaviors.This study aims to carry out an exhaustive inventory of hymenopteran families associated with this introduced crop and to evaluate their diversity and abundance.

2 MATERIALS AND METHODS

2.1 STUDY REGION

Fieldwork was conducted in El-Outayalocated in the north of Biskra province (34°55'58.27 '' N, 5°39'34 41" E, altitude,207 m) (Figure1). This region is characterized by an arid climate with mild winter and wet spring. Survey was done from February 2018 to February 2019 during plant life cycle. Collections were done every week to twice a month depending on the season.

2.2 SAMPLING METHODS

Two trap types were chosen for this study: yellow pan traps and pitfall traps. They were known to be good in catching insects. First trap type is a yellow colored plastic rectangular pan with 30 cm of diameter and a height of 20 cm.The pitfall traps were a plastic jar with a diameter of 10 cm and a height of 10 cm-16 cm. Each trap was filled with an immersing medium composed with a mixture of half liter of clean water and some detergent drops. Three traps of each trap type were distributeover crop area of 250 m^2 at a distance of about 2.5-5 m from each other.

2.3 IDENTIFICATION OF INSECTS

Collected insects were preserved in Eppendorf tubes that contain 70 %ethanol and taken to Entomological laboratory, Department of Agronomy, Biskra for identification. Several available keys of identification were used with reference to Delvare and Aberlenc, 1989; Goulet and Huber, 1993; Amiet et al., 2001; Amiet et al., 2004; Mikó et al., 2007, 2013; Farahani and Talebi, 2012; Paukkunen et al., 2015; Wu et al., 2016; Zi and Zaifu, 2016; Ghafouri Moghaddam et al., 2016; Farahani et al., 2016; Rousse and Villemant, 2012; Edmardashand et al., 2011; Schmid-Egger et al., 2017; Ferrer-Suay et al., 2015; Choi et al., 2012; Chen et al., 2017; Izadizadeh et al., 2015; Mokrousov, 2017; Aguirre et al., 2015; Yari et al., 2016; Zargaretal, 2019; Mikó et al., 2013; Prous et al., 2014, 2019. In order to justify insect trophic relationships, different plant species nearby the quinoa crop were sampled (Table1).

2.4 ANALYSIS

To evaluate insect biodiversity, the common measures were used, like species richness, Shannon-Wiener index and evenness measure.

2.4.1 Species richness (S)

It is a simple measure of total number of species in each sample in a given area. Thismeasurement is strongly dependent on sampling efforts.

2.4.2 Shannon-Wiener index (H')

It takes into account the number of individuals of each species within the local community.

$H' = -\Sigma Pi \log Pi$

Where:
H' = Shannon Wiener index
Pi = proportion of "ith" species and is calculated as

"ni/N", where, "ni" is the number of individuals in "ith" species and N is the total number of individuals in the sample.

2.4.3 Measure of evenness (E: Equitability)

It can be calculated by using species richness (S) and Shannon Wiener index (H')**.**It represents an important component of the diversity indices. It indicates how individuals are distributed among the different species.

$$E = H' / \log(S)$$

3 RESULTS

During this study, 1737 specimens were collected from quinoa crop. They were identified into 40 families and 166 species (Table2). Values of different indices calculated over all the seasons were: Shannon-Wiener index, H' = 3.72bits, Evenness (E) = 0.50. In the collection, 32 families were collected withpitfall traps and 29 families with yellow pan traps. Among these 1737specimens, 1377 were collected with thepitfall traps and represented 95 species, most of 1377 individuals belonged

Table 1: Cultivated and spontaneous plants collected infield-work

Plant species	Cultivated	Spontaneous
*Atriplex halimus*L.	-	+
Casuarina equisetifolia L.	+	-
Chenopodium vulvaria L.	-	+
Cupressus sempervirens L.	+	-
Diplotaxis harra (Forssk.) Boiss.	-	+
Hordeum vulgare L.	+	-
Malva parviflora L.	-	+
Medicago sativa L.	+	-
Moricandia arvensis DC.	-	+
Moringa oleifera Lam.	+	-
Olea europaea L.	+	-
Pinus halepensis Moulin	+	-
Salsola vermiculata L.	-	+
Sesbania aculeata (Willd.) Poir.	+	-
Sonchus asper L.	-	+
Suaeda mollis Delile	-	+
Tamarix gallica L.	-	+
Vitis vinifera L.	+	-
Zea mays L.	+	-

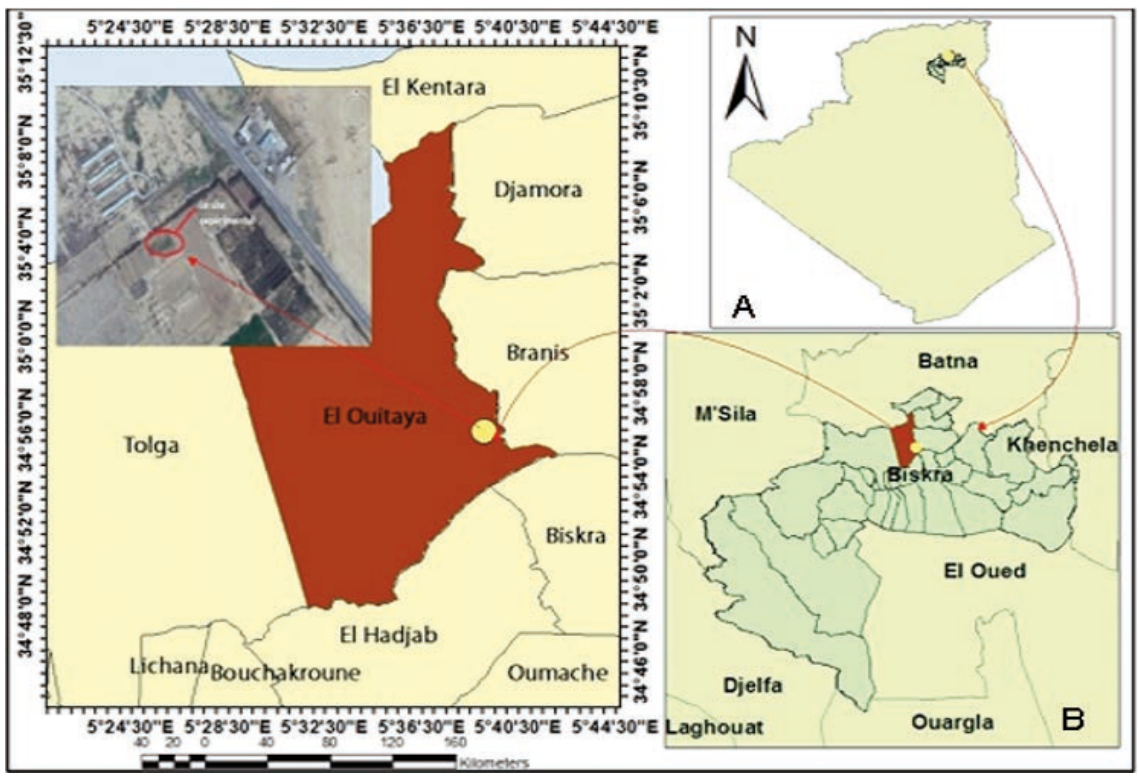

Figure 1: Situation of study site: Map of Algeria (A) showing the study province (B) and the study region

to Formicidae family with 1088 specimens. The rest 360 specimens were collected with the yellow pan traps. In this trap type, 126 species were recorded. 40 species were found in all trap types. The dominant family was Formicidae with 1175 specimens, followed by Braconidae with 124, Crabronidae with 80, Ichneumonidae with 55, Diapriidae 43 and Andrenidae with 35, whereas 17 families were represented with less number of specimens and 11 families with only one specimen (Figure 2). During autumn (17/10-22/12) 136 specimens were collected while 261 insects were collected inwinter (23/12-22/3) and 1340 in spring (23/3-17/5). The calculate values of various indices were presented in Table 3.

In autumn, in yellow pan traps two most abundant families were Ichneumonidae with 37 % and Vespidae with 20 %, but in pitfall traps, the Formicidae is the most abundant family with 58 %. In winter, the Diapriidae family was the most abundant with 21 % in yellow pan traps and the Braconidae family with 25 % in pitfall traps. In spring, in yellow pan traps; three families were the most abundant Formicidae with 40 %, Crabronidae

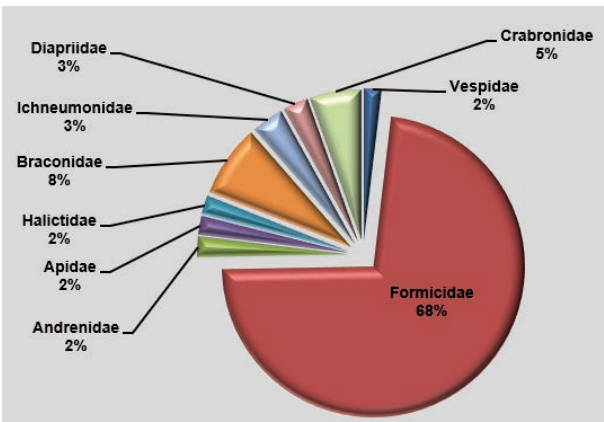

Figure 2: Population pie chart of abundant families given in relative abundance (percent)

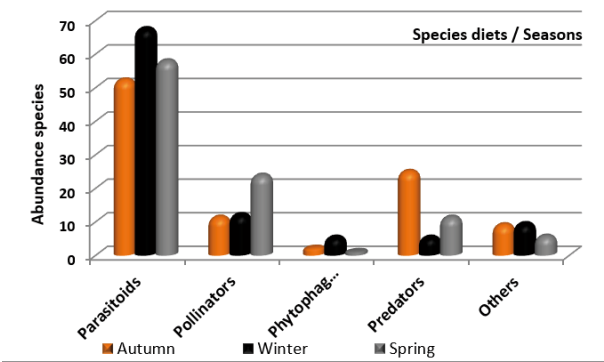

Figure 3: Hymenoptera species abundances of different functional groups

with 20 % and Braconidae with 10 % but in pitfall traps, there was an almost total dominance of Formicidae family with 83 %. Values of Shannon-Wiener index (H') and Evenness (E) indices presented in Table 4 and Table 5 indicate that yellow pan traps were more efficient.Spring and winter remain the best seasons of insect activity in study site. About the functional groups, results indicated the presence of three big groups; parasitoids, pollinators and predators Table 2. The parasitoid group is richer in all seasons. In collected data Crabronidae was the most abundant family of predator. Pollinator families showed no differences in abundance. However, various parasitoid species numbers were observed in all season (Figure3).

4 DISCUSSIONS

Available literature provides less information about entomofauna diversity of quinoa agro-ecosystem in native regions (Valoy et al., 2015). In Algeria, Biskra province is one of few production areas of quinoa and the only inventory done by Deghiche et al. (2021) in 2015-2016 at El-Outaya field may not reflect the true diversity at regional level. Deghiche et al. (2021) reported 5 hymenopteran families, 6 species and 174 specimens. As the second entomofauna listing on quinoa crop at the same site, present study showed a total of 40 hymenopteran families, 166 species and 1737 specimens. Among the collected families, the highest frequency was obtained for Formicidae (68 %). General calculate diversity indices (H'=3, 72 bits and E = 0.50) according to literature range indicated high rich hymenopteran families diversity and moderate distribution throughout the site study. In an ecosystem, various vegetation and favorable climatic conditions also maintain a high diversity of insect (Rasheed and Buhroo, 2018). At the trial site, different vegetation strata and types were present.Our results suggest that this agro-ecosystem harboured an important hymenopteran fauna. Comparison of the diversity indices between yellow pan traps and pitfall traps revealed that hymenopteran fauna composition collected by yellow pan is more important and evenly distributed (in terms of specimen abundance) than collected by pitfall traps. An analysis of the composition of hymenopteran families indicated that 77.77 % of the total specimen abundance is formed by 28 hymenopteran families but in the case of the pitfall traps, we found that 83.07 % of the total specimen abundance is formed by a single family (Formicidae). The winter and spring season results could be explained, in part, due to the key role of temperature as environmental factor ininsect's live cycle and in other part to high plant diversity nearby quinoa crop. Nonetheless, similar diversity could be not found in autumn, when all crop and spontane-

Table 2: Total number of species and specimens of Hymenoptera by families and their diets collected from Biskra province. Nomenclature of all hymenoptera families is follows that orders in Fauna Europaea (2021)

Order	Super-family	Families	Species Number	Specimen effectif	Diets
Hymenoptera	Pamphilioidea Cameron, 1890	Megalodontesidae Konow, 1897	1	4	phytophagous
	Tenthredinoidea Latreille 1803	Tenthredinidae Latreille, 1802	1	2	phytophagous
	Chrysidoidea Latreille, 1802	Chrysididae Latreille, 1802	7	7	Parasitoids
		Dryinidae, Haliday, 1833	1	1	Parasitoids
		Bethylidae, Forster, 1856	1	1	Parasitoids
	Scolioidea Latreille, 1802	Scoliidae, Latreille,1802	6	7	Parasitoids
	Vespoidea Latreille, 1802	Vespidae, Latreille, 1802	5	30	Predators
		Mutillidae, Latreille, 1802	1	1	Parasitoids
		Formicidae Latreille, 1809	8	1175	Variable diets
	Apoidea Latreille, 1802	Pompilidae, Harris, 1987	2	2	Parasitoids
		Crabronidae, Latreille, 1802	3	80	Predators
		Sphecidae, Latreille, 1802	10	1	Parasitoids
		Andrenidae, Latreille, 1802	3	35	Pollinators
		Apidae, Latreille, 1802	9	32	Pollinators
		Megachilidae, Latreille, 1802	6	7	Pollinators
		Melittidae, Michener, 2000	2	9	Pollinators
		Halictidae, Thomson, 1869,	5	32	Pollinators
		Colletidae, Lepeletier, 1841	1	6	Pollinator
	Ichneumonoidea Latreille, 1802	Braconidae, Latreille, 1829	21	124	Parasitoids
		Ichneumonidae, Latreille, 1802	29	55	Parasitoids
	Cynipoidea Latreille, 1802	Figitidae,Thomson, 1862	1	1	Parasitoid
	Diaproidea Halliday, 1833	Diapriidae, Haliday, 1833	3	43	Parasitoids
	Proctotrupoidea Latreille, 1802	Proctotrupidae, Latreille, 1802	2	6	Parasitoids
	Platygastroidea Haliday, 1833	Platygastridae, Haliday, 1833	5	12	Parasitoids
		Scelioninae, Haliday, 1839	5	6	Parasitoids
	Ceraphronoidea Haliday, 1833	Megaspilidae, Ashmead, 1893	2	23	Parasitoids
		Ceraphronidae, Haliday, 1833	3	5	Parasitoids
	Mymarommatoidea Debauche, 1948	Mymarommatidae, Debauche, 1948	2	2	Parasitoids

Continued on next page

Hymenoptera	Chalcidoidea Latreille, 1817	Aphelinidae, Thomson, 1876	1	2	Parasitoid
		Chalcididae, Latreille, 1817	1	1	Parasitoid
		Elasminae (Eulophidae)	1	1	Parasitoid
		Encyrtidae, Walker, 1837	2	2	Parasitoids
		Eulophidae, Westwood, 1829	2	6	Parasitoids
		Eupelmidae, Walker, 1833	1	1	Parasitoid
		Pteromalidae, Dalman, 1820	4	4	Parasitoids
		Tanaostigmatidae, Howard, 1890	1	1	Parasitoid
		Trichogrammatidae Haliday & Walker, 1851	1	1	Parasitoid
		Eurytomidae, Walker, 1832	3	3	Parasitoids
		Mymaridae, Haliday, 1833	3	5	Parasitoids
		Tetracampidae, Förster, 1856	1	1	Parasitoid

Table 3: Indices values of Hymenoptera families' diversity

Indices of Hymenopterafamilies diversity				
Name	Autumn	Winter	Spring	All Season
Richness S	19	28	27	40
Individuals	136	261	1340	1737
Shannon H (bits)	3.04	3.86	1.28	3.72
Evenness E	0.71	0.80	0.26	0.50

Table 4: Indices values of hymenoptera families collected by yellow pan in study seasons

Indices of yellow pan collected Hymenoptera families				
Name	Autumn	Winter	Spring	All Season
Richness S	12	20	22	29
Individuals	51	113	196	360
Shannon H (bits)	2.75	3.48	3.01	3.72
Evenness E	0.76	0.80	0.67	0.76

Table 5: Indices values of hymenoptera families collected by pitfall traps in study seasons

Indices of pitfall traps collected Hymenoptera families				
Name	Autumn	Winter	Spring	All Season
Richness (S)	15	21	21	32
Individuals	85	148	1144	1377
Shannon (H) (bits)	2.45	3.55	0.80	1.47
Evenness (E)	0.62	0.80	0.18	0.29

ous plants were in the early stages of their growth. In El-Outayasite, the average monthly temperatures during study in winter ranged from 12.1 to16.7 (with maximum of 27.1 ^{0}C) and the difference between the maximum and minimum temperature did not exceed 8 ^{0}C.Some observations were recorded in spring with mean monthly temperatures oscillated between 17.5 and 25 (with maximum of 30 ^{0}C).

With the exception of spring data collected by pitfall traps, various diversity indices calculated in all season (Tables 4 and 5) showed that great hymenopteran diversity were recorded and the detected families were evenly distributed throughout areas trial. At the quinoa field, scares infestation were observed. Moth was found on leaves in early stages, but the presence of aphid species and thrips were later. In the quinoa native region (Andes), aphids and thrips are considered occasional pests (Cruces et al., 2021).In trial field, quinoa harbours important hymenopteran diversity with different diets. The natural enemy complex formed by parasitoids and predatorsplays an essential role in plant protection. This crop-system proved very favorable for pollinator because they need good foraging resources.Surrounded by low use of pesticides and a variety of crops and herbs, the quinoa crop allows insects to have food and refuge Burgioet al. (2006).The results may also explain the greater diversity of entomophagous hymenopteran families. Parasitoids were the dominate group (Table 2).According to Rasmussen et al. (2003) in Andean region, up 45 % of quinoa pests were naturally controlled by parasitoids and predators. Apparently, our results were relatively similar to those in Cruces et al. (2020 a, 2020b); the introduced

quinoa crop appears with positive impact on local insects' diversity.

5 CONCLUSIONS

This study is the first research to emphasize the diversity of hymenopteran families present in the Biskra province. It has been shown the presence of 40 hymenopteran families and 166 species. Thus, the found richness may give more information about herbivore identity and entomophagous guilds function in relation to local plant diversity and introduced crops us quinoa at locality scale. It may be important to survey the component of each family. This data can be used as example for integrate pest management of quinoa cultivation in the future.

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Combining ability for morphological and nutritional traits in a diallel cross of tomato (*Solanum lycopersicum* L.)

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Combining ability for morphological and nutritional traits in a diallel cross of tomato (*Solanum lycopersicum* L.)

Abstract: Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown in Nigeria, either for fresh market or industrial purposes, necessitating the development of a robust tomato breeding programme aimed at maximizing genetic improvement on economically important traits. In this study, the combining ability, nature of gene action, heterosis, and heritability for morphological, nutritional, and physicochemical parameters of tomato were examined in five tomato parents and ten F_1 offsprings, generated with a 5 × 5 half diallel mating design in the greenhouse in 2017. The field evaluation was conducted at the Teaching and Research Farm of Ladoke Akintola University of Technology, Ogbomoso, Nigeria during the cropping season of 2018 using a randomized complete block design with three replications. Analysis of variance for combing ability revealed that both additive and nonadditive gene actions contributed to the fundamental genetic mechanism underlying the inheritance of the measured traits. The top two general combiner parents were UC-OP and Ib-local. Furthermore, the best tomato hybrid specific combiners were FDT_4 × UC-OP, FDT_2 × Ib-local and UC-OP × Ib-local which involved one parent having a high general combining ability effect for fruit yield and the other having other desirable traits. These hybrids may be further utilized in tomato breeding programmes.

Key words: combining ability; gene action; heritability; heterosis; hybrid; tomato; variation

Kombinacijske zmožnosti za morfološke in hranilne lastnosti paradižnika (*Solanum lycopersicum* L.) pri dialelnem križanju

Izvleček: Paradižnik (*Solanum lycopersicum* L.) je ena najpomembnejših vrtnin, ki se goji v Nigeriji za svežo porabo ali za industrijske namene. Za maksimiranje pridelave je potrebno razviti robustne žlahtniteljske programe, v katerih bi izboljšali njegove genetske in ekonomske lastnosti. V raziskavi je bila preverjena kombinacijska zmožnost delovanja genov, heteroze in dedovanja za morfološke, hranilne in fizikalno-kemijske lastnosti petih starševskih genotipov paradižnika in desetih F_1 potomcev, pridobljenih v 5 x 5 polovičnem dialelnem križanju v rastlinjaku leta 2017. Ovrednotenje v poljskem poskusu je bilo izvedeno na Teaching and Research Farm of Ladoke Akintola University of Technology, Ogbomoso, Nigeria v rastni sezoni 2018 v popolnem naključnem bločnem poskusu s tremi ponovitvami. Analiza variance za kombinacijske zmožnosti je pokazala, da je aditivno in neaditivno delovanje genov prispevalo k osnovnim mehanizmom dedovanja merjenih lastnosti. Dva najboljša starša za komibiniranje lastnosti sta bila 'UC-OP' in 'Ib-local'. Najboljša križanja za kombiniranje lastnosti so bila 'FDT_4' × 'UC-OP', 'FDT_2' × 'Ib-local' in 'UC-OP' × 'Ib-local', ki so vsebovala starše z velikimi splošnimi kombinacijskimi lastnostmi za pridelek plodov in druge zaželjene lastnosti. Ti hibridi bi lahko bili uporabljeni v nadaljnih žlahtiteljskih programih paradižnika.

Ključne besede: kombinacijska zmožnost; delovanje genov; sposobnost dedovanja; heteroza; hibrid; paradižnik; spremenljivost

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1 INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of Nigeria's most important vegetable crops, second only to onions, due to its high consumption, and is well adapted to a variety of climatic conditions, soil types, and altitudes (Osei et al., 2010). Tomatoes make an important contribution to human health and welfare because they are high in ascorbic acids (Vitamin C), minerals (calcium, phosphorus, and iron), and antioxidants (lycopene and *β*-carotene), which lower the risk of lung, breast, and prostate cancers (Willcox et al., 2003; Palozza et al., 2011; Rai et al., 2012). As a result, breeding programmes prioritize the nutritional and physico-chemical properties of tomato fruit (Panthee et al., 2015; Acharya et al., 2018). Tomato yield is a complex character that is affected by numerous factors. It is critical to note that, due to the geometric progression of human population and the rapid rate of urbanization, which is reducing cultivable land and increasing demand for tomato, breeding for high yield alone is insufficient to meet the demands of consumers and end-users. In Nigeria, there is still a significant gap in the development of high yielding and nutritive tomato hybrids. Several biotic and abiotic stresses are major impediments to the successful adoption and cultivation of improved tomato varieties (Soresa et al., 2020). The Nigerian tomato market is currently saturated with mixtures of diverse cultivar that are unable to meet the numerous demands. Consequently, it has become critical to assess the genetic potential of locally available tomato cultivars for their efficient utilization and further improvement.

In spite of the relatively high cost of hybrid seeds it has proven to be a successful approach for vegetable improvement (Kaushik & Dhaliwal, 2018) and usually characterized by high yield and homogeneity. Therefore, to obtain worthwhile information on the genetic makeup of cultivars useful as parental line in hybrid combination, the combining ability is primarily valuable (Sprague & Tatum, 1942). General combining ability (GCA) and specific combining ability (SCA) distinguishes between the average performance of parents in crosses (GCA) and the deviation of individual crosses from the average of the parents (SCA). Additionally, GCA basically involves additive gene action while SCA provides genetic information on the crosses, hence elucidates the existing nonadditive gene action which offers good choice for exploitation of heterosis (Ahmad et al., 2009; Senapati & Kumar 2015). The diallel mating design approach used in the expression of combining ability of lines provides information on the nature and magnitude of gene actions involved in the expression of quantitative and qualitative traits and helps to identify superior parents for hybrid development. Therefore, involving combining ability as a technique in the analysis and understanding of the genetic potential of parents and their hybrids is one of such possible ways in addressing tomato farmers' and consumers demands. Furthermore, diallel mating designs are useful in estimating genetic parameters, which contributes to a better understanding of the mechanism used to predict genetic progress when parental lines are chosen based on their own performance (Falconer, 1989).

Previous studies have used the diallel mating design to generate information on genetic parameters; GCA estimates (Patil et al., 2013; Singh et al., 2014; Kumar et al., 2018), identification of superior cross combinations with SCA estimates (de Souza et al., 2012; Yadav et al., 2013; Saleem et al., 2013a), heterosis relative to mid parents with potence ratio (THI, 2009; Shende et al., 2012; Agarwal et al., 2014) and heritability in broad and/or narrow sense (Osekita & Ademiluyi, 2014; Mohamed et al., 2018; Kumar et al., 2018) for diverse morphological traits in F_1 tomato hybrids. Results obtained from their studies provided essential information on gene actions controlling the inheritance of traits and crosses that can be utilized for developing high yielding tomato hybrid as well as for exploiting hybrid vigour.

Therefore, this experiment was carried out to determine general and specific combining abilities effects, nature of gene action, relationships between traits, and to estimate heritability, heterosis and the mean performance for qualitative and quantitative traits of tomato cultivars crossed in a half diallel mating design.

2 MATERIALS AND METHODS

The study was conducted at the Teaching and Research Farm of Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Nigeria and the soils are characterized as alfisol. The Global Positioning System coordinates of the experimental site was 8°10′ North, 4°10′ East, with an altitude of 341 m above sea level. The experimental site falls into the derived savanna agroecology of Nigeria, with annual mean rainfall of 1,100 mm and daily temperature ranges from 28–30 °C.

2.1 GENETIC MATERIALS

Five tomato cultivars with different traits, FDT_4 (P_1), FDT_2 (P_2), UC-OP (P_3), Ib-local (P_4) and Kerewa (P_5) representing the cultivars in the rain forest and derived savanna agro ecology of Nigeria were used in this study as the parental lines. Seeds of FDT_4 and FDT_2 were collected from the Federal University of Agriculture,

Abeokuta (FUNAAB), Nigeria and seeds of UC-OP and Ib-local were collected from the National Horticultural Research Institute (NIHORT), Ibadan, Nigeria. Kerewa a popular commercial cultivar was collected on farmers' field in Ogbomoso (Table 1). In the first growing season (2017), the parental tomato cultivars were grown in a greenhouse to conduct all needed crosses by hand in all possible combinations excluding reciprocals. In the second growing season (2018), tomato plants were evaluated on field.

2.2 NURSERY OPERATIONS

In 2017 growing season, seeds of each parental line were sown in nursery bed and watered regularly for six weeks. The seedlings were transplanted into a 4.5 kg soil-filled pot mixed with organic fertilizer (0.3 kg of poultry manure) in the greenhouse at six weeks after sowing and each cultivar was represented by 15 pots. The pots were laid out to fit into a diallel mating design and staking was done to keep the plants erect for easy crossing. Hybridization commenced at 7 weeks after transplanting (WAT). To achieve effective pollination, each parent lines with matured flowers that were ready to open within 24 hours were emasculated and crossed using the half diallel mating design of Griffing (1956) method II to produce the F_1's consisting of single crosses and parental lines (selfing). The pollinated flowers were carefully covered with pollinating bags and tagged for identification. The fruits from all successful pollinations were harvested at maturity and the seeds were extracted, dried and labeled for evaluation. The mating design produced 15 genotypes consisting of 10 hybrid crosses and 5 parental lines from selfing.

2.3 TRIAL EVALUATION AND DATA COLLECTION

Each of the 15 genotypes was raised as seedlings in nursery beds for six weeks and regularly watered before being transplanted to the evaluation plots. The parents (5) and F_1's (10) were evaluated on the field at the Teaching and Research Farm of LAUTECH in 2018, using a randomized block plot design with three replications. Each genotype was transplanted on a 5 m by 7.5 m plot with a spacing of 1 m between plots and 0.5 m between plants on a plot. N.P.K (15-15-15) fertilizer was applied at the rate of 120 kg N ha^{-1} three WAT. All other cultural practices, and plant protection against weeds, diseases and insects, were performed as recommended for commercial tomato production. Data collection commenced at 6 WAT and continued till harvesting. Data were recorded on plant height (PH) and stem width (SW), number of leaves per plant (NLPP), number of days to 50 % flowering (DTF), number of secondary branches (NSB), number of cluster per plant (CLPP), number of flower per cluster (NFPC). All harvested fruits of each plant were counted and weighed to determine number of fruits per plant (NFP), and total fruits mass per plant (FWP) measured in gram. Average fruit mass was estimated by dividing the total mass of all harvested fruits per plant by their total number.

Samples of five random ripe fruits per plant were taken from all replications of each genotype to measure pericarp thickness (PCAP) in mm and number of locules per fruit (NLOBE).

2.4 NUTRITIONAL AND PHYSICOCHEMICAL ANALYSES

Tomato fruit juice of each genotype was extracted from five random red ripe fruits per plant taken from all replicates. The extracted juice was filtered through double-layered muslin cloth and used for estimating total soluble solids (TSS), which was measured using a hand refractometer (RA-130-KEM, Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan). The readings were recorded as ^{o}Brix (0-32 $^{\circ}$C) at room temperature. For determination of vitamin C (VIT C) measured in mg kg^{-1}, 10 ml of juice was diluted in 100 ml of distilled water and titrated with NaOH 0.1 N till pH 8.2. The solution was titrated with iodine (0.1 N) till changes in colour occur (IPGRI, 1996). To determine lycopene (LPEN) content (mg kg^{-1}), 5 ml of acetone-n-hexane mixture in the ratio 4:6 was added to 0.8 g of tomato pulp for each genotype. The mix was centrifuged at 5000 rpm for 5 min at 4 °C; the supernatant was extracted and placed in spectrophotometre (model 6400, Jenway) and scanned at 503 nm using the acetone-n-hexane mix as blank (Rosales et al., 2006). Lycopene content was quantified using an extinction coefficient ($E^{\%}$) of 3150. All analysis was done in triplicate for each sample.

2.5 STATISTICAL ANALYSES AND ESTIMATION OF GENETIC PARAMETERS

Analysis of Variance (ANOVA) was conducted and estimate of the combining ability of the genotypes were calculated using SAS (SAS institute, 2011) statistical package according to Griffing's (1956) method II, model II for half diallel analysis which assumes that the genotype and the replicate are both random variables.

Table 1: Description of genetic materials used in the diallel crosses

Genotype	Source	Characteristics	Fruit colour
FDT_4	Federal University of Agriculture, Abeokuta	Oblong fruit shape with two slight lobes	Orange
FDT_2	Federal University of Agriculture, Abeokuta	Rectangle fruit shape	Orange
UC-OP	National Horticultural Research Institute, Ibadan	Rectangle shape, open pollinated variety	Orange
Ib-local	National Horticultural Research Institute, Ibadan	Flat shaped fruit, average-sized with five lobes	Red and yellow
Kerewa	Ogbomoso	Oblong shape, average sized with three lobes.	Pink

The relative importance of general combining ability (GCA) compared to specific combining ability (SCA) was calculated according to Baker (1978). If the ratio is closer to 1, it indicates predominance of additive gene action and greater predictability of progeny performance based on GCA effects (Gurmu et al., 2018). Least square mean were computed and separated using Fisher's least significant difference (LSD) test ($p < 0.05$). Mid-parent heterosis was calculated for all measured traits using the formula of Mather & Jinks (1971) and the student *t*-statistics was used to determine the statistical difference of F_1 hybrid means and the mid-parent according to Wynne et al. (1970) and Kolawole et al. (2019). Potence ratio was calculated according to Smith (1952) to determine the degree of dominance. Complete dominance is indicated when relative potence of gene set = +1.0; while partial dominance is indicated when the relative potence of gene set is between (-1 and +1); over-dominance is considered when potence ratio exceeds +1, whereas, the value zero, indicates absence of dominance. The positive and negative signs indicate the direction of dominance of either parent. Narrow (h^2_{ns}) and broad (H^2_{bs}) sense heritabilities were determined according to Mather & Jinks (1971). Estimates of heritability were categorized as low = < 0.50, moderate = 0.50 and high = > 0.50, (Robinson et al., 1949). Phenotypic correlation coefficients were computed for all pairs of traits using the PROC CORR in SAS (SAS Institute, 2011).

3 RESULTS

3.1 ANALYSIS OF VARIANCE AND MEAN PERFORMANCE

There were highly significant ($p < 0.001$) differences in the mean squares of the tomato parental lines and hybrids for all morphological traits, nutritional and physicochemical parameter measured except for number of leaves per plant (Table 2).

This implied the presence of considerable genetic variation which could be exploited in tomato breeding programme. The coefficient of variation (CV) showed good experimental precisions for most of the traits measured. The analysis of variance for combining ability partitioned genetic variation into GCA and SCA. General and specific combining abilities effects showed significant ($p < 0.001$) additive and nonadditive gene actions influencing all traits except for stem width and number of flower per cluster for GCA and number of leaves per plant, number of days to 50 % flowering and fruit mass per plant for SCA. The comparison between the genetic variance components showed higher values of GCA than those of SCA for 8 traits. The relative importance of GCA in comparison with SCA calculated based on Baker's Ratio ranged from 0.21 for number of flower per cluster to 0.93 for number of leaves per plant. The ratios were closer to unity for 9 traits out of 14, indicating the prevalence of additive gene action for plant height, number of leaves per plant, number of days to 50 % flowering, number of secondary branches, cluster per plant, pericarp thickness, number of locules per fruit, fruit mass per plant and vitamin C while nonadditive gene action was more important for stem width, number of flower per cluster, number of fruits per plant, lycopene and total soluble solid.

The mean performance of the 15 genotypes showed wide variabilities for seven of the traits measured and some hybrids had significantly higher vigour, yield and nutritional quality than the parental cultivars. The parental cultivar, Ib-local (P_4) was superior for 4 morphological traits such as stem width, cluster per plant, number of flower per cluster and fruit mass per plant (Table 3). Consequently, crosses involving Ib-local (P_4): FDT_4 × Ib-local, UC-OP × Ib-local and Ib-local × Kerewa had the highest mean value for number of fruits per plant, number of days to 50 % flowering, fruit mass per plant, cluster

Table 2: Mean squares, general and specific combining ability for morphological traits, nutritional and physicochemical parameters of five tomato parents and their 10 crosses

SOURCE OF VARIATION	df	PH (cm)	SW (mm)	NLPP	DTF	NSB	CLPP	NFPC
REPLICATION	2	0.07	0.01	580.96	0.47	0.07	1.49	0.16
GENOTYPE	14	3.83***	0.01***	1688.42	17.37***	16.99***	16.31***	0.85*
GCA	4	5.62***	0.004	4221.20***	38.49***	18.82***	20.44***	0.15
SCA	10	3.11***	0.012***	675.31	8.92	16.26***	14.65***	1.13**
ERROR	28	0.59	0.003	889.72	4.75	0.76	1.44	0.37
CV (%)		1.45	19.89	13.13	6.44	13.32	15.18	11.31
GCA/SCA		0.78	0.41	0.93	0.90	0.70	0.74	0.21
MEAN		52.88	0.31	227.16	33.87	6.53	7.91	5.38
MINIMUM		50.30	0.16	197.33	31.33	4.67	5.33	4.67
MAXIMUM		54.50	0.42	262.00	40.33	14.33	12.67	6.33
	df	NFP	PCAP (mm)	NLOBE	FMP (g)	LPEN (mg kg^{-1})	VIT C (mg kg^{-1})	TSS (°Brix)
REPLICATION	2	29.4	0.00	0.03	1024.21	0.12	0.03	0.01
GENOTYPE	14	452.00***	0.02***	2.82***	2328.83*	1836.91***	4773.07***	3.83***
GCA	4	111.48**	0.02***	3.57***	5191.15**	664.32***	5783.25***	1.56***
SCA	10	588.21***	0.02***	2.53***	1183.91	2305.95***	4368.99***	4.74***
ERROR	28	27.76	0.00	0.05	932.38	0.23	0.11	0.02
CV (%)		25.91	3.06	8.63	5.90	0.88	0.21	3.32
GCA/SCA		0.27	0.67	0.74	0.90	0.37	0.73	0.40
MEAN		20.33	0.55	2.67	517.47	54.05	160.23	4.61
MINIMUM		11.67	0.46	1.00	466.85	13.54	98.43	1.92
MAXIMUM		61.00	0.71	5.00	573.22	91.60	231.25	6.10

*, **, *** indicates significance at 0.05, 0.01, and 0.001 probability levels, respectively
GCA = general combining ability; SCA = specific combining ability; CV = Coefficient of variation
PH = plant height; SW = stem width; NLPP = number of leaves per plant; DTF = number of days to 50 % flowering; NSB = number of secondary branches; CLPP = cluster per plant; NFPC = number of flower per cluster; NFP = number of fruits per plant; PCAP = pericarp thickness; NLOBE = number of locules per fruit; FMP = fruit mass per plant; LPEN= lycopene; VIT C = vitamin C; TSS= total soluble solid

per plant, tallest plant and stem width. The parental cultivar, UC-OP (P_3) was superior for only three morphological traits. It had the highest number of secondary branches, number of fruits per plant and the thickest pericarp. F_1 hybrids with UC-OP (P_3) as one of the parents (FDT_2 × UC-OP, UC-OP × Ib-local and UC-OP × Kerewa) had the thickest pericarp, the tallest plant and the highest fruit mass per plant. The parental cultivar, Kerewa (P_5) had the highest mean value for nutritional and physicochemical quality, but with the lowest mean values for most of the morphological traits. Although crosses made to Kerewa (P_5) which includes: FDT_4 × Kerewa, FDT_2 × Kerewa, UC-OP × Kerewa and Ib-local × Kerewa had the highest mean value for number of secondary branches, number of flower per cluster, vitamin C content, number of locules per fruit, cluster per plant, early flowering and stem width. The parental cultivar, FDT_4 (P_1) was the tallest with the earliest flowers. Crosses involving of FDT_4 (P_1) such as: FDT_4 × FDT_2, FDT_4 × Ib-local and FDT_4 × Kerewa; had the highest mean value for number of leaves per plant, number of fruits per plant, number of secondary branches and number of flower per cluster. The parental cultivar, FDT_2 (P_2) had highest mean value only for number of leaves per plant. However Crosses of FDT_4 × FDT_2, FDT_2 × UC-OP, FDT_2 × Kerewa and FDT_2 × Ib-local; had the highest number of leaves per plant and early flowering. The F_1 hybrids morphological traits, nutritional and physicochemical parameters mean val-

Table 3: Mean performance of parents and their hybrids for morphological traits, nutritional and physicochemical parameters

Genotype	PH (cm)	SW (mm)	NLPP	DTF	NSB	CLPP	NFPC	NFP	PCAP (mm)	NLOBE	FMP (g)	LPEN	VIT C	TSS (°Brix)
Parents														
FDT_4 (P_1)	53.03	0.32	209.33	31.33	5.00	5.33	5.67	11.67	5.20	2.10	487.51	24.19	184.74	5.34
FDT_2 (P_2)	50.30	0.27	217.67	34.00	5.33	5.33	5.00	12.67	4.80	1.00	499.26	88.75	166.64	1.92
UC-OP (P_3)	52.87	0.35	206.67	33.33	5.67	5.33	5.67	61.00	5.30	2.00	494.29	14.26	204.18	5.10
Ib-local (P_4)	52.07	0.42	207.67	34.00	4.67	8.00	6.00	15.67	4.80	5.00	518.02	51.78	127.54	4.22
Kerewa (P_5)	51.03	0.20	201.00	34.67	5.33	6.00	4.67	14.33	5.20	2.83	466.85	91.60	231.25	5.82
Mean	51.86	0.31	208.47	33.47	5.20	6.00	5.40	23.07	5.06	2.59	493.18	54.11	182.87	4.48
Crosses														
$P_1 \times P_2$	53.33	0.30	262.00	33.33	6.00	8.00	5.00	16.67	5.30	2.33	517.67	57.75	110.69	5.32
$P_1 \times P_3$	53.13	0.31	259.33	33.33	6.00	11.00	6.00	17.33	6.10	2.00	516.16	42.73	164.67	3.69
$P_1 \times P_4$	54.07	0.23	250.00	33.67	5.67	9.67	5.33	33.33	5.60	3.17	570.95	83.40	176.45	5.25
$P_1 \times P_5$	52.70	0.31	257.33	31.67	14.33	8.67	6.33	15.00	6.60	3.10	512.35	54.51	98.43	4.81
$P_2 \times P_3$	53.00	0.37	253.00	32.33	7.67	8.33	5.00	15.67	7.10	2.00	518.69	57.39	123.66	6.10
$P_2 \times P_4$	52.87	0.16	223.00	33.33	5.00	11.00	4.67	17.33	5.20	2.00	509.04	76.12	188.44	4.81
$P_2 \times P_5$	53.40	0.33	216.67	31.33	6.00	6.33	5.00	18.67	4.60	3.00	524.84	37.50	200.43	3.83
$P_3 \times P_4$	54.50	0.29	197.33	40.33	6.00	7.00	5.00	18.33	5.80	2.33	573.22	55.43	115.13	2.84
$P_3 \times P_5$	54.40	0.34	240.33	38.00	8.33	6.00	6.00	19.33	4.90	4.00	515.43	13.54	131.07	5.26
$P_4 \times P_5$	52.47	0.38	206.00	33.33	7.00	12.67	5.33	18.00	6.30	3.20	537.75	61.75	180.17	4.82
Mean	53.39	0.30	236.50	34.07	7.20	8.87	5.37	18.97	5.75	2.71	529.61	54.01	148.91	4.67
LSD (0.05)	1.28	0.10	49.89	3.65	1.46	2.01	1.02	8.81	0.30	0.39	51.07	0.80	0.55	0.26

PH = plant height; SW = stem width; NLPP = number of leaves per plant; DTF = number of days to 50 % flowering; NSB = number of secondary branches; CLPP = cluster per plant; NFPC = number of flower per cluster; NFP = number of fruits per plant; PCAP = pericarp thickness; NLOBE = number of locules per fruit; FMP = fruit mass per plant; LPEN= lycopene (mg kg^{-1}); VIT C = vitamin C (mg kg^{-1}); TSS= total soluble solid

ues tended to be either more than their respective mid-or better parental values with few exceptions.

3.2 ESTIMATES OF GENERAL AND SPECIFIC COMBINING ABILITIES EFFECTS

The estimates of GCA effects varied among the five parental cultivar and they all showed good general combining abilities for diverse traits. The parental cultivars FDT_4 with highly significant ($p < 0.001$) and positive GCA effects was considered as good general combiner only for fruit vitamin C content (Table 4). Although, two other parents viz. Kerewa and UC-OP showed highly significant ($p < 0.001$) and positive GCA effect for this trait as well.

Moreover, for number of flower per cluster, UC-OP parental cultivar showed highly significant ($p < 0.001$) and positive GCA effect. Similarly, parental cultivar FDT_2 showed highly significant ($p < 0.001$) and positive GCA effect for number of leaves per plant and fruit lycopene content. The parental cultivar Ib-local showed highly significant ($p < 0.001$) and positive GCA effect for plant height, fruit mass per plant and fruit lycopene content. Parental cultivar Kerewa showed highly significant ($p < 0.001$) and positive GCA effects for number of secondary branches, cluster per plant and total soluble solid. On the other hand, considering number of days to 50 % flowering, UC-OP parental cultivar with significant ($p < 0.05$) and negative GCA effects was considered as good general combiner because desirable GCA effects for this trait must be negative for the development of early tomato hybrid. Likewise, parental cultivars UC-OP, FDT_2 and FDT_4 with significant ($p < 0.001$) and negative GCA effects for number of locules per fruit were considered as good general combiners. This is because a minimal number of fruit locules are desired for attractive shape and ease of processing in tomato.

None of the SCA effects were significant for number of leaves per plant and fruit mass per plant (Table 5). For plant height only the cross between Ib-local × Kerewa had positive and significant ($p < 0.01$) SCA effect. Likewise, only FDT_4 × Ib-local had positive and highly significant ($p < 0.001$) SCA effect for stem width, only the F_1 hybrid (FDT_2 × UC-OP) had positive and significant ($p < 0.05$) SCA effect for number of flower per cluster and only FDT_4 × UC-OP had positive and highly significant ($p < 0.001$) SCA effect for number of fruits per plant. Also, from the 10 F_1 hybrids, two crosses (FDT_2 × Kerewa and Ib-local × Kerewa) exhibited positive and highly significant ($p < 0.001$) SCA effects for number of secondary branches and the former reflected higher positive values for SCA effect. Similarly, the crosses between FDT_4 × Kerewa and FDT_2 × Kerewa had positive and highly significant ($p < 0.001$) SCA effect for pericarp thickness and the later reflected higher positive values for SCA effect. For cluster per plant, three crosses (FDT_4 × Ib-local, FDT_2 × UC-OP and UC-OP × Ib-local) showed positive and significant ($p < 0.001$) SCA effects whereas, the later reflected the highest positive values for SCA effect. The crosses FDT_4 × FDT_2, FDT_4 × Kerewa, UC-OP × Ib-local and Ib-local × Kerewa exhibited negative and highly significant ($p < 0.001$) SCA effects for number of locules per fruit but FDT_4 × FDT_2 had the highest negative value for SCA effect. Five out of the ten F_1 hybrids reflected positive and highly significant ($p < 0.001$) SCA effects for nutritional and physicochemical parameters. With respect to fruit lycopene content the best hybrid combination was found to be the cross FDT_4 × FDT_2, which gave the highest positive value for SCA effect. Comparing the estimated SCA effects for all crosses, the cross between FDT_2 × Ib-local could be considered as the best hybrid combination for vitamin C and total soluble solid since; it showed the highest and highly significant ($p < 0.001$) positive values for the SCA effects.

Table 4: Estimates of the GCA effects of five tomato parents for morphological traits, nutritional and physicochemical parameters

	Parents				
TRAITS	FDT_4	FDT_2	UC-OP	Ib-local	Kerewa
PH (cm)	-0.70***	-0.06	0.14	0.73***	-0.11
SW (mm)	0.01	-0.02	0.01	-0.01	0.01
NLPP	-15.90**	20.77***	6.96	-6.75	-5.09
DTF	-0.65	-0.55	-1.03*	2.35***	-0.12
NSB	-1.17***	0.59***	-0.17	-0.50**	1.26***
CLPP	-1.73***	0.46	0.41	0.17	0.70**
NFPC	0.06	0.06	-0.13	-0.04	0.06
NFP	0.71	-1.48	3.38***	0.05	-2.67**
PCAP (mm)	-0.04***	0.01**	0.03***	-0.02***	0.01***
NLOBE	-0.14***	-0.30***	-0.43***	0.40***	0.47***
FMP (g)	-21.63***	4.18	-3.30	22.15***	-1.41
LPEN (mg kg^{-1})	-4.22***	8.66***	-5.56***	1.63***	-0.52***
VIT C (mg kg^{-1})	19.67***	-19.12***	6.24***	-15.38***	8.59***
TSS (°Brix)	0.01	-0.19***	0.28***	-0.35***	0.24***

*, **, *** indicates significance at 0.05, 0.01, and 0.001 probability levels, respectively

PH = plant height; SW = stem width; NLPP = number of leaves per plant; DTF = number of days to 50 % flowering; NSB = number of secondary branches; CLPP = cluster per plant; NFPC = number of flower per cluster; NFP = number of fruits per plant; PCAP = pericarp thickness; NLOBE = number of locules per fruit; FMP = fruit mass per plant; LPEN= lycopene; VIT C = vitamin C; TSS= total soluble solid

Table 5: Estimates of the SCA effects involving morphological traits, nutritional and physicochemical parameters of 10 tomato crosses derived from a 5 × 5 half diallel

CROSS	PH (cm)	SW (mm)	NLPP	DTF	NSB	CLPP	NFPC
$FDT_4 \times FDT_2$	-1.82***	-0.03	-14.37	1.33	-0.62	-1.30*	-0.49
$FDT_4 \times$ UC-OP	0.55	0.03	-11.56	1.14	0.48	-1.25*	0.37
$FDT_4 \times$ Ib-local	-0.84*	0.12***	3.16	-1.57	-0.19	1.65**	0.60
$FDT_4 \times$ Kerewa	0.54	-0.13***	8.79	0.33	-0.48	0.02	-0.65*
$FDT_2 \times$ UC-OP	0.17	0.01	4.44	1.05	-0.95*	2.22***	0.70*
$FDT_2 \times$ Ib-local	0.51	-0.04	8.83	-2.00	-0.95*	1.13	-0.06
$FDT_2 \times$ Kerewa	0.56	0.03	7.79	-0.95	4.24***	-1.22*	0.35
UC-OP × Ib-local	-0.89*	-0.14***	-4.37	-1.86	-0.86	2.51***	-0.54
UC-OP × Kerewa	0.33	0.05	-0.44	-0.86	-0.14	-3.08***	-0.41
Ib-local × Kerewa	1.06**	0.05	8.70	3.67**	1.52***	-4.03***	0.30
	NFP	PCAP (mm)	NLOBE	FMP (g)	LPEN (mg kg^{-1})	VIT C (mg kg^{-1})	TSS (°Brix)
$FDT_4 \times FDT_2$	-6.90***	-0.04***	-1.23***	-0.76	30.27***	5.85***	-2.51***
$FDT_4 \times$ UC-OP	36.57***	-0.02*	-0.10	1.75	-30.02***	18.04***	0.20*
$FDT_4 \times$ Ib-local	-5.43*	-0.02**	2.07***	0.02	0.32	-36.99***	-0.05
$FDT_4 \times$ Kerewa	-14.14***	0.04***	-0.46***	-14.30	20.86***	27.92***	1.66***
$FDT_2 \times$ UC-OP	-0.71	0.02	0.06	-2.19	-14.42***	17.32***	-1.01***
$FDT_2 \times$ Ib-local	-4.90	0.01	0.39***	27.15	19.06***	50.71***	1.18***
$FDT_2 \times$ Kerewa	-1.90	0.05***	0.52***	-16.04	-21.29***	-62.58***	1.24***
UC-OP × Ib-local	-6.43*	-0.05***	-0.64***	-27.28	26.00***	37.35***	0.27***
UC-OP × Kerewa	-13.81***	-0.04***	0.48***	19.90	3.98***	-23.67***	-0.38***
Ib-local × Kerewa	-0.48	-0.003	-0.68***	-11.34	-43.51***	-36.72***	-0.32***

*, **, *** indicates significance at 0.05, 0.01, and 0.001 probability levels, respectively
PH = plant height; SW = stem width; NLPP = number of leaves per plant; DTF = number of days to 50 % flowering; NSB = number of secondary branches; CLPP = cluster per plant; NFPC = number of flower per cluster; NFP = number of fruits per plant; PCAP = pericarp thickness; NLOBE = number of locules per fruit; FMP = fruit mass per plant; LPEN= lycopene; VIT C = vitamin C; TSS= total soluble solid

3.3 BROAD-SENSE (H^2) AND NARROW-SENSE (h^2) HERITABILITIES ESTIMATES

Broad sense heritability estimates ranged from 0.28 for number of leaves per plant to 1.00 for lycopene and vitamin C (Table 6). The estimates were high for plant height, stem width, number of secondary branches, cluster per plant, number of fruits per plant, pericarp thickness, lycopene, vitamin C and total soluble solid, indicating low environmental influence. The remaining five traits had low broad sense heritability estimates. Narrow sense heritability estimates ranged from 0.04 for number of secondary branches to 0.50 for pericarp thickness.

The estimates were relatively low for number of leaves per plant, number of secondary branches, cluster per plant, number of flower per cluster and vitamin C whereas, stem width, number of days to 50 % flowering, pericarp thickness, lycopene and total soluble solid had moderate narrow sense heritability estimates, suggesting their importance in enhancing selection.

3.4 HETEROSIS AND POTENCE RATIO ESTIMATES OF TOMATO F_1 HYBRIDS

All the traits showed either or both significant positive or negative heterosis in different crosses, thereby reflecting that the parental cultivars are genetically diverse for traits measured except for number of secondary branches and pericarp thickness (Table 7). Mid-parent heterosis estimates for plant height were significant and positive for the ten tomato F_1 hybrids, and the highest (5.4 %) was observed for FDT_2 × Kerewa. The potence ratios for plant height ranged from 0.7 to 7.5, with nine crosses indicating overdominance and one indicating partial dominance in the inheritance of this trait. Simi-

Table 6: Narrow and broad sense heritability for morphological traits, nutritional and physicochemical parameters of tomato

Traits	Narrow-sense Heritability	Broad-sense Heritability
PH (cm)	0.14	0.66
SW (mm)	0.21	0.57
NLPP	0.08	0.28
DTF	0.25	0.49
NSB	0.04	0.88
CLPP	0.08	0.77
NFPC	0.09	0.42
NFP	0.15	0.87
PCAP (mm)	0.50	0.95
NLOBE	0.10	0.33
FMP (g)	0.13	0.33
LPEN (mg kg^{-1})	0.22	1.00
VIT C (mg kg^{-1})	0.09	1.00
TSS (°Brix)	0.25	0.99

PH = plant height; SW = stem width; NLPP = number of leaves per plant; DTF = number of days to 50 % flowering; NSB = number of secondary branches; CLPP = cluster per plant; NFPC = number of flower per cluster; NFP = number of fruits per plant; PCAP = pericarp thickness; NLOBE = number of locules per fruit; FMP = fruit mass per plant; LPEN= lycopene; VIT C = vitamin C; TSS= total soluble solid

larly, fruit mass per plant reflected desirable positive mid parent heterosis for the ten tomato F_1 hybrids, and the highest (13.7 %) was observed for FDT_4 × Ib-local. The potence ratios for fruit mass per plant ranged from -8.8 to 3.4 with nine crosses indicating overdominance and one cross combination (FDT_2 × Ib-local) indicating no dominance in the inheritance of this trait. For number of leaves per plant, a positive heterosis is desirable and was estimated for nine F_1 hybrids, and the highest (25.7 %) was observed for FDT_4 × UC-OP. These results were also confirmed by potence ratios, which had positive/negative values, indicating the presence of partial to over dominance effects. Regarding number of leaves per plant, 9 F_1 hybrids exhibited significant positive heterosis over mid parent and only the cross between UC-OP × Ib-local had significant negative heterosis but was also the only hybrid with significant positive heterosis over mid parent for cluster per plant. The potence ratios for number of leaves per plant ranged from -11.6 to 49.8 with eight crosses indicating overdominance and two indicating partial dominance in the inheritance of this trait whereas, the potence ratios for cluster per plant indicates partial dominance. For number of days to 50 % flowering, a negative heterosis is desirable and was estimated for half of the tomato F_1 hybrids, and the lowest (-8.7 %) was observed for FDT_2 × Kerewa indicating earliness, supported by the potence ratios signifying partial to over dominance effects. The estimates of heterosis, relative to mid parental values reflected significant mid parent heterosis but with only negative signs, on five and four tomato F_1 hybrids for number of flower per cluster and number of fruits per plant respectively, indicating the presence of the various degree of recessiveness involved in the inheritance of the two traits. This result was also confirmed by the potence ratios, which appeared with negative values for most of the hybrids. The range of significant mid parent heterosis in the desired direction for nutritional parameters varied from 2.3 to 28.1 % with the maximum (11.4 %) for lycopene content being found in FDT_2 × UC-OP while the best hybrid for tomato vitamin C content was FDT_2 × Ib-local with mid parent heterosis estimate of 28.1 %. These results were further confirmed with the potence ratios which were majorly described by partial to over dominance effects.

3.5 PHENOTYPIC CORRELATIONS BETWEEN TRAITS

Fruit mass per plant was significant ($p < 0.01$) and positively correlated with plant height (r = 0.46), number of days to 50 % flowering (r = 0.34) and cluster per plant (0.34) (Table 8). Number of flower per cluster had a significant ($p < 0.01$) positive association with stem width (r = 0.45) and number of secondary branches (r = 0.38).

A positive and significant ($p < 0.01$) correlation was observed between number of secondary branches and number of leaves per plant (r = 0.39). Number of locules per fruit also showed significant ($p < 0.05$) and positive correlation with stem width (r = 0.30) and number of flower per cluster (r = 0.33). On the other hand, correlation between the nutritional parameters and morphological traits were significant ($p < 0.01$) but negative, with correlation coefficient ranging from -0.30 to -0.51.

4 DISCUSSION

The significant variation among the tomato parental lines and their F_1 hybrids for all traits except number of leaves per plant shows inherent variability among the parental cultivars which support the report of Saleem et al. (2013b) and Kumar et al. (2018). These variations allowed combining ability analysis (Singh & Chaudhary, 1977). Considering all the traits measured in this study,

Table 7:Estimates of percent mid-parent heterosis and potence ratios for morphological traits, nutritional and physicochemical parameters of 10 tomato crosses

	Crosses									
	$FDT_4 \times FDT_2$		$FDT_4 \times$ UC-OP		$FDT_4 \times$ Ib-local		$FDT_4 \times$ Kerewa		$FDT_2 \times$ UC-OP	
	Parameters									
TRAITS	MPH (%)	Potence ratio	MPH (%)	Potence ratio	MPH (%)	Potence ratio	MPH (%)	Potence ratio	MPH (%)	Potence ratio
PH (cm)	3.2**	1.2	0.3**	2.2	2.9**	3.1	1.3**	0.7	2.8**	1.1
SW (mm)	-0.6	-0.1	-7.4	1.7	-37.5**	2.8	20.5	0.8	18.1	1.4
NLPP	22.7**	-11.6	24.7**	38.5	19.9**	49.8	25.4**	12.5	19.3**	-7.4
DTF	2.0**	-0.5	3.1**	-1.0	3.1**	-0.8	-4.0**	0.8	-4.0**	4.0
NSB	16.1	-5.0	12.5	-2.0	17.2	5.0	177.4	-55.0	39.4	13.0
CLPP	50.0	0.0	106.3	0.0	45.0	-2.3	52.9	-9.0	56.3	0.0
NFPC	-6.3**	-1.0	5.9	0.0	-8.6**	3.0	22.6	2.3	-6.3**	-1.0
NFP	36.9	-9.0	-52.3**	0.8	143.9	-9.8	15.4	-1.5	-57.5**	-0.9
PCAP (mm)	5.9	1.5	15.8	-25.0	12.0	2.6	27.6	43.0	39.5	8.6
NLOBE	50.5	1.4	-2.4	-1.0	-10.8**	0.3	25.7	-1.7	33.3	1.0
FMP (g)	4.9**	-4.1	5.2**	-7.5	13.7**	-4.5	7.4**	3.4	4.4**	-8.8
LPEN (mg kg^{-1})	2.3**	-0.1	122.3	4.7	119.6	-3.3	-5.9**	0.1	11.4**	-0.2
VIT C (mg kg^{-1})	-37.0**	-7.2	-15.3**	3.1	13.0**	0.7	-52.7**	4.7	-33.3**	-3.3
TSS (°Brix)	46.7	1.0	-29.3**	-12.7	9.9	0.9	-13.8**	3.2	74.0	1.6
	Crosses									
	$FDT_2 \times$ Ib-local		$FDT_2 \times$ Kerewa		UC-OP × Ib-local		UC-OP × Kerewa		Ib-local × Kerewa	
	Parameters									
TRAITS	MPH (%)	Potence ratio	MPH (%)	Potence ratio	MPH (%)	Potence ratio	MPH (%)	Potence ratio	MPH (%)	Potence ratio
PH (cm)	3.3**	1.9	5.4**	7.5	3.9**	5.1	4.7**	2.7	1.8**	1.8
SW (mm)	-54.1**	-2.5	39.0	-2.4	-26.2*	2.9	23.6	0.8	21.5	0.6
NLPP	4.9**	-2.1	3.5**	-0.9	-4.8**	19.7	17.9**	12.9	0.8**	0.5
DTF	-2.0**	0.0	-8.7**	-9.0	19.8**	-20.0	11.8**	-6.0	-2.9**	3.0
NSB	0.0**	0.0	12.5	0.0	16.1	1.7	51.5	17.0	40.0	-6.0
CLPP	65.0	3.3	11.8	2.0	5.0**	-0.3	5.9	-1.0	81.0	5.7
NFPC	-15.2**	-1.7	3.5	-1.0	-14.3**	5.0	16.1	1.7	0.0	0.0
NFP	22.4	2.1	38.3	6.2	-52.2**	-0.9	-48.7**	-0.80	20.0	4.5
PCAP (mm)	9.0	-13.0	-7.3	-2.2	15.2	2.9	-7.0	-5.5	26.9	-6.7
NLOBE	-33.3**	-0.5	56.5	1.2	-33.3**	0.8	65.5	-3.8	-18.3**	-0.7
FMP (g)	0.1**	0.0	8.7**	-2.6	13.3**	-5.7	7.3**	2.5	9.2**	1.8
LPEN (mg kg^{-1})	8.3**	-0.3	-58.4**	-37.1	67.9	-1.2	-74.4**	1.0	-13.9**	0.5
VIT C (mg kg^{-1})	28.1**	-2.1	0.8**	0.1	-30.6**	-1.3	-39.8**	6.4	0.4**	-0.0
TSS (°Brix)	56.9	1.5	-1.0	-0.0	-39.0**	-4.1	-3.7	0.6	-3.9	0.3

MPH = Mid-parent heterosis

*, **Significantly different from mid-parent at 0.05 and 0.01 probability levels, respectively, using *t*-test

Table 8: Pearson's correlation coefficients (r) between tomato morphological traits and nutritional parameters

	PH (cm)	SW (mm)	NLPP	DTF	NSB	CLPP	NFPC	NFP	NLOBE	FMP (g)	LPEN (mg kg^{-1})
SW	0.10										
NLPP	0.26	-0.08									
DTF	0.11	-0.13	-0.18								
NSB	0.11	0.18	0.39**	-0.08							
CLPP	0.12	-0.11	0.29*	-0.06	0.11						
NFPC	0.10	0.45***	0.14	-0.09	0.38**	0.01					
NFP	0.13	0.07	-0.17	0.06	-0.15	-0.12	0.09				
NLOBE	0.18	0.30*	-0.06	0.15	0.16	0.13	0.33*	-0.06			
FWP	0.46***	0.09	0.17	0.34*	0.10	0.34*	0.00	-0.01	0.15		
LPEN	-0.44***	-0.47***	0.00	-0.01	-0.13	0.28	-0.42***	-0.33*	-0.19	0.07	
VIT C	-0.30*	-0.30*	-0.35*	-0.22	-0.51***	-0.10	-0.28	0.28	-0.21	-0.30*	0.13

*, **, *** indicates significance at 0.05, 0.01, and 0.001 probability levels, respectively
PH = plant height; SW = stem width; NLPP = number of leaves per plant; DTF = number of days to 50 % flowering; NSB = number of secondary branches; CLPP = cluster per plant; NFPC = number of flower per cluster; NFP = number of fruits per plant; NLOBE = number of locules per fruit; FMP = fruit mass per plant; LPEN= lycopene; VIT C = vitamin C (mg kg^{-1})

the significant differences exhibited by GCA variance for number of leaves per plant, number of days to 50 % flowering and fruit mass per plant implies that only these three traits are controlled solely by additive gene action and the decision to improve those traits would be effective in early generations (Avdikos et al., 2021). The preponderance of additive variance in expression of morphological traits has been reported by Singh et al. (2010), Farzane et al. (2012), Shalaby (2013) and Vekariya et al. (2019). On the other hand, the exclusive significance of SCA variance for stem width and number of flower per cluster showed supremacy of nonadditive gene action the main cause of heterosis (Burdick, 1954) in the inheritance of these traits in agreement with the reports of (Govindarasu et al., 1981; Shankar et al., 2013).

The significance of both GCA and SCA variances for plant height, number of secondary branches, cluster per plant; number of fruits per plant, pericarp thickness, number of locules per fruit, lycopene, vitamin C and total soluble solid indicate the joint role of both additive and non-additive gene action which corroborate the report of Singh et al. (2010), Kumar et al. (2018) and Dufera et al. (2018). The magnitudes of GCA variances were higher than those of SCA variance for seven traits. Also, the ratio of GCA: SCA was greater than unity for those traits, indicating the preponderance of additive gene action in their inheritance (Christie & Shattuck, 1992). This is in agreement with Bakers' predictability ratio as the ratios for these traits were greater than 0.50. Therefore, selection for these traits could be an effective breeding approach in tomato improvement programmes. In addition, since GCA variances are higher than SCA variances, early generation selection of genotypes based on those traits becomes more efficient and promising hybrids can be identified (Smith et al., 2008). Conversely, the magnitude of SCA variances were higher than those of GCA variance for stem width, number of flower per cluster, number of fruits per plant, lycopene and total soluble solid as reported earlier (Farzane et al., 2012; Shende et al., 2012; de Souza et al., 2012; Yadav et al., 2013). Besides, the bakers' ratios were below 0.50 for these traits indicating the preponderance of nonadditive gene action in their inheritance (Christie & Shattuck, 1992). Thus, hybrid vigour can be exploited considering these traits in a tomato breeding programme but selection of superior genotypes may be delayed till later generations when the genes are fixed in the homozygous lines (Geleta & Labuschagne, 2006).

Out of the 14 traits measured, the overall parental mean value were significantly lower than the hybrids mean value for number of leaves per plant, number of secondary branches, cluster per plant, number of fruits per plant, fruit mass per plant and vitamin C which revealed an overall improvement in those traits through hybridization. It is important to mention that the parental lines and their offspring had similar gene for plant height which varied only by 2.9 %, as they both displayed the determinate growth habit. Additionally, the observed high number of leaves per plant was mainly because the data was collected at maturity which corroborates the report of Ibitoye et al. (2000). On the other hand, Ngosong et al. (2017) reported between 15 and 30 leaves per plant

but at three weeks after planting which implies that the stage of plant maturity determines the number of leaves. Thakur et al. (2017) and Vieira et al. (2019) previously described pericarp mean thickness for ripe tomatoes ranging between 4.1mm and 7.6mm, correlating with the value obtained in this study. The wide range for vitamin C observed in this study was in consonance with the report of Rivero et al. (2022) who reported 115.7 to 178.5 mg kg^{-1} in tomato cultivars commercialized in Cuba. With the exception of stem width, comparing the mean performance of the ten F_1 hybrids and their parental cultivar for other measured traits shows that more than eight of the hybrids tended to be either higher than their respective lower parent or deviated towards the higher parent. Superiority reflected by these hybrids was in agreement with the report of Pradeepkumar et al. (2001) and Hannan et al. (2007).

The estimations of general and specific combining abilities provided information on the breeding potential of the five tomato parents and their F_1 crosses. In crop improvement programmes, an astute selection of parental lines promotes a well-planned hybridization programme, and a parent with higher positive significant GCA effects (depending on the desired direction per trait) is considered a good general combiner. The estimates of significant GCA effects in the desired direction shows the reflection of the parental cultivars potential to transfer the traits to their progeny (Gayosso-Barragán et al., 2019). The parental cultivar UC-OP had the most significant GCA effects, with 5 traits in the desired direction, followed by FDT_2 and Kerewa with 4 traits each. By ranking the five parents according to the GCA effects, only Ib-local can be identified as promising general combiners for fruit mass per plant and plant height. Likewise, only FDT_2 was identified as general combiner for number of leaves per plant. Also, only Kerewa was superior general combiners for secondary branches and cluster per plant and UC-OP combines well for early flowering, number of fruits per plant and number of locules per fruit. Considering more than a parent with significant GCA effects in the desired direction for some traits; the parental cultivars FDT_2 and Ib-local seems to be better general combiners for lycopene. The parental cultivars, FDT_4, Kerewa and UC-OP were promising general combiners for vitamin C, Kerewa and UC-OP were identified as superior general combiners for total soluble solid while FDT_2, UC-OP and Kerewa are good combiner for pericarp thickness which makes them suitable for industrial use. These two parents (FDT_2 and FDT_4) also combine well for number of locules per fruit. Hence, the five parents were general combiners for diverse traits. Parents with high GCA effects for multiple traits could be used in breeding programmes to develop tomatoes with different combinations of traits because favourable additive genes would have accumulated (Bahari et al., 2012). Previous studies have reported significantly positive GCA effects for number of branches per plant (Singh and Nandapuri, 1974), number of fruits per plant (Dharmatti, 1996), plant height (Patil, 2013), fruit mass (Singh et al., 2014) and total soluble solid (Kumar et al., 2018) in tomato in spite of the different parents and environments used in their studies.

High and positive SCA effect estimates reveal the best combiner among the parental cultivars for the development of hybrids with specific target traits (Peña et al., 1998).

All the hybrid combinations were found to be good specific combiners for a minimum of two traits, indicating the significant role of nonadditive gene action in the inheritance of these traits. Tomato is a self-pollinated crop; hence SCA effect may not contribute much to improvement of traits but cross combinations with SCA in the desired direction coupled with good GCA may be utilized in breeding programmes (Wamm et al., 2010; Rewale et al., 2003). In this study, all cross combinations showed at least one desirable SCA effect, and none of the hybrids showed significant SCA effects for all traits. Tomato F_1 hybrids viz. UC-OP × Ib-local was the best specific combiners with the highest number of traits. The cross combinations between FDT_4 × FDT_2, FDT_4 × UC-OP, FDT_4 × Kerewa and UC-OP × Ib-local exhibited highly significant positive SCA effects for some morphological traits, nutritional and physicochemical parameters. According to Singh & Narayanan (1993) SCA effect refers to non-additive gene action which has positive relationship with heterosis. Therefore, hybrids FDT_4 × UC-OP, FDT_2 × Ib-local and UC-OP × Ib-local which involves one parent with a high GCA effect for number of fruits per plant and fruit mass per plant may be considered useful for the improvement of fruit yield, lycopene, vitamin C and total soluble solid and heterosis breeding may be recommended (Saleem et al., 2013a). These hybrids would be expected to produce segregants of a fixable nature in segregating generations through the simple pedigree method (Pandiarana et al., 2015).

Heritability estimates indicate the reliability with which traits can be passed down from one generation to the next. Estimates of broad-sense heritability were high for most of the traits measured indicating that the variation observed for those traits are genetically determined and the effect of environment on them were low, hence selection based on phenotypic expression will be efficient for genetic improvement of these traits. Moreover, selection for these traits at early segregating generation could lead to selection of elite genotypes (Bozokalfa et al., 2010). The broad-sense heritability estimates obtained in this study are in agreement with earlier reports (Haydar

et al., 2007; Sanjeev, 2010; Osekita & Ademiluyi, 2014; Kumar et al., 2018; Mohamed et al., 2018). Low narrow-sense heritability estimates for plant height, number of leaves per plant, number of secondary branches, cluster per plant, number of flower per cluster, number of fruits per plant, number of locules per fruit, fruit mass and vitamin C showed that they are primarily controlled by non-additive genes, and that selection for these traits may be ineffective.

The nature and magnitude of heterosis estimates help in the identification of promising hybrids (Pandiarana et al., 2015). The entire cross combinations were prominent for displaying highly significant heterosis over mid parent for only plant height and fruit mass per plant with the presence of various degrees of over dominance effects indicating the inherent genetic diversity between the parental cultivars and the newly developed hybrids that can be exploited through selection. This corroborates the report of previous researcher who found positive and significant heterosis for plant height and fruit mass per plant (Mageswari et al., 1999; Kurian et al., 2006; Shende et al., 2012; Agarwal et al., 2014). Thus, the best hybrid for plant height was FDT_2 × Kerewa (5.4 %) and superior hybrids for fruit mass per plant were FDT_4 × Ib-local (13.7 %) and UC-OP × Ib-local. Number of leaves per plant had mostly significant positive heterosis over mid parent with partial to over dominance effects. Out of 10 tomato F_1's, only one cross (UC-OP × Ib-local) expressed significant and positive heterosis with partial dominance for cluster per plant. The fewer the number of tomato locules the better for proper shape, firmness, processing, concentrations of solids and ascorbic acid (Dundi & Madalageri, 1991; Thamburaj, 1998). Heterosis in the negative direction with partial dominance effect with cross combinations FDT_2 × Ib-local and UC-OP × Ib-local (-33.3 %) are desirable hybrids for number locules per fruit. Considering the nutritional parameters, high lycopene and vitamin C are essential in the development of quality tomato because they add value to processed products as quality requirements desired by the consumers. Positive mid-parent heterosis estimates on few F_1 hybrids for lycopene and vitamin C found in this study were higher than the report of Pandiarana et al. (2015). These results were further confirmed with the potence ratios indicating partial to over dominance effects in the inheritance these parameters. Previous studies have reported significant positive heterosis for lycopene (YongFei et al., 1998), vitamin C (Makesh, 2002) and significant negative heterosis for number of locules per fruit (Sekhar et al., 2010). Early flowering in tomato is a desirable character; therefore negative heterosis is preferred over positive heterosis. Half of the cross combinations displayed significant negative heterosis over mid parent and the hybrids; FDT_2 × Kerewa depicted maximum significant negative heterosis. These results were strongly supported by the potence ratios, with two crosses each indicating partial to over-dominance effects, coupled with one cross combination signifying no dominance in the inheritance of this trait. Significant negative heterosis estimates were observed in the hybrids for number of fruits per plant and number of flower per cluster contrary to previous reports of Hannan et al. (2007). Various degrees of dominance were involved in the inheritance of the morphological and nutritional traits of tomato measured in this study and the negative values of potence ratio illustrated the presence of various degrees of recessiveness. Based on the significant percent mid parent heterosis estimate there is a potential to develop hybrids that are taller, early flowering, with fewer lobes, more number of leaves per plant, increased fruit mass per plant, higher lycopene and vitamin C content. In agreement with these findings, previous studies have reported significant performance of tomato hybrids above the parental lines (Singh et al., 2006; Dar et al., 2011; Singh et al., 2014; Pandey et al., 2015; Senapati & Kumar 2015).

Correlations between traits are critical in improving the efficiency of breeding programmes and assisting with appropriate selection indices (Nzuve et al., 2014). The positive relationship between fruit mass per plant, plant height, number of days to 50 % flowering, and cluster per plant is desirable, and it suggests that selecting taller tomato plants may result in larger fruits due to the stem reserve mobilization mechanism (Al-Tabbal & Al-Fraihat, 2012). Also, late flowering selection results in higher fruit yield and an increased number of tomato fruits due to a higher number of clusters per plant. Furthermore, the correlation between the number of flowers per cluster and the stem width and number of secondary branches, as well as the correlation between the number of leaves per plant and the number of secondary branches, show that traits with similar physiology were correlated and may be used for indirect selection. The relationship between the number of locules per fruit and stem width and the number of flowers per cluster suggests that selecting for a wider stem and flower cluster improves the capacity to support tomato fruits with many locules.

According to Mitchel et al. (1991) and Agong et al. (1997) nutritional and physicochemical properties are used as criteria to judge the organoleptic and processing qualities of tomato. Highly significant and negative correlations found between morphological traits and lycopene and even vitamin C corroborate the report of Agong et al. (2001) indicating that breeding programme would have to compromise some morphological traits to obtain better quality, particularly when nutrition is included as objectives in breeding programmes. On the

contrary, Kaushik & Dhaliwal (2018) reported lack of significant correlations between morphological traits and biochemical traits.

5 CONCLUSION

The half diallel analysis technique revealed the relative breeding potential of the parental cultivars and superior good combiner parents. The results from this study clarifies the nature and magnitude of gene action involved in the inheritance of the traits measured, provided information on the genetic worth of parental lines and possibility of selecting superior hybrids for further exploitation. The combining ability study confirms the presence of high variation among the genotypes with the preponderance of both additive and nonadditive gene actions influencing the inheritance of morphological traits, nutritional and physicochemical parameters measured. Parental line Ib-local was identified as potential donor for plant height, fruit mass, fruit lycopene content and UC-OP was superior for earliness, fruits per plant, number of locules per fruit, fruit vitamin C content. These two parents may be useful in tomato improvement programmes. Three promising hybrids (FDT_4 × UC-OP, FDT_2 × Ib-local and UC-OP × Ib-local) were selected on the basis of involvement of one parent with a high GCA effect for number of fruits per plant and fruit mass per plant, relevance of SCA effects and heterosis. These cross combinations may be considered useful for the improvement of fruit yield, lycopene, vitamin C and total soluble solid contents. The selected superior tomato hybrid may be released as varieties to growers for commercial cultivation. The findings of this study could be used to determine the best approach for tomato improvement in a breeding programme.

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Somatic embryogenesis and plant regeneration from radicles of olive (*Olea europaea* 'Chemlal') zygotic embryos

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Somatic embryogenesis and plant regeneration from radicles of olive (*Olea europaea* 'Chemlal') zygotic embryos

Abstract: Olive improvement by biotechnological tools such as genetic transformation requires an efficient *in vitro* regeneration system. Somatic embryogenesis seems the most suitable process. Our work describes for the first time the regeneration of whole plants in the main olive cultivar in Algeria 'Chemlal' via somatic embryogenesis induced from radicles of mature zygotic embryos. The obtained results showed that the establishment of a competent embryogenic culture is highly influenced by the chemical composition of the calli induction and maintenance media as well as addition of growth regulators. More than 10 and 13 % of nodular calli were obtained after callogenesis respectively on MS and OMc solid media containing IBA and zeatin followed by transfer to the same media without zeatin and a reduced concentration of auxin, while embryogenesis rates of 3.3 and 6.7 % were obtained respectively with IAA on MS medium and NAA on both tested media. However, no embryogenesis was observed with 2, 4-D or control which induced less callogenesis. Subsequently, an ECO medium with IBA, zeatin and BA particularly in liquid culture, allows better calli proliferation and embryogenic expression compared to OM and MS media. Finally, matured somatic embryos germinate quickly on a solid OM basal medium and generate normal well-developed plantlets easily acclimatized to natural conditions.

Key words: embryogenic callus; growth regulators; olive; proliferation rate; somatic embryo

Somatska embriogeneza in vzgoja rastlin iz radikule zigotskih embrijev oljke (*Olea europaea* 'Chemlal')

Izvleček: Izboljšanje oljke z biotehnološkimi orodji kot jer genetska transformacija zahteva učinkovit *in vitro* sistem vzgoje rastlin. V tem pogledu se zdi somatska embriogeneza najprimernejši način vzgoje. Raziskava opisuje prvič vzgojo rastlin najvažnejše sorte oljke Chemlal v Alžiriji s somatsko embriogenezo iz radikule zrelih zigotskih embrijev. Rezultati so pokazali, da je vzpostavitev primerne embriogene kulture močno odvisna od kemične sestave gojišča, v katerem poteka indukcija kalusa in gojišča za njegovo vzdrževanje kot tudi od dodatka rastnih regulatorjev. Več kot 10 in 13 % nodularnih kalusov je bilo pridobljenih na trdih MS in OMc gojiščih, ki sta vsebovali IBA in zeatin, čemur je sledil prenos v isto gojišče brez zeatina in zmanjšano koncentracijo auksina, 3,3 in 6,7 % embriogeneze pa je bilo dosežene z IAA na MS gojišču in dodatkom NAA na obeh preiskušenih gojiščih. Nasprotno pa ni bilo embriogeneze pri dodatku 2,4-D ali pri kontroli, kjer je bila indukcija kalogeneze slabša. Naknadno je ECO gojišče z IBA, zeatinom in BA, še posebej v tekoči kulturi, omogočilo boljšo proliferacijo in pojav embriogeneze v primerjavi z OM in MS gojiščem. Na koncu so zreli somatski embriji hitro vzkalili na trdnem OM osnovnem gojišču in dali normalne, dobro razvite rastlinice, ki so se zlahka prilagodile naravnim razmeram.

Ključne besede: embriogeni kalus; rastni regulatorji; oljka; hitrost proliferacije; somatski embrio

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1 INTRODUCTION

The olive tree (*Olea europaea* L.) is one of the most important crops in the Mediterranean region. In Algeria, several varieties of olive tree exist, of which the most cultivated are 'Chemlal' for oil extraction and 'Sigoise' as a table olive (Haddad et al., 2020) although other more interesting local cultivars also exist (Boukhari et al., 2020). Recently, the emergence of verticillium wilt caused by the fungus *Verticillium dahliae* Kleb. in newly established olive orchards has raised concerns about the development of the culture worldwide (Montes-Osuna and Mercado-Blanco, 2020) including in Algeria (Boutkhil et al., 2016). This soil-borne pathogen penetrates the plant via the roots and causes the necrosis of the organs as consequence of the obstruction of the vessels following the development of the fungal mycelium (Montes-Osuna and Mercado-Blanco, 2020). Indeed, the use of resistant genotypes remains the best solution to control this disease (Boutkhil et al., 2016; Narváez et al., 2019). However, the classical methods of breeding and multiplication achieved limited results and became unable to produce alone significant gains due to the long juvenile period of the species (10 to 15 years) specially in the current context of climate change (Rugini et al., 2020). Therefore, the use of biotechnological methods such as genetic transformation could be of great benefit for varietal creation in olive tree. However, the application of these techniques requires an effective *in vitro* regeneration system mainly for recalcitrant genotypes. Somatic embryogenesis seems to be the most appropriate and powerful *in vitro* method in several woody species including olive tree (Sánchez-Romero, 2019 and 2021).

Somatic embryogenesis has been induced from different explants of several olive cultivars. However, juvenile tissue is generally more successful for recalcitrant species (Von Arnold, 2008). Thus, the best results of olive embryogenic calli formation and regeneration were often obtained with juvenile tissues particularly radicles of mature zygotic embryos (Pires et al., 2020) because they include undifferentiated cells with a very high embryogenic activity (Sánchez-Romero 2019) while the petioles and the external parts of the leaf blade remain the most promising adult tissues for olive regeneration (Mazri et al., 2013) and even in wild olive (Narváez et al., 2019).

Nutritional and hormonal requirement for somatic embryogenesis depends directly on the type of explant and its degree of development and more on the genotype (Von Arnold, 2008). Induction and maintenance of olive embryogenic calli have often been achieved under dark conditions on solid medium based on the chemical composition of Olive Medium 'OM' (Rugini, 1984) or MS (Murashige and Skoog, 1962) supplemented with growth regulators. Later, the germination of somatic embryos can be successfully achieved under photoperiod on a simple medium even without hormones (Mazri et al., 2020) or a preliminary rigorous phase of maturation (Sánchez-Romero, 2019). Plants with a stable phenotype are regenerated despite some phenotypic variations observed after long periods of *in vitro* maintenance (Bradaï et al., 2016) and easily acclimatized to *ex vitro* conditions. However, the high genotype dependence limits the applicability and standardization of the pre-established protocols (Sánchez-Romero, 2021).

Thereby, our work describes for the first time an efficient protocol for *in vitro* regeneration of whole olive plants by somatic embryogenesis induced from radicles of zygotic embryos of the main cultivar in Algeria 'Chemlal', in order to apply some biotechnological techniques of genetic improvement particularly genetic transformation and induction of somaclonal variation. Thus, the effects of chemical composition of the culture medium and addition of growth regulators on the induction, proliferation of embryogenic calli as well as maturation and germination of somatic embryos were studied. In addition, acclimatization of the obtained plants to natural conditions was tested.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND DISINFECTION

Seeds extracted from the stones of olive cultivar 'Chemlal' mature fruits were sterilized with ethanol 70 % (v/v) for 1 min followed by soaking in sodium hypochlorite (NaClO 12°) at a concentration of 10 % (v/v) during 10 to 15 min. The disinfected seeds were rinsed three times with sterile distilled water for 5 min each time and kept immersed in water for 48 h at 25 ± 2 °C. After that, seeds were sterilized again as previous and conserved under sterile conditions, to extract the zygotic embryos from which radicles were carefully isolated to be used as explants for callus induction.

2.2 INDUCTION OF EMBRYOGENIC CALLI

Callogenesis was induced by culturing the previously isolated radicles during three weeks on two different solid media OMc based on Olive Medium 'OM' (Rugini, 1984) as adapted for callogenesis by Cañas and Benbadis (1988) or MS (Murashige and Skoog, 1962). Both media contain 0.5 mg l^{-1} of zeatin (Zea) and 5 mg l^{-1} of one of various auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphtyl-acetic acid (NAA)

or 2,4-dichloro-phenoxy-acetic acid (2,4-D). Then, explants were transferred during four weeks on the same media but without zeatin and with a reduced concentration of the auxin to 1/10th. The obtained calli were maintained for eight weeks on the cyclic embryogenesis olive medium (ECO) (Cerezo et al., 2011) with 0.1 mg l^{-1} of zeatin, 0.1 mg l^{-1} of benzylaminopurine (BA) and 0.05 mg l^{-1} of the previous auxin used during calli induction in addition to 0.55 g l^{-1} of glutamine and 1 g l^{-1} of casein hydrolyzate. All the used media during the establishment step of embryogenic calli contain 20 g l^{-1} of sucrose and 50 mg l^{-1} of myo-inositol. The pH was adjusted to 5.74 with NaOH or HCl (1 N) before adding 6 g l^{-1} of agar. Six radicles per Petri dish containing 25 ml of the culture medium and five dishes per treatment were incubated in the dark at 25 ± 2 °C. The changes of the radicle shape, appearance of callus as well as its texture and structure have been observed.

2.3 PROLIFERATION OF EMBRYOGENIC CULTURES

Three cell lines of calli induced on OMc with IBA and zeatin and considered as embryogenic for their good friability, nodular structure, low browning and well embryogenic expression were selected to estimate the embryogenic potential based on their proliferation rate (mass increase) in addition to embryo production and degree of necrosis on the maintenance medium. Thus, an embryogenic mass of 0.1 g was cultured on solid medium or in liquid culture with 100 rpm of stirring. Three chemical compositions (ECO, OM and MS) were tested. All the tested media contain 0.1 mg l^{-1} zeatin, 0.1 mg l^{-1} BA and 0.05 mg l^{-1} IBA in addition to 0.55 g l^{-1} glutamine and 1 g l^{-1} casein hydrolyzate. Furthermore, five combinations of growth regulators (including the control) composed of 0.1 mg l^{-1} of BA and 0.05 mg l^{-1} of IBA or NAA combined with 0.1 mg l^{-1} of zeatin or Thidiazuron (TDZ) were added to the ECO basal medium. At least three Petri dishes or three Erlenmeyer flasks of 100 ml containing 25 ml of medium were incubated in the dark at 25 ± 2 °C. After four weeks, the whole produced callus was recovered, weighed and the increase of the fresh mass in addition to the callus morphological traits such as texture, appearance of embryogenic structures, levels of friability and necrosis were determined, and a new inoculum of 0.1 g has been taken for the next subculture. The follow-up was carried out at least with three successive subcultures. Elsewhere, at the end of the liquid culture, the callus recovered by filtration was weighed and all the somatic embryos produced at different stages of development were determined.

2.4 MATURATION AND GERMINATION OF SOMATIC EMBRYOS

The pro-embryos used for maturation were produced by culturing a mass of 1 g of callus from each embryogenic line under total obscurity in 50 ml of liquid ECO medium containing growth regulators with stirring of 100 rpm. After four weeks, all the embryos at the globular, cordiform or torpedo stages exceeding 2 mm in size were transferred to a basal ECO solid medium supplemented with 1 g l^{-1} of activated charcoal for maturation during eight weeks while the cotyledonary embryos were directly germinated. More than three Petri dishes, containing at least nine pro-embryos each one, were incubated in total darkness at 25 ± 2 °C. Necrosis, cell proliferation and differentiation of mature embryos were recorded.

Germination of the matured and cotyledonary embryos has been achieved on OM basal medium with 20 g l^{-1} of sucrose, 100 mg l^{-1} of myo-inositol and solidified with 6 g l^{-1} of agar. More than sixteen embryos were cultivated individually in test tubes for eight weeks under a 16 h light photoperiod at a temperature of 25 ± 2 °C. Germination, root emergence, shoot length and number of developed leaves were observed. The obtained plantlets exhibiting an acceptable length with several well-developed leaves were acclimatized within laboratory for about two months on a humidified mixture of sand/potting soil/perlite at a rate of 2/2/1 (v/v/v). The reacting plants were transferred under natural conditions in a greenhouse on a substrate rich in organic matter and frequently irrigated before being permanently planted in the field.

2.5 STATISTICAL ANALYSIS

All statistical analyzes of the data (Analysis of variance and tests) were performed using the "XLSTAT" program version 2016.02.27444. In the case of a significant difference, the separation of the means was carried out by Fisher's LSD (Least Significant Difference) test. The percentages were analyzed by the chi-square test. The letters in the tables indicate homogeneous groups at significance level of 5 %.

3 RESULTS AND DISCUSSION

3.1 INDUCTION OF EMBRYOGENIC CALLI

Callogenesis from radicles of olive zygotic embryos was significantly influenced by the chemical composition of the induction medium as well as the used growth regu-

lators particularly the type of auxin. After three weeks, more calli were observed on OMc medium than on MS independently of the added growth regulators' balance except for 2,4-D with zeatin. The 'OMc' medium adapted for callogenesis by Cañas and Benbadis (1988) has been commonly used with olive juvenile tissues such as radicles and cotyledonary segments while the MS chemical composition was more beneficial for olive petioles and leaves segments (Trabelsi et al., 2011; Mazri et al., 2013) and even wild olive (Narváez et al., 2019). In fact, the best rate of callogenesis (100 %) was obtained on OMc with zeatin and IBA while less than 13 % of calli was obtained on controls and 26.7 % on OMc with 2,4-D (Table 1, Figure 1A and B). Mazri et al. (2012) noted that olive radicles on OMc medium containing IBA and 2iP (6-dimethylallylamino-purine) increase in size from the first week with formation of white calli on the injured part after three weeks in most of explants. However, Rugini (1995) indicated that callus can be formed even in the absence of auxins although obtaining embryogenic calli imperatively requires the combination of growth regulators inducing the conversion of somatic cells to a meristematic state (Maalej et al., 2002). Furthermore, Rugini (1988) indicated that 2, 4-D with BA induces less calli, inhibits cell differentiation and causes rapid and marked browning. These observations corroborate perfectly with our results where a weak amorphous callogenesis was observed on the controls media in addition to a rapid browning of the few calli obtained with 2, 4-D.

Transferring the explants to a medium without zeatin and with a reduced concentration of auxin induced their browning. However, 53.3 and 56.7 % of calli induced on OMc containing respectively IAA or IBA continue to proliferate better with production of nodules (Figure 1C) while 76.7 and 86.7 % of calli obtained respectively on OMc and MS containing 2,4-D showed a high browning with a low callogenesis (6 to 10 %) close to the control allowing 6.7 % of proliferation (Table 1). Mazri et al. (2012) noted that olive calli in the absence of cytokinin and reduced concentrations of auxins turn brown quickly due to a high accumulation of phenolic compounds reducing cell growth (Trabelsi et al., 2011). Nevertheless, calli produced in the presence of NAA were slightly browned (16 to 30 %) but with a significant loss of their proliferation capacity. Our results agree with the observation of Rugini (1988) indicating that NAA, despite the poor induced callogenesis, promotes embryogenic expression with less necrosis of cell masses.

Later, the passage of the cultures to the ECO solid medium including the appropriate combination of growth regulators indicated that obtaining and establishment of embryogenic calli were more determined by the auxin used during callogenesis than by the chemical composition of the induction medium. Pires et al. (2020) noted that a second transfer to an ECO medium rich in cytokinins allows a slow restart of cell proliferation with a significant embryogenic expression by formation of an easily separated nodules (Cerezo et al., 2011) considered as the initial marker of somatic embryogenesis (Mazri et al., 2013). However, low embryogenesis rates were often

Table 1: Effect of the chemical composition (OMc or MS) of the induction medium and growth regulators combination (different auxins + zeatin) on callogenesis, browning and somatic embryogenesis rates from radicles of olive zygotic embryos, 'Chemlal'*

		Callogenesis (%)		Browning (%)		Embryogenesis (%)
Induction medium	Auxins	3 weeks (+ Growth regulators)	4 weeks (- zeatin)	4 weeks (- zeatin)	+ 8 weeks (ECO)	8 weeks (ECO)
OMc	Control	13.3 d	6.7 d'	10.0 ef"	23.3 **de**	0.0 **c'**
	IAA	83.3 a	53.3 a'	73.3 ab"	90.0 **ab**	0.0 **c'**
	IBA	100.0 a	56.7 a'	70.0 ab"	83.3 **ab**	13.3 **a'**
	NAA	50.0 b	30.0 b'	30.0 de"	60.0 **c**	6.7 **abc'**
	2,4-D	26.7 cd	6.7 d'	86.7 a"	100.0 **a**	0.0 **c'**
MS	Control	10.0 d	6.7 d'	6.7 f"	20.0 **e**	0.0 **c'**
	IAA	60.0 b	20.0 bcd'	56.7 bc"	73.3 **bc**	3.3 **bc'**
	IBA	93.3 a	26.7 bc'	43.3 cd"	63.3 **c**	10.0 **ab'**
	NAA	46.7 bc	13.3 cd'	16.7 ef"	40.0 **d**	6.7 **abc'**
	2,4-D	43.3 bc	10.0 d'	76.7 ab"	100.0 **a**	0.0 **c'**

*Results are presented as the percentage to the total introduced explants. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %

obtained and vary between 7 and 13 % in the cultivars 'Picholine Marocaine', 'Dahbia' and 'Arbequina' (Mazri et al., 2012) while Cerezo et al. (2011) and Pires et al. (2020) reported 25 and 17 % of embryogenesis respectively in 'Picual' and 'Galega Vulgar'. Our results agree these observations since IBA combined with zeatin allowed the best rates of embryogenesis with 13.3 and 10 % respectively on OMc and MS (Figure 1D) while nearly 7 % of the calli obtained in the presence of NAA on both media and 3.3 % with IAA on MS were embryogenic (Table 1). In addition, the obtained embryogenic calli present a nodular and friable texture with white-yellowish globules of different sizes easy to separate allowing good proliferation during subculturing, whereas, some calli lines with strongly joined nodules often show marked browning and very slow proliferation capacity as well as few embryogenic structures. However, calli induced with 2,4-D were necrotic without any cell proliferation or subsequent embryogenic expression while apical browning followed by a total necrosis was observed in the radicles of the control media.

3.2 PROLIFERATION OF THE EMBRYOGENIC CULTURES

The obtained results indicated that proliferation capacity of olive embryogenic callus varied significantly among the genotype (cell lines) and according to the culture conditions, particularly the chemical composition of the maintenance medium and the followed method of culture, but much more by the added growth regulators.

3.2.1 Effect of the type and chemical composition of the proliferation medium

Regardless to the callus line and chemical composition of the medium; suspension culture allowed a significantly greater mass increase (Figure 1F) than solid medium (Figure 1E). Cell proliferation of the lines C1 and C3 was better on ECO liquid medium with 0.3 and 0.26 g of mass increase respectively while the OM chemical composition was more beneficial for the line C2 with nearly 0.2 to 0.3 g of mass increase respectively on solid and liquid media (Table 2). The low increases in mass of C1 and C3 calli with 0.2 and 0.08 g respectively were recorded on solid MS medium while proliferation of C2 seems to be less influenced by the chemical composition of the solid medium allowing between 0.16 and 0.18 g of mass increase (Table 2). Moreover, the best cell proliferation obtained on ECO medium was accompanied by good embryogenic expression and low necrosis of the cell masses (Figure 1E and F) whereas the other media particularly MS in addition to the low mass increase induced a marked browning and formation of compact calli weakly friable with large nodules.

The continued proliferation of embryogenic cultures is determined by several factors related to the nutrient and hormonal composition of the medium but highly by the genotype in culture (Merkle et al., 1995). As in our case, Cerezo et al. (2011) reported a pronounced browning in calli of olive cultivar 'Picual' on OMc with growth regulators whereas ECO liquid medium allowed a better production of less necrotic tissues, friable and nodular masses which contain several globular embryos despite the absence of a significant difference in the mass increase. Furthermore, ECO medium was more beneficial than MS for proliferation of calli induced from leaves and petioles of cultivar 'Dahbia' (Mazri et al., 2013). This beneficial effect of ECO medium may be due to its low content of macroelements, mainly nitrogen, which induces situation of stress favoring the proliferation of embryogenic structures (Cerezo et al., 2011). In addition, the improved result with suspension culture is the result of good cellular organization and synchronization

Table 2: Effect of the type (solid or liquid) and chemical composition (ECO, OM or MS) of the proliferation medium on the mass increase (in gram) of three lines (C1, C2 and C3) of embryogenic olive callus,'Chemlal' after four weeks of culture*

Type of culture	Solid medium			Liquid medium		
Chemical composition	C1	C2	C3	C1	C2	C3
ECO	0.29 ± 0.03 a	0.16 ± 0.02 b	0.14 ± 0.03 bc	0.29 ± 0.02 **ab**	0.20 ± 0.03 **d**	0.26 ± 0.01 **abc**
OM	0.28 ± 0.04 a	0.18 ± 0.01 b	0.09 ± 0.01 cd	0.22 ± 0.02 **cd**	0.30 ± 0.02 **a**	0.13 ± 0.02 **e**
MS	0.20 ± 0.03 b	0.18 ± 0.00 b	0.08 ± 0.01 d	0.20 ± 0.01 **d**	0.25 ± 0.03 **bcd**	0.11 ± 0.01 **e**

*Results are presented as mean ± standard deviation. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %. Liquid culture kept under 100 rpm of stirring

(Von Arnold, 2008) as well as a better oxygenation and availability of nutrients provided by continuous agitation (Neumann et al., 2009).

3.2.2 Effect of the growth regulators' combination

The calli of the three lines remained in proliferation even on the control media, although the presence of growth regulators and their combinations were essential for the proliferation of olive embryogenic calli, especially in suspension culture which allowed better proliferation compared to solid medium. According to Sánchez-Romero (2019) the embryogenic olive callus can proliferate even in the absence of growth regulators although these are important for the development of somatic embryos (Trabelsi et al., 2011). Indeed, our best results of mass increase were obtained in the presence of IBA and zeatin with formation of embryogenic masses less browned regardless to the followed type of culture for the line C1 producing more than 0.29 g of mass and line C3 on liquid medium with 0.26 g (Table 3). In concordance with previous studies; the combination of IBA, BA and 2iP instead of zeatin has been widely used for callus proliferation of several olive cultivars such as 'Picual' (Cerezo et al., 2011), 'Picholine Marocaine', 'Dahbia' and 'Arbequina' (Mazri et al., 2012) and 'Galega Vulgar' (Pires et al., 2020). However, Hegazi et al. (2017) observed that TDZ was more efficient when combined with IBA than with NAA to maintain calli obtained from radicles and cotyledons of the cultivar 'Coratina'. These authors suggested the existence of a relationship between the used auxin and cytokinin, which may be related to the endogenous hormonal balance of the explants directly varying with genotype (Mazri et al., 2012). Our results agree with this suggestion since that TDZ was more efficient when combined with IBA for the proliferation of lines C1 and C2 independently the culture method except for the C3 line for which TDZ combined with NAA allowed a better mass increase particularly in liquid culture with 0.23 g (Table 3). Furthermore, the cell proliferation of C2 and C3 was significantly reduced in presence of NAA combined with zeatin especially on solid medium with 0.04 and 0.06 g of mass increase respectively (Table 3) in addition of a marked browning preventing the appearance of embryogenic structures.

3.3 PRODUCTION AND MATURATION OF SOMATIC EMBRYOS

After one month in ECO liquid medium, the average number of recovered embryos and their degree of differentiation varied from one line of callus to another. Indeed, despite the low proliferation recorded in the lines C2 and C3 (Table 2 and 3); these lines produced more embryos (72.3 and 56 embryos/gfm) sufficiently differentiated (torpedo and cotyledonary) compared to C1 producing 33.3 embryos/gfm generally in the primary stages of differentiation (globular or cordiform) (Table 4). Subsequently, maturation of embryos varied significantly between the studied lines. About 15 % of the C1 pro-embryos turned necrotic more than the two other lines presenting 7.4 % of browning although no signifi-

Table 3: Effect of the growth regulators' combination (IBA or NAA combined with Zea or TDZ in addition to BA) added to the solid and liquid ECO proliferation medium on the mass increase (in gram) of three lines (C1, C2 and C3) of embryogenic olive callus, 'Chemlal' after four weeks of culture*

Type of culture	Solid medium			Liquid medium		
Growth regulators' combination	C1	C2	C3	C1	C2	C3
Control	0.04 ± 0.00 g	0.02 ± 0.00 g	0.02 ± 0.00 g	0.03 ± 0.00 **jk**	0.04 ± 0.01 **ijk**	0.02 ± 0.00 **k**
IBA-Zea	0.29 ± 0.03 a	0.16 ± 0.02 bc	0.14 ± 0.03 bcde	0.29 ± 0.02 **a**	0.20 ± 0.03 **cde**	0.26 ± 0.01 **ab**
IBA-TDZ	0.15 ± 0.03 bcd	0.11 ± 0.02 cdef	0.07 ± 0.01 efg	0.17 ± 0.03 **def**	0.21 ± 0.03 **bcd**	0.15 ± 0.01 **efg**
NAA-Zea	0.21 ± 0.04 b	0.04 ± 0.01 fg	0.06 ± 0.00 fg	0.1 ± 0.01 **ghi**	0.14 ± 0.02 **fg**	0.13 ± 0.01 **fgh**
NAA-TDZ	0.14 ± 0.02 bcde	0.08 ± 0.01 defg	0.17 ± 0.0 bc	0.16 ± 0.02 **def**	0.06 ± 0.02 **hij**	0.23 ± 0.02 **bc**

*Results are presented as mean ± standard deviation. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %. Liquid culture kept under 100 rpm of stirring

Table 4: Average number of immature somatic embryos (SE) produced per gram of fresh material (gfm) from three lines (C1, C2 and C3) of olive embryogenic callus, 'Chemlal' after four weeks in liquid culture with stirring and maturation after eight weeks on ECO basal solid medium with activated charcoal*

		Maturation			
Embryogenic callus lines	Average number of SE produced /gfm	Necrosis (%)	Proliferation (%)	Differentiation (%)	Average number of recovered matures SE
C1	33.3 ± 5.8 c	14.8 a'	74.1 a"	11.1 **b**	1.0 ± 0.0 **c'**
C2	72.3 ± 6.0 a	7.4 a'	44.4 b"	48.1 **a**	1.3 ± 0.2 **b'**
C3	56.0 ± 4.6 b	7.4 a'	48.1 b"	44.4 **a**	2.6 ± 0.4 **a'**

*Results are presented as mean ± standard deviation. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %

cant difference has been revealed statistically. Consequently, more than 74.1 % of the C1 embryos proliferated but only 11.1 % of explants differentiated few mature embryos while C2 and C3 explants presenting more differentiation with 48.1 and 44.4 % respectively produced 1.3 and 2.6 mature embryos recovered after eight weeks of incubation (Figure 1G; Table 4).

According to Rugini et al. (2005) olive pro-embryos on the maturation medium may show marked necrosis, amorphous cell proliferation or differentiate to advanced stages of development. Our results are close to those of Cerezo et al. (2011) and Narváez et al. (2019) indicating maturation rates varying from 49 to 59 % in pro-embryos of cultivar 'Picual' and wild olive respectively with an average of 1.7 to 2.2 mature produced embryos whereas about 6 % of embryos turned necrotic. Similarly, Bradaï et al. (2016) obtained about 5 mature embryos from young lines of 'Picual' callus.

3.4 GERMINATION OF SOMATIC EMBRYOS AND PLANTS REGENERATION

Germination of olive somatic embryos as well as the development of the resulted plantlets depend directly on the genotype (callus line). Under photoperiod, white-opaque embryos turn greenish with swelling and spreading of the two cotyledons in addition to the elongation of their roots, which turned yellow just before the emergence of a small shoot (Figure 1H). The same observations have been reported by Toufik et al. (2017) on embryos of the cultivar 'Dahbia' resulting in roots emergence and germination after two weeks under photoperiod conditions. In our study, embryos of C2 germinated with root emergence from the second week of introduction whereas no germination or rooting had been observed before two weeks with embryos of C3 (Data not shown). Rugini (1988) indicated that germination of olive somatic embryos is faster on OM medium. However, low germination rates have been often obtained with numerous cultivars where less than 13 % of germination obtained for 'Picual' (Cerezo et al., 2011) while Mazri et al. (2020) indicated that germination of 'Dahbia' somatic embryos was only achieved in the presence of growth regulators. This low conversion is mainly due to deficiencies in embryogenic maturation and development (Merkle et al., 1995). In our case, using cotyledonary somatic embryos after a maturation step, germination rates of 50 and 31.3 % respectively for C2 and C3 were obtained on OM basal medium. Consequently, C2 embryos resulted in well-developed plants (1.6 cm) with multiple leaves while small plants were obtained from C3 embryos (Data not shown). Finally, well-developed plantlets have been easily acclimatized and exhibited normal growth and phenotype *in vivo* even after transfer to field conditions (Figure 1I).

4 CONCLUSIONS

To our knowledge, our study describes for the first time an efficient regeneration of whole plants without morphological abnormalities in the main olive cultivar in Algeria 'Chemlal' via somatic embryogenesis induced from juvenile material, radicles of zygotic embryos. More embryogenic calli were induced on OMc medium containing IBA and zeatin, while the maintenance of cell lines on ECO medium particularly in liquid culture, allows better cell proliferation and production of embryos easily convertible into whole plantlets. The optimized approach will allow the application of biotechnological improvement methods particularly genetic transformation

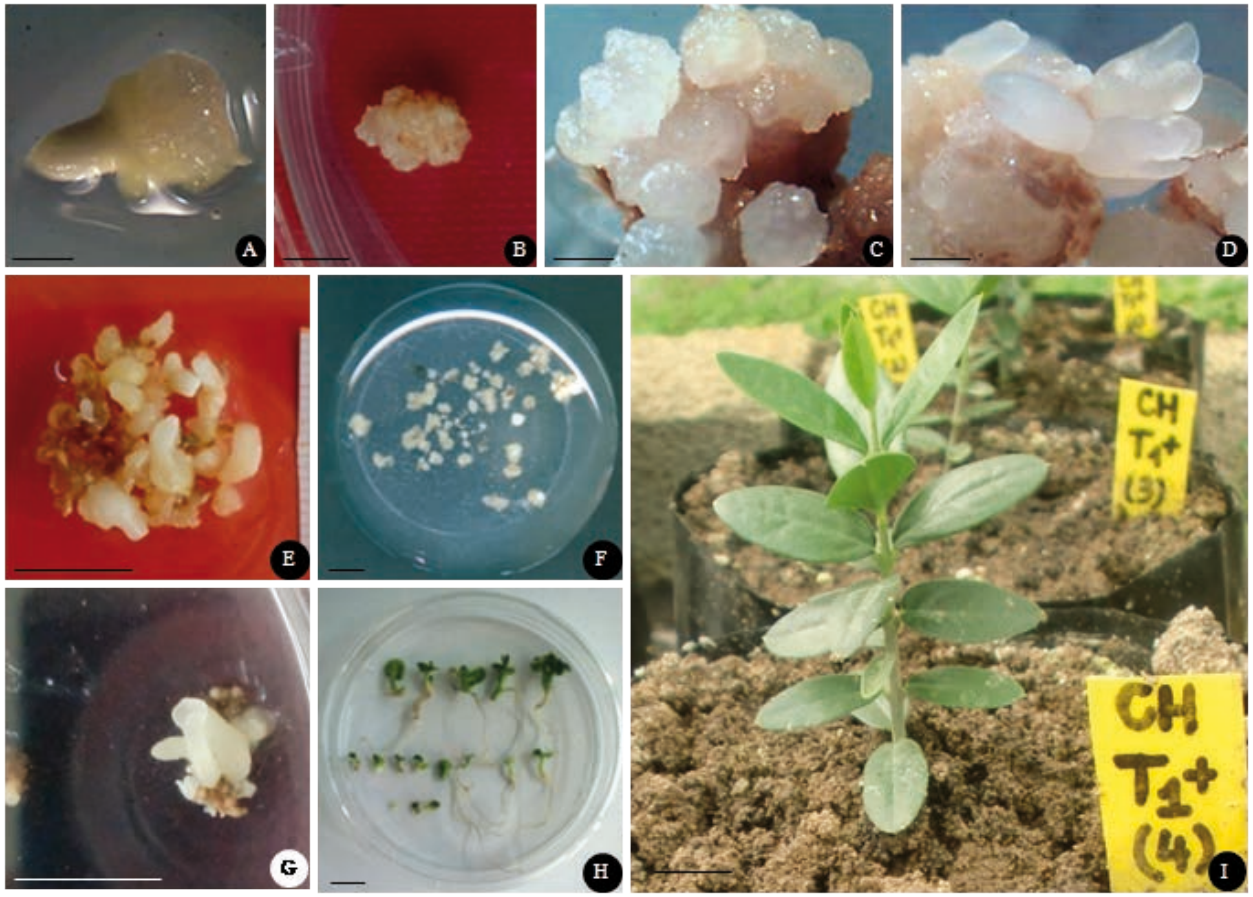

Figure 1: Plant regeneration via somatic embryogenesis induced from radicles of olive zygotic embryos, cv. 'Chemlal'. A and B. Aspect of radicles after 1 and 3 weeks on OMc solid medium containing IBA and zeatin. C. Appearance of nodules after 4 weeks on OMc without zeatin and reduced concentration of IBA. D, E and F. Somatic embryos (SE) appearance and callus proliferation after 4 weeks on solid or liquid ECO medium with IBA, zeatin and BA. G. Maturation of SE after 8 weeks on ECO solid basal medium with activated charcoal. H and I. Germination of SE on OM solid basal medium and acclimatized plantlets. (Bars correspond to 1 cm except for A, C and D the bar corresponds to 0.1 cm)

and induction of somaclonal variation as alternatives for varietal creation and improvement in olive tree against various stresses.

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Quality index based on fatty acids for Syrian pistachio cultivars (*Pistacia vera* L.) grown in Mascara (North-West of Algeria)

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Quality index based on fatty acids for Syrian pistachio cultivars (*Pistacia vera* L.) grown in Mascara (North-West of Algeria)

Abstract: Pistachio (*Pistacia vera* L.) is one of the most important hard-shelled nuts all over the world. Pistachio has a very high nutritional value with its quality index based on fatty acids composition, protein, mineral, vitamin-E, and antioxidant contents. In the current study, fatty acids values of the Syrian pistachio varieties cultivated in Algeria ('Adjmi', 'Bayadhi', 'Batouri', 'Achouri,' and 'Neb-djemel') were detected for the first time. Oil extraction of cultivars was performed using n-hexane in a Soxhlet apparatus, and the fatty acids composition of the oil was analyzed by gas chromatography coupled with a flame ionization detector (GC/FID) in the methyl ester form. The fatty acid composition of the pistachio cultivars was detected as: palmitic acid (8.23 % ± 0.36 to 9.49 % ± 0.07), palmitoleic acid (0.10 % ± 0.02 to 0.62 % ± 0.24), stearic acid (0.67 % ± 0.04 to 1.40 % ± 0.18), oleic acid (56.35 % ± 2.13 to 61.90 % ± 1.07), linoleic acid (19.48 % ± 0.27 to 26.76 ± 0.55) and linolenic acid (0.390 % ± 0, 03 to 0.59 % ± 0.01) in all samples. The results demonstrated that the five pistachio cultivars were rich in monounsaturated fatty acids (MUFA) (56.9 7% ± 1.88 to 62.10 % ± 1.02) and moderately low in saturated fatty acids (SFA) (9.90 % ± 0.04 to 10.37 % ± 0.23). 'Adjmi' has fatty acid composition lower than the other cultivars statically. Oleic acid value was determined higher than other fatty acid components. In the current study, the fat and fatty acid components of pistachio cultivars were determined and the results of this study can be used for future pistachio breeding programs.

Key words: kernels; pistachio; fatty acids composition

Na osnovi maščobnih kislin postavljen kakovostni indeks sirskih sort pistacije (*Pistacia vera* L.), ki se gojijo na območju Mascare (Severozahodna Alžirija)

Izvleček: Pistacija (*Pistacia vera* L.) je ena izmed najpomembnejših vrst oreškov, ki se goji širom po svetu. Ima zelo veliko hranilno vrednost, katere kakovostni indeks temelji na sestavi maščobnih kislin, beljakovin, mineralov, vitamina E in vsebnosti antioksidantov. V raziskavi je bila prvič preučena sestava maščobnih kislin v sortah sirske pistacije, ki se gojijo v Alžiri ('Adjmi', 'Bayadhi', 'Batouri', 'Achouri,' and 'Neb-djemel'). Ekstrakcija olja je bila narejena z n-heksanom v Soxhletovem aparatu, sestava maščobnih kislin v olju je bila analizirana s plinsko kromatografijo povezano s plamenskim ionizirajočim detektorjem (GC/FID) in metil estrom. V vseh vzorcih je bila ugotovljena naslednja sestava maščobnih kislin v olju pistacije: palmitinska kislina (8,23 % ± 0,36 do 9,49 % ± 0,07), palmitinsko-oleinska kislina (0,10 % ± 0,02 do 0,62 % ± 0,24), stearinska kislina (0,67 % ± 0,04 do 1,40 % ± 0,18), oleinska kislina (56,35 % ± 2,13 do 61,90 % ± 1,07), linoleijska kislina (19,48 % ± 0,27 do 26,76 ± 0,55) in linolenska kislina (0,390 % ± 0,03 do 0,59 % ± 0,01). Rezultati so pokazali, da je bilo olje vseh petih sort pistacije bogato na enkrat nenasičenih maščobnih kislinah (MUFA) (56,97 % ± 1,88 do 62,10 % ± 1,02) in sorazmerno revno na nasičenih maščobnih kislinah (SFA) (9,90 % ± 0,04 do 10,37 % ± 0,23). Sorta Adjmi je imela statistično slabšo sestavo maščobnih kislin, z večjo vsebnostjo oleinske kisline. Ugotovljeno je bilo, sa so oreški teh sort pistacije bogati na olju in maščobnih kislinah in da bi te sorte lahko uporabili v prihodnjih žlahtniteljskih programih.

Ključne besede: jedrca; pistacija; sestava maščobnih kislin

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1 INTRODUCTION

Pistacia genus has a dioecious flower habitat except for a few monoecious ones belonging to the order Sapindales, the family Anacardiaceae (Oukabli, 2005). It is included in more than 11 *Pistacia* species ranging from bush to tree form (Kafkas, 2006). *Pistacia vera* L. (pistachio) is the only *Pistacia* species in which nuts are edible and nutritional values are quite high since they are rich in fat and fatty acids ranging from 47.65 % to 63.31 % of the dry mass of the nuts (Agar et al., 1998., Yıldız et al., 1998; Küçüköner et al., 1998; Küçüköner and Yurt, 2001; Satil et al., 2003).

Several findings in the literature were reported regarding the biochemical composition of pistachio cultivars. Pistachio seeds contains 55.2-60.5 % oil, 15.0-21.2 % protein, and 14.9- 17.7 % carbohydrate and has a structure of fiber (10.3 g 100 g^{-1}), and 100 g of pistachio has 600 calories and it is one of the richest sources of energy (Krentz et al., 1994; Franz et al., 2002; Shahraki et al., 2014). The 100 g of pistachio nuts includes various micronutrients such as 4.0 mg sodium, 494-514.5 mg phosphorus, 120-150 mg calcium, 1,048-1,142 mg potassium, 494-514.5 mg phosphorus, 1.0-1.4 mg copper, 5.8-11.4 mg iron, 9.3 mcg selenium and 157.5-165.0 mg 100 g^{-1} magnesium (Franz et al., 2002; Shahraki et al., 2014). Pistachios are a serious nutritional source in terms of lutein and zeaxanthin (1,205 g 100 g^{-1}), vitamin B-6 (1.3 mg/100 g^{-1}), tocopherols (22.5 mg/100 g^{-1}), and carotenes (157g/100 g^{-1}), total phytosterols (279 mg /100 g^{-1}), sitosterol (210 mg/100 g^{-1}) (Kornsteiner et al., 2006; Bhagwat et al., 2008), and isoflavones (3.63 mg/100 mg) (Seeram et al., 2006; Gentile et al., 2007; Ballistreri et al., 2009).

The biggest producer countries all over the world were the US, Turkey, Iran, China, and Syria, respectively. Pistachio production in the world in 2020 was calculated as 1,205,532 Metric Tons (MTs). The highest production took place by the USA with 474,004 productions. Following, pistachio production was reported as 296,376 MT in Turkey, 190,000 MT in Iran, 80,000 MT in China, and 69,403 MT in Syria (Faostat 2022).

There are many cultivars that adapt well to the cultivated regions in pistachio (Karcı et al., 2022). There are a lot of pistachio cultivars in Iran, Turkey, and the US. Also, Syria is one of the oldest pistachio-growing countries and several cultivars grown in Syria were used commercially such as, 'Ajamy', 'Red Jalab', 'Adjmi', 'Bayadhi', 'Batouri', 'Neb-djemel', 'Bundouky', 'Marawhy', 'Lazwardy', 'White Oleimy', 'Nab Al-Djamal', 'White Jalab', 'Antaby', and 'Ein El-Tainah' (Kafkas, 2019). The oil composition of pistachio cultivars depends on environmental factors such as climate, geography, and soil type as well as cultivars (Chahed et al., 2007). There are serious differences in the biochemical traits of cultivars due to the climate conditions of the regions, because cultural application, rootstock, maturity at harvest, and moisture content also affect fat and fatty acid compositions.

Pistachio is a rich source of fixed oil and contains fatty acids such as oleic and linoleic acid, which are necessary for human nutrition (Garcia et al., 1992; Küçüköner and Yurt, 2003). The previous findings reported about positive effects on cardiovascular health, with significant reductions in total cholesterol (TC) and a dose-response improvement in TC/HDL, LDL/HDL, and non-HDL/HDL ratios (Sari et al., 2010; Koçyiğit et al., 2006; Gebauer et al., 2008). On the other hand, a pistachio-enriched diet is stated that to be an alternative drug agent and an effective hypoglycemic agent to protect against the prediabetic condition (Hernández-Alonso et al. 2014).

Several papers reported related to the detection of fat and fatty acids of pistachio cultivars exception of Syrian pistachio cultivars (Satil et al., 2003; Abdoshahi et al., 2011; Rabadan et al., 2018; Esteki et al., 2018; Pourian et al., 2019; Yahyavi et al., 2020). However, fat contents of Syrian pistachio cultivars have not been reported, to date. Thus, the results will be useful to provide a basis for the selection of parents and population construction, detection of the biochemical traits of all cultivars in the development of novel promising genotypes in terms of biochemical properties in future pistachio breeding.

Here, the objectives of this study were to identify the composition of the fat and fatty acids of Syrian pistachio cultivars. The fat and fatty acids results in Syrian cultivars can be used for future pistachio breeding programs.

2 MATERIALS AND METHODS

2.1 STUDY AREA

The study area is located at an altitude of 490 m above sea level with geographical landmarks (Latitude: 35°.22'N, Longitude: 00.11' 07"E). The climate conditions have a semi-arid Mediterranean climate which has a cool winter and hot summer. The annual rainfall was 380 mm irregularly distributed over the growing season. The soil of the plantation is sandy loam in texture with an alkaline pH (Figure 1).

2.2 PLANT MATERIAL

Pistachio samples ('Adjmi', 'Bayadhi', 'Batouri', 'Achouri' and 'Neb-djemel') were collected from experimental orchard of the Mascara University (Algeria) at

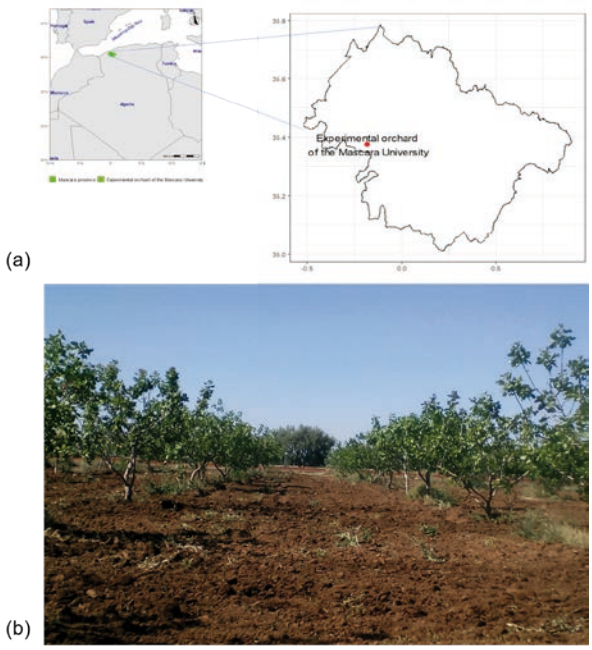

Figure 1: (a) Geographical location of pistachio cultivars of Syrian origin, (b) Pistachio orchard at the Mascara University experimental fields

harvest time. Pistachios' fruit exocarp tissues were removed and fruits were dried at room temperature condition. The samples were kept in a shell for one month at room temperature in the dark until analysis.

2.3 OIL EXTRACTION

Seed oil extraction was performed based on the Bligh and Dyer method (1959). Oils of 5 g nuts were extracted using hexane solvent for 2 h using automatic Soxhlet equipment (Gerhardt Soxtherm) and triplicate analysis was reported for each cultivar. The residue was dried until a constant mass was observed. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (FAMEs) (AOAC, 1990).

2.4 FATTY ACIDS ANALYSIS

Fatty acids were analyzed using a Clarus 500 Gas Chromatography with an autosampler (Perkin Elmer, Shelton, CT, USA) equipped with a flame ionization detector and a fused-silica capillary SGE column (100 m × 0.32 mm, ID 0.25 µm, BP20 0.25 UM; Perkin Elmer, Austin, TX, USA). The oven temperature was held at 140 °C for 5 min, and then raised to 200 °C at a rate of 4 °C min^{-1} and then to 220 °C at a rate of 1 °C min^{-1}, while the injector and the detector temperatures were set to 220 and 280 °C respectively. The sample volume was 1 µl, and the carrier gas was controlled at 16 psi. The split ratio was 1: 100. Fatty acids were detected by comparing their retention indices of the FAMEs with a standard 37- component FAME mixture (Supelco, Bellefonte, PA, USA).

2.5 STATISTICAL ANALYSIS

The Shapiro-Wilk test, using the shapiro.test function (R Core Team, 2020), showed satisfied data normality (p = 0.14). Analysis of variance (ANOVA) calculations was done using the l m function in R (R core Team, 2020). Tukey's post hoc Honesty Significance Difference (HSDT) test was applied to detect the source of the differences, with the help of the agricolae R package (Mendiburu, 2020).

2.6 PRINCIPAL COMPONENT ANALYSIS (PCA)

Principal Component Analysis (PCA) is an extremely powerful tool for synthesizing information, very useful when there is a large amount of quantitative data to process and interpret. Experimental data can be identified by PCA, which plays an important role in reducing the dimensionality of a large number of interdependent variables into a new set of uncorrelated variables called principal components (PC). PCA is applied to series to construct several groups according to similar variance properties at different time scales. The first PC represents the highest variance in the original variable, followed by the second, third, and other components. This method provides useful information using a smaller set of variables and is relatively easy to interpret. The objectives of a PCA are as follows (Westra et al., 2007); (i) the individuals' graphic representation, in a 2-dimensional plane, showing the similarities between them, (ii) the variables' graphic representation, on the same level by explaining at best the initial connections between them. Principal component analysis (PCA) was applied to data using the FactoMineR R package (Le et al., 2008).

3 RESULTS AND DISCUSSION

3.1 OIL CONTENT

The detected oil content values of Syrian cultivars in this study were ranging from 50.23 % to 65.42 %, while the oil content of Iranian varieties such as 'Badami', 'Ohadi', and 'Mumtaz' varies from 58.96 % to 60.10 %

Table 1: The oil content (%) and fatty acid composition (%) of the five varieties of pistachio

Cultivars	Oil Content	Myristic C14:0	Palmitic C16:0	Stearic C18:0	Total SFA	Palmitoleic C16:1	Oleic C18:1	Total MUFAs	Linoleic C18:2	Linolenic C18:3	Total PUFAs
Adjmi	50.23 ± 0.43	0.43 ± 0.01	8.23 ± 0.36	1.40 ± 0.18	10.06 ± 0.17	0.62 ± 0.24	56.35 ± 2.13	56.97 ± 1.88	19.87 ± 1.53	0.57 ± 0.08	20.45 ± 1.46
Bayadhi	65.42 ± 1.05	0.3 ± 0.12	9.20 ± 0.04	0.79 ± 0.07	10.29 ± 0.14	0.20 ± 0.04	61.90 ± 1.07	62.10 ± 1.02	20.98 ± 0.12	0.47 ± 0.01	21.45 ± 0.12
Batouri	57.29 ± 1.94	0.24 ± 0.01	9.49 ± 0.07	0.64 ± 0.17	10.37 ± 0.23	0.45 ± 0.02	60.46 ± 0.66	60.46 ± 0.66	19.48 ± 0.27	0.48 ± 0.01	19.96 ± 0.26
Achouri	63.13 ± 2.09	0.28 ± 0.07	8.73 ± 0.07	0.97 ± 0.02	9.98 ± 0.16	0.21 ± 0.04	60.41 ± 0.61	60.41 ± 0.61	20.73 ± 0.28	0.59 ± 0.01	21.32 ± 0.29
Neb-djemel	55.92 ± 2.49	0.21 ± 0.07	9.01 ± 0.15	0.67 ± 0.04	9.90 ± 0.04	0.10 ± 0.02	57.24 ± 0.33	57.24 ± 0.33	26.76 ± 0.55	0.39 ± 0.03	27.15 ± 0.59

(Kamangar et al., 1975). On the other hand, the researchers reported that the fat contents of five Iranian cultivars were calculated ranged from 52.48 to 60.65 % (Abdoshahi et al., 2011). A total of 17 pistachio cultivars from different locations in Iran were characterized according to fatty contents and they were determined ranged from 49.9 to 58.5 % (Yahyavi et al., 2020). In addition to different traits of cultivars, the climatic conditions of the cultivation region and the maintenance of the pistachio orchards could be the causes of the difference in the fat amount (Chahed et al., 2008). The fat content and fatty acids compositions of five pistachio cultivars ('Adjmi', 'Bayadhi', 'Batouri', 'Achouri' and 'Neb-djemel') were given in Table 1.

3.2 FATTY ACIDS COMPOSITIONS

The kernels of pistachio are rich in monounsaturated fatty acids followed by polyunsaturated fatty acids and saturated fatty acids. Although the highest percentage of MUFA was detected as 56.97 % in 'Adjmi', the lowest percentage of MUFA was calculated as 62.10 % in 'Bayadhi'. The lowest and the highest amount of PUFA were identified as 19.96 % and 27.15 % in 'Batouri' and 'Neb-djemel', respectively. The average content of unsaturated fatty acids was determined as 76.93 % in this study, while this value was found as 81.5 % of the total fatty acids in Iranian cultivars (Kamangar et al., 1975). The percentage of SFA were ranged between 9.90 and 10.37 %, and found in 'Neb-djemel' and 'Batouri' cultivars, respectively. The ratio of unsaturated/saturated acids in the kernel of these pistachio cultivars varied from 7.69 to 8.52 with an average of 8.05 (Table 2). This ratio was determined as 7.90 in Turkish cultivars and was found to be similar to the results of this study (Agar et al., 1995).

Table 2: The total saturated and unsaturated fatty acids content of pistachio cultivars

Cultivars	Saturated fatty acids (%)	Unsaturated fatty acids (%)	Unsaturated/ saturated
Adjmi	10.06 ± 0.17	77.42 ± 3.34	7.69
Bayadhi	10.29 ± 0.14	83.55 ± 1.14	8.11
Batouri	10.37 ± 0.23	80.42 ± 0.92	7.75
Achouri	9.98 ± 0.16	81.73 ± 0.9	8.18
Neb-djemel	9.9 ± 0.04	84.39 ± 0.92	8.52

One of the main fatty acid components of pistachio cultivars, oleic acid, was found in ranging from 56.35 to 61.90 %. This value was detected as slightly lower in Iranian cultivars (48.96-55.24 %) (Abdoshahi et al., 2011). On the other hand, while linoleic acid values were calculated (19.48-26.76 %) significantly lower than Iranian cultivars (30.48- 36.88 %), palmitic acid values were identified (8.23–9.49 %) similar to another research (Abdoshahi et al., 2011). The previous report demonstrated that high oleic content and lower levels of linoleic acid content make nut oil more stable against oxidative changes (Küçüköner and Yurt 2003). Shakerardekani et al. (2015) reported that pistachio is highly susceptible to lipid oxidation due to its high oil content, however that high oleic and palmitic oil content increased the oxidation stability.

The myristic (0.21-0.43 %), stearic (0.64-1.40 %), palmitoleic (0.10-0.62 %) and linolenic (0.39-0.59 %) were detected in traces in the current study. The previous findings related to fatty acids supported that oleic acid was the most common monounsaturated fatty acid, while linoleic acid was the most common polyunsaturated fatty acid in pistachio (Shokraii, 1977; Garcia et al., 1992; Kafkas et al., 1995; Agar et al, 1995; Okay, 2002; Satil et al., 2003; Esteki et al., 2019) and the similar results were found in this study.

The two-factor ANOVA (Cultivar and Type) dem-

Table 3: The results of two-way ANOVA analysis

	Df	Sum Sq	Mean Sq	F value	Pr (> F)
Cultivars	4	8.51	2.13	7.71	0.0001
Type	6	28418.79	4736.47	17150.98	3.60E-59
Cultivar:Type	24	107.44	4.48	16.21	1.23E-12
Residuals	35	9.67	0.28		

onstrated that there was a significant difference between cultivars ($p < 0.001$), types ($p < 0.001$). And, this analysis showed the existence of a significant interaction between cultivar and type ($p < 0.001$) (Table 3).

To detect the source of the differences, Tukey's post hoc Honesty Signicance Difference (HSDT) test was applied with the help of the agricolae R package (Mendiburu, 2020). The 'Neb-djemel', 'Bayadhi', 'Achouri' and 'Batouri' pistachio cultivars generated close results, while "Adjmi" cultivar created relatively different result from the others (Table 4).

The Tukey test revealed that the highest fatty acid compositions were identified as O leic.C18:1 (59.12), while the lowest results were calculated in Stearic.C18:0, Linolenic.C18:3, Palmitoleic.C16:1, Myristic.C14:0, respectively. The most efficient interaction was determined between 'Bayadhi', 'Achouri', 'Batouri' pistachio cultivars. On the other hand, the highest linoleic fatty acid composition was calculated as 26.76 in 'Neb-djemel' and the lowest linoleic value was found in 'Batouri' pistachio cultivar. Although the highest and the lowest palmitic acid compositions were detected as 9.49 and 8.24 in 'Batouri' and 'Adjmi', respectively. Although the lowest linolenic, myristic and palmitoleic fatty acid values were calculated as 0.40, 0.22 and 0.11 in 'Neb-djemel', respectively, the highest myristic, palmitoleic and stearic fatty acids were produced as 0.43, 0.62 and 1.40 in 'Adjmi' pistachio cultivar, respectively (Table 5).

Table 4: The results of multiple comparisons between cultivars with Tukey's test

Cultivars	Value	Groups
Neb-djemel	13.47	a
Bayadhi	13.41	a
Achouri	13.10	a
Batouri	12.97	ab
Adjmi	12.50	b

3.3 PRINCIPAL COMPONENT ANALYSIS OF FATTY ACIDS

Principal Component Analysis (PCA) is very helpful for processing and interpretation large amounts of quantitative data. It is a very important analysis in reducing the dimensionality of a large number of interdependent variables. The PCA analysis can illustrate the individuals' values, in a 2-dimensional plane, using the similarities or differences between them. In the present study, PCA was performed using the FactoMineR package (Le et al., 2008). The percent 86.47 of the total variability was explained by axes 1 and 2. The projection of the variables demonstrated that Linolenic.C18:3, Palmitoleic.C16:1, Myristic.C14:0 and Stearic.C18:0 were correlated significantly with axis 1, while Oleic.C18:1 (Linoleic.C18:2) was correlated (inversely correlated) with axis 2 (Figure 2).

The average of fatty acids belonging to all cultivars were calculated and grouped according to letters. Oleic.C18:1, Linoleic.C18:2 and Palmitic.C16:0 fatty acids had different groups from others, a, b, c; respectively (Table 5, Figure 2). However, statistically significant?? differences were detected between cultivars in Oleic.C18.1 and Linoleic.C18:2 fatty acids. The Oleic.C18:1 values of the cultivars were classified in two groups such as a and b. These values were determined similar in Batouri, Achouri and Bayadhi pistachio cultivars, while Adjmi and Neb-djemel were replaced in same group according to Oleic.C18:1 values of them. The Linoleic.C18:2 fatty acid values of Neb-djemel were calculated as higher than other pistachio cultivars and found different statistically. Adjmi and Neb-djemel pistachio cultivars were separated from the others due to differences among all pistachio cultivars according to Oleic.C18:1 and Linoleic.C18:2 fatty acids values in the analysis of PCA (Figure 3).

4 CONCLUSION

In the present study, fatty acid compositions of Syrian pistachio cultivars were determined. The 'Bayadhi' and 'Achouri' cultivars have the highest oil content, while 'Adjmi' has the lowest oil content. It determined that pistachio is rich in monounsaturated fatty acids such as, oleic acid and quite poor in saturated fatty acids. The highest SFA, MUFA, and PUFA values were detected in 'Batouri', 'Bayadhi', and 'Neb-jemel' pistachio culti-

Table 5: The results of multiple comparisons between cultivars-type with the Tukey's test

Cultivars	The fatty acids	Values	Groups	Average Values	Groups
Batouri	Oleic.C18:1	60.01	a	59.12	a
Achouri		60.21	a		
Bayadhi		61.90	a		
Adjmi		56.36	b		
Neb-djemel		57.13	b		
Neb-djemel	Linoleic.C18:2	26.76	c	21.56	b
Batouri		19.49	d		
Adjmi		19.88	d		
Achouri		20.73	d		
Bayadhi		20.98	d		
Adjmi	Palmitic.C16:0	8.24	e	8.93	c
Achouri		8.73	e		
Neb-djemel		9.02	e		
Bayadhi		9.20	e		
Batouri		9.49	e		
Neb-djemel	Linolenic.C18:3	0.40	f	0.50	d
Bayadhi		0.47	f		
Batouri		0.48	f		
Adjmi		0.58	f		
Achouri		0.59	f		
Neb-djemel	Myristic.C14:0	0.22	f	0.29	d
Batouri		0.24	f		
Achouri		0.29	f		
Bayadhi		0.30	f		
Adjmi		0.43	f		
Neb-djemel	Palmitoleic.C16:1	0.11	f	0.32	d
Bayadhi		0.20	f		
Achouri		0.21	f		
Batouri		0.46	f		
Adjmi		0.62	f		
Batouri	Stearic.C18:0	0.64	f	0.89	d
Neb-djemel		0.67	f		
Bayadhi		0.80	f		
Achouri		0.97	f		
Adjmi		1.40	f		

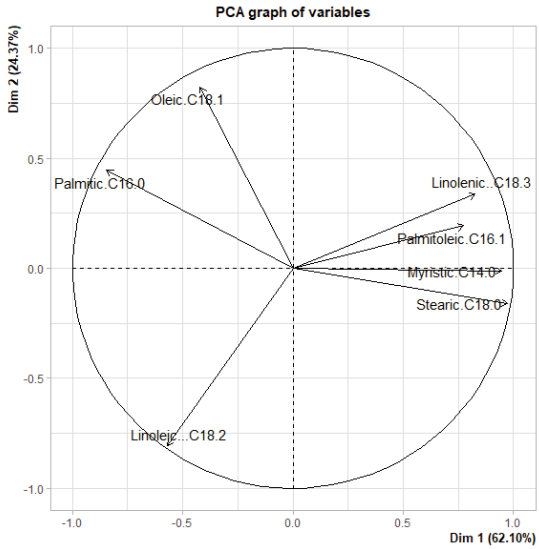

Figure 2: The projection of variables in the 1x2 plan

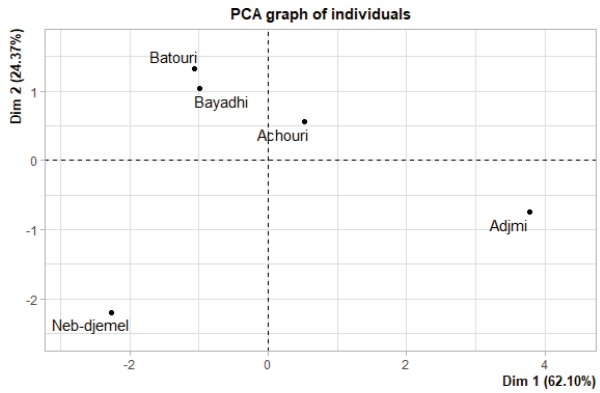

Figure 3: The projection of cultivars in the 1x2 plan

vars, respectively, and the lowest in 'Neb-jemel', 'Adjmi', and 'Batouri' cultivars. These results demonstrated that pistachio has a satisfying ratio of unsaturated and saturated fatty acids that provide superior nutritional value for consumers. As a result, the results obtained from this study are important in terms of using them for future pistachio breeding studies.

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Chemical composition and insecticidal effect of methanol extract of *Capparis spinosa* L. fruits on *Tribolium confusum* Jacquelin du Val, 1863 and *Sitophilus oryzae* (L., 1763) adults

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Chemical composition and insecticidal effect of methanol extract of *Capparis spinosa* L. fruits on *Tribolium confusum* Jacquelin du Val, 1863 and *Sitophilus oryzae* (L., 1763) adults

Abstract: *Tribolium confusum* and *Sitophilus oryzae* are stored product pests found worldwide. Environmental damages, human health issues and the emergence of resistance are driving scientists to seek alternatives to synthetic insecticides for its control. Under this scenario, plant secondary metabolites are being increasingly studied as bioinsecticides because they are renewable, natural, biodegradable, non-persistent in the environment and safe to non-target organism and humans. In this study, the chemical composition and lethal effects of methanol extract of *Capparis spinosa* fruits on *Tribolium confusum* and *Sitophilus oryzae* adults were studied. The LC_{50} of extract on *T. confusum* and *S. oryzae* in contact method were 14.7 and 10.5 mg cm^{-2}, respectively, whereas in the dip method, the LC_{50} value determined 41.3 and 34.3 mg ml^{-1} for *T. confusum* and *S. oryzae*, respectively. The most important identified compounds were the thymol (22.5 %), methyl sulfonyl heptyl isothiocyanate (13.3 %), butyl isothiocyanate (8.1 %), γ-terpinene (6.2%) and iso propyl isothiocyanate (5.8 %). The results confirmed the potential of the *C. spinosa* extract in controlling stored-product insects.

Key words: *Capparis spinosa*; GC-Mass; insecticidal plant extract; stored-product pest; erpenoides

Kemijska sestava in insekticidni učinki metanolnih izvlečkov plodov kaprovca *Capparis spinose* L. na odrasle osebke malega mokarja (*Tribolium confusum* Jacquelin du Val, 1863) in riževega žužka (*Sitophilus oryzae* (L., 1763))

Izvleček: Mali mokar (*Tribolium confusum*) in rižev žužek (*Sitophilus oryzae*) sta po vsem svetu razširjena skladiščna škodljivca. Okoljska škoda, škodljivi vplivi na zdravje ljudi in pojav odpornosti na insecticide vodijo raziskovalce k iskanju alternativ sintetičnim insekticidom za uravnavanje teh škodljivcev. V tem pogledu se povečuje preučevanje sekundarnih metabolitov rastlin, ker so obnovljivi, naravni, razgradljivi, neobstojni, okoljsko varni za neciljne organizme in človeka. V raziskavi so bili preučevani letalni učinki metanolnih izvlečkov plodov kaprovca (*Capparis spinose*) na odrasle osebke malega mokarja (*Tribolium confusum*) in riževega žužka (*Sitophilus oryzae*). LC_{50} vrednosti izvlečkov za malega mokarja in riževega žužka sta bili pri kontaktni metodi 14,7 in 10,5 mg cm^{-2}, pri metodi potapljanja pa 41,3 in 34,3 mg ml^{-1}. Najpomembnejše sestavine, določene v izvlečkih plodov kaprovca so bile timol (22,5 %), metil sulfonil heptil izotiocianat (13,3 %), butil izotiocianat (8,1 %), γ-terpinen (6,2 %) in izopropil izotiocianat (5,8 %). Rezultati so potrdili potencialno uporabo izvlečkov plodov kaprovca za uravnavanje škodljivih skladiščnih žuželk.

Ključne besede: *Capparis spinosa*; masni plinski kromatograf; insekticidni rastlinski izvlečki; skladiščni škodljivci; terpenoidi

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1 INTRODUCTION

Food production and preservation has always been a major concern of human beings. Likewise, the increasing trend of human population has made people experience numerous problems such as hunger and environmental pollution. In fact, over 600 species of beetles, 70 species of butterflies, and about 355 species of mites, which infest stored agricultural products, reduce the quantity and quality of these warehoused crops. As much as 50 %–60 % cereal yields can be lost during the storage stage due to the lack of technical possibilities for their proper harvesting and storage (Kumar and Kalita, 2017). In Iran, in rural areas, mainly due to the traditional and unsuitable conditions of warehousing, this rate arrives to 80 % (Forghani and Marouf, 2015).

Confused flour beetle (*Tribolium confusum* Jaqcquelin du Val, 1863) and rice weevil *Sithophilus oryzae* (L., 1763) are regarded two of the most important pests of stored cereals around the world, which not only causes significant losses due to feeding, but also, they infect the stored crop with their fecal pellets and larval shells, and reduces the crop's quality greatly (Forghani and Marouf, 2015).

In recent years, a great number of researchers have been attracted to the ways of pest management by using herbal essential oils and plant extracts. The plant extracts have useful insecticide properties such as less effect on natural enemies, no plant-burning, minimal lethality on the vertebrates and rapid degradation in the environment and can be partly replaced by artificial insecticides (Wink, 2018).

Capparis spinosa L. is a plant of the family Cappridaceae, a flowering bush with two bases branching from the base and broadly exfoliated on the ground. The leaves are plain and light green, and flowers are reddish-white with a large number of long flags and the fruit is oval and meaty and bright green, gradually reddish. It grows in different regions of Iran with different soils and has a high tolerance to heavy soil texture (Ahmadi and Saeidi, 2018).

Based on our knowledge, few studies have been reported previously relating to the activity of *C. spinosa* extract against insect pests. However, insecticidal activities of other species of *Capparis* genus were reported in various studies (Hussein et al., 2006; El-Shershaby, 2010; Upadhyay, 2012; Ladhari et al., 2013).

In this research, chemical composition and insecticidal effect of methanol extract of *Capparis spinosa* on two species of storage product pests (confused flour beetle and rice weevils) was investigated.

2 MATERIALS AND METHODS

2.1 INSECTS CULTURES

The two species of insects were obtained from the infected storages located in Zabol, Iran and maintained in the dark in incubators (27 ±1 °C; relative humidity = 65 ± 5%) for propagation. The larvae of *T. confusum* were picked up and reared in glass containers (0.5 l) containing wheat flour mixed with yeast (10:1, w/w). *S. oryzae* were reared on cracked rice seeds. Insects used in all of the experiments were one week old adults.

2.2 PLANT MATERIAL AND EXTRACTIONS

For the extraction of secondary metabolites, fresh fruits of *C. spinosa* were collected from the central part of Hirmand (61° 32' E and 31° 0' N), Sistan and Baluchistan province, Iran, during July and August, 2013. The fruits were dried naturally and grounded into powder. The powder was weighed and extraction was also done by soaking the powdered fruits in methanol solvent (ratio 1:10 w/v) and shaking the mixture on a shaker (350 rpm) for 24 hours at 25 °C.

After the completion of extraction, the liquid extract was filtered through Whatman No.1 filter paper fitted in a Buchner funnel using suction. The extracted plant substances were stored in airtight containers covered with aluminum foils in refrigerator at 4 °C. To determine the dry mass of pure extract at one milliliter of solution, the small parts of solution (5 ml) were collected in three replicate, separately and allowed to dry completely at 100 °C in oven (Ghaemi et al., 2006).

2.3 INVESTIGATION OF LETHAL EFFECT BY CONTACT METHOD (RESIDUAL TOXICITY)

For determination contact toxicity, each experimental unit consisted of a 6 cm Petri dish (with an area of 28.26 cm^2). In order to evaluate the lethal effect of the plant extract on both insect pests, different volumes ranging between 1.5 to 4.8 ml of the plant extract with the concentration of 117 mg ml^{-1} (equivalent to 6.21, 8.69, 11.8, 13.66, 14.91, 16.15, and 19.87 mg cm^{-2}) were uniformly poured by a 1 ml sampler into a Petri dish. In the control samples, various volumes of 1.5 to 4.8 ml of methanol were also poured through a Petri dish. The Petri dish cap was left open for 30 minutes to let the sol-

vent evaporate. Then, ten 1-7- day- old adult insects were put in each dish and the Petri dish was covered by selephon nylon. The Petri's patch was placed in the incubator at 27 ± 1 °C and relative humidity of 65 ± 5 °C in darkness for 24 h (Akbar et al., 2022; Tavares et al., 2021; Seremet et al., 2018).

The number of the dead insects was calculated after 24 hours based on the movement of the legs and tentacles. In case of observation of mortality in the control, the percentage of the lethality in different concentrations of the extract was corrected by Abbott's formula (Abbott, 1925).

2.4 INVESTIGATION OF LETHAL EFFECT BY INSECT-DIP METHOD (TOPICAL TOXICITY)

To evaluate the toxicity of metanol extract in dip method, different concentrations of extracts, including 26.5, 29.5, 35.4, 41.3, 44.2, 47.2 and 59 mg ml^{-1} were prepared, and the insects were immersed in each concentration for 10 seconds. Then, the insects were placed on the filter paper for 5 minutes to dry the bodies. The insects of each replicate were transferred into a 6 cm Petri dish on a filter paper and the Petri dish was closed. In the control, immersion was performed in the methanol solvent for 10 seconds. The number of dead insects was determined after 24 hours based on the movement of the legs and tentacles (Akbar et al., 2022; Tavares et al., 2021; Liu et al., 2016).

In both bioassay method, experiment was conducted on ten 1-7- day- old adult insects in a completely random design with four replicates for each concentration. To determine the 50 % lethal concentration (LC_{50}), five concentrations causing between 20 % and 80 % mortality were used mainly based on the preliminary test results of the contact toxicity of the extract. The experiment was carried out in the same way as the above test, and the number of live and dead insects was counted. The LC_{50} values were calculated using Probit analysis (IBM SPSS V21.0).

2.5 GAS CHROMATOGRAPHY–MASS SPECTROSCOPY OF EXTRACT

Gas chromatography–mass spectroscopy (GC–MS) analyses of metanol extract were carried out on a Hewlett-Packard 5890 gas chromatograph coupled to a HP 5970 mass-selective detector (MSD) using a fused silica ultra performance cross linked methyl silicone column (50 m × 0.2 mm i.d.; 0.25 µm film thickness) at 4 °C/min ramp rate. Helium was the carrier gas at 1 ml/min flow rate. Mass spectra were recorded over 40–500 amu range at 1 scan/s with ionization energy 70 eV and ion source temperature 250 °C. Constituent's identification was made by comparison of their mass spectra with those stored in NIST and Wiley libraries or with mass spectra from the literature (Ravan et al., 2019; Rojht et al., 2012).

2.6 STATISTICAL ANALYSIS OF DATA

Data were analyzed by SPSS software (IBM SPSS V21.0). Normality of raw data was surveyed using Non-Parametric One-Sample Kolmogorov-Smirnov test. Statistical analysis of the mortality data was performed by one-way analysis of variance (ANOVA) with a post-hoc Tukey test at $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 INSECTICIDIAL ACTIVITIES

The results showed that by increasing the concentration of metanol extract of plant, the mortality rate of the tested insects increased significantly in both bioassay methods (Figure 1, $F_{5, 23} = 27$, $p < 0.001$ for *T. confusum* and $F_{4, 19} = 39.6$, $p < 0.001$ for *S. oryzae* in contact method and $F_{5, 23} = 32.6$, $p < 0.001$ for *T. confusum* and $F_{5, 23} = 108$, $p < 0.001$ for *S. oryzae* in insect-dip method).

The 50 % lethal concentration (LC_{50}) in contact method was determined as 10.5 and 14.7 mg cm^{-2} for *S. oryzae* and *T. confusum*, respectively. LC_{50} was calculated as 34.3 and 41.3 mg ml^{-1}, 24 hours after dipping in the metanol extract of *C. spinosa*, for *S. oryzae* and *T. confusum*, respectively (Table 1). Due to the non-overlapping of the 95 % confidence level of LC_{50}, the toxicity of metanol extract of *C. spinosa* on *S. oryzae* was significantly more than that of *T. confusum* (Table 1).

No study has been reported concerning the contact toxicity of *Capparis spinosa* extract on confused flour beetle and rice weevil. However, insecticidal activities of other species of *Capparis* genus were reported in various studies. The essential oil of another species *Capparis* genus, *Capparis decidua* (Forssk.) Edgew., had an insecticide and repellency effect on *Sitophilus oryzae* and *Rhyzopertha dominica* (Fabricius, 1792) (Upadhyay 2012). Besides, the lethality and repellency of *Capparis aegyptia* Lam. on *Tetranychus urticae* Koch, 1836 (Acari, Tetranychidae) was reported (Hussein et al. 2006).

Moreover, the biological and toxic effects of the leaf extract of *Capparis aegyptia* on *Agrotis segetum* (Denis and Schiffermüller, 1775) (Lepidoptera: Noctuidae) and *A. ipsilon* (Hufnagel, 1766) was studied and according to

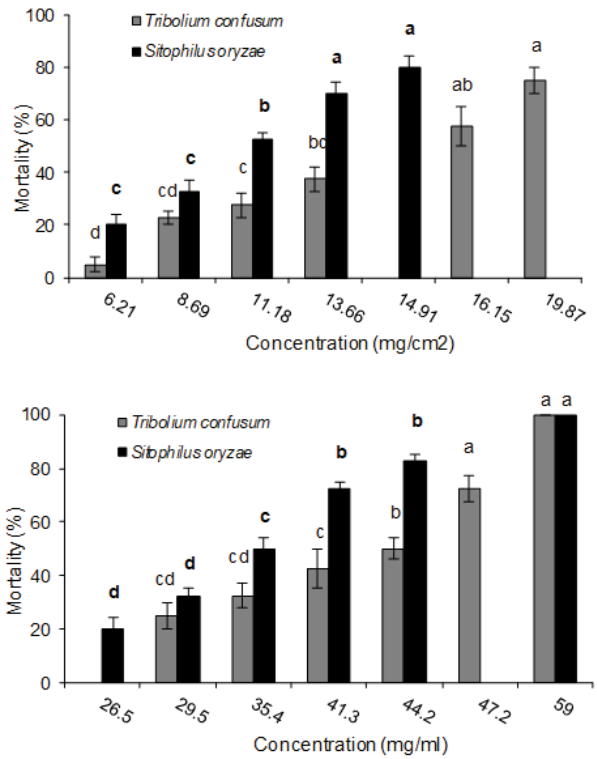

Figure 1: Mortality percentage (Mean ± SE) of *T. confusum* and *S. oryzae* exposed to metanol extract of *C. spinosa* in contact (above) and dip method (below) bioassay
Means with the different letters above the columns for each pest species are significantly different ($p < 0.05$) (Tukey post-hoc test after analysis of variance)

the results, its extract reduced the fertility of adult female insects by 50 % (El-Shershaby 2010).

The metanol extracts of *C. spinosa* leaf in the concentration of 10000 ppm caused 46 % mortality of the third instar larvae of *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera, Noctuidae) after three days of treatment. This rate increased by 100 % after a 7- day treatment. The anti-nutritional index of the metanol extract of this plant in the same concentration on larvae was 49.7 % (Ladhari et al. 2013).

3.2 CHEMICAL COMPOSITION OF PLANT EXTRACT

Gas chromatography-mass spectrometry analysis indicated that there were 33 compounds in the metanol extract of *C. spinosa* fruits, comprising 98.6 % of the total mass, among which the most notably were thymol (22.5 %), methyl sulfonyl heptyl isothiocyanate (13.3 %), butyl isothiocyanate (8.1 %), γ-terpinene (6.22 %) and isopropyl isothiocyanate (5.8 %) (Table 2).

The present result was similar to the other literature reported previously. The main components of *C. spinosa* leaf oil were thymol (26.4 %), isopropyl isothiocyanate (11 %), 2- hexenol (10.2 %) and butyl isothiocyanate (6.3 %). The volatile oils of the fruits composed mainly of isopropyl isothiocyanate (52.2 %) and methyl isothiocyanate (41.6 %) (Afsharypuor et al., 1998).

Existence of the glucosinolates degradation products as isothiocyanate derivatives in metanol extract of *C. spinosa* fruits, are of pharmacological interest because they have insecticidal effect and so contribute to the plant's overall defense mechanism (Bohinc et al., 2014; Gupta et al., 1999).

In addition, investigation of the plant extract showed the presence of terpenoid compounds such as thymol (22.5 %), γ-terpinene (6.2 %), carvacrol (3.1 %), caryophyllene (2.3 %), linalool (1.5 %) α-thujene (1.3 %), β-pinene and 1,8-cineole (1.1 %) in extract of *C. spinosa* fruits that have been well documented as active fumigants, repellents, and insecticides toward stored-product insects (Rojht et al., 2012; Papachristos et al., 2004).

4 CONCLUSIONS

The metanol extracts of *C. spinosa* fruits showed insecticidal activities against *S. oryzae* and *T. confusum* adults. The main components of plant extract have insecticidal properties such as isothiocyanate derivatives and terpenoid compounds. However, further tests are needed

Table 1: LC_{50} values of methanol extract of *C. spinosa* against *T. confusum* and *S. oryzae* after 24 h

Bioassay method	Insect	LC_{50}	95 % CL	P-value	Slope ± SE	x^2(df)
Contact	*T. confusum*	14.7*	(13.4-16.6)	0.67	4.24 ± 0.61	2.33 (4)
	S. oryzae	10.5	(9.5-11.6)	0.49	4.56 ± 0.73	2.73 (3)
Dipping	*T. confusum*	41.3	(38.3-45.8)	0.34	5.47 ± 1.29	3.34 (3)
	S. oryzae	34.3	(32.3-36.4)	0.92	7.68 ± 1.18	0.48 (3)

* Ten individuals per replicate, four replicates per concentration, 5 or 6 concentrations per assay, total number of tested insects were 200 or 240 adults for every test; LC: lethal concentration (mg cm^{-2} in Contact method and mg ml^{-1} in insect-dip method), CL: confidence limits

to develop a formulation and to improve the potency and stability of these potential insecticides for practical use.

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Table 2: Chemical constituents of methanol extract of *Capparis spinosa* fruits

Peak No	Compounds	Rate (%)	Kovats index
1	Methyl isothiocyanate	2.7	741
2	Isopropyl isothiocyanate	5.8	822
3	2-Hexeneal	1.5	858
4	Benzyl aldehyde	1.1	869
5	Methyl sulfonyl heptyl isothiocyanate	13.3	902
6	Methyl furan	2	919
7	Butyl isothiocyanate	8.1	936
8	α-Thujene	1.3	957
9	Comphene	0.9	971
10	β-pinene	1.1	988
11	3-octanone	3.4	1011
12	α-Terpinene	1.5	1020
13	Ocymene	1	1051
14	1,8-cineole	1.1	1080
15	γ-Terpinene	6.2	1106
16	Linalool	1.5	1128
17	Cis-sabinene hydrate	1.8	1175
18	n-Dodecane	2.6	1220
19	Comphor	1	1248
20	Para-menta-6,8,dien-2-ol-acetate	1.3	1270
21	Carvone	1.4	1311
22	Thymol	22.5	1345
23	Caryophyllene	2.3	1382
24	n-tetradecane	1	1460
25	α -farnesene	0.9	1481
26	Carvacrol	3.1	1495
27	Cadinol	1	1520
28	Geranyl acetone	0.8	1588
29	Hexadecanoic acid	2.5	1707
30	Frulic acid	0.8	1731
31	Octa decanoic acid	1.2	1815
32	n-Eicosane	1.1	1878
33	Beta-sesquiphellandrene	0.8	1897
	Total	98.6	

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Molecular diversity of rice (*Oryza sativa* L.) genotypes in Malaysia based on SSR markers

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Molecular diversity of rice (*Oryza sativa* L.) genotypes in Malaysia based on SSR markers

Abstract: Rice crop improvement is determined by the degree of genetic variability and the heritability of favorable genes. A total of twenty-five SSR markers were used to measure the level of polymorphism and genetic variation among the 65 rice genotypes. Twenty-one of the twenty-five SSRs were discovered to be polymorphic, whereas the rest were determined to be monomorphic. A total of 91 alleles were found in 21 SSR markers, with an average of 4.00 alleles which ranged from 3 (RM335, RM551, RM538 RM190, RM242 and RM270) to 7 (RM263). The average PIC value was 0.62 ranging from 0.28 (RM 270) to 0.76 (RM 481). The rice genotypes were divided into nine primary clusters by a dendrogram based on NTSYS software's UPGMA analysis. The cluster analysis revealed that these genotypes were divided into nine clusters where cluster IB-1a has the most genotypes (31) followed by cluster IB-1b (24). The genotype BR24 and Utri as well as Pukhi and WANGI PUTEH had the highest dissimilarity coefficient values indicating genotype diversity. These accessions have a lot of genetic diversity among the constituents; thus, they could be used directly in a hybridization program to improve yield-related parameters.

Key words: molecular diversity; SSR markers; polymorphic information content; rice

Molekularna raznolikost genotipov riža (*Oryza sativa* L.) v Maleziji, določena na osnvi SSR označevalcev

Izvleček: Izboljšanje pridelka riža temelji na njegovi genetski variabilnosti in zmožnosti dedovanja primernih genov. Za meritev ravni polimorfizma in genetske variabilnosti med 65 genotipi riža je bilo uporabljenih 25 SSR označevalcev. Od 25 SSR je bilo 21 prepoznanih kot polimorfnih, med tem, ko so bili ostali določeni kot monomorfni. V 21 SSR označevalcih je bilo celokupno 91 alelov s poprečno vrednostjo 4,00, ki je variirala od 3 (RM335, RM551, RM538 RM190, RM242 in RM270) do 7 (RM263). Poprečna vrednost PIC je bila 0,62, z razponom od 0,28 (RM 270) do 0,76 (RM 481). Genotipi riža so se v dendrogramu razdelili v devet primarnih grozdov na osnovi programa NTSYS in UPGMA analize. Analiza grozdov je odkrila devet grozdov, kjer je grozd IB-1a vseboval največ genotipov (31), temu je sledil grozd IB-1b (24). Genotipi kot so BR24, Utri, Pukhi in WANGI PUTEH so imeli največji koeficient neenakosti, kar nakazuje veliko genetsko raznolikost. Te akcesije imajo v svojih lastnostih veliko genetsko raznolikost in bi lahko bile neposredno uporabljene v programih križanja za izboljšanje s pridelkom povezanih lastnosti.

Ključne besede: molekulaska raznolikost; SSR označevalci; informacija o polimorfnosti; riž

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1 INTRODUCTION

Rice is a key crop in the world, and its production is dependent on the development of superior varieties with greater productivity and adaptability, which is largely dependent on the presence of sufficient genetic variation in rice germplasm (Osekita et al., 2015). This is normally performed through hybridization following that in segregating populations in selecting plants with favorable qualities (Becerra et al., 2017)Chile, has a rice (Oryza sativa L.. Rice accessions include a large number of beneficial genes that rice breeders can employ to improve the crop and genetic heterogeneity exists among rice accessions allowing for a wide range of agricultural improvements. Any crop development effort needs genetic diversity because it aids genotype collection, monitoring, and identification. The generation of segregating progenies with significant genetic variety with potential development of recombinants for further selection and introgression of desirable genes from these diverse genotypes. A major goal in evolutionary biology has long been to characterize and quantify genetic variation. Analyzing morphological or molecular data can be used to estimate genetic diversity. One method to understanding their diversity is to use modern molecular technology (Linda Mondini et. al., 2009)

Any crop improvement initiative must start with a genetic diversity assessment since it aids in the development of improved recombinants. Genetic divergence across genotypes is significant in identifying parents with a wide range of characteristics. Genetic diversity can be measured using morphological traits, iso-enzymes, and DNA markers. The morphological changes in physical features that result from genetic differentiation may not be enough to identify between closely related species, races, or ecotypes. As a result, genetic characterization of natural resources is a vital step toward a better understanding of genetic resources and their use in future breeding programmes. Molecular markers have generally outperformed physical pedigree, heterosis and biochemical evidence in assessing genetic diversity (Nadeem et al., 2018). Factors affecting genetic gain include genetic variation available in breeding materials, heritability for traits of interest, selection intensity, and the time required to complete a breeding cycle. Genetic gain can be improved through enhancing the potential and closing the gaps, which has been evolving and complemented with modern breeding techniques and platforms, mainly driven by molecular and genomic tools, combined with improved agronomic practices (Xu et al., 2017). Genetic diversity is frequently quantified in terms of genetic distance or genetic similarity, both of which indicate the existence of genetic differences or similarities.

Microsatellite (SSR) markers are being one of the most reliable and effective DNA molecular markers. They are employed in a variety of applications including genetic diversity studies and rice breeding programmes (Bohra et al., 2017). Due to their multi-allelic and highly polymorphic nature even a small number of SSR markers can provide a better genetic diversity spectrum (Singh et al., 2016). The genetic diversity of crop kinds introduced throughout time fluctuates over time. SSR markers have been demonstrated to be a dependable technique for assessing genetic diversity in both wild and cultivated rice species as well as for detecting genetic polymorphism and genotype differentiation (Krupa, 2017). Microsatellite markers (SSR) are superior to other PCR-based markers used in genetic mapping research because they are highly informative, co-dominant in nature, highly reproducible, plentiful, easy to analyses and cost effective (Nadeem et al., 2018).

Genetic characterization clearly outperforms existing methods when it comes to detecting variety, both genotypes and genes (Koskey et al., 2018)there is paucity of data on rhizobia diversity and genetic variation associated with the newly released and improved mid-altitude climbing (MAC. Similarly, genetic characterizations using molecular technologies has a higher detection power than phenotypic approaches. This is due to the fact that molecular technologies reveal disparities in genotypes or the ultimate level of diversity embodied by an individual's DNA sequences that is unaffected by their environment. Standard characterization and evaluation of accessions can be carried out using a variety of ways, including classic procedures like the use of morphological character descriptor lists. They may also include agronomic performance evaluations under a variety of environmental situations. Genetic characterizations refer to the description of traits that follow a Mendelian inheritance pattern or that include specific DNA sequences. Molecular markers rather than morphological features can indicate significant differences between more direct, reliable, and effective technique for germplasm characterization, conservation, and management that is not impacted by environmental influences (Toppo et al., 2018). Molecular markers have been broadly utilised to investigate the genetic diversity and a wider selection of breeding materials that are less impacted by time, geographical, and environmental factors. Molecular markers can be used to identify genetic variation in rice cultivars (Yadav et al., 2017).

Therefore, the goal of the current study was to use SSR markers to examine the trend in genetic diversity in 65 rice genotypes. In view of the points, the current study was conducted to examine molecular diversity across 65

rice genotypes in order to discover varied genotypes that could benefit rice breeding programmes.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND DESIGN

The plant material for the present research work includes rice genotypes. Table 1 shows a complete list of genotypes used in the current study. The research evaluation took place at the Field 10, Universiti Putra Malaysia, Serdang, Selangor in 2019 The seedlings of 65 rice genotypes were raised on tray and appropriate agronomic practices were done. Twenty-one days old seedlings were transplanted in the plastic pot with three replications following randomized complete design. All the agronomic practices were carried out in the pot to grow a healthy crop**.**

2.2 MOLECULAR DIVERSITY ANALYSIS

A total 25 SSR markers were selected on the basis of polymorphism shown by markers in screening (Table 2).

2.3 ISOLATION OF GENOMIC DNA AND SCORING OF DNA BANDS

Young fresh leaves of individual genotypes were collected from 14 days old seedlings and the DNA was extracted using Doyle & Doyle (1987) CTAB extraction method with minor modifications. The DNA quality estimation was done using spectrophotometrically (Spectronic® Genesis™). The resulting ratio (OD260/OD280) was used to determine the nucleic acid purity in various DNA samples. A polymerase chain reaction (PCR) was used to amplify a specific region of total genomic DNA to a billion-fold in vitro. The Eppendorf Thermo-cycler (Mastercycler® X50) was used for all amplifications. On 2 % agarose gel, the amplified DNA products generated by SSR primers were resolved in TAE buffer [242 g Tris-base, 57.3 ml glacial acetic acid, and 100 ml 0.5 M EDTA (pH 8.0) diluted in distilled water and final volume made to 1000 ml]. Approximately 15 µl of PCR product was combined with 2 µl of 6X loading dye (bromophenol blue) and placed into an agarose gel slot for electrophoresis. The gels were loaded with 1µg of a 50 bp DNA marker to assess the molecular size of amplified products (Fermentas, USA). In a gel documentation system (Gel DocTM XR+, BIO-RAD, USA), the gels were visualized under

Table 1: List of genotypes used in the present study

Sl. No.	Genotypes	Sl. No.	Genotypes	Sl. No.	Genotypes	Sl. No.	Genotypes
1	Pukhi	18	Dhala saitta	35	KUNYIT	52	BRRI dhan82
2	Panbira	19	Morich boti	36	GHAU	53	BRRI dhan72
3	Dharial	20	Saitta	37	LALAMG	54	BRRI dhan28
4	Utri	21	Lal Dular	38	MGAWA	55	BRRI dhan39
5	Luanga	22	Nayan moni	39	SUNGKAI	56	BRRI dhan42
6	Kaisa panja	23	Kalabokra	40	UGAN	57	BRRI dhan43
7	Vandana	24	HUA1003	41	TADOM	58	BRRI dhan46
8	Dular	25	Takanari	42	BANGKUL	59	BRRI dhan75
9	Sondhumoni	26	Kachalath	43	NMR151	60	BRRI dhan55
10	Hasikamli	27	Wkhi1	44	NMR152	61	BRRI dhan69
11	Dumai	28	Hukurikul193	45	MR297	62	B370
12	Parija	29	ML6	46	Putra 1	63	BINASAIL
13	Kataktara	30	ML9	47	Putra 2	64	BINA dhan7
14	Balirdia	31	Wanxiam-P10	48	MR 303	65	BINA dhan5
15	Binnatoa	32	RENGAN WANG	49	MR 309		
16	Parangi	33	PETEH PERAK	50	BR24		
17	Chengri	34	WANGI PUTEH	51	BRRI dhan48		

Table 2: List of primers used for varietal characterization of 65 rice genotypes

Sl no.	Markers	Forward primers (5'-3')	Reverse primers (3'-5')
1	RM1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
2	RM84	TAAGGGTCCATCCACAAGATG	TTGCAAATGCAGCTAGAGTAC
3	RM424	TTTGTGGCTCACCAGTTGAG	TGGCGCATTCATGTCATC
4	RM174	AGCGACGCCAAGACAAGTCGGG	TCCACGTCGATCGACACGACGG
5	RM263	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG
6	RM 231	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG
7	RM232	CCGGTATCCTTCGATATTGC	CCGACTTTTCCTCCTGACG
8	RM335	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG
9	RM551	AGCCCAGACTAGCATGATTG	GAAGGCGAGAAGGATCACAG
10	RM168	TAGCAAGCTTGGAGAAGTGATGG	CAGAAGAAGTCAGCTCTATGCTTGG
11	RM87	CCTCTCCGATACACCGTATG	GCGAAGGTACGAAAGGAAAG
12	RM39	GCCTCTCTCGTCTCCTTCCT	AATTCAAACTGCGGTGGC
13	RM334	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC
14	RM528	GGCATCCAATTTTACCCCTC	AAATGGAGCATGGAGGTCAC
15	RM103	GTTGCGTCCTACTGCTACTTC	GATCCGTGTCGATGATAGC
16	RM190	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG
17	RM481	TAGCTAGCCGATTGAATGGC	CTCCACCTCCTATGTTGTTG
18	RM264	CATCTCCGCTCTCCATGC	GGAGTTGGGGTCTTGTTCG
19	RM434	GCCTCATCCCTCTAACCCTC	CAAGAAAGATCAGTGCGTGG
20	RM242	CACTCACACAAGCGACTGAC	CGCAGGTTCTTGTGAAATGT
21	RM216	GCATGGCCGATGGTAAAG	TGTATAAAACCACACGGCCA
22	RM202	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA
23	RM270	GGCCGTTGGTTCTAAAATC	TGCGCAGTATCATCGGCGAG
24	RM277	CTGGTTCTGTCTGGGAGCAG	CTGGCCCTTCACGTTTCAGTG
25	RM453	GGCCAACGTGTGTATGTCTC	TTAATGCCAAGACGGATGGG

UV light after 1.5 hours of electrophoresis at a constant voltage of 80 volts. Finally, polymorphism was graded on the images of amplification products generated from all primers in order to assess genotype diversity.

2.4 STATISTICAL ANALYSIS

2.4.1 Molecular Diversity

Molecular Marker-based Genetic Diversity Analysis (MMGDA) has the ability to examine changes in genetic diversity through time and place. Each genotype and primer combination's band position in the comparative SSR profile was assessed using the gel images. This analysis includes SSR profiles from only genotype × primer combinations that gave steady amplification for all genotypes and no blank lane per unclear bands. The existence of a band was scored as a '1' while the lack of a band was scored as a '0,' resulting in the 0 and 1 matrix. The genetic similarity data among the 65 rice genotypes was generated using this binary data matrix.

The impact of different scales of measurement for different quantitative traits were decreased by standardising data for each trait separately prior to cluster analysis. Standardization was done by dividing the deviation of mean for a line from the mean for 65 genotype with the standard deviation for the given trait; the NTSYS (Rohlf Fj, 1987) software's STAND module was utilised to provide the information.

2.4.2 Genetic dissimilarity and cluster analysis based on UPGMA

NTSYS-pc version 2.2 W (Rohlf Fj, 1987) was used

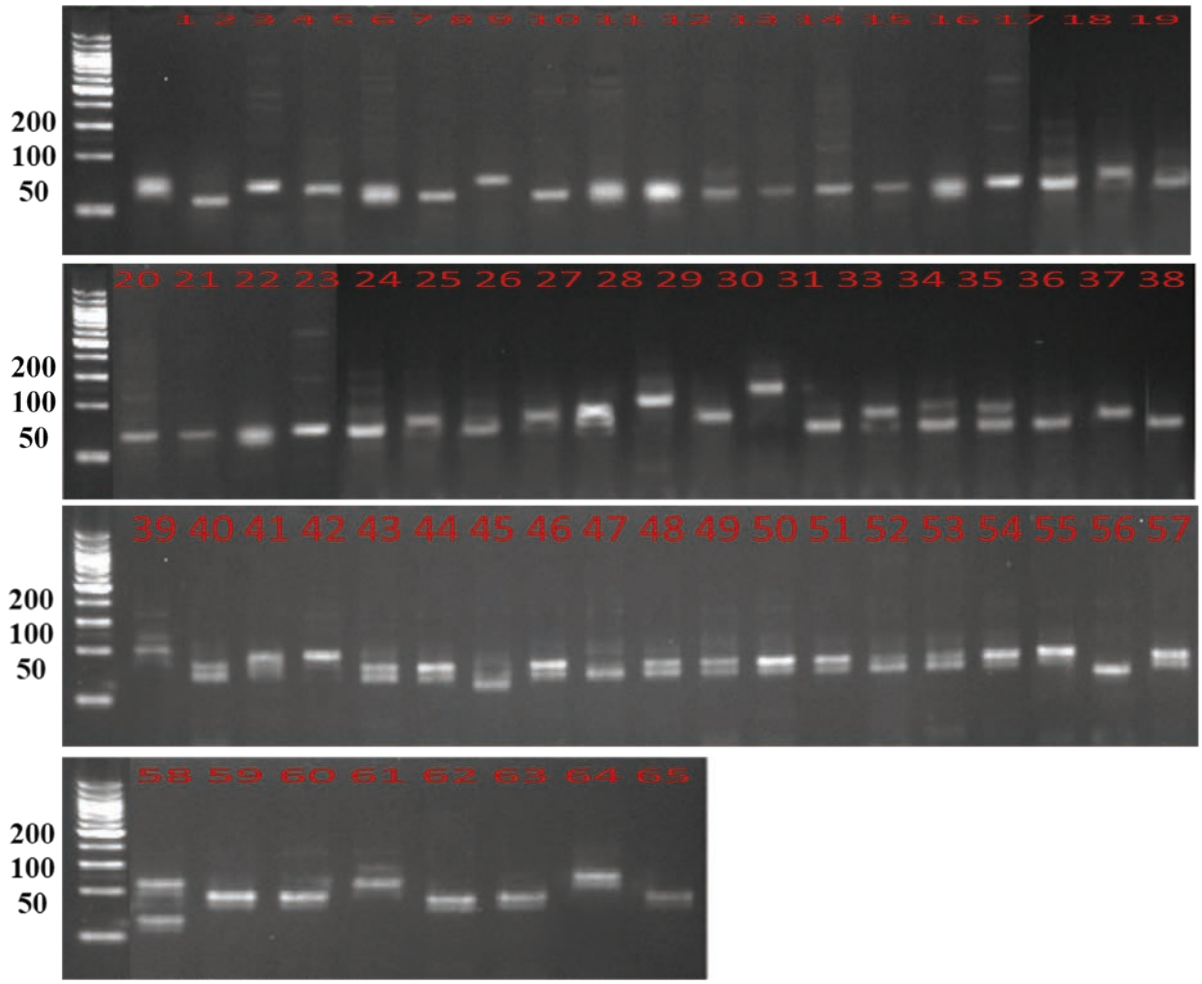

Figure 1: SSR banding profile obtained by marker RM481. Lane 1-65 represents rice genotype used in the present study; M = 50 bp DNA size marker

to analyse binary data matrix generated by polymorphism SSR markers. The Jackard's dissimilarity coefficient was calculated using the SIMQUAL programme. Cluster analysis was done using the dissimilarity matrix as an input. The Sequential Agglomerative Hierarchical Non-Overlapping (SAHN) module was used to do UPGMA-based clustering with Jackard's Coefficient of NTSYS-pc being used for dendrogram generation. The average distance between all individuals in the two groups was calculated using the unweighted pair-group technique with arithmetic averages (UPGMA) to connect clusters.

2.5 POLYMORPHIC INFORMATION CONTENT (PIC)

The ability of markers to detect polymorphisms is measured by their polymorphism information content (PIC). The number and frequency distribution of detectable alleles determine the PIC of a marker for identifying polymorphism within a population (Anderson et al., 1993)different populations are required to fulfill different objectives. Clones from the linkage map(s. PIC for the i_{th} marker is calculated as follows:

$$PIC = 1 - \Sigma P_{ij} \; (j = 1, 2, \ldots..n)$$

where P_{ij} is the frequency of the j_{th} pattern for the i_{th} marker and the summation extends over (n) patterns.

3 RESULTS

The molecular data on preferences of the genotypes were analyzed to find out the variation among genotypes for different characters. The following sub heads show

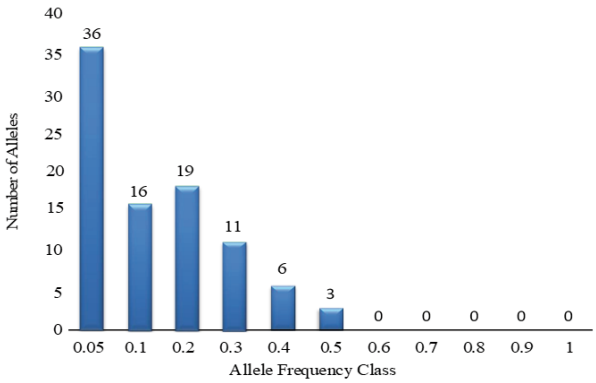

Figure 2: Histogram of allele frequency for all 91 alleles in the 65 rice genotypes

the experimental results from the current investigation for 65 traditional and improved genotypes.

3.1 ALLELIC POLYMORPHISM

The molecular diversity of 65 rice genotypes was assessed using an SSR marker. Out of 25 primers, the 21 primers created a polymorphic pattern that was repeatable, while the four monomorphic primers produced a monomorphic pattern. These SSR markers identified 91 alleles across all rice genotypes. The number of alleles per locus varied greatly amongst markers are ranging from 3 to 7 with an average of 4.00 alleles per locus (Table 3). The highest number of alleles (7) was detected in the marker RM263, and lowest number of alleles (3) were detected in the markers RM335, RM551, RM538 RM190, RM242 and RM270. Monomorphic markers identified on RMRM39, RM103, RM277 and RM453. The diverse genotypes and SSR markers used by different researchers could explain the discrepancies in average allele per locus. Rice genotypes shared a common major allele at 65 loci from 0.26 (RM202) to 0.74 (RM232). A moderate level of allele's frequency exists in these loci of rice genotypes with the average 0.42 (Table 3). Gel image showing SSR banding profile obtained by primer RM481 is presented in Figure 1. The rare alleles (frequency < 0.05) comprised 39.56 %, whereas intermediate (frequency 0.1-0.5) comprised 60.44 % and there were no abundant alleles (frequency > 0.5) (Figure 2). Out of 21 polymorphic, only one marker RM168 provided the highest number of 4 rare alleles, followed by RM174 (3), RM263 (3), RM232 (30, RM84 (20, RM424 (2), RM87 (2), RM481 (2), RM264 (2), RM216 (2), RM1 (1), RM231 (1), 335 (1),RM551 (1), RM334 (1), RM528 (1), RM190 (1), RM434 (1), RM242 (1), RM202 (1) and RM270 (1).

3.2 POLYMORPHIC INFORMATION CONTENT

The degree of polymorphism found by 21 loci markers could not be associated with the number of alleles or the Polymorphic Information Content (PIC) values in this investigation. The PIC values of several loci that produced equivalent numbers of alleles were not statistically different. For instance, 3 alleles were detected at each six loci on RM335, RM551, RM528, RM190, RM242 and RM270; 4 alleles were detected at each of the three loci on RM231, RM334 and RM434; 5 alleles were detected at each of the seven loci on RM1, RM424, RM174, RM87, RM481, RM264 and RM202, 6 alleles were detected at each of the four loci on RM84, RM232, RM168 and RM216, and 7 alleles were found of the loci on RM263. However, no significant difference was detected in their PIC values.

The average PIC value was 0.62 with values ranging from 0.28 (RM 270) to 0.76 (RM 481). (Table 3). The PIC values which reflect allele diversity and frequency among cultivars differed from site to locus. For all of the SSR loci, the PIC values derived from allelic diversity and genotype frequency were inconsistent. This meant that all of the genotypes included in the study were judged to be sufficiently diverse. The study found the highest PIC value 0.76 (RM481) followed by 0.75 (RM551), 0.72 (RM174, RM168 & RM434), 0.71 (RM263), 0.70 (RM216), 0.67 (RM424 & RM202), 0.65 (RM190), 0.64 (RM335), 0.62 (RM84 & RM232), 0.61 (RM87), 0.60 (RM528), 0.59 (RM264) and 0.58 (RM1). The PIC values of eighteen SSR markers found to be polymorphic and utilized in this study were more than 0.5 (Table 3). Low PIC values for the RM270 SSR marker may be the result of closely related genotypes while high PIC values for the RM481 SSR marker may be the result of diverse genotypes of cluster analysis implying that the shared allele distance and cluster analysis were appropriate methods for using SSR marker information. The study found that 18 SSR markers were considered to be the finest and highly informative. Therefore, it can be employed for molecular characterization and QTL analysis.

3.3 GENETIC RELATIONSHIPS

The rice genotype is rich reservoir of valuable genes that plant breeders exploit it for crop improvement. The primers have the ability to differentiate different rice genotypes based on the differences in their genomic region and their number of alleles. The average Genetic Diversity (GD) value was 0.69 with values ranging from 0.33 (RM270) to 0.81 (RM481) (Table 3). The significant rate

Table 3: Number of alleles, polymorphic information content and genetic diversity index for 25 simple sequence repeat (SSR) loci in the 65 rice genotypes

Marker	Chr. No	No. of observation	MAF	NA	RA	GD	PIC
RM1	1	53.00	0.32	5	1	0.66	0.58
RM84	1	53.00	0.39	6	2	0.70	0.62
RM424	2	65.00	0.46	5	2	0.74	0.67
RM174	2	65.00	0.58	5	3	0.80	0.72
RM263	2	65.00	0.69	7	3	0.76	0.71
RM 231	3	64.00	0.40	4	1	0.54	0.49
RM232	3	64.00	0.74	6	3	0.69	0.62
RM335	4	57.00	0.28	3	1	0.72	0.64
RM551	4	57.00	0.42	3	1	0.80	0.75
RM168	4	57.00	0.34	6	4	0.78	0.72
RM87	5	49.00	0.30	5	2	0.69	0.61
RM334	5	49.00	0.36	4	1	0.74	0.39
RM528	6	65.00	0.44	3	1	0.65	0.60
RM190	6	65.00	0.49	3	1	0.70	0.65
RM481	7	65.00	0.46	5	2	0.81	0.76
RM264	8	65.00	0.50	5	2	0.64	0.59
RM434	9	54.00	0.29	4	1	0.78	0.72
RM242	9	54.00	0.34	3	1	0.54	0.54
RM216	10	64.00	0.44	6	2	0.69	0.70
RM202	11	51.00	0.26	5	1	0.72	0.67
RM270	12	57.00	0.38	3	1	0.33	0.28
			0.42	91	36	0.69	0.62

MAF = Major Allele Frequency, NA = Number of alleles, Rare allele (RA) = Number of alleles that frequency < 0.05, GD = Gene Diversity, PIC = Polymorphic Information Content

of interchange of genetic materials across the rice genotypes tested particularly during their genetic improvement could explain the average genetic diversity value. Germplasm conservation, characterization, and breeding effects are all influenced by genetic diversity.

The genetic distance was calculated using the Unweighted Paired Group Method Using Arithmetic Averages (UPGMA) clustering method. UPGMA is a basic grouping algorithm for phylogenetic dendrograms based on genetic distance. Based on Jackard's dissimilarity coefficient the UPGMA was used to generate a dendrogram (Figure 3). Sixty-five rice genotypes were grouped into two main groupings (Table 4) based on dissimilarity coefficients: cluster I and cluster II (0.15). Cluster I was further sub-divided into two minor sub-groups IA and IB were further sub-divided into two sub-groups i.e. IA-1 and IA-2 (0.31) and IB-1 and IB-2 (0.32) respectively. Two minor sub-groups were created from the second main cluster i.e. IIA and IIB with dissimilarity coefficient 0.16. This indicated that the genotypes analyzed had a lot of variability. It is critical to have the most diverse genotypes when selecting desirable cultivars for use in breeding programmes.

The dissimilarity coefficient varies from 1 to 0, close to one shows high similarity while close to zero shows high dissimilarity. The average of dissimilarity coefficient varies from 0.77 to 0.51. The dissimilarity coefficient of all sixty-five genotypes is 0.61 on average. The highest dissimilarity coefficient varied between the genotype BR24 and Utri (0.9429) followed by the Pukhi and WANGI PUTEH (0.9286). The lowest value was found between Sondhumoni and Dumai (0.150). The most diverse genotype was Utri and Pukhi. These genotypes were grouped into nine clusters. Cluster indicated that 31 genotypes out of sixty-five belong to the cluster IB-1a followed cluster IB-1b which has 24 genotypes and cluster IB-1c with

Table 4: Grouping of sixty-five rice genotypes into different clusters based on Jackard's IJ coefficient

Cluster	Number of Genotypes	Name of the Genotypes
IA-1	1	Utri
IA-2	1	BRRI dhan42
IB-1a	31	Vandana, Parija, Luanga, Chengri, Nayan moni, Wkhi1, BRRI dhan55, Binatoa, BRRI dhan69, B370, ML6, BRRI dhan82, BINASAIL, BINA dhan7, Wanxiam-P10, RENGAN WANG, BRRI dhan72, BRRI dhan28, TADOM, BRRI dhan48,Dular,Kataktara, Sondhumoni, Dumai, Balirdia, Kaisa panja, Dharial, Parangi, Dhala saitta, Panbira, Hasikamli
IB-1b	24	Lal Dular, UGAN, HUA1003, ML9, PETEH PERAK, Kachalath, BR24, MR297, BRRI dhan39, BRRI dhan75, MR 309, BRRI dhan46, BINA dhan5, Hukurikul193, WANGI PUTEH, LALAMG, MGAWA, MR 303, GHAU, BANGKUL, NMR151,BRRI dhan43, KUNYIT, NMR152
IB-1c	3	Kalabokra, Takanari, Putra 1
IB-2	2	Morich boti, Saitta
IIA-1	1	SUNGKAI
IIA-2	1	Putra 2
IIB	1	Pukhi

3 genotypes. Cluster IA-1, IA-2, IIA-1, IIA-2 and IIB were monogenic in nature containing single genotypes each i.e. Utri, BRRI dhan42, SUNGKAI, Putra 2 and Pukhi, respectively.

3.4 ANALYZE THE POPULATION STRUCTURE

Using 21 SSR polymorphic markers across 65 rice genotypes the model-based clustering method was applied. The relatively high value of K for 65 genotypes was for K = 9 (Figure 4). At K = 9, the population structure analysis in the present study resulted in nine populations (Figure 5 & Table 5). The distribution of rice genotypes between genetic groups is quite different among genotypes. The mixture is most likely the product of a long history of breeding and domestication, both of which have had significant impacts on the diversity structure. Human-mediated gene flow may play an important role within a population due to breeding in rice for its self-fertilization nature. In other words, in the absence of human-mediated gene flow across populations by breeding one would expect a larger partitioning of diversity among rather than within populations in self-pollinated species.

In present study, the average distances between individuals in the same population (excluding for heterozygosity) were 0.3764 (population A), 0.4318 (population B), 0.6219 (population C), 0.3442 (population D), 0.4735 (population E), 0.2951 (population F), 0.5146 (population G), 0.3369 (population H), and 0.3114 (population I). The genetic diversity of different rice was assessed using average genetic distances (Table 5).

Data on genetic diversity and population structure can be utilised to create successful breeding processes for expanding the genetic base of commercial cultivars, identifying molecular markers, and preserving germplasm. In the generation of necessary derived varieties, distinguish, uniformity, and stability (DUS) testing of plant varietal characteristics as well as the discovery of molecular markers will be critical.

4 DISCUSSION

Molecular markers have been employed in genetic improvement programmes to examine genetic diversity to choose parents for cross-breeding, and learn about marker trait associations (Uba et al., 2021)West, Central, Southern and East Africa. The findings of DNA-based genetic diversity analyses could be used to develop effective breeding programmes aimed at extending the genetic base of commercially grown cultivars (Brumlop & Finckh, 2011)and poor neurodevelopment in children. We carried out a comprehensive literature review to examine the neurotoxicity of BaP. The data were used to identify potential point of departure (POD. The molecular diversity and characterization of rice genotypes were examined using twenty-five microsatellite simple sequence repeats (SSRs) in this study. SSR is more polymorphic than most other DNA markers as well as be-

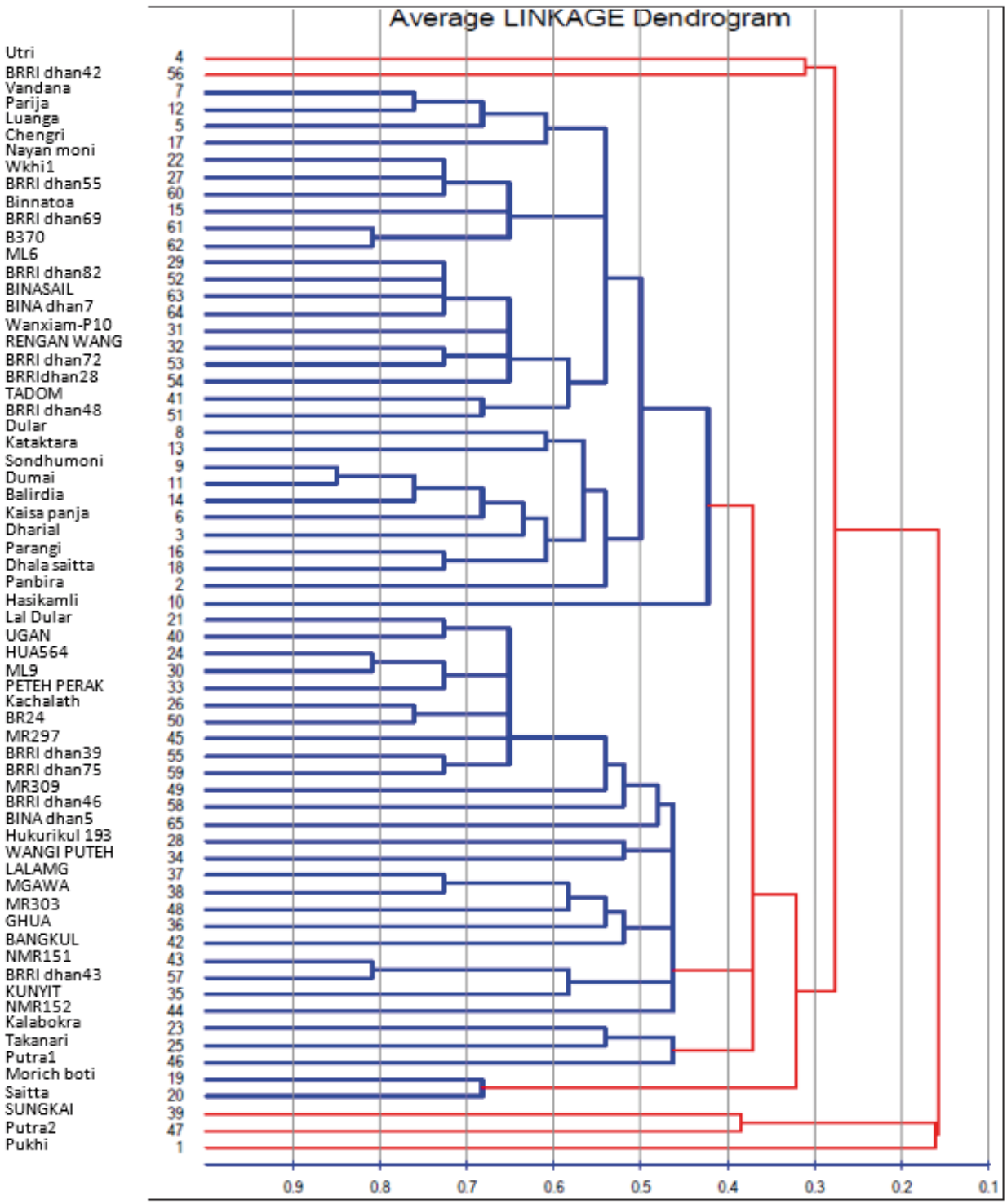

Figure 3: Cluster study utilizing the UPGMA method employing SSR fingerprint data and a Jaccard's IJ coefficient in 65 rice genotypes

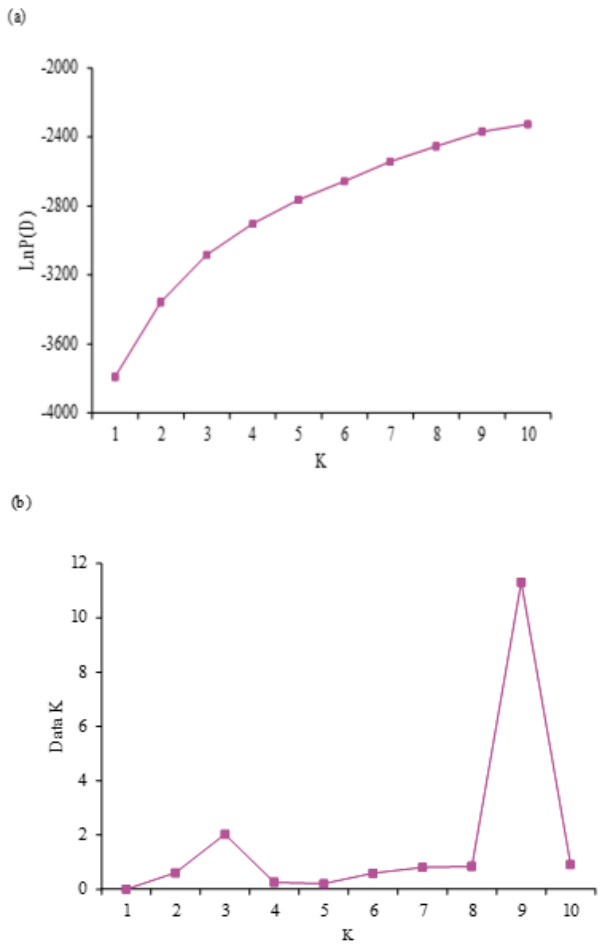

Figure 4: (a) The Bayesian log probability data [LnP (D)] by increasing K
(b) Magnitude of K as a function of K

ing co-dominant and having a bigger amount. As a result of SSR's high polymorphism information richness so that microsatellite markers have been used as molecular markers in fingerprinting (Sun et al., 2020)91 poplar cultivars belonging to four sections (Aigeiros, Tacamahaca, Populus and Turanga.

The SSR markers utilized in this study were both scorable and clear. Out of twenty-five markers used twenty-one were polymorphic allele and remaining four markers were monomorphic in nature. The number of alleles detected per marker were 3 to 7 with an average of 4.57. The alleles showed high degree of polymorphism, with major percent polymorphic bands in twenty-one SSR markers. It suggests that the genotypes used in present study shown as genetic divergence. Similar results were also reported (Ashraf et al., 2016) SSR primers were used to explore genetic variability of various rice landraces. However, when compared to values reported in other parts of the collections, this figure is extremely low. Thomson et al. (2010) reported that the average number of alleles per locus was 4.86, with the number of alleles per locus ranging from 3 to 8. The knowledge on these alleles in various genotypes will be tremendously useful in developing mapping populations for genome study and in applied breeding programmes. Molecular-based biological and geographical diversity differed in allelic richness, frequency of uncommon alleles, common and most frequent alleles, and group-specific unique alleles.

Genetic diversity is required for the selection of various plant breeding programmes and genomic diversity can be determined using a variety of methods. The environment has a significant impact on the expression of a variety of plant morphological features that are currently available and used to distinguish genotypes (Buzatti et al., 2019). Investigations at the molecular level reveal the true distinctions between genotypes. Molecular markers have been employed in genetic improvement programmes to research genetic diversity and to select parents for cross-breeding between parents with different backgrounds, as well as to determine marker trait associations (Gedil & Menkir, 2019).

This analysis showed the incidence of significant diversity in the traditional and improved rice genotypes studied. Based on the dendrogram the maximum degree of genetic resemblance between genotypes Sondhumoni and Dumai followed by BRRI dhan69 and B370, HUA564 and ML9 and NMR151 and BRRI dhan43. The most diverse genotype was Utri and Pukhi. Similar result was found also by (Nachimuthu et al., 2015) where the cultivars were grouped in two major groups and 14 sub-groups. As a result, the diversity of genotypes is critical for selecting suitable genotypes for use in breeding programmes.

The dissimilarity coefficient was calculated by Jackard IJ distance analysis, and result showed value ranged from 0 to 1. According to this dissimilarity coefficient we can understand the dendrogram and their relatedness. So, the highest diverse genotypes can be used as parents in breeding programme. The average of dissimilarity coefficient varies from 0.7689 to 0.5079. The total average of all sixty-five genotype's dissimilarity coefficient is 0.6119. The dissimilarity coefficient varied from the largest value 0.9429 between the genotype BR24 and Utri followed by the genotype value 0.9286 between the genotype Pukhi and WANGI PUTEH which shows high similarity between them and it may be expected that both of them may have arouse from the same parents. The lowest value 0.150 was found between Sondhumoni and Dumai. Similar result was reported by (Siva et al., 2013).

The power of distinction or polymorphism information content (PIC) is a useful statistic for comparing

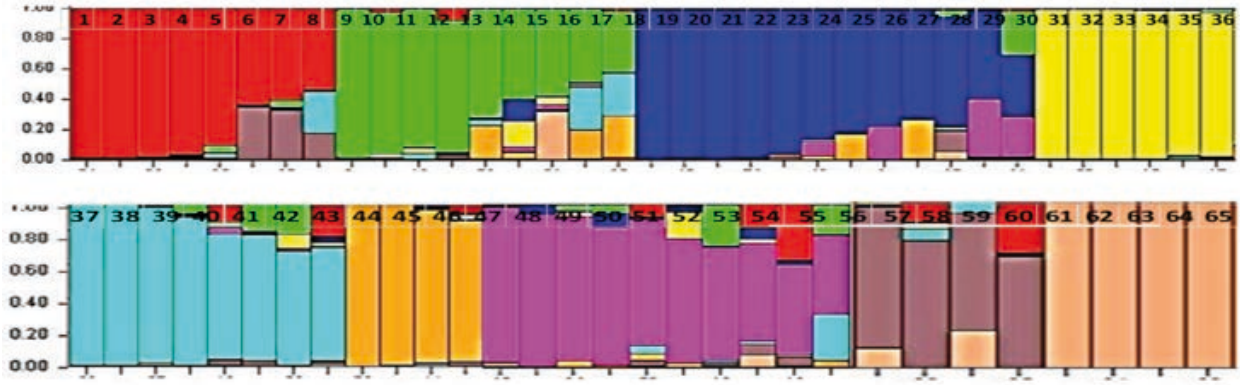

Figure 5: Model-based membership of 65 rice genotypes using STRUCTURE. Colors represent model based population for 9 inferred cluster

Table 5: The average distances between individuals of the same population (without heterozygosity)

P-A	P-B	P-C	P-D	P-E	P-F	P-G	P-H	P-I
0.3764	0.4318	0.6219	0.3442	0.4735	0.2951	0.5146	0.3369	0.3114

P-A = Population A, P-B = Population B, P-C = Population C, P-D = Population D, P-E = Population E, P-F = Population F, P-G = Population G, P-H = Population H, P-I = Population I

various markers (Serrote et al., 2020). This criterion's high levels indicate that the locus has a lot of polymorphisms and that uncommon alleles play a substantial influence in individual difference. Therefore, a marker with a high PIC will be particularly beneficial for differentiating genotypes with tight relationships. The allele diversity and frequency among genotypes are reflected in the PIC value (Ashraf et al., 2016). Each marker's PIC value can be calculated based on its alleles. All of the SSR loci investigated had different PIC values. Calculating PIC values for each of the SSR loci was used to assess the level of polymorphism among the 65 genotypes in the current investigation. In this study, the PIC values ranged from 0.28 (RM 270) to 0.76 (RM481) with an average of 0.52. PIC values of 0.5-mark markers are especially useful in genetic studies for determining the polymorphism rete of a marker at a particular locus. The presence of polymorphism between genotypes indicated that genetic variation exists at the molecular level. Microsatellite analysis was compared to three previous estimations in rice 0.26 to 0.65 with an average of 0.47 (Singh et al., 2014), 0.28-0.50 with a mean of 0.45 (Ashraf et al., 2016) and 0.239 to 0.765 with an average of 0.508 (Pathak et al., 2020). The number of alleles found was proportional to the PIC value of the locus. So PIC value is totally related polymorphism of markers. The highest PIC value of 0.76 at RM481 was shown to be the best marker for differentiating across rice cultivars. A number of other researchers have conducted similar investigations (Tarang et al., 2020).

5 CONCLUSIONS

Twenty-five SSR markers were used out of which twenty-one were found polymorphic. 21 polymorphic markers detected a total of 91 alleles among 65 rice genotypes, with an average of 4.00 alleles per polymorphism marker. The PIC values ranged from 0.28 to 0.76 and the highest PIC value of 0.76 was determined to be marker RM481 which was proven to be the best appropriate marker for discriminating among rice genotypes. The genetic divergence analysis divided 65 rice genotypes into nine groups with cluster IB-1a having the most cultivars (31) and clusters IB-1b and V having the least. Based on dendrogram the maximum degree of similarity was observed between genotype Sondhumoni and Dumai followed by BRRI dhan69 and B370, HUA564 and ML9 and NMR151 and BRRI dhan43. The most diverse genotype was Utri and Pukhi. The maximum value of the dissimilarity coefficient was discovered between the genotype BR24 and Utri (0.0429) and between Pukhi and WANGI PUTEH (0.9286) whereas lowest value was seen between Sondhumoni and Dumai (0.150) showing highly diverse genotypes. Breeders may attempt hybridization among the above genotypes that demonstrated the most diversity in the hopes of increasing genetic variability in rice and assisting in the development of promising rice genotypes. It is important to remember that both morphological and molecular techniques for studying genotypes have been demonstrated to be beneficial and one meth-

odology complements the other and offers a trustworthy result.

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Problematika hladnega skladiščenja pri proizvodnji potaknjencev zelnatih in lesnatih okrasnih rastlin

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Issues relating to the use of cold storage method in the production of herbaceous and woody cuttings of ornamental plants

Abstract: In ornamental horticulture, propagation by cuttings is the most important and most commonly used method of vegetative propagation of plants. During propagation, growers use various techniques to ensure or maintain the highest quality of material. With the relocation of the world's major ornamental plant growers to tropical and subtropical areas, maintaining the quality of the material during transport has become crucial for further plant production. The method of cold storage of plant material is used in vegetative propagation and in the transport of cuttings of herbaceous and woody ornamental plants from equatorial areas to the areas where they are to be rooted. Cold storage lowers the temperature of the plant material, thus slowing down the metabolism of the plants during storage, preserving the growth potential and quality of the cuttings and extending their shelf life. This paper reviews the management of cuttings of various ornamental plant species by cold storage, focusing on cuttings of herbaceous plants, cuttings of woody plants, and *in vitro* production.

Key words: cold storage; cuttings; vegetative propagation; ornamental plants

Problematika hladnega skladiščenja pri proizvodnji potaknjencev zelnatih in lesnatih okrasnih rastlin

Izvleček: Razmnoževanje s potaknjenci je najpomembnejša in najpogosteje uporabljena metoda vegetativnega razmnoževanja rastlin v okrasnem vrtnarstvu. V verigi proizvodnje sadik se pridelovalci poslužujejo različnih tehnik in metod, da bi zagotovili oziroma ohranili karseda kakovosten rastlinski material. Zaradi selitve večjih svetovnih pridelovalcev zelnatih okrasnih rastlin v tropska in subtropska območja, kjer je pridelava enostavnejša, cenejša in lažja, je ohranjanje kakovosti materiala v času transporta postalo ključnega pomena za nadaljnjo proizvodnjo rastlin. Metoda hladnega skladiščenja se uporablja pri vegetativnem razmnoževanju in pri transportu potaknjencev zelnatih in lesnatih okrasnih rastlin iz ekvatorialnih delov do območij, kjer jih nato koreninijo. Hladno skladiščenje zniža temperaturo rastlinskega materiala, posledično se upočasni metabolizem rastlin med skladiščenjem, ohrani se rastni potencial in kakovost potaknjencev ter podaljša se njihovo obstojnost. V prispevku je predstavljen pregled na področju hladnega skladiščenja potaknjencev različnih vrst okrasnih rastlin, s poudarkom na potaknjencih zelnatih, lesnatih rastlin in rastlin iz *in vitro* proizvodnje.

Ključne besede: hladno skladiščenje; potaknjenci; vegetativno razmnoževanje; okrasne rastline

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1 UVOD

Okrasne rastline se uporabljajo za dekoracijo in popestritev notranjih in zunanjih prostorov v najrazličnejših oblikah. Proizvodnja okrasnih rastlin je vsako leto močnejša, predvsem v vodilnih državah Evropske Unije glede pridelave okrasnih rastlin Nizozemski, Angliji, Italiji, Franciji, Španiji. V Sloveniji so se, po podatkih Statističnega urada RS pridelovalne površine (ha), namenjene za pridelavo okrasnih rastlin, od leta 2010 (229,6 ha) do leta 2019 (117,1 ha) zmanjšale za 49 % (Preglednica 1) (Statistični urad RS, 2021).

Leta 2006 je bilo v Sloveniji po številu pridelanih sadik največ balkonskih rastlin, enoletnic, dvoletnic in sobnih rastlin (Slika 1). Kljub temu pa je poraba okrasnih rastlin v Sloveniji relativno velika in primerljiva z omenjenimi evropskimi državami.

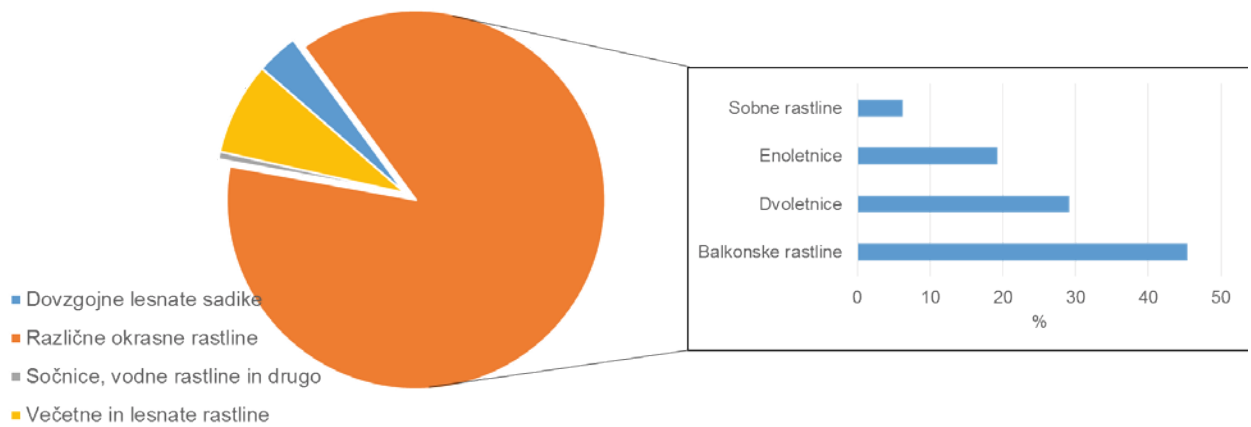
Sortiment okrasnih rastlin se z vsakim letom povečuje in obsega preko dva tisoč rodov (Chen, 2021). Žlahtnitelji iščejo nove, zanimive sorte, z glavnim ciljem obilnejšega cvetenja, atraktivne barve ali barvne kombinacije ter polnosti cvetov ter manjše občutljivosti na abiotske in biotske dejavnike.

Posebnost panoge okrasnega vrtnarstva je raznolikost pridelave. Pridelavo okrasnih rastlin lahko razdelimo na pridelavo na prostem in/ali v zavarovanem prostoru. Na prostem se večinoma prideluje lesnate rastline (drevesa, grme in polgrme), zelnate rastline (trajnice in dvoletnice, npr. mačehe) ter okrasno trato. V zavarovanih prostorih pa poteka predvsem razmnoževanje okrasnih rastlin in pridelava lončnih rastlin (balkonske rastline, sobne cvetoče in zelene rastline) ter rastlin za rezano cvetje in dekoracijo.

Poznamo dva načina razmnoževanja okrasnih rastlin, generativno (iz semen) in vegetativno. Vegetativno razmnoževanje je razmnoževanje z vegetativnimi deli

Preglednica 1: Pridelovalne površine (ha) okrasnih rastlin leta 2019 v Sloveniji (Statistični urad RS, 2021)
Table 1: Cultivated area (ha) of ornamental plants in 2019 in Slovenia (Statistical Office of the Republic of Slovenia, 2021)

	Pridelovalna površina (ha)
Okrasne rastline	117,1
Sobne rastline in lončnice	5,6
Balkonske rastline	18,4
Enoletnice	7,4
Dvoletnice	3,9
Večletne in lesnate rastline	78,6
Dovzgojne sadike cvetja, okrasnih ali lesnatih rastlin za prodajo	3,2
Rezano cvetje	6,7



Slika 1: Struktura števila pridelanih sadik v Sloveniji (%) leta 2006 (Statistični urad RS, 2021)
Figure 1: Structure of the number of seedlings produced in Slovenia (%) in 2006 (Statistical Office of the Republic of Slovenia, 2021)

rastlin (poganjek, del poganjka, del korenine, del lista, rastni vršiček idr.), kjer se izkorišča gensko določene lastnosti rastlinskega tkiva in celice - sposobnost regeneracije. Vsaka vegetativna celica je v določenih razmerah sposobna podvajanja (mitotske delitve) v določeni fazi ali starosti. Pri vegetativnem razmnoževanju tako izkoriščamo t.i. totipotentnost rastlin (Hartmann in sod., 1997; Smole in Črnko, 2000).

Najenostavnejši način neposrednega vegetativnega razmnoževanja je razmnoževanje s potaknjenci, če se vrsta oz. sorta lahko tako razmnožuje, zato je razmnoževanje s potaknjenci najpomembnejša in najpogosteje uporabljena metoda vegetativnega razmnoževanja v okrasnem vrtnarstvu. Mnoge celice imajo možnost, da se remeristematizirajo in proizvedejo korenine in poganjke. Vsaka rastlinska totipotentna celica vsebuje potreben genetski zapis, da proizvede novo rastlino in to lastnost pri razmnoževanju s potaknjenci pridelovalci s pridom izkoriščajo (Hartmann in sod., 1997; Druege, 2009). V zadnjih desetletjih so se tudi za razmnoževanje lesnatih rastlin, predvsem zaradi možnosti hitrega razmnoževanja v velikem številu, začeli močno uporabljati *in vitro* postopki.

Namen prispevka je pregled relevantne svetovne literature s področja proizvodnje potaknjencev s poudarkom na pregledu uporabnosti metode hladnega skladiščenja potaknjencev zelnatih in lesnatih okrasnih rastlin.

2 PRIDELAVA POTAKNJENCEV

Razmnoževanje s potaknjenci je zelo razširjen način pridobivanja novih rastlin, tako v vrtnarijah kot tudi med ljubitelji okrasnih rastlin in ima številne prednosti: iz nekaj matičnih rastlin lahko vzgojimo mnogo novih rastlin. Metoda je poceni, hitra in enostavna glede na ostale vegetativne metode, ne potrebujemo posebnih tehnik in veliko prostora ter dobimo rastline enotnega (uniformnega) videza (Hartmann in sod., 1997; Druege, 2009). Potaknjenec je opredeljen kot del enoletnega ali toletnega poganjka zelnatih ali lesnatih (drevesa ali grmi) rastlin, ki jih lahko režemo v različnih razvojnih obdobjih (fenofazah) rastline. Pri nekaterih potaknjencih kombiniramo celo toletni les z večletnim lesom pri sami osnovi potaknjenca. To pomeni, da te potaknjence pripravimo tako, da pri toletnem poganjku pri osnovi pustimo ostanek večletnega lesa (tim. ‚peto'). Ta način priprave potaknjencev je v uveljavi predvsem pri vrstah, ki razvijajo kratke poganjke, pri takšnih vrstah včasih potaknjence v celoti pripravljamo iz večletnega lesa (nekatere sorte smrek, pacipres, klekov, tudi kutina) (Osterc in Rusjan, 2013). Pri pridelavi okrasnih rastlin ločimo zelene potaknjence (režemo jih med rastno dobo) in lesnate potaknjence (režemo jih v mirovanju), listne potaknjence (del lista, iz katerega se lahko razvije nova rastlina) ter koreninske potaknjence (odrezan del korenine), iz katerega se v določenih razmerah razvije nova rastlina (Smole in Črnko, 2000).

Med dejavnike, ki vplivajo na uspeh razmnoževanja s potaknjenci štejemo kakovost matične rastline, čas rezi potaknjencev, oskrbo in skladiščenje potaknjencev, nivo rastlinskih endogenih dejavnikov, rastne razmere ter način utrjevanja potaknjencev.

Ustrezna starost, kakovost (vitalne in zdrave) matične rastline, del rastline od koder odvzamemo potaknjence imajo pomembno vlogo, še posebej pri lesnatih rastlinah. S starostjo matične rastline se uspeh koreninjenja potaknjencev zmanjšuje, kar je v večini povezano s starejšimi meristemi, naraščanjem inhibitorjev koreninjenja in manjšo sposobnostjo tvorbe in akumulacije avksinov in drugih snovi, potrebnih za nastanek nadomestnih korenin (Hartmann in sod., 1997; Osterc, 2009). Ko potaknjenec ločimo od matične rastline, se ksilemske in floemske poti v rastlini prekinejo, potaknjenec na odrezanem delu opluteni zaradi nalaganja suberina, celice na tem delu pa se začnejo večati in deliti. Razvije se kalus, novonastalo tkivo, ki zaraste rano in ga imenujemo kalus rane. Nato se prične dediferenciacija zrelih celic v bližini prevajalnih tkiv in začno se tvoriti koreninske zasnove, ki kasneje razvijejo koreninske primordije. Ti rastejo skozi razviti kalus na zunanjo stran, nazadnje se oblikuje še koreninska čepica. To je običajna fiziološka pot razvoja nadomestnih korenin (Hartmann in sod., 1997; Sinkovič, 2000).

Razvoj debele plasti kalusa na bazi potaknjencev, ki se včasih tudi pojavi, je nasprotno izreden pojav in je povezan večinoma z neustrezno fiziološko starostjo matičnih rastlin (predvsem pri lesnatih rastlinah), neprimernega termina rezi potaknjencev, pa tudi uporabe neustrezne rastlinske vrste. Plast kalusa ne sme biti predebela, saj lahko tako predstavlja oviro pri nastanku nadomestnih korenin. Tvorba nadomestnih korenin je končno, poleg drugih dejavnikov, odvisna tudi od začetnega statusa preskrbe matične rastline z dušikom in ogljikovimi hidrati (Blazich, 1988; Veierskov, 1988).

Čas rezi in potika je odvisen od vrste rastline, razvojne faze, načina potika in sistema gojenja (Hartmann in sod., 1997). V splošnem velja pomlad (vse do junija) za najprimernejši čas rezi potaknjencev, ko rastline pričenjajo z rastjo in je vsebnost avksinov v njih največja (Osterc, 2009), prav tako je takrat v rastlinah največja vsebnost ogljikovih hidratov (Rapaka in sod., 2005). Še posebej pri nekaterih vrstah rastlin (mnogi iglavci) se kot uspešen čas potika pojavlja tudi avgust (po koncu največje poletne vročine), pri čemer se ukoreninjeni potaknjenci presajajo naslednjo spomlad. Pomembno je, da

matične rastline ob rezi niso v vodnem stresu (Behrens, 1988; Hartmann in sod., 1997). Oskrba potaknjencev po rezi vključuje odstranjevanje odvečnih listov, prilistov, odstranitev cvetov, cvetnih brstov, trajanje in razmere v času skladiščenja, mineralna prehrana in izguba hranil, vsebnost avksinov in drugih endogenih spojin ter okoljskih dejavnikov (vlage, temperature, svetlobe, patogenov, rastnega substrata, uporabe rastnih regulatorjev). Razmnoževanje je uspešno, če potaknjenec preživi in uspešno raste poleg tega pa razvije dober koreninski sistem (Hartmann in sod., 1997).

Ker se pogosto dogaja, da potaknjencev ne moremo potakniti takoj po odstranitvi iz matične rastline, jih je potrebno do potika primerno skladiščiti. Primerno skladiščenje lahko predstavlja ključen dejavnik za nadaljnji uspeh gojenja rastline (uspešno koreninjenje in kasnejšo rast in razvoj). Pred več desetletji, je začela večina največjih svetovnih pridelovalcev zelnatih okrasnih rastlin, proizvodnjo seliti v tropska in subtropska območja Severne Afrike, Srednje Amerike, Azije in nekaj tudi Južne Evrope. Razlog za to je predvsem ekonomski, nižji proizvodni stroški, nižji davki in podpora lokalnih vlad. Globalizacija proizvodnje okrasnih rastlin je povzročila dodatne prilagoditve pridelovalcev, ki vključujejo transport in skladiščenje potaknjencev. Neukoreninjene potaknjence se iz matičnih nasadov transportira po vsem svetu in lahko traja nekaj dni, da material prispe do vrtnarij in centrov, kjer jih ukoreninjajo in sadijo v gojitvene posode (Rapaka in sod., 2007).

3 SKLADIŠČENJE POTAKNJENCEV

Zaradi izrazite sezonske narave hortikulturne industrije, je največje povpraševanje po potaknjencih in sadikah s strani vrtnarij in centrov v ozkih prodajnih obdobjih. Proizvajalci se zato večkrat srečujejo s težavami pri zagotavljanju potreb povpraševanja v tem kratkem času. Ena izmed možnosti, s katero pridelovalci rešujejo naval povpraševanja, je proizvodnja in rez potaknjencev nekaj tednov prej in skladiščenje do vrhunca povpraševanja. Prav tako je zelo pomembna uporaba hladnega skladiščenja pri zelnatih rastlinah pri samem transportu potaknjencev do držav oz. območij, kjer jih nato koreninijo. Uspešen sistem skladiščenja mora minimizirati metabolizem rastlinskega materiala med samim skladiščenjem, ohraniti fotosintezno aktivnost in rastni potencial, obenem pa ohraniti kakovosten izgled materiala.

Preživetje in tvorba nadomestnih korenin pri takih potaknjencih pogosto predstavlja težavo in je odvisna, ne samo od razmer med skladiščenjem in transportom, ampak tudi od sposobnosti prilagoditve potaknjencev, ki so gojeni v razmerah velikih jakosti svetlobe v območjih blizu ekvatorja in niso prilagojeni na razmere majhne jakosti svetlobe med zimskim obdobjem koreninjenja v steklenjakih Srednje Evrope (Forschner in Reuther, 1984; Druege in sod., 2004).

Oblikovanje korenin pri neukoreninjenih rastlinskih organih (tvorba nadomestnih korenin), je kompleksen fiziološki proces, na katerega vpliva veliko endogenih in okoljskih dejavnikov. Tvorba nadomestnih korenin je proces, ki zahteva energijo, ta pa večinoma izhaja iz zalog ogljikovih hidratov v delu nastanka korenin (Agullo-Antón in sod., 2011). Uspešnost koreninjenja potaknjencev je torej odvisna od stanja matičnih rastlin, časa rezi potaknjencev, trajanja in oskrbe v času skladiščenja, izgube hranil in vode, vsebnosti endogenih spojin ter razmer v času potika in po njem. Ustrezno skladiščenje potaknjencev pred potikom je ključnega pomena za njihovo uspešno koreninjenje in kasnejšo rast in razvoj (Hartmann in sod., 1997; Druege in sod., 2004; Kadner in Druege, 2004; Klopotek in sod., 2016).

Med skladiščenjem in transportom potaknjencev je pomembno preprečiti oziroma zmanjšati izgubo vlage in preprečiti vdor patogenov. Med transportom do ciljne države oziroma vrtnega centra se lahko, sprva visoka kakovost potaknjencev pridobljenih v ugodnih klimatskih razmerah, zelo poslabša, kar se odraža na rumenenju listov, vsebnosti vode, rastnih regulatorjev in zalog hranil v potaknjencih in tudi na propadu med obdobjem koreninjenja. Takšen odziv je lahko posledica neprimernega skladiščenja in transportnih razmer, vključno z visoko temperaturo in nezadostno prilagoditvijo potaknjencev na razmere, ki prevladujejo v končni državi (Forschner in Reuther 1984; Behrens, 1988; Kadner in Druege, 2004).

Hladno skladiščenje potaknjencev se pogosto uporablja pri vegetativnem razmnoževanju okrasnih rastlin, med drugim tudi pri transportu potaknjencev in ima pomembno vlogo pri proizvodnji okrasnih rastlin po Evropi (Agullo-Antón in sod., 2011; Klopotek in sod., 2016). Upočasnjuje rastlinski metabolizem, podaljša obstojnost potaknjencev, zmanjša vsebnost ogljikovih hidratov v potaknjencih, ki pri razvoju nadomestnih korenin sodelujejo kot signalne molekule, vir energije in ogljikovih skeletov, predvsem pa ohranja kakovost potaknjencev okrasnih rastlin (Behrens, 1988; Bredmose in Nielsen, 2009; Klopotek in sod., 2010; Rudnicki in sod., 1991). Nižja temperatura med skladiščenjem upočasni dihanje, zmanjša fotosintezo v listih potaknjenca, kar pospeši transport saharoze v bazalni del, poveča uspešnost koreninjenja ter zavre širjenje predvsem glivičnih okužb potaknjencev (Behrens, 1988; Paton in Schwabe, 1987; Druege, 2009; Druege in Kadner, 2008). Sun in sod. (2022) ter Skutnik in sod. (2020) poročajo, da

se metoda hladnega skladiščenja uspešno uporablja pri potaknjencih potonik (*Paeonia lactiflora* Pall.), in lahko podaljša življenjsko dobo potaknjencev za več kot 20 dni.

Nepravilno skladiščenje rastlinskega materiala lahko povzroči zmanjšanje klorofila v listih (Conover, 1976), odpadanje listov (Curtis in Rodney, 1952), pospešeno porabo zalog ogljikovih hidratov (Behrens, 1988) in povečano občutljivost rastlin za bolezni in škodljivce (Smith, 1982). Vsi ti dejavniki lahko vplivajo na kakovost rastlin in povzročijo nadaljnje težave pri pridelavi. Kot poročajo Arteca in sod. (1996) se je pri pelargonijah (*Pelargonium* × *hortorum* L.H. Bailey) zmanjšala vsebnost klorofila v listih (za 59 %), ogljikovih hidratov (zlasti škroba) in masa korenin (za 98 %), ko je temperatura v času transporta v 5-ih dneh skladiščenja narasla iz 4 na 25 °C. Takšen odziv pripisujejo pospešenim presnovnim procesom, zlasti dihanju. Pri prenizkih temperaturah skladiščenja, lahko pride do poškodb potaknjencev (Behrens, 1988; Kadner, 2005; Van der Hoeven, 1990). Hawramee (2019) prav tako poroča o negativnem vplivu nepravilnega hladnega skladiščenja potaknjencev navadne robinije (*Robinia pseudoacacia* L.).

Raziskave na tem področju potekajo v smeri iskanja novih metod, s katerimi bi med skladiščenjem ohranili dobro fiziološko stanje potaknjencev za nadaljnje koreninjenje in zavrli katabolne procese ter preprečili morebitne okužbe (Arteca in sod., 1996; Behrens, 1988; Brondani in sod., 2012; Hausman in sod., 2000).

3.1 ZELNATE RASTLINE

Pri zelikah oziroma zelnatih rastlinah je najbolj razširjen način vegetativnega razmnoževanja razmnoževanje z zelenimi potaknjenci. Potaknjenci so različni deli rastlin, niso enako dozoreli in so rezani v času vegetacije ali mirovanja. To razmnoževanje je razširjeno tako v vrtnarijah kot tudi med ljubitelji okrasnih rastlin. Metoda je relativno hitra, enostavna, poceni, vzgojene rastline pa so, če je metoda optimizirana, enotnega videza. Po poročanjih nekaterih avtorjev, se na hladno skladiščenje dobro odzivajo potaknjenci nageljnov (*Dianthus* spp.) (Agullo-Antón in sod., 2011; Garrido in sod., 1996; Garrido in sod., 1998), krizantem (*Chrysanthemum* spp.), pelargonij (*Pelargonium* spp.) (Druege in sod., 2000) in petunij (*Petunia* spp.) (Klopotek in sod., 2010).

Kadner (2005) je v raziskavi preučeval vpliv temperature na skladiščenje potaknjencev moljevke (*Plectranthus coleoides* Benth). Potaknjence, zavite v polietilensko folijo so skladiščili pri temperaturi 1, 5 ali 12 °C različno dolgo časa (3, 4, 5, 6, 7 in 14 dni). Ugotovili so, da je skladiščenje pri temperaturi 1 °C, ne glede na čas, negativno vplivalo na delež koreninjenja potaknjencev, prav tako pa je veliko potaknjencev propadlo. Izpostavili so, da mora biti temperatura skladiščenja in transporta potaknjencev moljevke prilagojena razmeram, v katerih so bili potaknjenci proizvedeni, ter se tako ohrani kakovost potaknjencev do 7 dni. Temperatura skladiščenja in transporta potaknjencev iz južnih geografskih širin, zraslih pri večjih jakostih svetlobe, naj bi bila enaka ali večja od 10 °C, medtem ko je za potaknjence pridelane v centralno evropskih območjih pozimi in zgodaj spomladi najprimernejša temperatura za skladiščenje in transport 5 °C.

S povečanim povpraševanjem po pelargonijah na svetovni ravni, se z vsakim letom povečuje tudi transport potaknjencev teh rastlin iz proizvodnih obratov v ekvatorialnih delih sveta. S tem se povečuje tudi uporaba dolgotrajnejšega skladiščenja potaknjencev v obdobju neposredno po rezi in pred potikom v substrat. Potaknjenci so tako izpostavljeni različnim stresnim dejavnikom, ki lahko negativno vplivajo na kakovost. Tu so pomembni predvsem tema, temperaturna nihanja, vodni stres in tresenje pri prevozu, kar lahko povzroči mehanske poškodbe rastlinskega materiala (Arteca in sod., 1996).

Sorte pelargonij se razlikujejo glede občutljivosti na skladiščenje in transport, pri večini pa se opaža relativno hitra razgradnja klorofila in staranje listov, kar poveča dovzetnost potaknjencev za glivične okužbe in zato v splošnem pelargonije ne veljajo za rastlinsko vrsto, pri kateri se da potaknjence daljši čas uspešno skladiščiti (Behrens, 1988). Arteca in sod. (1996) so v svoji raziskavi ugotovili, da temperatura skladiščenja vpliva na stanje potaknjencev različnih sort pelargonij. Po petdnevnem skladiščenju pri 4 °C ni bilo razlike v izgledu, masi listov in korenin ter vsebnosti klorofila v listih med kontrolnimi rastlinami in potaknjenci, medtem ko je že po prvem dnevu skladiščenja pri 20 °C in 25 °C vsebnost škroba v potaknjencih različnih sort pelargonij močno upadla. Druege in sod. (2004) poročajo, da skladiščenje potaknjencev v temi pri 10 °C za 1 teden značilno vpliva na zmanjšano vsebnost škroba in saharoze v listih potaknjencev pelargonij (*P.* × *hortorum* L.H. Bailey), medtem ko je v stebelnih tkivih ta vpliv manjši. Prav tako so Rapaka in sod. (2005) ugotovili močan upad vsebnosti ogljikovih hidratov (glukoze, fruktoze in škroba) v listih potaknjencev dveh sort pelargonij po štiridnevnem skladiščenju pri 10 °C.

V raziskavi, ki so jo izvedli Druege in Kadner (2008) so ugotavljali vpliv hladnega skladiščenja potaknjencev pelargonij (10 °C za 4 dni) na razvoj listov in razvoj nadomestnih korenin. Ugotovili so, da je hladno skladiščenje vplivalo na zmanjšanje vsebnosti sladkorjev v bazalnem delu poganjkov. Začetna velika vsebnost škroba v bazalnem delu potaknjenca se je med skladiščenjem zmanjšala.

Druege in sod. (2004) so prav tako pri pelargonijah (*P. ×·hortorum* L.H. Bailey) raziskovali vpliv hladnega skladiščenja potaknjencev (10 °C za 1 teden) na vsebnost ogljikovih hidratov. Ugotovili so, da je nivo ogljikovih hidratov v rastlini odvisen od preskrbe rastline z dušikom, ki močno vpliva na asimilacijo in razporeditev ogljika. Dušik in preskrbljenost z ogljikovimi hidrati imata velik vpliv na vzdržljivost rastlin po prodaji. To igra pomembno vlogo, ne samo pri preživetju in razvoju, ampak predvsem takrat, ko predstavlja odvzeti del rastline začetni material za novo rastlino in je potrebna tvorba nadomestnih korenin. Ugotovili so, da je skladiščenje vplivalo na zmanjšanje vsebnosti škroba pri vseh obravnavanjih, medtem ko se je vsebnost sladkorjev zmanjšala samo pri potaknjencih, katerih matične rastline niso bile tretirane z dušikom. Zmanjšanje vsebnosti ogljikovih hidratov med skladiščenjem, zlasti škroba, je v skladu tudi z drugimi rezultati, pridobljenimi pri isti vrsti, po kratkotrajnem skladiščenju pri višjih temperaturah in se pripisuje metabolnim procesom, vključno z dihanjem (Arteca in sod., 1996; Behrens, 1988; Druege, 2000; Purer in Mayak, 1989).

Matične rastline petunije (*Petunia* spp.) gojijo v ekvatorialnih območjih, kjer pobrane potaknjence spravijo v embalažo in pošljejo do glavnih centrov za koreninjenje potaknjencev v Srednji Evropi in ZDA (Klopotek in sod., 2016). Tam potaknjence koreninijo v razmerah majhne jakosti svetlobe čez zimo, za zagotovitev rastlin kupcem spomladi in zgodaj poleti. Obdobje transporta potaknjencev petunij je pomemben del pri proizvodni verigi mladih rastlin in običajno poteka v temi. Prav tako potaknjence takoj shranijo običajno pri nizki temperaturi, preden vstopijo v fazo koreninjenja (Klopotek in sod., 2016). Klopotek in sod. (2010) so v raziskavi proučevali vpliv hladnega skladiščenja potaknjencev petunij v temi na tvorbo nadomestnih korenin. Potaknjence so po odstranitvi iz matične rastline skladiščili v temi, 7 dni in pri temperaturi 10 °C. Podobno raziskavo, prav tako na hibridih pri petuniji (*Petunia* spp.), so izvedli še Klopotek in sod. (2016). Oboji so ugotovili, da takšno skladiščenje izboljša rast nadomestnih korenin in dostopnost ogljikovih hidratov v potaknjencih petunij. Kmalu po končanem skladiščenju, so vsebnosti sladkorjev in škroba v potaknjencih narasle, predvsem v listih in v bazalnem delu potaknjenca.

Pozitiven učinek hladnega skladiščenja (0,5, 4 ali 5 °C) v temi na rast nadomestnih korenin so ugotovili pri potaknjencih krizantem in nageljnov (Druege in sod., 2000; Garrido in sod., 1996). Agullo-Antón in sod. (2011) so proučevali vpliv sladkorjev in avksinov na tvorbo nadomestnih korenin pri potaknjencih nageljna (*Dianthus caryophyllus* L.) skladiščenih na hladnem. Potaknjence so shranili v plastične vrečke, pri relativni zračni vlagi 100 %, skladiščenih pri 5 °C v temi ali pri majhni jakosti svetlobe. Tedensko so merili koncentracijo glukoze, fruktoze, saharoze in škroba v bazalnih delih potaknjencev (0,5 cm). Po enem tednu skladiščenja so opazili bazipetalni transport predvsem saharoze iz mest nastanka v zelenih listih do mesta porabe v bazalnem delu stebla, kjer se tvorijo nadomestne korenine.

Podobno raziskavo so izpeljali Garrido in sod. (2002), ki so pri potaknjencih nageljnov (*D. caryophyllus* L.) skladiščenih 1 - 2 tedna pri temperaturi 4 °C podobno ugotovili, da tako skladiščenje povzroči akumulacijo IAA (indol-3-ocetna kislina) na mestu koreninjenja, kar pospeši koreninjenje. Nivo ogljikovih hidratov med skladiščenjem je bil uravnavan z izpostavitvijo potaknjencev manjšim jakostim svetlobe ali temi. Potaknjenci so bili tretirani z avksinom, potaknjeni, nato pa so spremljali rast nadomestnih korenin. Eksogeni avksin, apliciran na bazalni del, spodbudi metabolizem sladkorjev za sprostitev energije in za preskrbo z ogljikovimi skeleti za sintezo ostalih pomembnih komponent. Hladno skladiščenje potaknjencev v temi za 4 tedne je povečalo delež potaknjencev, ki so zgodaj oblikovali nadomestne korenine, večje je bilo število in dolžina nadomestnih korenin, kljub zmanjšani vsebnosti sladkorjev v potaknjencu. Svetloba med hladnim skladiščenjem pa je očitno povečala vsebnost sladkorjev v potaknjencih, predvsem v bazi poganjka (Haissig, 1986).

Raziskavo o skladiščenju potaknjencev kalanhoje (*Kalanchoe blossfeldiana* Poelln.) sta izvedla Kirk in Andersen (1986), ki sta ugotovila, da je bilo preživetje potaknjencev kalanhoje, skladiščenih pri 100 % zračni vlagi in temperaturi 15,8 °C za 5 tednov, večje od 50 % in več kot 90 % potaknjencev je tvorilo nadomestne korenine.

Lopez in Runkle (2008) so v poskusu ugotavljali, kako temperatura (0 - 30 °C) med skladiščenjem in čas skladiščenja (0 do 5 dni) potaknjencev vodenk (*Impatiens hawkeri* W. Bull) vpliva na fluorescenco klorofila, dihanje, izgled, kakovost in nadaljnje preživetje, koreninjenje in razvoj rastlin. Ugotovili so, da so potaknjenci vodenk lahko skladiščeni do 5 dni pri temperaturi 10 - 20 °C, pri tem pa se nič ali le malo spremeni fotosinteza pri potaknjencih, preživetje, izgled, kakovost potaknjencev, koreninjenje in nadaljnji razvoj rastline.

3.2 LESNATE RASTLINE

Lesnate rastline uspešno razmnožujemo z zelenimi in lesnatimi potaknjenci. Zelene potaknjence (iz toletnih poganjkov) pri lesnatih rastlinah režemo sredi rastne sezone, ko je vegetacija bujna, ko ima rastlina liste in, ko poganjki v rastni dobi že dosežejo določeno zrelost in tudi ustrezno razmerje med dušikovimi snovmi

in ogljikovimi hidrati. Rastline lahko tako uspešno razmnožujemo samo v zavarovanih prostorih (rastlinjakih ali gredah), kjer jim zagotovimo ustrezne razmere (povečana zračna vlaga) in ob dodatku rastnih regulatorjev ob predpostavki, da smo potaknjence v razmnoževalni prostor prenesli neposredno po rezi v matičnem nasadu. Za lesnate rastline v splošnem velja, da dolgotrajnejše skladiščenje, še posebej zelenih potaknjencev ni ustrezno (Smole in Črnko, 2000).

Pri lesnatih rastlinah je nekaj objavljenih raziskav, v povezavi s hladnim skladiščenjem zelenih potaknjencev evkalipta (*Eucalyptus benthamii* Maiden & Cambage) (Brondani in sod., 2012), topola (*Populus tremula* L. × *P. tremuloides* Michx.) (Hausman in sod., 2000), javorja (*Acer grandidentatum* Nutt. in Torr. & A. Gray) (Richards in Rupp, 2012), kanadskega topola (*Populus* × *canadensis* Foug.) (Shibuya in sod., 2013) in kriptomerije (*Cryptomeria japonica* D. Don) (Shibuya in sod., 2014).

Pri razmnoževanju lesnatih rastlin z zelenimi potaknjenci so Brondani in sod. (2012) izvedli raziskavo, kjer so preverjali vpliv časa trajanja hladnega skladiščenja zelenih potaknjencev evkalipta (*E. benthamianae* Brooker), na indukcijo rasti nadomestnih korenin. Potaknjence treh klonov omenjene vrste so izpostavili temperaturi 4 °C za 24, 48, 72, 96 in 120 ur. Ugotovili so, da čas izpostavitve potaknjencev hladnemu skladiščenju ni imel vpliva na preživetje potaknjencev. Poleg tega so tudi ugotovili, da je skladiščenje pozitivno vplivalo na koreninjenje potaknjencev, čeprav so se vseeno najbolje izkazali potaknjenci, ki niso bili skladiščeni in so bili takoj potaknjeni v substrat, saj so tako ohranili najboljšo kakovost in razvili kakovosten nadomestni koreninski sistem.

Hausman in sod. (2000) so izvedli raziskavo na hibridnem topolu (*P. tremula* L. x *P. tremuloides* Michx.), kjer so preverili vpliv kratkotrajnega hladnega skladiščenja zelenih potaknjencev. Te so izpostavili 10 °C za 14 dni. Rezultati so pokazali, da skladiščenje ni vplivalo na preživetje rastlin, je pa med skladiščenjem pri nizkih temperaturah prišlo do sinteze različnih polipeptidov, kar pripisujejo biokemični prilagoditvi, ki jo je v svoji raziskavi že predhodno opisal Guy (1990).

Shibuya in sod. (2013, 2014) so zelene potaknjence pri kanadskem topolu (*P.* x *canadensis* Foug.) in pri japonski kriptomeriji (*C. japonica* D. Don) namakali v topli vodi (30 °C, 10 mm nad rezom, 28 dni) in tako segrevali bazalni del potaknjenca, ob temperaturi zraka 10 °C. Namen raziskave je bil preveriti, kako tako tretiranje vpliva na nadaljnje koreninjenje potaknjencev in vitalnost rastlin po potiku. Ugotovili so, da bi takšno tretiranje lahko izboljšalo razmnoževanje potaknjencev lesnatih rastlin, predvsem zaradi tega, ker je razmnoževanje lesnatih rastlin težje in traja dlje, kot razmnoževanje zelnatih rastlin. Rezultati so pokazali, da tako tretiranje omogoča, da so potaknjenci pripravljeni na razvoj korenin z minimalno izgubo zalog ogljikovih hidratov, znižana temperatura zraka na 10 °C pa je najverjetneje prispevala k zmanjšani senescenci listov. Poleg prej omenjenega, so Shibuya in sod. (2014) pri japonski kriptomeriji ugotovili, da se je tvorba korenin začela hitreje pri potaknjencih, hranjenih pri temperaturi zraka 5 ali 10 °C in namakanih v vodi temperature 20 – 30 °C. To nakazuje na dejstvo, da temperaturni gradient, ustvarjen s segrevanjem baze in hlajenjem apikalnega dela potaknjenca, stimulativno vpliva na tvorbo nadomestnih korenin.

Pri lesnatih potaknjencih uporabimo enoletne olesenele dele rastline. Te režemo ob koncu rastne dobe, od jeseni do pomladi, ko je tkivo popolnoma dozorelo. Ker jih v praksi zelo pogosto potikamo kasneje, kot jih na matični rastlini režemo, jih čez zimo shranjujemo v vlažnem substratu in v hladnih razmerah. Lesnati potaknjenci v osnovi tehnološko temeljijo na tem, da jih koreninimo v hladnem delu leta in skladiščimo pri konstantnih temperaturah, ki niso nujno posebej nižje kot zunaj (1 ali 2 °C). Tako so potaknjenci odpornejši, a se pri mnogih vrstah slabše ukoreninjajo. Tudi pri lesnatih potaknjencih so zato v preteklosti, v želji bo izboljšanju uspešnosti koreninjenja, pri nekaterih vrstah (med drugim različni kloni jablane (*Malus*), uporabni kot podlaga za jablano) preizkušali vmesno, nekajtedensko skladiščenje baze potaknjencev pri višji temperaturi (med 15 in 20 °C). Glede na naravo rastlinskih vrst sicer lesnate potaknjence delimo v dve skupini: neolistane potaknjence (listopadne vrste) in olistane potaknjence (vednozelene vrste). Uporaba lesnatih potaknjencev je cenejša in velja za najbolj razširjen način razmnoževanja nekaterih vrst lesnatih rastlin (npr. topol, posamezne vrste hortenzij, bezeg, ribez idr.) (Hartmann in sod., 1997).

3.3 IN VITRO PROIZVODNJA

Da pridelovalci zadostijo potrebam povpraševanja trga in so dobro pripravljeni na konico sezone, potrebujejo veliko število rastlin v kratkem časovnem obdobju. Kljub temu, da gre pri razmnoževanju s potaknjenci za relativno hitro metodo, se pridelovalci poslužujejo tudi *in vitro* metod razmnoževanja. Gre za zelo uporaben način gojenja okrasnih rastlin, kjer lahko v kratkem časovnem intervalu dobimo veliko število, zdravega sadilnega materiala (Jain in Ochatt, 2010).

Pri metodi meristemske kulture, ki je ena pomembnejših metod *in vitro* proizvodnje rastlin kot osnovni razmnoževalni material uporabljamo zelo majhne rastlinske dele –meristeme, rastni vršiček, ki je v brstu ali pa celo samo skupino nekaj celic, pri nekaterih rastlinah celo cvetni prah. Lahko uporabimo tudi embrio (tudi če ni

povsem razvit, npr. pri zgodaj zorečih sortah), iz njega lahko na posebnih hranilnih raztopinah vzgojimo novo rastlino. V širšem smislu se pri rastlinskih transformacijah večinoma uporabljata dve metodi rastlinske regeneracije: somatska embriogeneza in organogeneza (Slater in sod., 2008). V tkivni kulturi je potrebno natančno kontrolirati zunanje dejavnike, saj na rast in razvoj rastlin pomembno vplivajo tudi svetloba, njena intenziteta, sestava in dolžina dnevnega osvetljevanja, temperatura in vlaga. Te dejavnike kontroliramo z gojenjem rastlin v rastnih komorah (Ravnikar, 1996).

Somatski embriji so lahko hranjeni pri nizki temperaturi ali zmrznjeni in so potem sposobni kalitve in razvoja v sadike. Pomemben dejavnik, ki vpliva na fiziologijo embrijev, sadik in rastlin, je temperatura med embriogenezo. Z uporabo somatskih embrijev razmnožujejo npr. veliko vrst iglavcev (von Aderkas in sod., 2007).

Pomen temperature na kakovost somatskih embrijev ni zelo raziskano področje, predvsem v povezavi s kalitvijo embrijev in razvojem sadik. Znano je, da ima izpostavitev nizkim temperaturam embrijev več učinkov na somatsko embriogenezo. Somatski embriji so lahko hranjeni več mesecev pri temperaturi 4 – 5 °C, lahko pa je takšno daljše skladiščenje tudi tvegano.

Pond in sod. (2002) so v raziskavi ugotovili, da je izpostavitev embrijev, vrste *Picea glauca* (Moench) Voss, nizkim temperaturam (5 °C) za 4 in 8 tednov, pozitivno vplivalo na razvoj nedozorelih embrijev. Podobno sta Beardmore in Charest (1995) ugotovila koristen vpliv dvodnevne izpostavitve somatskih embrijev smreke *P. mariana* Britton, Sterns & Poggenb. temperaturi 2 °C (hladen šok).

Von Aderkas in sod. (2007) so pri vrsti *P. glauca* (Moench) Voss ugotovili, da je izpostavitev nizkim temperaturam (5 °C) v času pozne somatske embriogeneze najučinkovitejša metoda. Hkrati pa avtorji ugotavljajo, da izpostavitev nizkim temperaturam v zgodnjih fazah razvoja embrija, zavre diferenciacijo tkiva v samih embrijih. Temperatura vpliva na vsebnost in vrsto akumulacije rezervnih zalog. Nizka temperatura v času zgodnje embriogeneze zmanjša akumulacijo rezervnih zalog v embriju (predvsem lipidov) (von Aderkas in sod., 2007).

Hladno („šok“) skladiščenje se uporablja za povečanje tolerance somatskih embrijev za izsuševanje. Izpostavitev embrijev vrste *P. glauca* (Moench) Voss za 2 - 14 dni temperaturi 2 °C je izboljšala toleranco za počasno izsuševanje embrijev, na dolgi rok pa se to tretiranje ni izkazalo kot najboljše (Pond in sod., 2002).

4 ZAKLJUČEK

Globalizacija proizvodnje okrasnih rastlin je povzročila prilagoditve pri pridelavi z namenom ohranjanja vrhunske kakovosti in vitalnosti rastlin. Pogosto se dogaja (predvsem pri zelnatih vrstah rastlin), da potaknjencev ne potaknejo takoj v substrat, ampak so ti lahko izpostavljeni tudi daljšemu transportu iz območij proizvodnje do pridelovalcev. Za ohranitev kar se da dobre kakovosti potaknjencev, se pridelovalci pri tem poslužujejo več različnih tehnik. Najobičajnejša metoda hladnega skladiščenja potaknjencev se je pri tem izkazala kot uporabna metoda pri ohranjanju vitalnosti potaknjencev različnih zelnatih in lesnatih okrasnih rastlinskih vrst pri transportu potaknjencev, pri zelnatih vrstah zelo pogosto iz ekvatorialnih delov do območij, kjer jih nato koreninijo. V preglednem prispevku smo predstavili proizvodnjo okrasnih rastlin s potaknjenci. Povzeli smo najpomembnejše dejavnike pri proizvodnji potaknjencev, delitev potaknjencev in predstavili metodo hladnega skladiščenja potaknjencev, ki se uporablja pri vegetativnem razmnoževanju okrasnih rastlin. Na podlagi pregledane relevantne svetovne literature s tega področja smo ugotovili, da je metoda hladnega skladiščenja ključna za ohranjanje optimalne kakovosti potaknjencev zelnatih in lesnatih okrasnih rastlin, še posebej takrat, ko gre za transport sadilnega materiala na daljše razdalje.

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Vloga malih RNK pri odzivu rastlin na okužbo s patogenimi organizmi

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The role of small RNA molecules in plant response to pathogen infection

Abstract: Plants have evolved diverse and complex mechanisms to regulate gene expression. Recently, a new mechanism called RNA interference (RNAi) has been discovered. At the core of RNAi are small non-coding RNAs (sRNAs), 21-24 nucleotides in length, that prevent the translation of transcripts into proteins by binding to complementary sites in transcripts. Because sRNAs are determined by origin, precursor structural properties, and sequence characteristics, they are classified into several classes like microRNAs (miRNAs) and secondary small interfering RNAs (siRNAs), which include tasiRNAs and phasiRNAs. They play important roles in regulating gene expression in a wide range of biological processes and in plant responses to biotic or abiotic stresses. Despite the numerous conserved sRNAs among plant species and the characterization of their function, there is still no comprehensive understanding of their role in plant defense responses against phytopathogens. This review summarizes the current understanding of Verticillium wilt pathogenesis, plant defense mechanisms against phytopathogens, and the biogenesis and roles of miRNAs, tasiRNAs, and phasiRNAs in plant defense responses against fungal pathogens. Further studies on plant sRNAs and their expression in response to various phytopathogens are needed to clearly define their roles. New sequencing approaches, bioinformatic analysis, and prediction of the role of miRNA targets during infection may allow us to develop new forms of plant protection in non-model organisms.

Key words: biotic stress; microRNA; *Verticillium nonalfalfae*; small RNAs

Vloga malih RNK pri odzivu rastlin na okužbo s patogenimi organizmi

Izvleček: Rastline imajo razvite raznolike in kompleksne mehanizme za regulacijo izražanja genov. Nedavno je bil odkrit nov mehanizem, imenovan RNK interferenca (RNKi). Osrednjo vlogo v RNKi imajo male nekodirajoče RNK (sRNK) dolge od 21-24 nukleotidov, ki z vezavo na komplementarna mesta v transkriptih preprečijo njihovo prevajanje v proteine. Ker sRNK definira izvor, strukturne lastnosti prekurzorjev ter sekvenčne lastnosti, jih delimo v več različnih razredov. mikroRNK (miRNK) ter sekundarne male interferenčne RNK (siRNK), med katere prištevamo tasiRNK in phasiRNK imajo pomembno vlogo v regulaciji izražanja genov v številnih bioloških procesih ter odzivu rastlin na biotske ali abiotske dejavnike stresa. Kljub številnim ohranjenim sRNK med rastlinskimi vrstami ter karakterizaciji njihovega delovanja, do danes še ni celovitega razumevanja njihove vloge v obrambnem odzivu rastlin pred fitopatogeni. Ta pregled povzema trenutno razumevanje patogeneze verticilijske uvelosti, obrambnega mehanizma rastlin pred fitopatogeni in biogeneze ter vloge miRNK, tasiRNK ter phasiRNK v obrambnem odzivu rastlin pred glivnimi patogeni. Nadaljnje raziskave rastlinskih sRNK in njihovo izražanje v odzivu rastlin na različne fitopatogene organizme so potrebne za jasno določitev njihove vloge. Novi pristopi sekvenciranja ter bioinformacijske analize in napovedovanja vloge miRNK tarč v času okužb nam lahko pri nemodelnih organizmih omogočijo razvoj novih načinov varstva rastlin.

Ključne besede: biotski stres; mikroRNK; *Verticillium nonalfalfae*; male RNK

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1 UVOD

Male RNK (sRNA; angl. small RNA) so nekodirajoče RNK, ki jih glede na sekvenčne in funkcijske lastnosti delimo v male interferenčne RNK (siRNK), sekundarne male interferenčne RNK (sekundarne sRNK) in mikroRNK (miRNK). Za male RNK je znano, da so vpletene v številne biološke in fiziološke procese rastlin, prav tako pa imajo pomembno vlogo v odzivu rastlin na dejavnike abiotskega stresa in tudi v odzivu na biotski stres, ki ga povzročajo različni patogeni organizmi. V zadnjem času se vse več študij ukvarja z vlogo miRNK v procesih odziva rastlin na glivne patogene in potencialnih aplikacijah miRNK za boj proti glivnim boleznim (Gupta in sod. , 2014).

Patogeni mikroorganizmi rastlin so, skupaj z rastlinojedimi živalmi in pleveli, odgovorni za kar 20 do 40 % izgube svetovne pridelave gospodarsko pomembnih kmetijskih rastlin (Savary in sod., 2012). K velikemu deležu izgube pridelka prispevajo različna glivna obolenja, še posebej pa tista, ki jih povzročajo talni fitopatogeni, kot sta glivi *Verticillium nonalfalfae* Inderbitzin et al. in *Verticillium dahliae* Kleb. (Neve, 1991).

V Sloveniji gliva *V. nonalfalfae* povzroča veliko izgubo v izplenu storžkov ženskih rastlin hmelja (*Humulus lupulus* L.), ki se uporabljajo v pivovarski industriji. Izguba pridelka je še posebej izrazita od leta 1997, ko smo v Sloveniji prvič zaznali pojav agresivnejšega, letalnega patotipa glive, ki se v hmeljiščih sporadično pojavlja še danes. Letalna oblika te bolezni je prav tako prisotna v Angliji, Nemčiji in na Češkem. Bolezen verticilijske uvelosti je bila sprva uvrščena na seznam karantenskih bolezni, danes pa sodi med glivne bolezni razreda II/A2, kar pomeni, da so potrebna dodatna prizadevanja za spremljanje in preprečevanje te bolezni, kar prinaša tudi večje stroške pridelave hmelja (Radišek in sod., 2004; IHGC, 2019).

Hmelj se goji predvsem za uporabo v pivovarstvu, saj storžki ženskih rastlin vsebujejo snovi, ki pivu zagotavljajo značilen okus in aromo, hkrati pa delujejo kot stabilizatorji in konzervansi. V zadnjih letih se hmelj uporablja tudi v farmacevtske namene, saj študije kažejo številne pozitivne učinke na zdravje (Steenackers in sod., 2015; Hrnčič in sod., 2019). Programi žlahtnjenja hmelja so osredotočeni predvsem na povečanje donosa, kakovosti ter sestavo in vsebnost sekundarnih metabolitov v storžkih, medtem ko so študije odpornosti hmelja proti verticilijski uvelosti manj obširne. Trenutno ni na voljo učinkovite metode oziroma fitofarmacevtskega sredstva, ki bi širjenje bolezni uspešno omejila. Molekularna patogeneza je premalo poznana zaradi tega je razumevanje bioloških procesov, ki so vključeni v občutljivost oziroma toleranco rastlin ob okužbi z glivo, izrednega pomena.

2 PATOGENEZA VERTICILIJSKE UVELOSTI

Simptomi bolezni verticilijske uvelosti, ki jo povzroča gliva *V. nonalfalfae*, se razlikujejo glede na patogenost seva. Ob okužbi rastlin z blagim patotipom lahko opazimo neznatne simptome uvelosti rastlin, medtem ko okužba z letalnim patotipom vodi v pojav simptomov, kot so kloroze, nekroze, odebelitev stebla in odmiranje tkiva, kar v končni fazi patogeneze vodi v propad rastline oziroma propad celotnega nasada (Neve, 1991; Radišek in sod., 2006).

Ob zaznavi koreninskih eksudatov gostitelja, gliva preide iz dormantne v parazitsko fazo in prične s kolonizacijo korenin. Hife glive prodrejo preko rizoderma ali eksoderma v primarno skorjo korenine. Endoderm, kot zadnja plast primarne skorje predstavlja prvo bariero pred vstopom glive v ksilem, saj so njegove celične stene trakasto okrepljene s plastjo lignina in tvorijo tako imenovan Kasparijev trak. Prav tako se lahko v endodermalnem tkivu nalaga suberin, ki dodatno otežuje prodiranje glivnih hif proti prevajalnemu sistemu. Ob vstopu hif v prevajalno tkivo, gliva začne tvoriti konidiospore, katere se nato s ksilemskim sokom sistematično razširijo iz koreninskega v stebelno tkivo (Yadeta & Thomma, 2013). Rastline v tej fazi sprožijo procese za tvorbo različnih fizičnih barier v prevajalnem sistemu, s katerimi poskušajo ustaviti prenos konidiospor. Eden takšnih procesov so tiloze, v katerem prihaja do vraščanja mehurjastih izrastkov celic parenhimov v traheide in traheje in nastajanja til, ki preprečijo pretok ksilemskega soka in s tem konidiospor (Talboys, 1958a). Slednji proces je bil opažen predvsem pri občutljivih sortah, medtem ko je bil proces nalaganja suberina v endodermalni plasti bolj intenziven pri odpornih sortah hmelja (Talboys, 1958a; Cregeen in sod., 2015). Ko konidiospore naletijo na fizične bariere, pričnejo tvoriti hife, s katerimi lahko zaobidejo bariere in vstopijo v sosednje žile, preko katerih se dalje s ksilemskim sokom prenašajo kot konidiospore (Chen in sod., 2004). Tekom kolonizacije gliva izloča različne hidrolitične encime, ki razgradijo komponente rastlinskih celičnih sten, kar ji omogoča lažji prehod med žilami prevajalnega sistema (Talboys, 1958b). Prav tako pa ji v zadnji fazi patogeneze encimi omogočajo prehod iz prevajalnega tkiva v preostala tkiva ter s tem popolno kolonizacijo gostitelja, pri čemer pri občutljivih sortah prihaja do pojava izrazitih simptomov kot so kloroze in nekroze listov in odmiranje tkiva (Talboys, 1958a).

3 OBRAMBNI MEHANIZEM RASTLIN

Ksilem si lahko torej predstavljamo kot bojišče, na

katerem gliva napada in poskuša zaobiti bariere, ki jih ustvarja gostitelj, ta pa prav tako z različnimi obrambnimi mehanizmi poskuša zaustaviti nadaljnjo kolonizacijo glive. Odziv hmelja na okužbo z glivo sledi modelu interakcij med rastlinami in patogeni, ki ga predstavlja večplastni imunski sistem rastlin (Jones & Dangl, 2006).

Prvi nivo obrambnega odziva tega modela se navezuje na receptorje rastlin za prepoznavo molekularnih vzorcev (angl. pattern recognition receptors - PRRs), ki se nahajajo na površini celičnih membran in prepoznajo s patogeni povezane molekularne vzorce (angl. pathogen associated molecular patterns - PAMPs). PAMP direktno z vezavo na PRR-je ali posredno z vezavo na druge molekule, ki so v interakciji s PRR-ji, stimulirajo bazalni imunski odziv rastline, ki vključuje tako fizično kot kemično obrambo pred patogeni. Patogeni lahko zaobidejo bazalni imunski odziv in sprostijo t.i. efektorje v apoplast ali citoplazmo rastlinskih celic. Efektorji so raznolika skupina sekretornih proteinov, ki jih patogeni prenesejo v gostiteljeve celice, kjer povzročajo motnje v signalizaciji, ter s tem onemogočajo prepoznavo proteinov, s katerimi patogen napada gostitelja, prav tako pa lahko patogena obvarujejo pred obrambnimi molekulami gostitelja (Jones & Dangl, 2006). Njihovo prisotnost v citoplazmi zaznajo receptorji, imenovani odpornostni proteini oziroma z rezistenco povezani proteini, ki jih kodirajo *R* geni (angl. resistance (R) proteins). Slednji obsegajo raznolike znotrajcelične receptorje, katerim je skupna predvsem domena za vezavo nukleotidov (angl. nucleotide-binding domain - NB) ter domena bogata s ponovitvami levcina (angl. leucine-rich repeat domain - LRR), ki je odgovorna za prepoznavo efektorskih proteinov (Padmanabhan in sod., 2009). Imunski odziv rastlin, sprožen z odpornostnimi proteini v citoplazmi, je drugi nivo obrambe pred patogeni in predstavlja močnejšo obliko odziva, ki zagotavlja z efektorji sproženo odpornost (angl. effector-triggered immunity – ETI) (Jones & Dangl, 2006; Thomma in sod., 2011).

Ko rastline preko zgoraj opisanega mehanizma zaznajo patogena v ksilemu, se sprožijo obsežne metabolne spremembe v celicah parenhima, ki se nahajajo v neposredni bližini okuženega prevodnega sistema in povzročijo akumulacijo različnih proteinov ter sekundarnih metabolitov v rastlinskem ksilemu ali tvorjenje til. Znano je, da lahko številne molekule in spojine, ki se tvorijo v času patogeneze, prispevajo k obrambi rastlin in popolnoma zavrejo ali upočasnijo razrast in širjenje glive v rastlini (Gayoso in sod., 2010). Nekatere spojine, ki se po okužbi nakopičijo v ksilemskem soku, spremenijo morfologijo ksilemskega tkiva in s tem zavrejo vertikalno in lateralno širjenje patogena po rastlini, medtem ko druge nakopičene spojine delujejo protimikrobno in tako tudi eliminirajo povzročitelja bolezni (Yadeta & Thomma, 2013).

Pomembno vlogo pri regulaciji izražanja genov ob okužbi, ki se odvija pred ali med prepisom DNK v mRNK, imajo transkripcijski faktorji in sekvenčni motivi ali regulatorne regije, ki se nahajajo pred začetkom gena. Nekateri izzovejo začetek transkripcije, drugi pa delujejo kot njeni aktivatorji ali represorji. Nov način regulacije genske ekspresije je bil potrjen z odkritjem sRNK, ki delujejo na post-transkripcijskem nivoju in vplivajo na stabilnost transkriptov (mRNK). Slednje je bilo pri prokariontih opisano v devetdesetih letih prejšnjega stoletja, njena funkcija, tako v homeostatskih procesih, kot v procesih odziva na različne biotske in abiotske dejavnike, pa je bila pri evkariontih potrjena šele desetletje kasneje (Wagner & Simons, 1994). Danes vse procese regulacije izražanja genov z sRNK uvrščamo v mehanizem interference RNK (RNKi), ki jo vodita obe glavni skupini sRNK, mikroRNK in siRNK (Saurabh in sod., 2014).

4 BIOGENEZA mIRNK, tasiRNK IN phasiRNK TER Z NJIMI VODENA INTERFERENCA RNK

miRNK so nekodirajoče RNK molekule, ki so v svoji zreli oziroma aktivni obliki dolge 20-24 nukleotidov (nt). Geni miRNK (*MIR*) so v rastlinskih genomih kodirani večinoma v med-genskih regijah, redki pa so locirani tudi v intronskih regijah protein kodirajočih genov. *MIR* geni spadajo v razred II genov, kar pomeni, da so samostojne transkripcijske enote, ki se v primarne transkripte (pri-miRNK) prepisujejo s pomočjo RNK polimeraze II (Lee in sod., 2004). Pri-miRNK so podobno kot protein-kodirajoči geni stabilizirani z 7-metilgvanozinsko kapo na 5‘ koncu in poli(A) repom na 3‘ koncu ter zviti v obliko lasnične zanke, ki meri do 1000 nukleotidov. Proces zorenja rastlinske pri-miRNK do končne zrele in aktivne oblike miRNK v celoti poteka v celičnem jedru, kjer ga usmerja več-proteinski kompleks v dvostopenjskem procesu. Slednjega sestavlja encim RNaza III Dicer-ju podobni protein (DCL1), ki je odgovoren za razrez pri-miRNK v krajše, prav tako v lasnico zvite prekurzorske miRNK (pre-miRNK) ter spremljajoča proteina HYL1 (angl. HYPONASTIC LEAVES 1) in SE (angl. SERRATE). V drugem koraku procesiranja strukturne in sekvenčne lastnosti pre-miRNK lasnice proteinu DCL1 narekujejo mesta za endonukleolitično cepljenje stebla lasnice pre-miRNK iz katerega nastane dvoverižni dupleks sestavljen iz zrele miRNK ter njene komplementarne miRNK*. Za nastali dupleks je značilen 3‘-štrleči konec z dvema nukleotidoma. Za večjo stabilnost oziroma preprečitev razgradnje dupleksa, ga metiltransferaza

HEN1 (angl. HUA ENHANCER 1) na 3‘ štrlečem koncu metilira (Song in sod., 2010). V naslednjem koraku se nato metiliran dupleks s pomočjo proteina HST1 (angl. HASTY) prenese v citoplazmo (Yang in sod., 2006; Ren in sod., 2014), kjer se naloži v kompleks RISC (z RNK induciran kompleks utišanja genov, angl. RNA-induced silencing complex), ki je sestavljen iz različnih proteinov. Med njimi protein Argonavt (AGO) razcepi komplementarno verigo dupleksa in jo usmeri v pot razgradnje v eksosome, zrela oblika miRNK, vezana v kompleks RISC, pa le-tega vodi na njej komplementarno mesto v tarčnem transkriptu. Ob vezavi na tarčno mesto protein AGO, ki predstavlja glavno katalitično komponento, izvede cepitev tarčnega transkripta (Khraiwesh in sod., 2012). Družina proteinov Argonavtov ima torej osrednjo vlogo v procesih utišanja RNK, pri čemer vsak protein kaže preferenčno vezavo določenih miRNK in s tem delno narekuje vlogo miRNK v določenih procesih; npr. procesih rasti, razvoja, obrambnih odzivih, z malimi RNK vodeni metilaciji DNK in podobno (Henderson in sod., 2006).

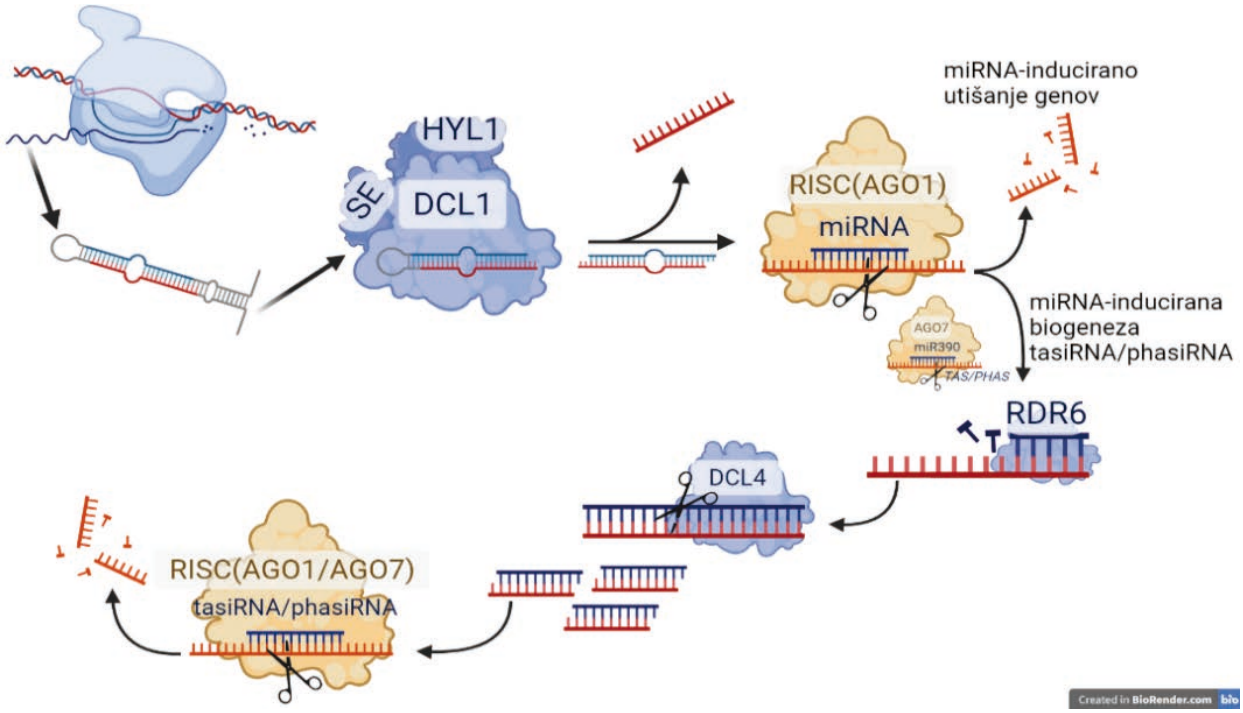
Za razliko od interakcij med miRNK in tarčo pri živalih, kjer je dopuščeno neujemanje, je za interakcije pri rastlinah značilno skoraj popolno ujemanje. Prepoznava vezavnega mesta temelji na ohranjenosti nukleotidnega zaporedja tarče in miRNK, kar omogoča, da lahko eno tarčo regulira več miRNK ter obratno, torej ena miRNK regulira več tarč s podobno ohranjenim mestom vezave (Axtell & Meyers, 2018). Na podlagi ohranjenosti nukleotidnega zaporedja in posledično podobnosti njihove vloge pri različnih rastlinskih vrstah, lahko miRNK uvrščamo v družine. Po drugi strani pa v številnih študijah opisujejo tudi vrstno-specifično ali tkivno-specifično pojavljanje miRNK, tako v fizioloških procesih, kot tudi obrambnih odzivih na abiotske in biotske dejavnike stresa (Dezulian in sod., 2005).

V zadnjih letih je prišlo do izrednega napredka pri razumevanju malih RNK pri rastlinah. Karakterizacija genov, ki kodirajo komponente poti RNKi, kot sta proteina DCL in AGO ter od RNK-odvisna RNK polimeraza (RDR), je potrdila obstoj več različnih poti utišanja, ki jih vodijo sRNK. siRNK so druga skupina sRNK, za katero je značilno, da nastajajo iz popolnoma ujemajoče prekurzorske dvoverižne RNK (dsRNK). Te pa izvirajo s prepisom protismiselne verige ali pa z obratnim prepisom z miRNK razrezanega transkripta s pomočjo RDR. Slednje uvrščamo med sekundarne siRNK, saj njihov nastanek povzroči delovanje specifičnih miRNK. Sekundarne siRNK delimo glede na njihov izvor oziroma biogenezo in delovanje na fazne siRNK (angl. phased siRNA; phasiRNA) in trans-delujoče male interferenčne RNK (tasiRNK). phasiRNK nastajajo iz RNK, ki se prepišejo iz *PHAS* lokusov (lahko so kodirajoči ali ne kodirajoči), njihovo biogenezo pa sprožijo specifične miRNK, ki razrežejo transkripte *PHAS* lokusov. Po razrezu transkripta se le-ta obratno prepiše s pomočjo RDR6, DCL4 pa nato nastalo dvoverižno RNK razreže na 21-24 nukleotidov dolge fragmente, s čimer nastajajo popolnoma ujemajoči se dsRNK dupleksi (Fei in sod., 2013). Enako kot pri delovanju miRNK, se ena od verig inkorporira v AGO proteine, ki phasiRNK usmerjajo na transkripte *PHAS* lokusov iz katerih izhajajo, torej delujejo v *cis* načinu (Axtell & Meyers, 2018). V eksperimentalnih in bioinformacijskih študijah ugotavljajo, da phasiRNK nastajajo iz genov, ki kodirajo proteine vpletene v imunski odziv (z rezistenco povezani geni), transkripcijske faktorje iz družine MYB ter proteine, vpletene v signalizacijo z avksinom (angl. transporter inhibitor response/auxin F-box gene - TIR/AFB) (Yu in sod., 2019). Za razliko od phasiRNK, *trans*-delujoče siRNK izhajajo izključno iz ne-kodirajočih lokusov *TAS* (angl. trans-acting siRNA) in kot njihovo ime nakazuje, delujejo v trans načinu, kar pomeni, da utišajo transkripte iz katerih same ne izhajajo. miRNK, ki sprožijo cepitev *TAS* lokusov, so večinoma vezane v AGO7 ali AGO2. Najbolj natančno je karakterizirana biogeneza tasiRNK, ki izhajajo iz lokusa *TAS3*. Te imajo pomembno funkcijo v regulaciji izražanja dejavnikov odziva na avksin (angl. auxin response factors; ARF). Njihovo biogenezo povzroči miR390 vezana v AGO7 (Montgomery in sod., 2008). Po cepitvi z miR390-AGO7 se 3′-konec razrezanega *TAS* transkripta obratno prepiše s pomočjo RDR6, s čimer nastane popolnoma ujemajoča dsRNK, ki jo nadalje protein DCL4 zaporedno razreže v 21 nt dolge fragmente, s čimer nastajajo tasiRNK, ki dodatno ojačajo utišanje z RNKi (Cuperus in sod., 2010) (Slika 1).

5 miRNK V OBRAMBNEM ODZIVU RASTLIN PRED GLIVNIMI PATOGENI REGULIRAJO HORMONSKO SIGNALIZACIJO

Ena izmed prvih opisanih družin miRNK, ki sodeluje pri odzivu na biotski stres, je bila miR393. Navarro in sod. (2006) so dokazali, da peptid, ki izvira iz flagelina, povzroči izražanje rastlinske miRNK, ki negativno uravnava transkripte za receptorje avksina TIR1 (angl. transport inhibitor response1), AFB2 in AFB3 (angl. auxin-signalling F-Box corepressor). Utišanje signalizacije z avksinom vodi v omejevanje rasti bakterije *Pseudomonas syringae* Van Hall, 1904 pri navadnem repnjakovcu (*Arabidopsis thaliana* (L.) Heynh.), kar nakazuje, da je avksin odgovoren za dovzetnost na bolezen, pri čemer z miRNK-posredovano utišanje signalizacije avksina vodi v odpornost (Navarro in sod., 2006).

Hormonska signalizacija modulira odziv rastlin na biotski stres ter posredno ali neposredno narekuje kompromis med primarno rastjo in odzivnimi mehanizmi

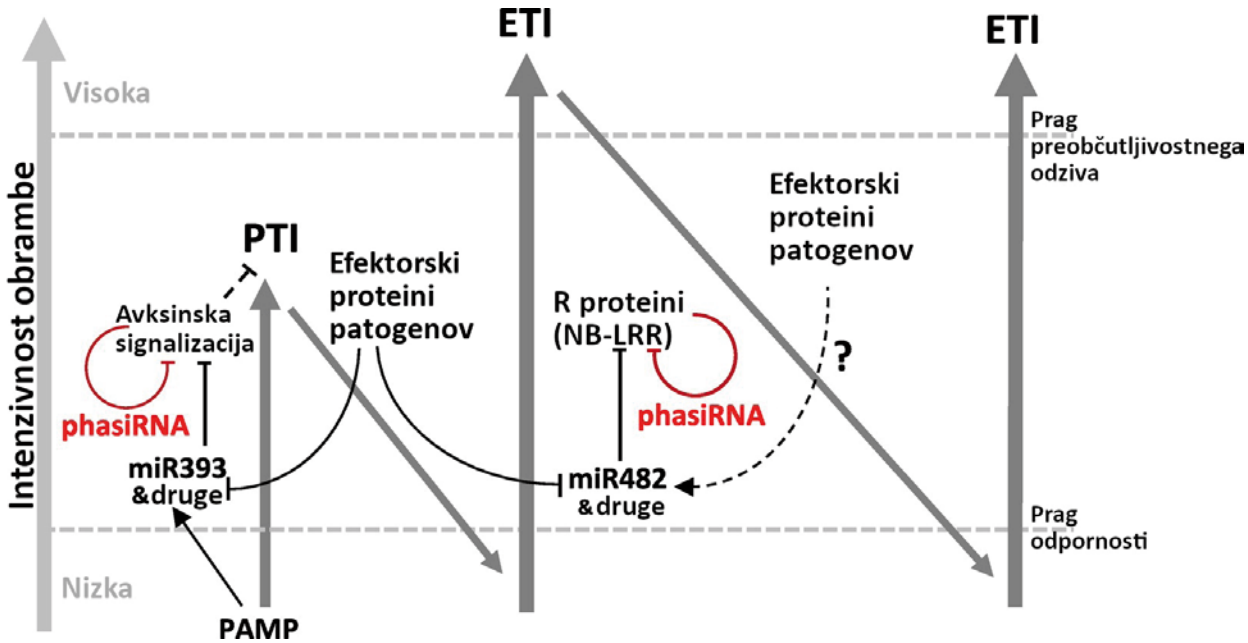


Slika 1: Shematski prikaz biogeneze miRNK, tasiRNK in phasiRNK. Po prepisu *MIR* genov v primarne miRNK, se le-ta zvije v delno komplementarno obliko lasnice, ki se v večih korakih z Dicer-ju podobnimi proteini procesira do nastanka deloma ujemajočega dupleksa. Ena od verig slednjega se inkorporira v AGO1 protein, ki predstavlja glavno katalitično komponento RISC kompleksa. miRNK vodi celoten kompleks na njej komplementarno mesto v tarčnem transkriptu ter s pomočjo AGO1 proteina izvede razrez (miRNK-inducirano utišanje genov). V primeru izražanja specifičnih miRNK (npr. miR390), se lahko te inkorporirajo v druge AGO proteine (npr. AGO7) ter povzročijo razrez nekodirajočih *TAS* transkriptov ali kodirajočih *PHAS* transkriptov (miRNK-inducirana biogeneza sekundarnih malih interferenčnih RNK – tasiRNK ali phasiRNK). Za te transkripte je značilno, da se obratno prepišejo s pomočjo RDR v dsRNK. Tako nastalo dsRNK DCL4 razreže v popolnoma ujemajoče se duplekse, katerih ena od verig se inkorporira v AGO protein in dodatno ojača signal utišanja genov, ki je značilen za siRNK

Figure 1: Schematic representation of the biogenesis of miRNAs, tasiRNAs, and phasiRNAs. After transcription of *MIR* genes into primary miRNAs, the latter is coiled into a partially complementary hairpin form that is processed in several steps by Dicer-like proteins to form a partially matched duplex. One of the strands of the resulting duplex is incorporated into the AGO1 protein, which is the major catalytic component of the RISC complex. The miRNA guides the entire complex to its complementary site in the target transcript and, with the help of the AGO1 protein, performs the cut (miRNA-induced gene silencing). When specific miRNAs are expressed (e.g. miR390), they can be incorporated into other AGO proteins (e.g. AGO7) and induce cleavage of non-coding *TAS* transcripts or coding *PHAS* transcripts (miRNA-induced biogenesis of secondary small interfering RNAs - tasiRNAs or phasiRNAs). These transcripts are characterized by their reverse transcription by RDR into dsRNAs. The resulting dsRNA is cut by DCL4 into perfectly matched duplexes, one strand of which is incorporated into the protein AGO, further amplifying the gene silencing signal characteristic of siRNA

med patogenezo glivnih obolenj (Bari & Jones, 2009). Signalizacija posredovana z avksinom temelji na prisotnosti ali odsotnosti avksina v jedru celice. Ob odsotnosti avksina so proteini Aux/IAA (avksin/indolocetna kislina, angl. auxin/indole acetic acid) vezani na ARF, s čimer jih inaktivirajo. Ob zaznavi avksina se tvori kompleks proteina TIR1 ter spremljajočih proteinov AFB1, AFB2 in AFB3, ki povzroči ubikvitinacijo proteinov Aux/IAA, s čimer se slednji sprostijo iz dejavnikov ARF, kar vodi v aktivacijo (lahko pa tudi represijo – odvisno od vloge družine ARF proteinov) transkripcije na avksin odzivnih genov (Quint & Gray, 2006). Prav tako pa avksinska signalizacija utiša delovanje signalizacije posredovane s salicilno kislino. To nakazuje, da lahko povečano izražanje miR393 ter s tem utišanje avksinske signalizacije vodi v kopičenje salicilne kisline, ki dodatno zagotavlja večjo odpornost na fitopatogene (Wang in sod., 2007) (Slika 2).

Prav tako v študijah opisujejo regulacijo avksinske signalizacije z drugimi miRNK, ki delujejo na transkripte različnih genov vpletenih v signalizacijo. Pri navadnem repnjakovcu miR160 nadzoruje nastanek koreninske čepice z uravnavanjem izražanja genov *ARF10* in *ARF16*. Zaradi spremembe v izražanju *ARF16*, ki jo povzroči miR160, se je pri rastlinah zmanjšala vitalnost ter število



Slika 2: Model „zig-zag-zig" rastlinskega imunskega sistema. Izvirni model Jones and Dangl (2006) opisuje stopničast – več nivojski, evolucijski model obrambe rastlin pred patogeni, ki opisuje kvantitativno naravo in molekularno evolucijo odpornosti proti boleznim pri rastlinah. Z odkritjem regulatorne vloge malih RNK v obrambnem odzivu rastlin so številne mikroRNK in fazne sekundarne male interferenčne RNK (phasiRNK) v „zig-zag-zig" modelu rastlinskega imunskega sistema dobile svoje mesto, bodisi na nivoju PTI ali ETI. V različici tega modela molekularni vzorci, povezani s patogeni (PAMP), povzročijo izražanje miRNK, ki prek hormonske signalizacije sodelujejo v imunosti sproženi s PAMP (angl. PAMP triggered imunity - PTI). Na primer, tretiranje s flagelinom poveča izražanje miR393, ki regulira izražanje genov, vključenih v avksinsko signalizacijo (*TIR1*, *AFB2* in *AFB3*). Utišanje signalizacije avksina v času okužbe posledično izboljša gostiteljevo PTI. Prav tako pa miR393 sproži biogenezo phasiRNK, ki okrepijo aktivnost te miRNK, saj prav tako delujejo na gene, vključene v pot signalizacije avksina. Efektorski proteini patogenov lahko zavirajo delovanje rastlinskih miRNK, kar poveča občutljivost rastlin na okužbo. Pri tem lahko rastline aktivirajo drugi nivo obrambe v katerem sodeluje miR482. Slednja je negativni regulator rastlinskih genov za odpornost (*R* genov) in se ji ob zaznavi efektorjev zmanjša izražanje, kar poveča odpornost sproženo z efektorji (angl. effector triggered immunity - ETI). Nekatere miRNK lahko sprožijo biogenezo phasiRNK, ki izhajajo iz *R* genov, te phasiRNK pa lahko delujejo sinergistično z miRNK bodisi v *cis* ali *trans* načinu in dodatno zmanjšujejo nivo transkriptov *R* genov. Nekateri efektorji lahko spodbudijo stabilnost oziroma izražanje miRNK z delovanjem na RNKi komponente, vključene v biogenezo miRNK. Mehanizmi tega modela še niso popolnoma razjasnjeni in so v sliki označeni z vprašajem. Na primer, ali lahko efektorji aktivirajo izražanje miRNK vključenih v ETI in s tem oslabijo imunski odziv gostitelja (povzeto po Fei in sod. (2013), Copyright © 2016 The American Phytopathological Society DOI: 10.1094/MPMI-09-15-0212-FI)

Figure 2: A "zig-zag-zig" model of the plant immune system. The original model proposed by Jones and Dangl (2006) describes a stepwise, multi-level, evolutionary model of plant defense against pathogens that describes the quantitative nature and molecular evolution of disease resistance in plants. With the discovery of the regulatory role of small RNAs in plant defense responses, a number of microRNAs and secondary sRNAs, namely phased small interfering RNAs (phasiRNAs), have found their place in the 'zig-zag-zig' model of the plant immune system, either at the PTI or the ETI level. In a version of this model, pathogen-associated molecular patterns (PAMPs) induce the expression of miRNAs that participate in PAMP-triggered immunity (PTI) via hormonal signalling. For example, flagellin treatment increases the expression of miR393, which regulates the expression of genes involved in auxin signalling (*TIR1*, *AFB2* and *AFB3*). Consequently, suppression of auxin signalling during infection enhances host PTI. In addition, miR393 triggers the biogenesis of phasiRNAs that enhance the activity of this miRNA, as they also act on genes involved in the auxin signalling pathway. Pathogen effector proteins can inhibit the activity of plant miRNAs, increasing the susceptibility of plants to infection. In this context, plants may activate a second level of defense involving miR482. The latter is a negative regulator of plant resistance genes (*R* genes) and its expression is reduced when effectors are detected, increasing effector triggered immunity (ETI). Some miRNAs can trigger the biogenesis of *R* gene-derived phasiRNAs, and these phasiRNAs can act synergistically with miRNAs in either *cis* or *trans* mode to further reduce the level of *R* gene transcripts. Some effectors may promote miRNA stability or expression by acting on RNAi components involved in miRNA biogenesis. The mechanisms of this model are not yet fully understood and are indicated by a question mark in the figure. For example, whether effectors can activate the expression of miRNAs involved in ETIs and thereby attenuate the host immune response (reproduced from Fei et al. (2013), Copyright © 2016 The American Phytopathological Society DOI: 10.1094/MPMI-09-15-0212-FI)

stranskih korenin (Wang in sod., 2005). Prav tako pa so nepravilno rast koreninskega tkiva opazili pri mutantih navadnega repnjakovca, ki so izražali različico *ARF17*, na katero miR160 ni imela vpliva. Rastline, ki so izražale različico *ARF17*, odporno na miRNK, so imele povečane ravni transkriptov *ARF17* in spremenjeno kopičenje mRNK GH3-podobnih proteinov (YDK1/GH3.2, GH3.3, GH3.5 in DFL1/GH3.6), katerih izražanje inducira avksin in so odgovorni za konjugacijo avksina. Spremembe v izražanju teh genov so povezane z drastičnimi razvojnimi okvarami, vključno z anomalijami simetrije embrijev in rastočih listov, okvarami oblike listov, prezgodnjim razvojem socvetja, spremenjeno filotaksijo, zmanjšano velikostjo cvetnih listov, nenormalnimi stebli, sterilnostjo in okvarami rasti korenin (Mallory in sod., 2005). V naši nedavni študiji smo v koreninskih vzorcih odporne sorte hmelja, ki je bila inokulirana z letalnim patotipom glive *V. nonalfalfae*, opazili povečano izražanje hlu-miR160b. V analizi napovedovanja tarč, smo tarčno mesto te miRNK določili v transkriptih genov *ARF10* in *ARF18* (Kunej in sod., 2021). Za slednjega je znano, da zavira signalizacijo z avksinom, kar vodi v podaljševanje hipokotilov pri rastlinah, ki rastejo v senčnih razmerah (Jia in sod., 2020). Povečano izražanje miR160 ter uravnavanje transkriptov ARF je bilo dokazano tudi med patogenezo bolezni stebelnega raka pri topolu vrste *Populus trichocarpa* Torr. & A.Gray ex. Hook. (Zhao in sod., 2012) in pri krompirju, pri katerem je bilo izražanje *StARF10* utišano. Slednji se veže na promotorje gena *DFL1*/*GH3.6*, ki posreduje v navzkrižni povezavi poti signalizacije s salicilno kislino in avksinom ter je tako s tem vpleten v lokalno obrambo in sistemsko pridobljeno odpornost proti krompirjevi plesni (*Phytophthora infestans* (Mont.) de Bary) (Natarajan in sod., 2018). Prav tako pa so v študiji na rižu dokazali, da lahko prekomerna ekspresija miR160a ali miR398b poveča odpornost riža na glivo *Magnaporthe oryzae* (Li in sod., 2014).

Nasprotno pa so v študijah na jajčevcu (Yang in sod., 2013) ter oljni ogrščici (Shen in sod., 2014) opazili, da okužba z glivama *V. dahliae* oziroma *V. longisporum* povzroči zmanjšano izražanje miR160. Slednje nakazuje, da je izražanje te miRNK potencialno vrstno- ali sortno-specifično oziroma povezano z občutljivostjo ali odpornostjo rastlin na glivne patogene. Večina odpornih sort kaže povečano izražanje miR160, medtem ko v občutljivih opazimo zmanjšano ali nespremenjeno izražanje tako miRNK, kot njihovih tarč (Yang in sod., 2013; Li in sod., 2014; Shen in sod., 2014).

Tako kot miR393 in miR160, imajo tudi miR167 vlogo v regulaciji avksinske signalizacije. Za slednje so pri navadnem repnjakovcu dokazali, da regulirajo izražanje *ARF6* in *ARF8* (Jones-Rhoades & Bartel, 2004). Pri rižu, okuženem z glivo *M. oryzae,* je občutljiva sorta kazala manjše izražanje miR167a/b/c, odporna sorta pa znatno povečano, kar nakazuje, da so te miRNK potencialni pozitivni regulatorji odpornosti riža proti omenjeni glivi (Li in sod., 2014).

Dodaten nivo regulacije oziroma vzdrževanja homeostaze avksina zagotavljajo tudi miR164, ki regulirajo izražanje transkripcijskih faktorjev z domenam značilnimi za družino NAC. Med njimi NAC1 pozitivno uravnava razvoj stranskih korenin preko signalizacije z avksinom, pri čemer miR164, ki jo inducira avksin, nadzoruje raven transkriptov *NAC1* (Guo in sod., 2005). Regulacijo te miRNK so opazili pri bombažu in rižu kot odziv na okužbo z glivama *V. dahliae* in *M. oryzae* (Li in sod., 2014; Hu in sod., 2020). To lahko nakazuje na rast in razvoj novih stranskih korenin v času okužbe, saj rastlina z mašenjem prevajalnega tkiva (tvorbo til) poskuša omejiti širjenje glive po rastlini (Talboys, 1958a).

Nadaljnja karakterizacija interakcije miR164-NAC100 pri bombažu je pokazala, da povečano izražanje miR164 v odzivu na okužbo z *V. dahliae* vodi v neposredno cepitev transkriptov *NAC100*. Prav tako pa so dokazali, da izražanje miR164 in tudi izbitje *NAC100* pozitivno prispeva k odpornosti na glivo, saj je slednji represor gena *GhPR3* (s patogenezo povezan gen 3; angl. pathogenesis related gene 3). Slednje nakazuje, da interakcija miR164-NAC100 igra pomembno vlogo v obrambo rastlin prek RNKi (Hu in sod., 2020).

Povečano izražanje miR164 so opazili tudi pri odpornih sortah riža, okuženih z glivo *M. oryzae*, pri čemer so opazili, da ta cepi transkripte gena »*s salicilno kislino induciran protein 19*« (angl. *Salicylic acid-induced protein 19* – LOC_Os12g41680). To dodatno nakazuje na vlogo te miRNK v hormonski signalizaciji in potencialni odpornosti na glivne patogene (Li in sod., 2019).

Prav tako pa obstajajo še druge miRNK, ki so odzivne na okužbo z glivnimi patogeni in katerih tarče niso vpletene v hormonsko signalizacijo, ampak lahko posredno vplivajo na odpornost rastlin na okužbo. Študije kažejo, da je povečana odpornost rastlin povezana s povečanim izražanjem miRNK, ki so vpletene v rast in razvoj tkiv (Guo in sod., 2005; Mallory in sod., 2005; Wang in sod., 2005; Singh in sod., 2014). Po drugi strani pa v študijah opisujejo različno izražanje miRNK, ki so posredno ali neposredno vpletene v regulacijo obrambnih mehanizmov in imunost (Yi & Richards, 2007; Gupta in sod., 2012; Zhao in sod., 2012; Wong in sod., 2014). Pri miRNK, ki so vpletene v obrambne mehanizme, lahko opazimo različno izražanje, ki je odvisno od patosistema. Medtem ko miRNK, ki so neposredno vpletene v imunost, t.j. regulirajo izražanje s patogenezo povezanih genov ali genov za odpornost proti patogenom, kažejo znižano izražanje.

6 miRNK SO VPLETENE V OBRAMBNE MEHANIZME

V mehanizme odpornosti vključujemo tudi nastajanje strukturnih barier, sintezo sekundarnih metabolitov in protimikrobnih encimov in proteinov. Rastline lahko širjenje patogenov omejijo s tvorbo fizičnih barier v prevajalnem tkivu ter nalaganjem lignina v endodermne plasti. Slednje procese so v odzivu na glivo *V. nonalfalfae* opazili pri občutljivi sorti hmelja, medtem ko so pri odporni opazili intenzivno nalaganje suberina (Talboys, 1958a; Cregeen in sod., 2015). Omenjeni procesi so zelo dobro uravnavani na različnih ravneh, pri čemer imajo miRNK pomembno vlogo v njihovi post-transkripcijski regulaciji. Na primer za miRNK iz družin miR397, miR398 in miR408 je znano, da so v času patogeneze glivnih obolenj preko uravnavanja bakrove superoksidne dismutaze, lakaz in plantacianinov vpletene v regulacijo bakra, ki ima biocidno aktivnost (Abdel-Ghany & Pilon, 2008). Še posebej pomembni sta slednji dve skupini, lakaze in plantacianini, ki so tarče miR408. Ti so ključni encimi v biosintezi lignina, saj katalizirajo zadnji korak polimerizacije monolignolov (Huang in sod., 2016). Pri občutljivi in odporni sorti pšenice so v odzivu na okužbo z glivo *Puccinia graminis* f. sp. *tritici* patotip 62G29-1 ugotovili zmanjšano izražanje miR408 in miR1138, kar se je odražalo v povečanem izražanju tarč in povečani biosintezi kaloze in lignina v času preobčutljivostnega odziva na mestu okužbe (Gupta in sod., 2012). Karakterizacija interakcije ghr-miR397 in njene tarče *GhLAC4* je pri bombažu v času okužbe z glivo *V. dahliae* pokazala njegovo pomembno vlogo v odpornosti. Po okužbi z glivo *V. dahliae* so raziskovalci ugotovili večjo vsebnost lignina pri rastlinah z izbito miR397 v primerjavi s kontrolnimi rastlinami, medtem ko je prekomerno izražanje miR397 in izbitje gena *GhLAC4* zmanjšalo vsebnost lignina, pri čemer so rastline kazale večjo občutljivost na okužbo z glivo. To nakazuje na pomembno vlogo biosinteze in nalaganja lignina v času okužbe (Wei in sod., 2021).

Nekatere tarče teh miRNK so negativni regulatorji mehanizmov odpornosti, kar pomeni, da lahko povečano izražanje miRNK prispeva k manjšemu izražanju tarč in s tem povečani odpornosti rastlin. Na primer, miR166 in miR165 delujeta tarčno na transkripte genov iz družine HD-ZIP III (angl. Class III homeodomain-leucine zipper). Povečano izražanje miR166 so opazili v apikalnem meristemu korenin pri navadnem repnjakovcu ter opazili večjo aktivnost apikalnega meristema in rast korenin, hkrati pa tudi razvoj sekundarne celične stene in prevajalnega tkiva (Singh in sod., 2014). Progar in sod. (2017) so primerjali transkriptomska profila odporne in občutljive sorte hmelja po okužbi z glivo *V. nonalfalfae* in opazili 2,3-krat manjše izražanje genov družine HD-ZIP III v okuženih rastlinah odporne sorte hmelja Wye Target šesti dan po inokulaciji, česar pa niso opazili pri občutljisorti Celeia. Slednje nakazuje, da so v proces regulacije genov družine HD-ZIP III v času okužbe potencialno vpletene miR165 in miR166, kar bi lahko botrovalo k opaženi večji odpornosti sorte Wye Target. miR166 je še posebej zanimiva, saj spada v kategorijo miRKA, ki se lahko prenaša med kraljestvi. (Zhang in sod.(2016) so dokazali, da rastline bombaža v odzivu na okužbo z glivo *V. dahliae* povečajo izražanje miR166 in miR159 ter ju prenesejo v hife glive. Prav tako so tudi dokazali, da delujeta tarčno na transkripte glivnih genov *Clp-1* (od Ca^{2+}odvisna cisteinska proteaza) in *HiC-15* (izotrihodermin C-15 hidroksilaza), ki sta odgovorna za virulenco glive.

7 miRNK REGULIRAJO IZRAŽANJE GENOV Z REZISTENCO POVEZANIH PROTEINOV

V primeru, da patogeni preidejo prvi nivo obrambe, t.j. PTI in začnejo sproščati efektorske proteine v celice, se aktivira izražanje znotrajceličnih receptorjev, ki jih kodirajo *R* geni. Ti prepoznavajo efektorske proteine in sprožijo ETI (Jones & Dangl, 2006; Thomma in sod., 2011). Glavna skupina R proteinov je sestavljena iz NB domene za vezavo nukleotidov in LRR domene bogate s ponovitvami levcina. Kodirajo jih NB-LRR *R*-geni. NB domena preferenčno veže ATP/ADP ali GTP/GDP, domena LRR pa je pogosto vključena v interakcije med proteini (protein-protein) in vezavo ligandov. NB-LRR *R*-gene lahko nadalje, glede na vrsto N-terminalne domene, delimo na receptorje z N-terminalno domeno podobno toll interlevkinu-1 (TIR-NB-LRR) in receptorje z domeno v obliki vijačnice (CC-NB-LRR) (Knepper & Day, 2010).

Tako kot v prejšnjih poglavjih opisane miRNK, ki so odzivne na s patogeni-povezanimi molekularnimi vzorci v prvem nivoju odziva rastlin na patogene (PTI) ter imajo vlogo obrambnih mehanizmih, obstajajo miRNK, ki neposredno ali posredno – z aktivacijo biogeneze sekundarnih siRNK - regulirajo izražanje *R* genov in so odgovorne za uravnavanje prirojene imunosti (Fei in sod., 2013).

Leta 2007 sta Yi in Richards prva poročala o morebitnih učinkih post-transkripcijskega utišanja pri blaženju prekomernega izražanja *R* genov iz kompleksnega lokusa pri navadnem repnjakovcu. Lokus RPP5 (angl. recognition of *Peronospora parasitica* 5) pri navadnem repnjakovcu vsebuje sedem genov iz TIR-NB-LRR družine *R* genov. Dva gena na tem lokusu, imenovana *RPP4* in *SNC1*, zagotavljata odpornost proti bakteriji *P.*

syringae in oomiceti *Hyaloperonospora parasitica* (Pers.) Constant., V študiji sta potrdila, da endogene male RNK, ki nastanejo s prepisom protismiselne verige transkriptov *SNC1*, zavirajo izražanje ostalih genov tega lokusa. Pri mutantih z okvarjenimi komponentami RNKi, kot sta *dcl4* in *ago1*, se je povečalo kopičenje *SNC1* (Yi & Richards, 2007). V nadaljnjih študijah pa so odkrili tudi ostale specifične miRNK, ki posredno, preko indukcije biogeneze sekundarnih sRNK (phasiRNK), regulirajo izražanje NB-LRR genov ter eksperimentalno potrdili in natančneje karakterizirali celotno RNKi pot (Shivaprasad in sod., 2012).

Pri paradižniku so opisali 15 lokusov iz katerih izhajajo phasiRNK, od katerih jih 6 kodira NB-LRR proteine. Avtorji so ugotovili, da miR482 vezana v AGO1 povzroči cepitev mRNK LRR1, proteina tipa CC-NR-LRR, ki se nato obratno prepiše s pomočjo RDR6. Tako nastalo dsRNKA nato DCL4 od začetka mesta cepitve, ki ga določa vezavno mesto miR482, v fazah razreže v 21 nt dolge phasiRNK. Ob okužbi paradižnika z bakterijo *P. syringae* so opazili zmanjšano izražanje miR482 ter sočasno indukcijo *R* genov, kar potencialno ščiti rastline pred napadom patogena (Shivaprasad in sod., 2012). Povečano izražanje miR482 so opazili tudi v interakciji med bombažem in glivo *V. dahliae*, kar je verjetno vodilo do de-represije *R* genov po okužbi ter s tem specifičnega imunskega odziva - ETI na glivno okužbo (Zhu in sod., 2013). Podobno so zmanjšano izražanje te miRNK opazili tudi pri soji, okuženi z oomiceto *Phytophthora sojae* Kaufm. & Gerd. (Wong in sod., 2014). Dodatno so avtorji opisali še dve miRNK; miR2109, ki se veže na motiv TIR1 v mRNK *TIR-NB-LRR* in miR1507, ki deluje na motiv kinaza-2 v mRNK CC-NB-LRR. Za slednji miRNK so ugotovili, da kažeta povečano izražanje pri soji okuženi z *P. sojae*, ne pa pri soji inokulirani z neaktivno obliko seva. To kaže na njuno vlogo uravnavanja ETI odziva, saj lahko dodatno zmanjšanje izražanje teh miRNK vodi v hitro povečanje vsebnosti NB-LRR proteinov ter s tem še bolj intenzivnega odziva na okužbo (Wong in sod., 2014). Na podlagi teh analiz lahko domnevamo, da je regulacija NB-LRR, ki jo posredujejo male RNK, ključna za imunske odzive rastlin.

8 ZAKLJUČEK

Razumevanje delovanja malih RNK je odvisno od identifikacije tarčnih genov ter njihove vloge. Identifikacija siRNK in miRNK ter njihovih tarč postavlja temelje, ki so potrebni za boljšo karakterizacijo kompleksnega omrežja regulatornih interakcij, ki nadzorujejo rast in razvoj ter druge fiziološke procese rastlin in so prav tako vključeni v odziv rastlin na biotske in abiotske dejavnike stresa. Kljub temu, da so bile miRNK odkrite pred približno tremi desetletji, nam je razvoj tehnologije sekvenciranja zelo kratkih RNK omogočil hitro odkritje in postavitev modela delovanja poti RNK interference, ki jo vodijo male interferenčne RNK. Z dobro identifikacijo delovanja nekaterih sRNK pa se vzpostavljajo metode, ki kažejo velik potencial za izboljšave nekaterih lastnosti rastlin. Predvsem pa je poudarek na razvoju strategij za izboljšanje lastnosti rastlin, ki pripomorejo k večji odpornosti agronomsko pomembnih rastlin, ki nam zagotavljajo prehransko varnost.

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