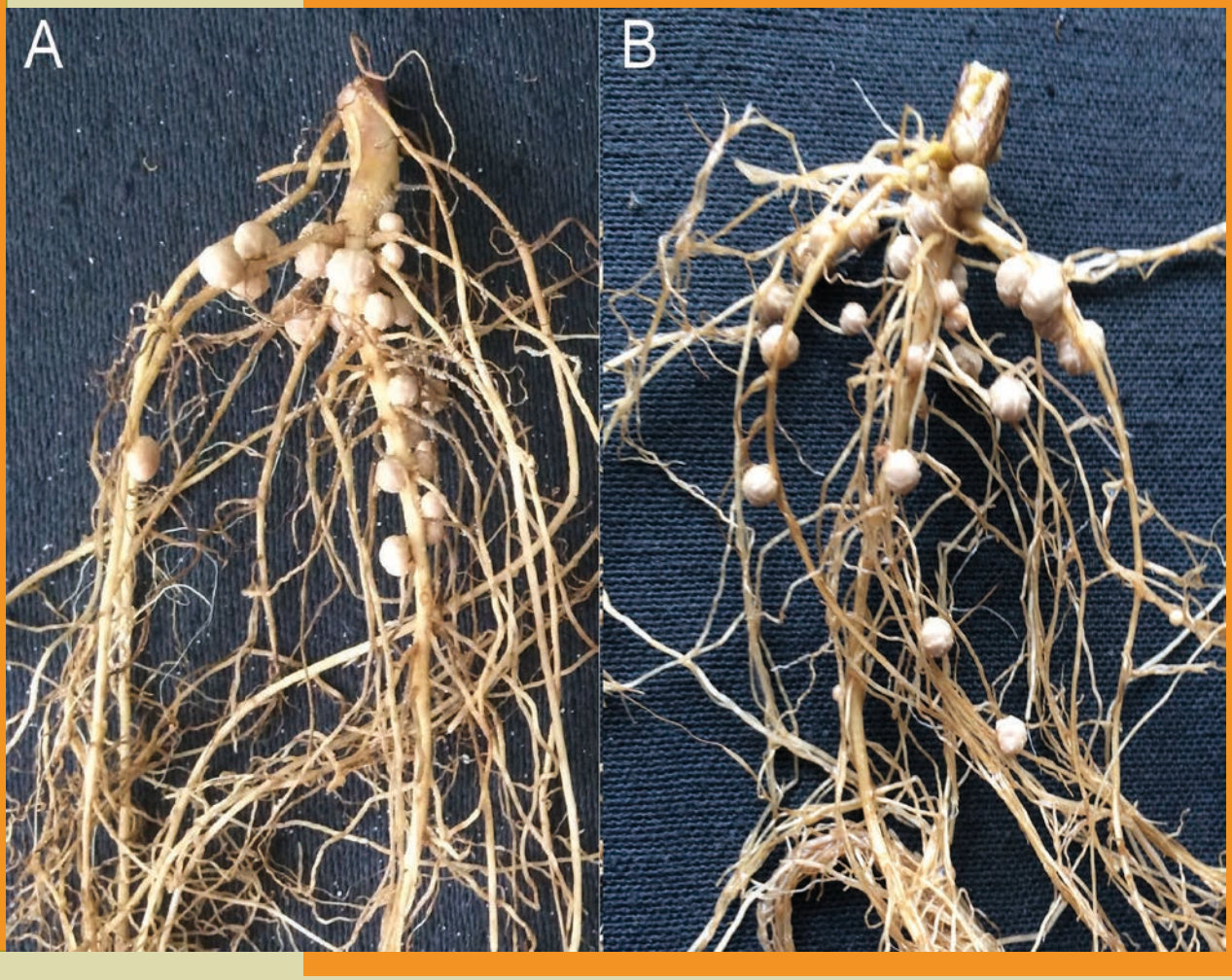

118 • 2
2022

ACTA AGRICULTURAE SLOVENICA

Acta agriculturae Slovenica • eISSN 1854-1941 • 118 – 2 • Ljubljana, junij 2022

Univerza *v Ljubljani*
Biotehniška fakulteta

Acta agriculturae Slovenica **eISSN 1854-1941**
Letnik / Volume 118 · Številka / Number 2 · 2022

Založnik in izdajatelj / *Publisher and Issuer*	*Založila / Published by: Založba Univerze v Ljubljani / University of Ljubljana Press* *Za založbo / For the Publisher: Gregor MAJDIČ, rektor Univerze v Ljubljani / the Rector of the University of Ljubljana* *Izdala / Issued by: Univerza v Ljubljani, Biotehniška fakulteta / University of Ljubljana, Biotehnical Faculty* *Za izdajatelja / For the Issuer: Nataša POKLAR ULRIH, dekanja Biotehniške fakultete UL / the Dean of the Biotehnical Faculty UL*
Naslov Uredništva / *Editorial Office Address*	Univerza v Ljubljani, Biotehniška fakulteta, Acta agriculturae Slovenica Jamnikarjeva ulica 101, SI-1000 Ljubljana
E-naslov / *E-mail*	Acta.Agriculturae.Slovenica@bf.uni-lj.si
Spletni naslov / *Web address*	http://ojs.aas.bf.uni-lj.si

Acta agriculturae Slovenica izhaja samo kot spletna revija, skupni letnik pa praviloma obsega štiri številke. / *Acta agriculturae Slovenica is published only as an online journal with four issues per year in one common volume.*

Trenutno revija ne zaračunava stroškov za predložitev in obdelavo vključenih prispevkov. / *The journal does not charge APCs or submission charges.*

Acta agriculturae Slovenica izhaja s finančno pomočjo / *is published with the financial support:* Javne agencije za raziskovalno dejavnost Republike Slovenije / *Slovenian Research Agency*.

Acta agriculturae Slovenica je vključena v / *is included into*: Scopus (SJR, SNIP), DOAJ, WOS Zoological Records, CrossRef, CAB Abstracts, FSTA, Google Scholar, dLib, COBISS.

Ovitek: Noduli na koreninah soje po inokulaciji semen z aktivnim sevom bakterije *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 D87 v razvojni stopnji začetka cvetenja: A – inokulacija semen na dan setve, B – inokulacija semen 7 dni pred setvijo (Foto: Petro Pukhtaievych, 1–11)
Cover: Nodules on soybean roots at seeds inoculation by the active strain Bradyrhizobium japonicum *(Kirchner, 1896), Jordan, 1982 D87 at the budding-beginning of flowering stage: A – inoculation at the sowing day, B – inoculation in 7 days before sowing (Photo: Petro Pukhtaievych, 1–11)*

Acta agriculturae Slovenica
Volume / Letnik **118** · Number / Številka **2** · **2022**

Table of Contents / Kazalo

Original Scientific Article / Izvirni znanstveni članek

Review Article / Pregledni znanstveni članek

doi:10.14720/aas.2022.118.2.2272 Original research article / izvirni znanstveni članek

Open vertical farms: a plausible system in increasing tomato yield and encouraging natural suppression of whiteflies

Suleiman MUSTAPHA [1, 2, 3], Abdulrasak Kannike MUSA [1], Oluropo Ayotunde APALOWO [4], Abdrahaman Adebowale LAWAL [1], Olaniyi Israel OLAYIWOLA [5], Helen Olaide BAMIDELE [1], Robert Omotayo UDDIN II [1]

Received July 07, 2021; accepted March 21, 2022.
Delo je prispelo 7. julija 2021, sprejeto 21. marca 2022

Open vertical farms: a plausible system in increasing tomato yield and encouraging natural suppression of whiteflies

Abstract: This study evaluated the effectiveness of open vertical farming in increasing tomato yield and also recruiting the presence of ecological service providers in the control of whiteflies. The experiment compared the horizontal farming approach to novel outdoor vertical farm design. Using both raised and flatbeds to represent horizontal farm, tomato plants were grown in a spacing of 3.6 and 2.4 m^2 respectively while the vertical farm covered a land space of 1.8 m^2 having three arrays with array 1 at ground level, array 2 and 3 were elevated at 110 and 220 cm high respectively. Data collected included the numbers of *Bemisia tabaci* (Gennadius, 1889) and predatory spiders and; tomato fruit yield (g). Results indicated that the mean number of predatory spiders in the vertical farm from 6 – 10 weeks after transplanting were able to supress *B. tabaci* populations when compared to the horizontal farm. The total fruit yield harvested indicated that the vertical farm produced more tomato fruit yield compared to the horizontal farm. It is plausible that the practice of outdoor vertical farming may be a step approach solution to land shortages and also a sustainable system for integrated pest management.

Key words: Vertical farm; *Bemisia tabaci*; predator-prey interaction; biological control; tomato; insect pest

Navpični način gojenja: racionalen sistem za povečanje pridelka paradižnika in vzpodbuda za sonaravno zatiranje tobačnega ščitkarja

Izvleček: V raziskavi je bilo ovrednoteno gojenje paradižnika v navpičnem sistemu z namenom povečanja pridelka in kot način ekološkega uravnavanja tobačnega ščitkarja. V poskusu sta bila primerjana dva načina gojenja in sicer običajen vodoraven in navpičen sistem gojenja na prostem. Pri vodoravnem načinu gojenja so bile uporabljene visoke in navadne grede, kjer je posamezna rastlina paradižnika pokrivala 3,6, oziroma 2,4 m^2. Pri navpičnem načinu gojenja je posamezna rastlina zavzemala 1,8 m^2 v treh višinah in sicer na tleh (1), na višini 110 cm (2) in 220 cm (3). Parametri, ki so bili merjeni so obsegali število osebkov škodljivca (*Bemisia tabaci* (Gennadius, 1889) in predatorskih pajkov ter pridelek paradižnika (g). Rezultati so pokazali, da je številu predatorskih pajkov v navpičnem sistemu gojenja v 6 do 10 tednih po sadnji bolje uspelo zatreti populacijo škodljivca v primerjavi z vodoravnim načinom gojenja. Tudi celokupen pridelek paradižnika je bil pri navpičnem načinu gojenja večji kot pri vodoravnem. Iz izsledkov lahko zaključimo, da je gojenje paradižnika v navpičnem načinu gojenja na prostem racionalen korak pri reševanju pomanjkanja zemljišč kot pri trajnostnem uravnavanju škodljivcev.

Ključne besede: navpišni način gojenja**;** *Bemisia tabaci*; interakcija plenilec-plen; biološki nadzor; paradižnik; škodljive žuželke

1 Department of Crop Protection, University of Ilorin, Nigeria
2 Division of Crop Protection, ICAR-Indian Institute of Horticultural Research, Bengaluru, India
3 Corresponding author, e-mail: juniorsuleiman78@gmail.com
4 Department of Crop Science and Horticulture, Nnamdi Azikiwe University, Awka, Nigeria
5 Institute of Global Food Security, School of Biological Sciences, Queen's University Belfast, UK

1 INTRODUCTION

Food insecurity is fast becoming an increasingly vital matter worldwide (Al-Kodmany, 2018). It has been predicted that the urban population will constantly rise in the coming decades. At the same time, land experts (i.e., ecologists, agronomists, and geologists) warn of intensifying shortages of farmland (Corvalan et al., 2005; Healy and Rosenberg, 2013; Thomaier et al., 2015). With a rapidly expanding population and changing climate, pressures on food production systems are expected to increase in the coming years (FAO, 2018). Traditional farming methods cannot produce enough food to feed the world's growing population and may fail in future (Despommier, 2013, Touliatos et al., 2016; Muller et al., 2017). Therefore, there is urgently the need for transformative solutions in food production. Vertical farming has been proposed as a way out in addressing the problem of farmland shortages because of its promises in maximizing small spaces to grow more crops and its sustainability to the environment (Corvalan et al., 2005; Despommier, 2014; Healy and Rosenberg, 2013; Thomaier et al., 2015) although, the effects it has on different insect pest complex is still not fully studied.

Herein, we examined how outdoor vertical farming might be used to increase yields and also in the sustainable management of insect pests by investigating the fruit crop tomato which easily adapts to this technique. Tomato, having lots of culinary and nutritional benefits is attacked by different pests a major of which are the whiteflies (Varela et al., 2003; Waiganjo et al., 2006). Whiteflies are highly polyphagous and are also known vectors of the tomato yellow leaf curl viruses (TYLCV) (Scholthof et al., 2011). The whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) larvae produces honeydew on which sooty moulds grows, reducing the photosynthetic capabilities of the plant and resulting in defoliation and stunting (European and Mediterranean Plant Protection Organization, 2004). The primary method used to control the insect pest is by the application of insecticides which unfortunately being practiced mainly in traditional horizontal farming has led to the development of resistance to numerous types of insecticides, reduction of beneficial arthropods and causing negative impacts on human health and the environment at large (Denholm et al., 1998; Matthews, 2008).

Natural enemies are helpful in curtailing the destructiveness of the insect pest with previous reports on predators such as wasps, lacewings, mites and also spiders effective in bringing whitefly population down (Gerling, 2001). Although, with limited information on its application and implication in vertical farming approach (Roberts et al., 2020). It is also not clear if the technique of vertically growing crops outdoors could be useful in checking the population of *B. tabaci* by natural intervention of predators as observed in other farming practices. Therefore, the current study is an attempt to answer the following questions: Can outdoor vertical farms increase the yields of crops?, and, would it be sustainable enough to support natural suppression of key insect pests?

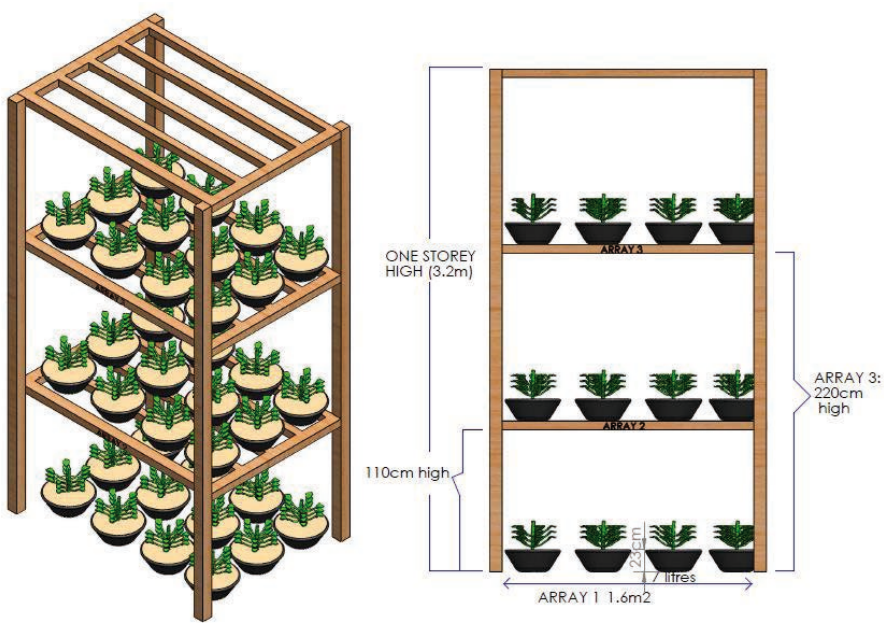

Fig. 1: Schematic diagram of the open-air vertical structure used for this experiment

2 MATERIALS AND METHODS

2.1 STUDY SITE

The study was conducted at the Department of Agronomy, Faculty of Agriculture, University of Ilorin (latitude 8°29'9N and longitude 4°35'38E), Kwara State. This area is located in the Southern Guinea Savannah Ecological Zone of Nigeria. The area was characterized by a bimodal rainfall with peaks in June and September and an annual rainfall between 1000-1240 mm. The study was carried out from December 2016 to March 2017.

2.2 ARRANGEMENTS FOR THE HORIZONTAL AND VERTICAL FARMS

The flatbeds were made in a size of 150 cm × 160 cm (2.4 m^2) each with an inter-bed spacing of one metre (1 m). The plot spacing for each raised bed was 240 × 150 cm (3.6 m^2) with an inter-bed spacing of one metre (1 m). The vertical farm was built using open-air vertical structures (Fig. 1) 3.2 m high using 8 cm wide and 5 cm thick wood (Garg and Balodi, 2014). It was built in a spacing of 120 × 150 cm (1.8 m^2) with an inter vertical farm spacing of 1 m apart.

2.3 TREATMENTS AND EXPERIMENTAL DESIGN

The experiment was laid out in a randomized complete block design with four replicates. The horizontal farm was made into two types of beds; raised beds and flatbeds, these served as the treatments in the horizontal farm. While the vertical farm was made having three arrays; with Array 1 at ground level (GL), Array 2 at 110 cm high and Array 3 at 220 cm high and these served as treatments in the vertical farm.

2.4 LAND PREPARATION AND PLANTING ON THE HORIZONTAL AND VERTICAL FARMS

The raised and flatbeds in the horizontal farm were prepared on farmland previously cultivated for tomato having sandy loam soil that was well drained. The tomato variety used for this study was UC82B packaged and supplied by the trademark company Technisem. Tomato seedlings were grown in the nursery in a screen house and were transplanted 4 weeks after sowing on the horizontal farm at a spacing of 50 × 80 cm on both beds according to the manufacturer's spacing instruction, with each bed containing a total of twelve tomato seedlings and 36 seedlings for the three replicates per bed type. In the vertical farm, tomato seedlings were transplanted from the nursery into 7 litre buckets with a diameter of 25 cm and a height of 23 cm filled with sandy loam soil with also history of tomato cultivation. Each vertical array contained twelve (12) buckets with a total of thirty-six (36) buckets per vertical farm and 108 buckets for the three replicates. Tomato seedlings that were introduced to the third array of a growing height of 220 cm high were gradually introduced to this height from ground level to 110 cm high and finally to 220 cm high within four days interval.

At 2 weeks after transplanting (WAT), tomato seedlings were lightly pruned by cutting off a few branches to encourage its growth and acclimation in both the vertical and horizontal farm. N.P.K (15-15-15) fertilizer was applied at the rate of 120 kg ha^{-1} 3WAT to boost the growth of the crops in both farms. The fertilizer was applied by ring placement into drills 5 cm deep and 7 cm away from the plant and covered with soil (Olaniyi et al., 2010). Watering of tomato was done daily in both the vertical and the horizontal farm at 8:00 am using a watering can.

2.5 COLLECTION AND IDENTIFICATION OF ASSOCIATED ARTHROPODS

Adult whiteflies were collected using aspirator and yellow sticky traps while a x10 magnifying lens was used for the observation of the presence of puparia or pupal cases underneath tomato leaves before taking leaf samples for viewing under a stereo microscope. All collected whitefly samples were identified to the species level on the basis of morphological characters of adults, puparium and/or pupal case (Simala et al., 2009).

The observed spiders in this experiment were not identified to species, genus, nor family level due to the lack of taxonomist specialized in arachnology in the country hence difficulties were experienced in speciating spiders.

2.5.1 Data collection

Nine (9) tomato plants were selected at random from each replicate from both the horizontal and vertical farm and data was collected for the number of adult whiteflies, predatory spiders and total fruit yield (g). The numbers of whiteflies were estimated by dividing the crop canopy into three layers: upper (> 40 cm),

intermediate (20-40 cm) and lower (0-20 cm) and selecting five leaves from each layer per plant which were gently turned over to the abaxial side to count the total number of adult whiteflies (Sequeira and Naranjo, 2008; López et al. 2010). This was performed from 7:30-9:30 am. Numbers of spiders were estimated by counting the total number of spiders seen per crop. This was carried out from 7-9:30 pm when spiders were observed to be very active and could be easily spotted with a flashlight. The number of *B. tabaci* on the tomato plants in both the vertical and horizontal farm were determined to have reached action threshold when above 5 of the insect were counted per leaf according to Ellsworth and Martinez-Carrillo (2001). Tomato fruits harvested from both farms at the end of the experiment were measured on a weighing scale calibrated in grams.

2.5.2 Data Analysis

Data was presented in mean and standard error of mean (SEM) and significant differences between means were separated according to Kruskal-Wallis one-way analysis of variance by allocating ranks to means. Spearman correlation analysis was done to determine the association between population of spiders, *B. tabaci* and fruit yield using SPSS 20th Edition.

3 RESULTS

3.1 *Bemisia tabaci* ON BOTH HORIZONTAL AND VERTICAL FARMS

Table 1 shows that the infestation of *B. tabaci* started at 1 week after transplanting (WAT) in both the horizontal and vertical farms with the flatbed having a significantly (H (4) = 11.500, p = 0.021) higher mean number of 12.81. There were no significant differences between the mean rank of *B. tabaci* in both the horizontal and vertical farms from 2 to 4 WAT. The horizontal farm, on the other hand, reached its peak population of *B. tabaci* at 5 WAT in the flat bed with a mean number of 14.69 (H (4) = 11.360, p = 0.023) and 9 WAT in the raised bed with a mean population of 13.35 (H (4) = 13.745, p = 0.008) significantly higher than the vertical farm (Table 1). Throughout the experiment, the horizontal farm experienced the most numbers of *B. tabaci* adults affecting tomato plants when compared to the vertical farm as seen in Table 1.

3.2 SUPPRESSION OF *Bemisia tabaci* BY SPIDERS

Table 2 shows that there were no significant differences between the mean rank numbers of the predatory spiders observed at 3 (H (4) = 6.222, p = 0.183) and 4 (H (4) = 8.038, p = 0.090) WAT in both the vertical and horizontal farms. Even though a significant population of predatory spiders was observed at 5 WAT (H (4) = 10.678, p = 0.030) in the vertical farm, it was not able to bring the population of *B. tabaci* below the action threshold (Tables 1 and 2).

Tables 1 and 2 also revealed that biological suppression by natural intervention of spiders (Fig. 2) was initially achieved only in the vertical farm at 6 WAT and started with the mean spider number of 13.00 in Array 3 significantly (H (4) = 12.616, p = 0.013) able to bring down the population of *B. tabaci* to the mean number of 0.00 (Table 1). This continued further as the number of spiders gradually increased in the vertical farm from 7 to 10 WAT and reached its peak at 10 WAT in array 3 with the mean number of 35.00 which was highly significantly (H (4) = 12.994, p = 0.011) effective in suppressing the population of *B. tabaci* below the action threshold when compared to the horizontal farm which was above it throughout the period of the experiment (Table 1 and 2), this was as a result of the

Table 1: Number of *Bemisia tabaci* on the vertical and horizontal farm

		Population of *B. tabaci* in horizontal and vertical farms (WAT)									
Farm Type		1	2	3	4	5	6	7	8	9	10
Horizontal	Raised bed	9.15^{d}	8.61^{c}	7.26^{b}	11.75^{e}	13.22^{d}	9.54^{d}	8.28^{b}	9.51^{c}	13.35^{c}	8.84^{b}
	Flat bed	12.81^{e}	9.75^{d}	10.64^{d}	10.51^{c}	14.69^{e}	11.84^{e}	9.12^{c}	7.04^{b}	9.38^{b}	10.80^{c}
Vertical	Array 1	8.72^{c}	7.75^{b}	13.79^{e}	6.54^{a}	7.64^{c}	4.99^{c}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	Array 2	6.52^{b}	7.68^{a}	6.96^{a}	8.87^{d}	7.38^{b}	3.73^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	Array 3	5.30^{a}	11.00^{e}	8.08^{c}	6.33^{b}	5.80^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
SEM		0.82	0.50	1.44	1.35	1.24	1.05	1.28	0.98	0.60	0.61

Superscripts within column indicates mean rank number according to Kruskal-Wallis Test, with a = rank 1, b = rank 2, c = rank 3, d = rank 4 and e = rank 5; 1 being the lowest to 5 the highest rank, SEM = Standard error of mean

Table 2: Number of predatory spiders on the vertical and horizontal farm

		Population of predatory spiders in horizontal and vertical farms (WAT)							
Farm Type		3	4	5	6	7	8	9	10
Horizontal	Raised bed	0.00[a]	0.33[a]	0.00[a]	0.67[a]	1.00[b]	1.00[b]	6.30[b]	0.00[a]
	Flat bed	0.00[a]	0.33[a]	0.33[b]	0.67[b]	1.70[a]	0.30[a]	4.30[a]	0.00[a]
Vertical	Array 1	0.00[a]	0.33[a]	9.33[e]	5.00[c]	15.00[c]	21.00[c]	23.70[e]	16.30[b]
	Array 2	0.67[c]	1.00[b]	7.67[c]	10.33[d]	20.30[d]	22.30[d]	21.70[d]	20.00[c]
	Array 3	0.33[b]	3.33[c]	8.67[d]	13.00[e]	34.00[e]	27.00[e]	21.00[c]	35.00[d]
SEM		0.19	0.73	1.25	1.10	4.02	2.49	3.61	2.31

Superscripts within column indicates mean rank number according to Kruskal-Wallis Test, with a = rank 1, b = rank 2, c = rank 3, d = rank 4 and e = rank 5; 1 being the lowest to 5 the highest rank, SEM = Standard error of mean

significantly low numbers of spiders recorded in the horizontal farm which were less than that of the vertical farm as shown in Table 2.

3.3 MASS OF TOMATO FRUIT YIELD IN HORIZONTAL AND VERTICAL FARM

The total tomato fruit yield (g) harvested from both the horizontal and vertical farms are shown in Table 3. There was no significant ($H(4) = 7.767$, $p = 0.101$) differences between the vertical and the horizontal farm. Further observations on the mean rank of the fruit mass indicated that array 3, array 2 and array 1 of the vertical farm had the highest tomato fruit mean mass of 67.10, 61.20 and 55.10 respectively when compared to the horizontal farm (Table 3).

Table 3: Mass of tomato fruits (g) in both horizontal and vertical farms

Farm Type		Mass of tomato fruit (g)
Horizontal	Flat bed	16.90[a]
	Raised bed	30.50[a]
Vertical	Array 1	55.10[a]
	Array 2	61.20[a]
	Array 3	67.10[a]
SEM		14.03

3.4 CORRELATION BETWEEN NUMBER OF *B. tabaci*, NUMBER OF SPIDERS AND FRUIT YIELD

Significantly negative correlation ($R_s = -0.999$) was observed between the number of spiders and the number of *B. tabaci* in the vertical farm as shown in Table 4. The horizontal farm, on the other hand, the numbers of spiders observed did not have a significantly negative correlation effectively enough to reduce the population of *B. tabaci* ($R_s = -0.318$) compared to the vertical farm.

Table 4 also showed that the fruit yield in both the horizontal and vertical farm was affected by the population of *B. tabaci* having a negative correlation of -0.28 and -0.813 respectively. However, the correlation of the number of spiders in the vertical farm with the fruit yield ($R_s = 0.806$) showed that the presence of the spiders positively influenced the fruit yield in the vertical farm compared to the horizontal farm which had a negative correlation of -0.256 in its fruit yield as seen in Table 4.

4 DISCUSSION

The current study is a novel approach in indicating the usefulness of outdoor vertical farming technique in sustainable crop production. Despommier as described by Corvalan et al. (2005) and Al-Kodmany (2018) hinted that vertical farming system will succeed only if they function by imitating natural ecological processes. The outdoor vertical farming system could be useful in supporting the practice of growing crops organically. The design allows for the natural use of sunlight and also encourages the natural interactions with ecological service providers as observed in this research. The experiment indicated that spiders acted as positive service providers in terms of natural suppression against whiteflies affecting tomato grown using open-air vertical farms. The technique of outdoor vertical farm unlike the indoor ultra-modernized versions allows for the interaction of plants with beneficial arthropods. Plants' evolutionary response to pest damage is to emit

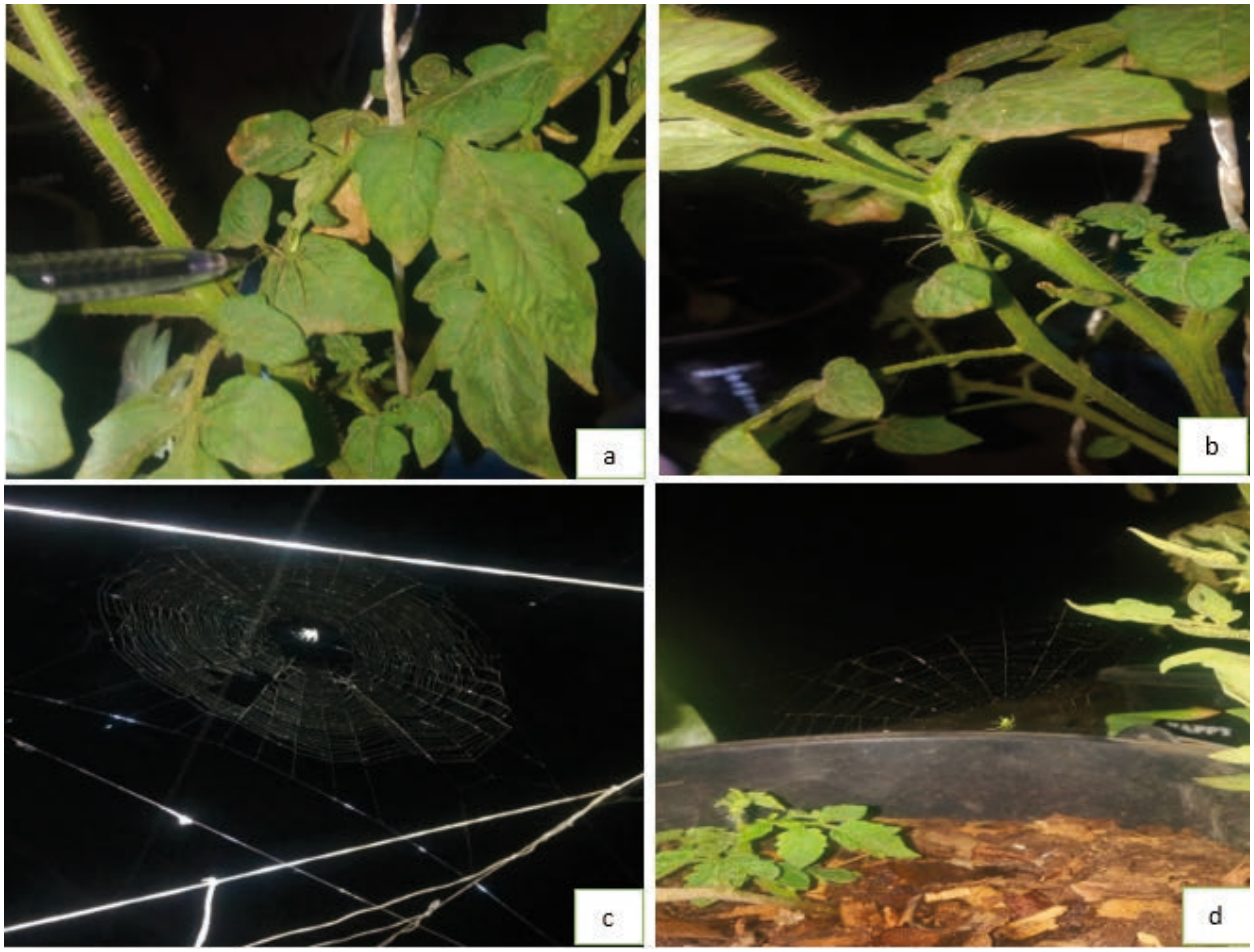

Figure 2: Showing presence of spiders in the vertical farm. Pictures a, b: green spiders observed using camouflage to hunt for preys; pictures c, d: two different species of spider on their web to capture prey

Table 4: Correlation between no. of *B. tabaci*, no. of spiders and fruit yield in the vertical and horizontal farm

		Horizontal farm		Vertical Farm	
		BTH	SPH	BTV	SPV
Horizontal farm	BTH	-	-	-	-
	SPH	-0.318	-	-0.788	-
	FYH	-0.28	-0.256	0.693	-0.699
Vertical farm	BTV	0.363	-	-	-
	SPV	-0.333	0.802	-0.999**	-
	FYV	-0.083	0.303	-0.813	0.806

*Correlation is significant at the 0.05 level (2 tailed), ** Correlation is significant at the 0.01 level (2-tailed)
BTH=*B. tabaci* horizontal farm, BTV= *B. tabaci* vertical farm, SPH= Spiders horizontal farm, SPV= Spiders vertical farm, FYH= Fruit yield horizontal farm, FYV= Fruit yield vertical farm, - = no correlation

a unique chemical signal known as herbivore induce plant volatiles- a distress signal to recruit the services of predatory arthropods who feed off these pest (Karban and Baldwin, 1997; Thaler, 1999; Kessler and Baldwin, 2001; Lou et al., 2006; Pickett et al., 2006). This study positively indicated that the ability of crops to emit this substance is not restricted using open vertical farming techniques. The vertical farm created a favourable niche

for the increase in the population of spiders by having adequate platforms where spiders could set up webs and could easily move around to reach and capture their prey within the varying heights (Jayakumar and Sankari 2010). Spiders, being a carnivorous arthropod, typically preying on insects, positively provided an important service in keeping the population of *B. tabaci* below the action threshold by natural intervention in the vertical farm in all three arrays (Marshall, 2006; Oyeniyi and Oyeseyi, 2014).

Sahu et al. (1996) and Jayakumar and Sankari (2010) studied the predatory efficiency of spiders in the suppression of pests in some field crops. In their study, there was no comparison to different growing heights to the predatory potential of spiders as shown in this research that growing heights using vertical farm could also be resourceful in influencing the increase of spiders to control pests. Correlation analysis also revealed that the rise in the population of spiders has a strong effect in suppressing the population of *B. tabaci* in the vertical farm when compared to the horizontal farm. The increased population of spiders in the vertical farm limited the population of whiteflies (Jayakumar and Sankari, 2010). The practice of vertical farming is considered to promote sustainable agricultural practices more than that adopted by conventional farming method (horizontal farm), which refers to large scale, outdoor agriculture that embraces techniques that engage heavy irrigation, intensive tillage and excessive use of fertilizers, and pesticides (Despommier, 2007; Healy and Rosenberg, 2013).

The fruit yield data collected from both the vertical and horizontal farm indicated that even though the horizontal farm produced fruit yield that was not significantly different from the different arrays of the vertical farm, the same was not enough yield in comparison to the vertical farm that gave better fruit yield. The increased fruit yield harvested from the vertical farm may be due to the ability of the farm to grow crops in arrays in limited space by stacking crops above each other which is an advantage over the horizontal farming method that makes use of huge expanse of land (Garg and Balodi, 2014; Hossain et al., 2015). With a little utilized space of about 1.8 square metre in the vertical farm, more fruit yield was gotten when compared to the horizontal farm space of 3.6 square metre and this is indicative of the facts that this technique could be used to increase food yield where land is fast becoming a limited resource. Also, the different heights of the vertical arrays in an outdoor situation would ensure that elevated plants have greater and better access to ambient amount of sunlight which will positively affect the performance of the crop to produce more fruit (Garg and Balodi, 2014).

By vertically growing crops, it would not only mitigate the need for more land, it would also produce available growing space in the air where crops could be grown in arrays to get more yield as shown in this study (Sarkar and Majumder, 2015; Hossain et al., 2015; Despommier, 2009; Garg and Balodi, 2014). This method also ensured the maximum use of land for tomato production without wastage and could address the loss of cultivable land by utilizing the spaces around households by suspending crops vertically and may eliminate the need to create additional farmland and also help create a cleaner environment with the use of less crop protection products such as pesticides that contaminates the environment and by encouraging the activities of natural enemies like spiders against the activities of insect pests (Despommier, 2009; Hossain et al., 2015).

We would like to put some caution on the interpretation of our result. While we did not identify the species of spiders and also report direct consumption of whiteflies by them through molecular analysis of their gut contents, the increased presence of spiders in the vertical farm may have had a threatening effect on the whiteflies and as such reduced their numbers significantly. Similarly, Southon et al. (2019) in an experiment they conducted by studying biological control of predatory wasps against the insect pest fall armyworm, observed that the presence of wasps negatively affected the feeding habit of fall armyworm, reduced their body mass and also kept them in hiding. It is plausible to infer here that whiteflies may have noticed the increasing population of spiders in the vertical farm and as such felt threatened and would rather derived nutrition elsewhere far from the presence of predators.

5 CONCLUSION

This study observed the use of open vertical farming in increasing the presence of predatory arthropods such as spiders in the natural suppression of *B. tabaci*, a major pest of tomato and also to increase yield. Since vertical farming is fast becoming an acceptable trend worldwide due to the overwhelming population increase, the technique could be practiced to produce crops in tight spaces to boost yield to feed the growing populace. Horizontal farming, on the other hand, is just not sufficient enough to meet the needs of this ever-increasing population due to the rapid rate of urbanization. Outdoor vertical farming in comparison to the traditional horizontal technique indicated that natural suppression by ecological service providers

could be plausible on crops grown using the technique. Although this is just a pilot trial, further investigations are necessary to ascertain the level of effectiveness open vertical farms will pose in the future to ensure continuous sustainable production of food as an alternative to the dwindling agricultural land resources. The practice could be encouraged to minimizing the dependency on chemical pesticides which have been studied to have deleterious effects. Also, the presence of predatory arthropods could be further influenced for future integrated pest management in open vertical farms. There is, therefore, the need to begin considering this technique as an urban approach to the lack of cultivable lands for food production.

6 CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 REFERENCES

Al-Kodmany, K. (2018). The vertical farm: a review of developments and implications for the vertical city. *Buildings*, *8*(24), 2-36. https://doi.org/10.3390/buildings8020024

Corvalan, C., Hales, S. & McMichael, A. J., (2005). *Ecosystems and human well-being: Health synthesis;* World Health Organization: Geneva, Switzerland.

Denholm, L., Cahill, M., Byrne, F. J. & Devonshire, A.L. (1996). Progress with documenting and combating insecticide résistance in *Bemisia*. In: Gerling, D. and Mayer, R.T., (eds.), *Bemisia 1995: Taxonomy, Biology, Damage, Control and Management,* Intercept Ltd. Andover, Hants. UK, pp. 577-603.

Denholm, L., Cahill, M., Dennehy, T. J. & Horowitz, A. R. (1998). Challenges with managing insecticide résistance in agricultural pests, exemplified by the whitefly *Bemisia tabaci*. *Philosophical Transactions of the Royal Society* (Lond. B), *353*:1757-1767. https://doi.org/10.1098/rstb.1998.0328

Despommier, D. (2007). The vertical farm essay 1: Reducing the impact of agriculture on ecosystem function and services. Retrieved November 8, 2011, from http://www.verticalfarm.com/more?essay1

Despommier, D. (2009). The rise of vertical farms. *Scientific American*, *301*, 80-87. https://doi.org/10.1038/scientificamerican1109-80

Despommier, D. (2010). *The Vertical Farm: Feeding the World in the 21st Century*; Thomas Dunne Books: New York, NY, USA.

Despommier, D. (2013). Farming up the city: The rise of urban vertical farms. *Trends in Biotechnology*, *31*, 388–389. https://doi.org/10.1016/j.tibtech.2013.03.008

Despommier, D. (2014). *Encyclopedia of Food and Agricultural Ethics (Vertical Farms in Horticulture)*; Springer: Dordrecht, The Netherlands. https://doi.org/10.1007/978-94-007-0929-4_88

Ellsworth, P. C. & Martinez-Carrillo, J. L. (2001). IPM for *Bemisia tabaci*: a case study from North America. *Crop Protection*, *20*, 853-869. https://doi.org/10.1016/S0261-2194(01)00116-8

European and Mediterranean Plant Protection Organization (2004). Diagnostic protocols for regulated pests. *Bulletin*, *34,* 281–288. https://doi.org/10.1111/j.1365-2338.2004.00729.x

FAO, 2018. Food and Agriculture Organization of the United Nations: FAOSTAT. http://www.fao.org/faostat/en/#data/EL (last access: 9 March 2018).

Garg, A. & Balodi, R. (2014). Recent Trends in Agriculture: Vertical Farming and Organic Farming. *Advances in Plants & Agriculture Research*, *1*(4), 142-144. https://doi.org/10.15406/apar.2014.01.00023

Gerling, D., Alomar, O. & Arnô, J., (2001). Biological control of *Bemisia tabaci* using predators and parasitoids. In Naranjo, S. E. and Ellsworth, P. C. (eds.), Spécial Issue: Challenges and Opportunities for Pest Management of *Bemisia tabaci* in the New Century. *Crop Protection*, *20***,** 779-799. https://doi.org/10.1016/S0261-2194(01)00111-9

Healy, R. G. & Rosenberg, J. S. (2013). *Land Use and the States*; Routledge: New York, NY, USA. https://doi.org/10.4324/9781315064406

Hossain, E., Nabi, S. N. & Kaminski, A. (2015). Vertical agriculture: Homestead horticulture suspended in sacks. Penang, Malaysia: WorldFish, Program Brief

Jayakumar, S. & Sankari, A. (2010). Spider population and their predatory efficiency in different rice establishment techniques in Aduthurai, Tamil Nadu. *Journal of Biopesticides*, *3*, 020-027.

Karban, R. & Baldwin, I. T. (1997). *Induced Responses to Herbivory*. Chicago University Press, Chicago, Illinois, USA. https://doi.org/10.7208/chicago/9780226424972.001.0001

Kessler, A. & Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, *291*, 2141–2144. https://doi.org/10.1126/science.291.5511.2141

López, R., Carmona, D., Vincini, A. M., Monterubbianesi, G. & Caldiz, D. (2010). Population dynamics and damage caused by the leafminer *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) on seven potato processing varieties grown in temperate environment. *Neotropical Entomology*, *39*(1), 108-114. https://doi.org/10.1590/S1519-566X2010000100015

Lou, Y., Xiaoyan, H., Turlings, T. C. J., Cheng, J., Xuexin, C. & Gongyin, Y. (2006). Differences in induced volatile emissions among rice varieties result in differential attraction and parasitism of *Nilaparvata lugens* eggs by the parasitoid, *Anagrus nilaparvatae* in the field. *Journal of Chemical Ecology*, *32*, 2375–2387. https://doi.org/10.1007/s10886-006-9151-7

Marshall, S. A. (2006). *Insect: Their Natural History and Diversity*. Firefly Books

Matthews, G. A. (2008). Attitudes and behaviours regard-

ing use of crop protection products—A survey of more than 8500 smallholders in 26 countries. *Journal of Crop Protection*, *27*, 834-846. https://doi.org/10.1016/j.cropro.2007.10.013

Muller, A., Ferré, M., Engel, S., Gattinger, A., Holzkämper, A., Huber, R., Müller, M. & Six, J. (2017). Can soil-less crop production be a sustainable option for soil conservation and future agriculture? *Land Use Policy*, *69*, 102–105. https://doi.org/10.1016/j.landusepol.2017.09.014

Olaniyi, J. O., Akanbi, W. B., Adejumo, T. A. & Akande, O. G., (2010). Growth, fruit yield and nutritional quality of tomato varieties. *African Journal of Food Science*, *4*(6), 398–402.

Oyeniyi, A. O. & Oyeseyi, J. O. (2014). Diversity and distribution of spiders in southwestern Nigeria. *Natural Resources*, *5*, 926-935. https://doi.org/10.4236/nr.2014.515079

Pickett, J. A., Bruce, T. J. A., Chamberlain, K., Hassanali, A., Khan, Z. R., Matthes, M. C., Napier, J. A., Smart, L. E., Wadhams, L. J. & Woodcock, C. M. (2006). Plant volatiles yielding new ways to exploit plant defence. In: Dicke, M., Takken, W. (Eds.), *Chemical Ecology: From Gene to Ecosystem*. Springer, Netherlands, pp. 161–173. https://doi.org/10.1007/978-1-4020-5369-6_11

Roberts, J. M., Bruce, T. J. A., Monaghan, J. M., Pope, T. W., Leather, S. R. & Beacham, A. M. (2020). Vertical farming systems bring new consideration for pest and disease management. *Annals of Applied biology*, *176*, 226-232. https://doi.org/10.1111/aab.12587

Sahu S., Sing, R. & Kumar, P. (1996). Host preference and feeding potential of spiders predaceous in insect pests of rice. *Journal of Entomological Research*, *20*(2), 145-150.

Sarkar, A. & Majumder, M. (2015). Opportunities and challenges in sustainability of vertical eco farming: A review. *Journal of Advanced Agricultural Technologies*, *2*, 98-105. https://doi.org/10.12720/joaat.2.2.98-105

Scholthof, K. B. G., Adkins, S., Czosnek, H., Palukaitis, P. & Jacquot, E. (2011). Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology*, *12,* 938–954. https://doi.org/10.1111/j.1364-3703.2011.00752.x

Sequeira, R. V., Naranjo & S. E. (2008). Sampling and management of *Bemisia tabaci* (Genn.) biotype B in Australian cotton. *Crop Protection*, *27*, 1262–1268. https://doi.org/10.1016/j.cropro.2008.04.002

Šimala, M., Milek, T. M. & Korić, B. (2009). Whitefly species (Hemiptera: Aleyrodidae) recorded on imported ornamental plants in croatia from 2005–2008. *Institute for plant protection in agriculture and forestry of Republic of Croatia*, 389-396.

Southon, R. J, Fernandes, O. A., Nascimento, F. S. & Sumner, S. (2019). Social wasps are effective biocontrol agents of key lepidopteran crop pests. *Proceedings of the Royal Society B. Soc. B.*, 286, 20191676. https://doi.org/10.1098/rspb.2019.1676

Thaler, J. (1999). Jasmonic acid mediated interactions between plants, herbivores, parasitoids and pathogens: a review of field experiments in tomato. In: Agrawal, A. A., Tuzun, S., Bent, E. (Eds.), *Induced Plant Defenses Against Pathogens and Herbivores* (pp.319-334). APS Press, St. Paul, Minnesota.

Thomaier, S, Specht, K, Henckel, D, Dierich, A, Siebert, R, Freisinger, U. B & Sawicka, M. (2015) Farming in and on urban buildings: Present practice and specific novelties of zero-acreage farming (Z Farming). *Renewable Agriculture and Food Systems*, *30*, 43–54. https://doi.org/10.1017/S1742170514000143

Touliatos, D., Dodd, I. C. & McAinsh, M. (2016). Vertical farming increases lettuce yield per unit area compared to conventional horizontal hydroponics. *Food and Energy Security*, *5*, 184–191. https://doi.org/10.1002/fes3.83

Varela, A. M., Serf, A. & Lohr, B. (2003). *A guide to IPM in tomato production in eastern and southern Africa.* International Centre of Insect Physiology and Ecology

Waiganjo, M. M., Wabule, N. M., Nyongesa, D., Kibaki, J. M., Onyango, I., Wepukhulu, S. B. & Muthoka, N. M. (2006). *Tomato production in Kirinyaga district*, Kenya. A baseline Survey report. 3-4.

doi:10.14720/aas.2022.118.2.2417 Original research article / izvirni znanstveni članek

Comprehensive seed priming assessment of *Hibiscus sabdariffa* L. in germination and seedling growth stage under salt stress

Mostafa AHMADIZADEH [1, 2], Ashkan ASGARI [1, 3], Hossein PASALARI [1]

Received November 10, 2021; accepted March 22, 2022.
Delo je prispelo 10. novembra 2021, sprejeto 22. marca 2022

Comprehensive seed priming assessment of *Hibiscus sabdariffa* L. in germination and seedling growth stage under salt stress

Abstract: This study was performed to appraise the effects of several seed pretreatment solutions and priming time on seed germination indices and growth characteristics of *Hibiscus sabdariffa* L. in various salt stress levels. Seed priming was accomplished by KCl (1 and 2 %), Na_2SO_3 (0.5 and 1 %), KNO_3 (0.5 and 1 %), and Ca_2CO_3 (1 and 2 %) as halopriming and distilled water as hydropriming at 12 and 24 h priming durations and control (non-primed), then primed seeds exposed to four levels (0, 50, 100, 200 mM) of NaCl solutions. The highest germination percentage was observed in 12 and 24 h hydropriming (63.3 and 53.3 %) and non-primed (56.6 %) under normal condition, respectively. Besides, there was no germinated seed at 24 h priming by 0.5 and 1 percentage of KNO_3. Under saline condition, 24 h 2 % Ca_2CO_3 had the highest germination percentage (43.3 %) in 50 mM, while 12 h treatment with 0.5 % Na_2SO_3 (33.3 %) had high germination percentage in 100 mM levels of saline conditions. Also, the highest germination rate index was observed in 0.5 % Na_2SO_3 with 12 h treatment time (4.05 and 3.95 respectively) in 50 and 100 mM levels of saline conditions. Overall, salt stress considerably reduced germination and growth traits of *Hibiscus sabdariffa* L. seedlings. Considering the effect of various seeds priming of *Hibiscus sabdariffa* L. on germination indices like germination percentage and mean germination time, the importance of priming duration and type of priming solutions could be concluded.

Key words: abiotic stress; medicinal plant; roselle; seed treatment

Ocena predobravnavanja semen vrste osleza *Hibiscus sabdariffa* L. v razvojnih stopnjah kalitve in kalice v razmerah solnega stresa

Izvleček: V raziskavi so bili ocenjeni učinki predobravnavanja semen z različnimi raztopinami in časi obravnavanja na kalitveni indeks in rastne lastnosti vrste *Hibiscus sabdariffa* L. v razmerah različnega solnega stresa. Predobravnavanje semen je bilo izvedeno z raztopinami KCl (1 in 2 %), Na_2SO_3 (0,5 in 1 %), KNO_3 (0,5 in 1 %), in Ca_2CO_3 (1 in 2 %) kot obravnavanje s solmi in z destilirano vodo kot vodno obravnavanje za 12 in 24 h ter kontrolo (brez predobravnavanja). Po tem so bila ta semena izpostavljena raztopinam štirih koncentracij natrijevega klorida (0, 50, 100, 200 mM NaCl). Največji odstotek kalitve je bil ugotovljen pri semenih, ki so bila predobravnavana z vodo za 12 in 24 ur (63,3 in 53,3 %) in pri netretiranih semenih (56,6 %) v normalnih razmerah. Pri predobravanavanju semen za 24 ur z 0,5 in 1 % raztopino KNO_3 ni vzklilo nobeno seme. V razmerah slanosti je imelo 24 urno obravnavanje z 2 % raztopino Ca_2CO_3 največji odstotek kalitve (43,3 %) pri 50 mM med tem, ko je imelo12 urno obravnavanje z 0,5 % raztopino Na_2SO_3 (33,3 %) še vedno velik odstotek kalitve v razmerah 100 mM slanosti. Največja vrednost indeksa kalitve je bila ugotovljena pri obravnavanju z 0,5 % raztopino Na_2SO_3, z 12 urnim časom obravnavanja (4,05 in 3,95) v razmerah 50 in 100 mM slanosti. Nasplošno je solni stres znatno zmanjšal kalitev in rastne parameter sejank osleza *Hibiscus sabdariffa* L.. Upoštevaje učinke različnih predobravnavanj semen osleza *Hibiscus sabdariffa* L. na kalitvena indeksa kot sta odstotek kalitve in poprečni čas kalitve je potrebno pri tem posebej upoštevati pomen časa obravnavanja in vrsto raztopine za obravnavanje.

Ključne besede: abiotski stres; zdravilna rastlina; *Hibiscus sabdariffa* L.; obravnava semen

1 Areas, Minab Higher Education Center, University of Hormozgan, Bandar Abbas, Iran; Research Group of Agroecology in Dryland
2 Corresponding author, e-mail: ahmadizadeh.mostafa@gmail.com
3 Corresponding author, e-mail: asgariashkan6@gmail.com

1 INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a member of the Malvaceae family (Shruthi et al., 2018), which originally belonged to Malaysia and India (Mahadevan et al., 2009), and cultivated in tropical and subtropical climates (Da-Costa-Rocha et al., 2014). It is perennial or annual sub-shrub or woody-based herb, and widely grown in subtropical and tropical zones (Ibrahim et al., 2013). These plant species have played a key role in people's living because they provide humanity's needs that are food, clothes, shelter, and medicines (Riaz & Chopra, 2018)polysaccharides and organic acids thus having enormous prospective in modern therapeutic uses. The study aimed to review and document all the available evidence and information about the calyces of Hibiscus sabdariffa (roselle. Roselle is used in traditional medicine, due to overfill in phytochemicals like polyphenols, particularly anthocyanins, polysaccharides, and organic acids; hence, it has significant potential in modern medicinal applications (Riaz & Chopra, 2018; Sukkhaeng et al., 2018). It is traditionally cultivated owing to the usage of calyces, stems, leaves, and seeds as all organs have pharmacological and other uses (Wright et al., 2007). Calyx products are applied in indigenous medicine to treat high blood pressure, liver diseases and fever (Ali et al., 2005). Roselle extracts are increasingly developed for medications, food, and cosmetics (Farnsworth & Bunyapraphatsara, 1992).

The presence of salt in the water or soil is considerable challenge for plant production in the world. It is most prevalent in dryland and coastal areas. Due to unsuitable irrigation and drainage management, limited rain, high evaporation, and saline irrigation water, salt concentration in the soil and water is increasing inland (Ibrahim, 2016). This problem takes about 3.7 million acres of the area of food production every year (Munns & Tester, 2008). Therefore, half of the cultivation area will be lost by the Mid-21st century (Wang et al., 2003). Salt stress became a limitation factor to the production of the crops, and the majority of crops are extremely sensitive to saline soil and water (Lin et al., 2017; Ahmadizadeh et al., 2016). Seed germination and seedling growth are the susceptible stages to abiotic stress, and abiotic stress can be slowed or stopped the germination of seeds (Ahmadizadeh et al., 2011; Galal, 2017). Rouhi et al. (2011), Ahmadizadeh et al. (2011), Ansari et al. (2013), and Ebrahimi et al. (2014) stated that raising the stress had a negative impact on the germination rate.

In the past decade, several strategies have been applied to improve abiotic stress tolerance in crops. There are various methods to enhance crop growth and development in salt-affected conditions (Hussain et al., 2016; Feghhenabi et al., 2020). One of the appropriate methods is pretreatment, like prime the seeds with various materials before sowing (Ali et al., 2017; Subramanyam et al., 2019). Seed pretreatment as a practical, cost-effective, and low-risk enhancing germination of seed and seedling growth through pre-germinating metabolic processes improvement (Jime'nez-Arias et al., 2015; Migahid et al., 2019). Priming of seed is moderate stress, which activates a stress-reaction mechanism (Bhanuprakash & Yogeesha, 2016). Priming of seed is a physiological method of seed hydration and drying to ameliorate the pre-germinate metabolism under stressed conditions. The primed seeds exhibit quicker and normal seed germination (Hasanuzzaman & Fotopoulos, 2019), and seed priming adjusts the biochemical and physiological of the embryo. Priming also decreases the seeds sensitivity to unfavorable conditions (Afzal et al., 2016).

Several researchers have indicated that seed priming enhances the well establishment and growth of plants (Farooq et al., 2010; Kerchev et al., 2020; Feghhenabi et al., 2020). The beneficial impacts of seed priming in saline conditions have been shown in several crops for instance, pepper (Khan et al., 2009), okra (Dkhil et al., 2014), tomato (Ebrahimi et al., 2014), rosella (Galal, 2017), and *Silybum marianum* (L.) Gaertn. (Migahid et al., 2019). Latef et al. (2020) studied the impact of priming with Al_2O_3 nanoparticles on the growth of roselle, and the results showed that Al_2O_3 nanoparticles influenced growth traits, like dry mass, fresh mass, root, and shoot length. Shruthi et al. (2018) concluded pretreatment with GA_3, KNO_3, and hot water to study the influence of seed priming on germination of Roselle (*Hibiscus sabdariffa* L.), they indicated the positive impact of seed pretreatment on the properties of germination speed and germination percentage. Nassar (2010) reported the positive results of seed priming and organic fertilizer on the yield and quality of roselle. Sheyhakinia et al. (2020) showed ameliorate of salt stress tolerance by jasmonic acid in roselle. Their results showed that jasmonic acid protected roselle seedlings against salinity damage. Germination indices and seedling traits of two tomato cultivars are influenced by the great potential value of seed treatment with $CaCl_2$ and KNO_3 solution under salinity conditions. In contrast, enhanced salinity concentrations led to a significant reduction in germination indices and seedlings growth (Ebrahimi et al., 2014).

Soil salinity is one of the principal widespread abiotic stresses, which has adverse effects on crop production (Ismail et al., 2007; Ahmadizadeh et al., 2021). Appropriate seed germination is a prerequisite for the successful stand establishment of plants in unfavorable

environments such as low moisture, saline water, and soil, which are limiting germination factors (Ahmadizadeh, 2013). The fast and uniform germination and seedling establishment are influential factors for plant performance. There are several priming techniques, which are helpful for successful stand establishment of plants in abiotic stress conditions. Therefore, the study aimed to investigate the effects of various priming including KCl, Na_2SO_3, KNO_3, and Ca_2CO_3 and hydropriming of seeds on germination at different priming durations under normal and various levels of salinity conditions.

2 MATERIAL AND METHODS

In order to assess the effects of various seed priming compounds and priming durations on seed germination indices and growth characteristics of *Hibiscus sabdariffa* L. seedlings in various levels of salt stress conditions were studied at the agricultural laboratory of Minab higher education center, university of Hormozgan. An experiment was conducted in a factorial experiment based on a completely randomized design with three replications. Priming treatments consisted of halopriming with KNO_3 (0.5 and 1 %), Na_2SO_3 (0.5 and 1 %), KCl (1 and 2 %) and Ca_2CO_3 (1 and 2 %), and hydropriming with distilled water and control (non-primed), priming durations was 12 and 24 h, then seeds exposed to four levels (0, 50, 100, 200 mM) of NaCl solution. For any treatments, disinfected seeds were immersed in 50 ml of the prepared solution for 12 and 24 h in covered glass containers to preserve evaporation loss. The seeds were then rinsed with distilled water several times, afterward dried back at room temperature (25 °C) for 24 h to be dried (Ebrahimi et al., 2014; Ibrahim, 2016; Aghdaei et al., 2019).

Fifteen healthy primed seeds of roselle were placed in petri dishes on two layers of filter paper, then 8 ml of the salinity solutions (0, 50, 100, and 200 mM NaCl). To germinate the seeds, the petri dishes were put in an incubator at 26 ± 1°C. Germination of *Hibiscus sabdariffa* L. the seeds were counted as a germinated seed once they displayed extension of radicle almost 2 mm. The germination count was recorded every 24 h up to 7 days. At the end of the first week, and germination percentage (*GP*), germination rate index (*GRI*), mean germination time (*MGT*), germination index (*GI*), and vigor index (*VI*) were calculated based on the following equations (Al-Mudaris, 1998):

$$GP = (N/M) * 100 \quad (1)$$

where *GP* is germination percentage, *N* is the total number of germinated seeds at the end of seven days, and *M* is the total number of cultivated seeds.

$$GRI = (G1/1) + (G2/2) + \ldots + (Gx/x) \quad (2)$$

G1 = germination percent in first day. *G2* = germination percent in second day to final experiment,

$$MGT = (fx) / f \quad (3)$$

where *f* is the number of newly germinated seeds on each day and *x* is the day of counting,

$$GI = (7 * N_1) + (6 * N_2) + (5 * N_3) + \ldots \quad (4)$$

N1, N2, . . . = the number of germinated seeds in first day, second day and . . .,

$$VI = GP \times \text{Seedling length } (SL) \quad (5)$$

(El-ouaer & Hannachi, 2012),

Also, fresh shoot mass, fresh root mass, shoot length, root length, dry shoot mass and dry root mass were measured. Mass of root and shoot were measured from the sample mass before and after drying at 70 °C for 12 h. the data were analyzed using SAS software, and means comparisons were done by the least significant difference test (LSD) at $p < 0.05$ level of confidence. The Excel software was used to draw figures.

3 RESULT AND DISCUSSION

Seed germination and early establishment of seedling are the crucial stages for the crops, and these two stages are the delicate growth stages in unfavorable environments (Begcy et al., 2018). Seed germination is sometimes prevented or delayed under different abiotic stresses (Fazlali et al., 2013; Muhie et al., 2020a,b). Roselle is sensitive to germination and early seedling development in saline conditions (Bahaeldeen et al., 2012; Al-Tohafi et al., 2015; Kadamanda, 2019). Considering to the value and privilege of the *Hibiscus sabdariffa* products, particularly medicinal value, this study was conducted to evaluate the influence of various priming approaches in different NaCl concentrations on germination indices and seedling traits of roselle plants in petri dish at a controlled experimental environment. The analysis of variance showed a highly significant ($p < 0.01$) difference between various priming treatments in terms of germination indices and seedling growth traits (Table 1). Also, the result revealed a significant difference among the salinity levels. The priming × salinity interaction effect was significant for all studied traits (Table 1). High concentrations of salinity prevent and reduce the performance of most plants, but seed emergence is the utmost momentous process for well seed germination in medicinal plants (Nadjafi et al., 2006; Reed et al., 2022). The highest germination

Table 1: Analysis of variance for salinity and priming effects on some germination characteristics of *Hibiscus sabdariffa*

S.O.V	DF	Mean Square										
		GP	*GRI*	*MGT*	*GI*	*VI*	*ShL*	*RL*	*FShM*	*FRM*	*DShM*	*DRM*	*Sh/R*
Prim	18	861.39**	15.25**	4.78**	907.27**	49425.72**	14.93**	5.62**	0.02**	0.00044**	0.00033**	0.0000035**	202.13**
Salinity	3	12156.73**	235.69**	33.82**	884.45**	1001023**	213.34**	57.56**	0.34**	0.0048**	0.0034**	0.0000372**	1394.11**
P*S	54	197.69**	4.51**	1.19**	13323.17**	21367.41**	2.73**	1.33**	0.004**	0.00014**	0.000068**	0.00000077**	88.44**
Error	152	35.41	0.98	0.69	43.14	3152.86	0.50	0.18	0.001	0.00001	0.0000089	0.00000013	25.12

**: is significant at 1 %,
GP: Germination Percentage, GRI: Germination Rate Index, MGT: Mean Germination Time, GI: Germination Index, VI: Vigor Index, *ShL:* Shoot length, *RL:* Root Length, *FShM:* Fresh Shoot Mass, *FRM:* Fresh Root Mass, *DShM:* Dry Shoot Mass, *DRM:* Dry Root Mass, *Sh/R:* Shoot/Root ratio

rate and percentage of T. polium seeds were obtained at concentrations of 500-2500 ppm GA3. Washing and chilling (5°C. The highest germination percentage (*GP*) (36.93 %) was observed in normal condition, while under the salinity conditions the highest *GP* (23.24 %) was observed under 50 mM salinity condition (Table 2).

The decreasing in the percentage of germination may be associated with the increase of external osmotic pressure that has an impact on the water absorption of the seed, as well as, owing to the accumulation of some ions in the embryo, which may result in stimulation of the metabolic processes of germination and ultimately leading to cells death in the embryo (Maher et al., 2013; Feghhenabi et al., 2020). Afkari Bajehbaj (2010) and Shereiwy et al. (2021) demonstrated that enhancing salinity levels decreased final germination in seeds, but, the adverse impact of salinity on primed seeds was less than unprimed seeds. The highest germination rate index (*GRI*) (5.06) was observed in normal condition, while under the salinity conditions the highest *GRI* was observed under 50 mM salinity condition that had significantly different from normal condition (Table 2).

The highest *GI* was observed in normal condition, under the low level of salinity conditions the *GI* reduction was 46 percentage, and the highest *GI* in stress condition was observed under 50 mM salinity condition (Table 2). The highest *VI* was observed in normal condition, and there was significantly difference between the various levels of salinity stress (Table 2). The means comparison under different levels of salinity stress revealed the highest *MGT* under 50, and 100 mM salinity conditions (Table 2). In this respect, Kaveh et al. (2011), Thiam et al. (2013), and Ibrahim (2016) indicated that enhancing the concentration of salinity improved germination time and reduced the germination percentage. In general, low levels of salinity cause a dormancy and low impact on the germination rate, but ascending concentration of salt prevents the seed germination and reduces the percentage of germination (Khan & Weber, 2006; Shannon & Grieve, 1998).

There was a significant difference between control and salinity conditions in terms of shoot and root mass. The highest of shoot fresh mass (0.19 g), root fresh mass (0.023 g) and root dry mass (0.002 g) were observed at non-salinity condition (control) but the highest shoot dry mass (0.019) was achieved at salinity condition (Table 2), suggesting that root growth is more sensitive to salinity than shoot growth. Amiri et al. (2010) with the study of germination characteristics of *Cynara scolymus* L. and *Echinacea purpurea* (L.) Moench under salinity stress demonstrated that shoot dry mass was reduced by enhancing salt concentration in studied medicinal plants. The similar finding was reported by research-

Table 2: Mean comparison of salinity levels on germination indices and seedling growth traits in *Hibiscus sabdariffa*

Salinity *mM*	*GP* (%)	*GRI*	*MGT* (day)	*GI*	*VI*	*ShL* (cm)	*RL* (cm)	*FShM* (g)	*FRM* (g)	*DShM* (g)	*DRM* (g)	*Sh/R*
0	36.93 a	5.067 a	1.666 b	38.63 a	314.5 a	4.859 a	2.628 a	0.1900 a	0.0236 a	0.0167 b	0.00202a	8.350 c
50	23.24 b	2.297 b	2.367 a	20.84 b	136.7 b	3.363 b	1.601 b	0.1673 b	0.0161 b	0.0181 a	0.00157b	11.711 b
100	19.38 c	1.869 c	2.274 a	17.49 c	80.3 c	2.394 c	1.217 c	0.1273 c	0.0114 c	0.0190 a	0.00137c	14.611 a
200	1.49 d	0.161 d	0.693 c	1.35 d	2.84 d	0.243 d	0.196 d	0.0156 d	0.0015 d	0.0026 c	0.00013d	3.092 d
LSD	2.2024	0.3665	0.3092	2.4309	20.78	0.2642	0.161	0.0122	0.0012	0.0011	0.0001	1.8549

GP: Germination Percentage, *GRI*: Germination Rate Index, *MGT*: Mean Germination Time, *GI*: Germination Index, *VI*: Vigor Index, *ShL*: Shoot length, *RL*: Root Length, *FShMW*: Fresh Shoot Mass, *FRM*: Fresh Root Mass, *DShM*: Dry Shoot Mass, *DRM*: Dry Root Mass, *Sh/R*: Shoot/Root ratio

ers in other species consisting of roselle (Galal, 2017), safflower (Kaya et al., 2003; Khodadad, 2011), triticale (Atak et al., 2006), and tomato (Ebrahimi et al., 2014).

There are multiple pretreatment approaches applied and classification based on the priming compounds. These comprise halo-priming, hydro-priming, hormone priming, osmo-priming, solid matrix, hardening, stratification, and thermal shock and humidification. The hydropriming, osmo-priming, halopriming, and hormone priming techniques commonly had been used for seed treatment (Ashraf & Foolad, 2005; Eskandari, 2013; Paparella et al., 2015). Water potential, temperature, seed vigor, priming duration, seed primed storage condition, and plant species are the factors that influence the response of the seed to priming. Therefore, the optimization and fine-tuning of the priming approach is substantial to obtain the best outcome (Ratikanta & Kalipada, 2013).

Hydropriming affect some of the required metabolic processes for germination to happen without germination be accomplished, faster imbibition, further, softening of seed coat led to lesser mechanical prevention as a result of priming (Askari-Nejad & Farahmand, 2012). Seed pretreatment by inorganic salts enhances the enzymes activity engaged in the germination of seed and changes the mobilization of organic substances' to various embryo parts (Aghdaei et al., 2019). Priming for 12 h with 0.5 % Na_2SO_3, 12 and 24 h with 2 % Ca_2CO_3, 12 h with 1 % KCl, 12 h with 1 % Na_2SO_3 and various hydro-prim showed the highest *GP* (Table 3). The highest *GRI* was in 12 h priming with 0.5 % Na_2SO_3, 1 % Na_2So_3 and hydro-prim (Table 3). The *GRI* illustrate the percentage of germination on every day of the germination period. Higher *GRI* values display prompt and high germination (Fuller et al., 2012). Priming with 0.5 % Na_2SO_3 and 1 % Ca_2CO_3 in 12 h revealed the lowest median germination time (*MGT*) (Table 3). The lower *MGT* showed the faster germination of a seeds population (Fuller et al., 2012). The highest *GI* was in 12 h priming with 0.5 % Na_2SO_3, hydro-prim, and 24 h with 2 % Ca_2CO_3 (Table 3). Priming with 12 h 0.5 % Na_2SO_3, hydro-prim, and 24 h with 2 % Ca_2CO_3 showed the highest *VI* (Table 3). In contrast, 12 and 24 h priming with two percentage KCl had the lowest *GRI, GI, VI*, and *GP* (Table 3).

The highest shoot length was in 24 h priming with 2 % Ca_2CO_3 (4.19 cm), 1 % Ca_2CO_3 (3.91 cm), 1 % Na_2SO_3 (3.89 cm), and 12 h priming with 1 % KCl (3.74 cm), there were no statistically significant difference among these treatments. Priming with 24 h with 2 % Ca_2CO_3 showed the highest root length (Table 3). In terms of shoot mass at 24 h priming with 2 % Ca_2CO_3 (0.19 g), 1 % Ca_2CO_3 (0.18 g), and 1 % Na_2So_3 (0.17

g) had the highest shoot mass that there were no statistically significant difference among these treatments. Also, 24 h priming with 2 % Ca_2CO_3 root mass (Table 3). The highest dry shoot mass was in 24 h priming with 1 % Ca_2CO_3, and 2 % Ca_2CO_3, priming with 24 h 2 % Ca_2CO_3, hydro_prim, and 12 h with 2 % Ca_2CO_3 showed the highest dry root mass (Table 3). The positive effects of various priming approaches like priming the tomato seed by potassium nitrate on germination (Lara et al., 2014), hydropriming on sorghum and rice germination percentage (Farooq et al., 2006; Moradi & Younesi, 2009), and salicylic acid on *Solanum melongena* L. seed germination percentage (Mahesh et al., 2017), as well as, enhance of the shoot and root length of cotton (*Gossypium hirsutum* L.) in hydropriming (Shaheen et al., 2015) have been reported.

Plant cell turgor reduction and decrease of shoot and root length caused by salinity stress (Werner & Finkelstein, 1995). Also, it was suggested that salinity stress acts firstly on water uptake. Moreover, Na^+ and Cl^- accumulation prevent the metabolism of cells dividing and expanding (Neumann, 1997), less germination, and even resulting in seed or embryo death. In addition, salt stress leads prevent and decrease the enzymes activities that may be significantly associated with seed germination (Katembe et al., 1998). Priming approaches have been utilized for better germination of seeds in both normal and unfavorable environments (Jisha et al., 2012). The positive and affirmative effects of priming were observed in adverse condition than optimal conditions (Ashraf & Foolad, 2005; Chen & Arora, 2011; Ibrahim, 2016). Suggested priming mechanisms were consisting of the incidence of epigenetic alterations, also the transcription factors accumulation and inactive and inhibition of signaling proteins. These mechanisms are induced against the stress, hence improved resulting in a well and effective defense mechanism (Tanou et al., 2012). Some treatments and techniques were able to develop well establishment of crops in stressful conditions (Soeda et al., 2005; Patade et al., 2009).

The highest *GP* was in 0, 12, 24 hours hydropriming under normal condition. Also, 2 % Ca_2CO_3, 0.5 % Na_2So_3, and 1 % KCl in 12 hours treatment showed high GP under normal condition (Figure 1). enhancing the growth characteristics resulting from seed priming by water soaking could be owing to the impact of seed hydropriming on the fast and sound establishment of plants (Ashraf & Foolad, 2005). The lowest *GP* was observed in 0.5 % KNO_3 in 24 hours pretreatment in all studied conditions, 0.5 % KNO_3 in 12 hours pretreatment under 200 mM salinity condition, as well as, the same results were observed by 24 pretreatments of 1 %

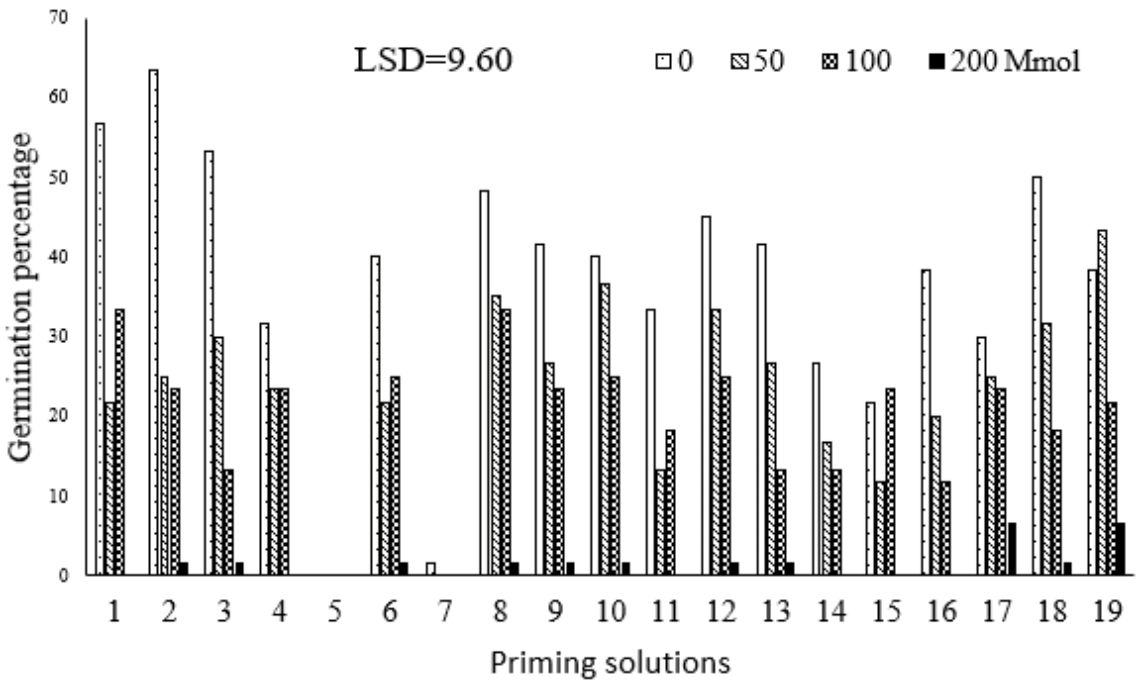

Figure 1: Effect of various priming under four level of salt stress on germination percentage of *Hibiscus sabdariffa* L.
1: non-primed, 2: Hydro 12h , 3: Hydro 24 h, 4: KNO_3_0.5 % 12 h , 5: KNO_3_0.5 % 24 h , 6: KNO_3_1 % 12 h, 7: KNO_3_1 % 24 h, 8: Na_2SO_3_0.5 % 12 h, 9: Na_2SO_3_0.5 % 24 h , 10: Na_2SO_3_1 % 12 h, 11: Na_2SO_3_1 % 24 h, 12: KCl_1 % 12 h, 13: KCl_1 % 24 h, 14: KCl_2 % 12 h , 15: KCl_2 % 24 h, 16: Ca_2CO_3_1 % 12 h, 17: Ca_2CO_3_1 % 24 h, 18: Ca_2CO_3_2 % 12 h , 19: Ca_2CO_3_2 % 24 h

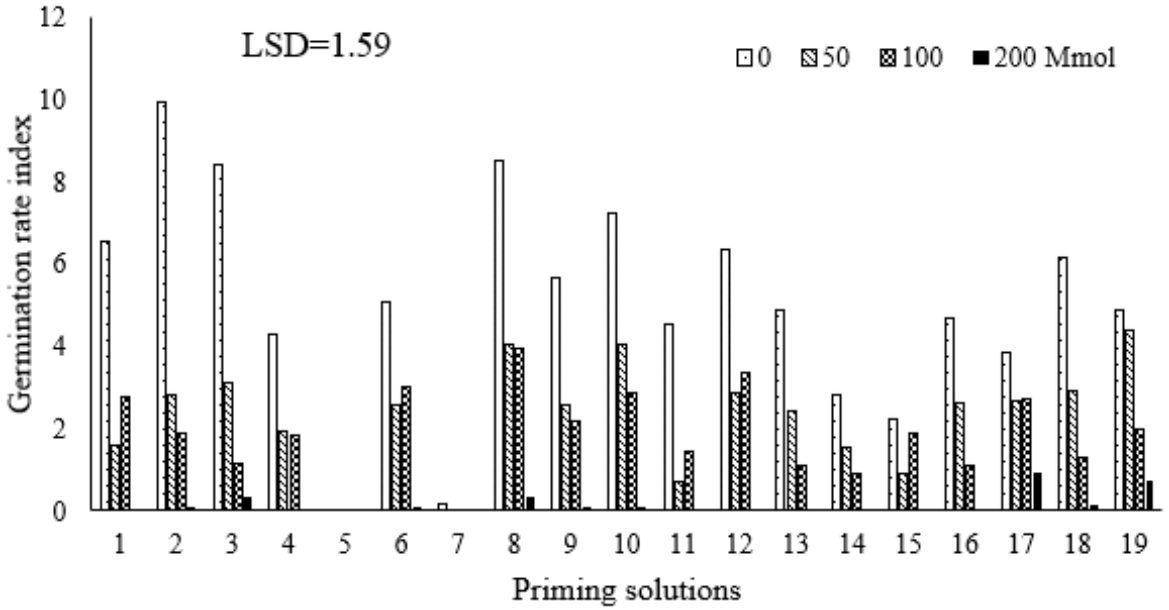

Figure 2: Effect of various priming under four level of salt stress on germination rate index of *Hibiscus sabdariffa* L.
1: non-primed, 2: Hydro 12h , 3: Hydro 24 h, 4: KNO_3_0.5 % 12 h , 5: KNO_3_0.5 % 24 h , 6: KNO_3_1 % 12 h, 7: KNO_3_1 % 24 h , 8: Na_2SO_3_0.5 % 12 h, 9: Na_2SO_3_0.5 % 24 h , 10: Na_2SO_3_1 % 12 h, 11: Na_2SO_3_1 % 24 h, 12: KCl_1 % 12 h, 13: KCl_1 % 24 h , 14: KCl_2 % 12 h , 15: KCl_2 % 24 h, 16: Ca_2CO_3_1 % 12 h, 17: Ca_2CO_3_1 % 24 h, 18: Ca_2CO_3_2 % 12 h , 19: Ca_2CO_3_2 % 24 h

Na_2SO_3, and 2 % KCl under 200 mM salinity condition, and 1 % Ca_2CO_3 in 12 hours pretreatment under 200 mM salinity condition (Figure 1). In saline conditions, 2 % Ca_2CO_3, 0.1 % Na_2SO_3 under 50 mM salinity, and 0.1 % Na_2SO_3 in 100 mM salinity showed the high *GP*. However, there was significantly different with the control condition (Figure 1). The result implied that the best treatments in terms of *GP* were 12 h Hydro, 12 h Na_2SO_3_0.5 %, 24 h Ca_2CO_3_1 %, 24 h Ca_2CO_3_2 % in control, 100 mM salinity, 200 mM salinity, and 50 mM salinity conditions, respectively (Figure 1).

Hydropriming (12 and 24 h), 0.5 % Na_2SO_3, and 1 % Na_2SO_3 in 12 h had the highest *GRI* in the normal condition. In saline conditions, 12 h seed priming by 0.5 % Na_2SO_3 under 50 mM salt stress condition and 12 h priming with 1 % KCl in 200 mM salt stress condition, while there were zero *GRI* in 24 h pretreatment of seeds with 0.5 % KNO_3 and 1 % KNO_3 under salinity conditions, as well as, 1 % Na_2SO_3, 1 % Ca_2CO_3 and 2 % KCl under 200 mM salinity condition (Figure 2). The highest *MGT* was in 1 % Ca_2CO_3 and 2 % KCl with 12 and 24 priming hours under 200 and 100 mM salinity conditions, respectively. Also, there were no significant differences in 24 h priming with 2 % Ca_2CO_3 under 50 mM salinity conditions, 0.5 % Na_2SO_3, hydro-prim and 1 % Na_2SO_3 under 100 mM salinity conditions, and 12 h priming with 1 % Na_2SO_3 and 1 % Ca_2CO_3 in 100 mM salinity condition (Table 4). Similar results were reported by (Farooq et al., 2006) and (Qadir et al., 2011), who reported reducing *MGT* using $CaCl_2$ primed seeds.

Hydropriming (12 and 24 h) had the highest *GI* in normal conditions, and there were no significant differences with hydropriming (control), 0.5 % Na_2So_3, and 1 % Na_2SO_3 in 12 h in normal condition (Table 4). The seeds with twelve-hour priming of 1 % KCl in 100 mM salinity, 0.5 % Na_2So_3, and 0.1 % Na_2SO_3 under 50 mM salinity showed high *GI*, but there were significant differences with the control condition (Table 4). Enhancing the germination rate in treatment seeds can be illustrated through the enhanced synthesis of protein, the less term of metabolism in the germination stage, the influence on cell membrane phospholipids (Ansari et al., 2013), enhance of cell division rate (Taylor & Harman, 1990), and faster absorption of water, better development in these seeds, which all eventually lead to enhancing of seed germination duration. Shahverdi et al. (2017), with priming the stevia seeds, indicated a considerable correlation between the germination percentage enhancement and seed germination improvement factors. It seems that the efficiency of pretreatment of seed is affiliated with the elements like type and concentration of priming compound, duration of seed treatment by compounds (duration of priming).

The 12 h treatment with hydro-prim, 1 % Na_2So_3,

Table 3: Mean comparison of various priming on germination indices and seedling growth traits in *Hibiscus sabdariffa* L.

Prim	%	Time	*GP* (%)	*GRI*	*MGT* (day)	*GI*	*VI*	*ShL* (cm)	*RL* (cm)	*FShM* (g)	*FRM*(g)	*DShM* (g)	*DRW* (g)	*Sh/R*
Hydro	-	Control	27.917	2.7319	1.9104	25.75	143.33	1.9417	1.2058	0.13333	0.015333	0.016375	0.001633	8.833
		12	28.333	3.6903	1.9492	28.167	200.46	2.6833	1.8083	0.13583	0.015417	0.016367	0.001642	7.608
		24	24.583	3.2569	1.7405	25.083	155.67	2.3917	1.625	0.1375	0.017833	0.011717	0.001883	4.772
KNO_3	0.5	12	19.58	2.0139	1.825	18.333	88.29	2.4667	0.775	0.13167	0.013333	0.015667	0.001458	8.277
		24	0	0	0	0	0	0	0	0	0	0	0	0
	1	12	22.08	2.6875	1.7681	22.083	126.83	2.825	1.075	0.125	0.014992	0.015125	0.001333	12.993
		24	0	0	0	0	0	0	0	0	0	0	0	0
Na_2SO_3	0.5	12	29.583	4.2111	1.3682	31.583	184.92	3.3083	0.1	0.135	0.015158	0.018292	0.001367	11.886
		24	23.333	2.6319	2.0141	22.417	160.33	3.5083	1.7417	0.1425	0.012792	0.016933	0.001367	13.181
	1	12	25.833	3.5514	2.0231	24.75	158.67	2.925	1.475	0.12167	0.011342	0.015167	0.001217	10.36
		24	16.25	1.6764	2.3915	13.917	155.67	3.8917	1.9583	0.17417	0.01435	0.013575	0.00105	11.225
KCl	1	12	26.25	3.1625	1.967	25.917	195.5	3.7417	1.525	0.14167	0.013375	0.014875	0.001175	10.414
		24	20.833	2.1208	2.2943	19.667	139	2.725	1.425	0.13167	0.01155	0.01655	0.001325	11.548
	2	12	14.167	1.3125	1.9494	12.75	63.88	2.2833	0.9083	0.10583	0.007083	0.015258	0.000898	13.817
		24	14.167	1.25	2.0431	12.25	67.17	2.2917	1.1083	0.10833	0.008408	0.012975	0.000733	14.143
Ca_2CO_3	1	12	17.5	2.1042	1.6296	17.333	133.24	3.175	1.5575	0.12917	0.016833	0.012475	0.001492	7.633
		24	21.25	2.5486	1.9756	21.167	145.25	3.9167	2.1917	0.18083	0.017833	0.019433	0.001733	12.976
	2	12	25.417	2.6375	2.0192	23.833	189.04	3.1417	1.875	0.1375	0.019417	0.015967	0.0018	6.902
		24	27.5	2.9986	2.2199	26.583	239.38	4.1917	2.9417	0.19	0.02525	0.020867	0.002075	12.033
LSD	5%		4.8001	0.7987	0.6738	5.298	45.289	0.5758	0.3508	0.0265	0.0027	0.0024	0.0003	4.0427

GP: Germination Percentage, *GRI*: Germination Rate Index, *MGT*: Mean Germination Time, *GI*: Germination Index, *VI*:Vigor Index, *ShL*: Shoot length, *RL*: Root Length, *FShM*: Fresh Shoot Mass, *FRM*: Fresh Root Mass, *DShM*: Dry Shoot Mass, *DRM*: Dry Root Mass, *Sh/R*: Shoot/Root ratio

and 1 % KCl had the highest *VI* in normal condition, and there were no significant differences with 0.5 % Na_2SO_3, 1 % Ca_2CO_3, 2 % Ca_2CO_3, and 1 % KNO_3 in 12 h treatments under normal condition, and 0.5 % Na_2SO_3 (12 h priming) in 50 mM salinity condition, 1 % Na_2SO_3, and hydro-prim in 24 h treatment at normal condition (Figure 3). The 12 h treatment with 0.5 % Na_2SO_3 in 50 mM salinity condition, 1 % KCl, 1 % Ca_2CO_3, 1 % Na_2SO_3, and the 24 h treatment with 1 % Na_2SO_3 had the highest shoot length in normal condition. Also, there were no significant differences with 12 h treatment by 1 % KNO_3 under 100 mM salinity, 1 % KNO_3,

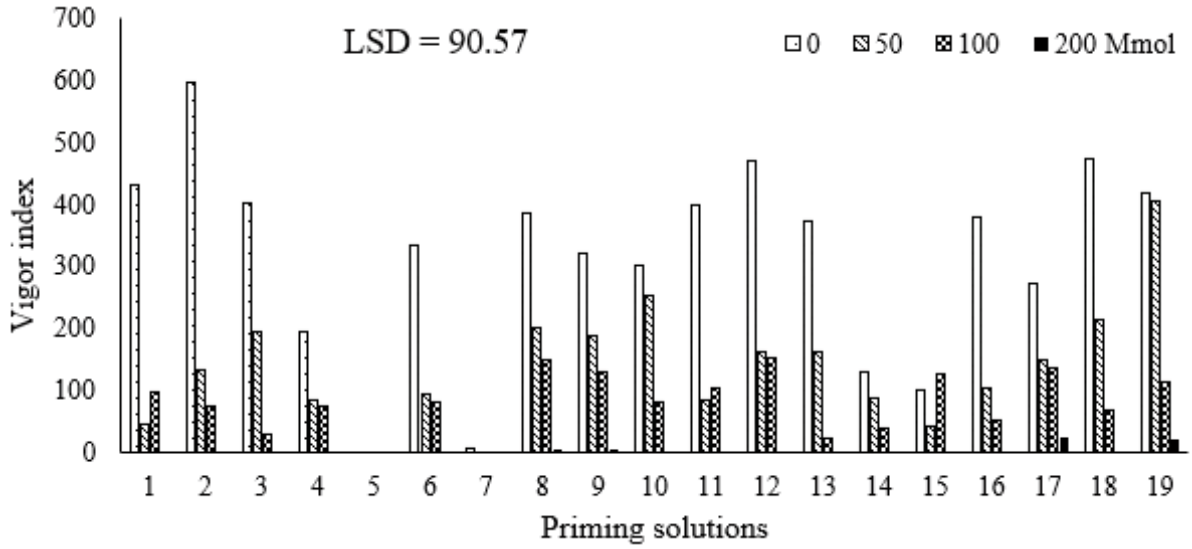

Figure 3: Effect of various priming under four level of salt stress on vigor index of *Hibiscus sabdariffa* L.
1: non-primed, 2: Hydro 12h , 3: Hydro 24 h, 4: KNO_3_0.5 % 12 h , 5: KNO_3_0.5 % 24 h , 6: KNO_3_1 % 12 h, 7: KNO_3_1 % 24 h , 8: Na_2SO_3_0.5 % 12 h, 9: Na_2SO_3_0.5 % 24 h , 10: Na_2SO_3_1 % 12 h, 11: Na_2SO_3_1 % 24 h, 12: KCl_1 % 12 h, 13: KCl_1 % 24 h , 14: KCl_2 % 12 h , 15: KCl_2 % 24 h, 16: Ca_2CO_3_1 % 12 h, 17: Ca_2CO_3_1 % 24 h, 18: Ca_2CO_3_2 % 12 h , 19: Ca_2CO_3_2 % 24 h

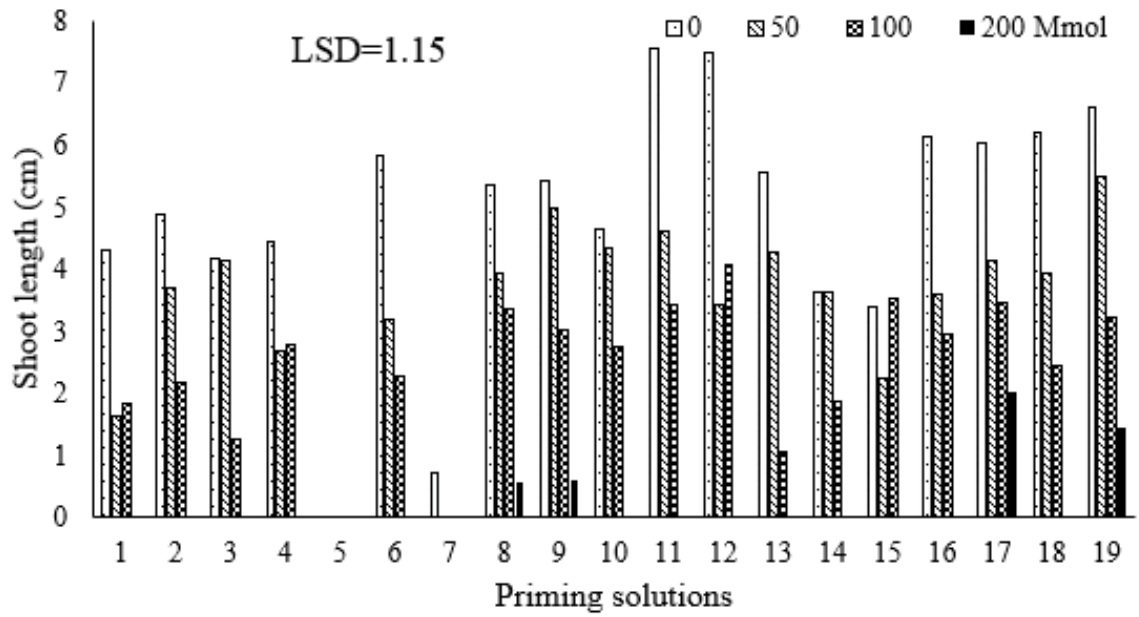

Figure 4: Effect of various priming under four level of salt stress on shoot length of *Hibiscus sabdariffa* L.
1: non-primed, 2: Hydro 12h , 3: Hydro 24 h, 4: KNO_3_0.5 % 12 h , 5: KNO_3_0.5 % 24 h , 6: KNO_3_1 % 12 h, 7: KNO_3_1 % 24 h , 8: Na_2SO_3_0.5 % 12 h, 9: Na_2SO_3_0.5 % 24 h , 10: Na_2SO_3_1 % 12 h, 11: Na_2SO_3_1 % 24 h, 12: KCl_1 % 12 h, 13: KCl_1 % 24 h , 14: KCl_2 % 12 h , 15: KCl_2 % 24 h, 16: Ca_2CO_3_1 % 12 h, 17: Ca_2CO_3_1 % 24 h, 18: Ca_2CO_3_2 % 12 h , 19: Ca_2CO_3_2 % 24 h

2 % Ca_2CO_3 in normal condition, 24 h treatment by 1 % KCl, and 0.5 % Na_2SO_3 in normal condition (Figure 4).

The highest root length was in hydro-prim, 1 % Ca_2CO_3 at 12 h in normal condition, and 0.5 % Na_2SO_3 at 12 h in 50 mM salinity condition, and there were no significant differences with 2 % Ca_2CO_3 at 12 h, 1 % Na_2SO_3, and hydroprim at 12 h in normal condition (Figure 5). Neumann (1995) also reported that salinity could quickly prevent root growth and thus the ability to uptake water and essential mineral nutrition. The 12 h treatment with 1 % Na_2SO_3, in 50 mM salinity condition, 1 % Ca_2CO_3 and 1 % KCl had the highest shoot mass in normal condition. Also, there were no significant differences with 1 % Na_2SO_3 at 24 h, 2 % Ca_2CO_3 in 12 h treatment in normal condition and 1 % KNO_3 in 12 treatment under 100 mM salinity (Table 4).

The 24 h treatment with 1 % Na_2SO_3, 1 % KCl, and the 12 h treatment with 2 % Ca_2CO_3 had the highest root mass in normal condition. Also, there were no significant different with 12 treatment of 1 % Na_2SO_3, 0.5% Na_2SO_3 in normal condition and 1 % Ca_2CO_3 under 50 mM salinity (Table 4). The results showed several priming had high dry shoot mass under various salinity conditions, for instance 2 % Ca_2CO_3, 1 % Ca_2CO_3, 1 % Na_2SO_3, 0.5 % Na_2SO_3, 0.5 % KNO_3, and 1 % KCl illustrated the highest dry shoot mass under 100 mM salinity condition. Besides, 0.5 % Na_2SO_3, 1 % Ca_2CO_3, 0.5 % KNO_3, 2 % Ca_2CO_3, 1 % KCl, 1 % KNO_3 and 2 % KCl had the highest dry shoot mass under 50 mM salinity condition (Table 4). Seed priming by $CaCl_2$, KCl, and NaCl were figured out to be effective in diminishing the negative impact of salinity on wheat via their effects on changing the levels of various plant phytohormones (Iqbal et al., 2006).

The highest dry root length was in hydro-prim, 1 % Na_2SO_3, and 1 % KCl at 24 h, and 1 % Ca_2CO_3 at 12 h in normal condition, also, there were no significant differences with hydro-prim (12 h), 2 % Ca_2CO_3 (12 h), 0.5 % Na_2SO_3 (12 and 24 h), and 1 % KCl (24 h) in 50 mM salinity condition, 0.5 % Na_2SO_3 (24 h), 1 % Na_2SO_3 (24 h), and 1 % KNO_3 (12 h) in 100 mM salinity condition (Table 4). Abdollahi & Jafari (2012) demonstrated that KNO_3 3 % treatment enhanced root length to primary shoot ratio more than NaCl 1 % under saline condition. This enhances the water uptake by the plant that may help the growth development of seedlings in saline conditions. In addition, application of the four potassium nitrate concentrations (0, 0.5, 1, and 2 %) on time to 50 percentage germination, and germination percentage of amaranth seeds revealed that using 0.5 percent of potassium nitrate decreased time to 50 % seed germination (Musa & Lawal, 2015).

The 24 h treatment with 1 % KCl, 1 % Ca_2CO_3, and 2 % Ca_2CO_3 had the highest shoot and root ratio in 100 mM salinity condition. Also, there were no significant differences with 12 treatments of 1 % Ca_2CO_3, 2 % KCl,

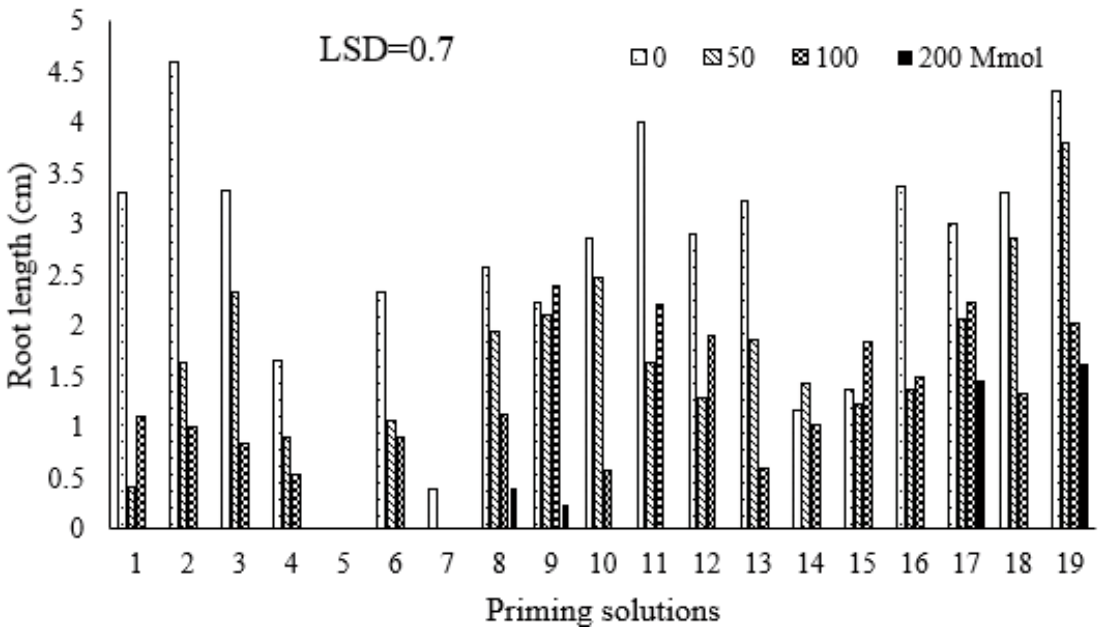

Figure 5: Effect of various priming under four level of salt stress on root length of *Hibiscus sabdariffa* L.
1: non-primed, 2: Hydro 12h , 3: Hydro 24 h, 4: KNO_3–0.5 % 12 h , 5: KNO_3–0.5 % 24 h , 6: KNO_3–1 % 12 h, 7: KNO_3–1 % 24 h , 8: Na_2SO_3–0.5 % 12 h, 9: Na_2SO_3–0.5 % 24 h , 10: Na_2SO_3–1 % 12 h, 11: Na_2SO_3–1 % 24 h, 12: KCl_1 % 12 h, 13: KCl_1 % 24 h , 14: KCl_2 % 12 h , 15: KCl_2 % 24 h, 16: Ca_2CO_3–1 % 12 h, 17: Ca_2CO_3–1 % 24 h, 18: Ca_2CO_3–2 % 12 h , 19: Ca_2CO_3–2 % 24 h.

Table 4: Mean comparison of priming and salinity interaction effect on germination indices and seedling growth traits in *Hibiscus sabdariffa* L.

Prim (%)	Time	salt	*MGT* (day)	GI	*FShM* (g)	*FRM* (g)	*DShM* (g)	*DRM* (g)	Sh/R
Hydro	Control	0	2.05	56.33	0.19	0.020	0.0192	0.0025	7.79
		50	2.85	18	0.19	0.021	0.0209	0.0017	16.36
		100	2.73	28.66	0.14	0.02	0.0253	0.0023	11.17
		200	0	0	0	0	0	0	0
	12 h	0	1.58	68.66	0.15	0.025	0.0227	0.0023	10.07
		50	2.30	23.33	0.21	0.023	0.0213	0.0020	10.52
		100	2.9	19.33	0.17	0.013	0.0213	0.0022	9.82
		200	1	1.33	0	0	0	0	0
	24 h	0	1.49	59	0.19	0.03	0.0196	0.0033	5.90
		50	2.19	28.66	0.21	0.021	0.0155	0.0023	6.71
		100	2.94	10.66	0.14	0.016	0.0116	0.0018	6.46
		200	0.33	2	0	0	0	0	0
KNO_3_0.5 %	12 h	0	1.72	33.66	0.18	0.016	0.0201	0.00196	10.35
		50	2.72	20.33	0.18	0.023	0.0221	0.0022	10.28
		100	2.85	19.33	0.16	0.013	0.0203	0.0016	12.47
		200	0	0	0	0	0	0	0
	24 h	0	0	0	0	0	0	0	0
		50	0	0	0	0	0	0	0
		100	0	0	0	0	0	0	0
		200	0	0	0	0	0	0	0
KNO_3_1 %	12 h	0	2.02	40.33	0.21	0.043	0.0189	0.003	6.34
		50	1.94	22	0.19	0.011	0.0216	0.0013	17.68
		100	2.10	24.66	0.096	0.005	0.0198	0.0010	27.94
		200	1	1.33	0	0	0	0	0
	24 h	0	0.66	1.66	0.06	0.001	0.005	0.00053	3.12
		50	0	0	0	0	0	0	0
		100	0	0	0	0	0	0	0
		200	0	0	0	0	0	0	0
Na_2SO_3_0.5 %	12 h	0	1.26	55.66	0.20	0.023	0.0189	0.00163	11.57
		50	1.97	35	0.17	0.018	0.0239	0.00206	11.61
		100	1.89	33.66	0.11	0.015	0.0228	0.00126	19.35
		200	0.33	2	0.04	0.003	0.0075	0.0005	5
	24 h	0	1.64	44.66	0.18	0.017	0.0202	0.0018	12.81
		50	2.85	22.66	0.2	0.016	0.0211	0.00203	10.45
		100	2.55	21	0.15	0.015	0.0236	0.00156	15.62
		200	1	1.33	0.03	0.002	0.0027	0.0002	13.83

Continued on the next page

Na_2SO_3_1 %	12 h	0	1.36	45.33	0.14	0.016	0.0136	0.002	6.78
		50	3.09	30	0.19	0.018	0.0217	0.00163	13.32
		100	2.63	22.33	0.15	0.009	0.0252	0.00123	21.32
		200	1	1.33	0	0	0	0	0
	24 h	0	2.12	33.33	0.31	0.034	0.0188	0.0021	9.12
		50	4.77	6.66	0.20	0.009	0.0116	0.00073	16.92
		100	2.66	15.66	0.17	0.013	0.0238	0.0013	18.85
		200	0	0	0	0	0	0	0
KCl_1 %	12 h	0	1.66	48	0.27	0.031	0.016	0.0018	8.91
		50	2.70	28.66	0.14	0.010	0.0207	0.00173	12.33
		100	1.83	26	0.15	0.011	0.0228	0.00117	20.40
		200	1.66	1	0	0	0	0	0
	24 h	0	1.89	42.66	0.21	0.025	0.0202	0.00243	8.64
		50	2.66	23.66	0.19	0.015	0.0251	0.00203	12.32
		100	2.61	12	0.12	0.005	0.0207	0.00083	25.22
		200	2	0.333	0	0	0	0	0
KCl_2 %	12 h	0	2.21	25.66	0.15	0.007	0.0162	0.001267	13.56
		50	2.58	15	0.18	0.009	0.0223	0.001467	15.48
		100	3	10.33	0.09	0.0116	0.0224	0.00086	26.21
		200	0	0	0	0	0	0	0
	24 h	0	2.36	19.33	0.15	0.0103	0.0172	0.0012	15.12
		50	3.22	9	0.12	0.0143	0.0173	0.00073	24.05
		100	2.58	20.66	0.16	0.009	0.0174	0.001	17.4
		200	0	0	0	0	0	0	0
Ca_2CO_3_1 %	12 h	0	1.96	38.66	0.23	0.041	0.0172	0.0032	5.33
		50	2.05	20.33	0.14	0.0143	0.0137	0.001	13.67
		100	2.5	10.33	0.13	0.012	0.0189	0.001767	11.51
		200	0	0	0	0	0	0	0
	24 h	0	1.81	31	0.24	0.032	0.0171	0.00213	8.66
		50	2.11	24	0.20	0.009	0.0198	0.0015	13.54
		100	2.13	22.66	0.14	0.019	0.0206	0.00233	8.88
		200	1.83	7	0.13	0.010	0.0201	0.000967	20.81
Ca_2CO_3_2%	12 h	0	1.91	50.66	0.21	0.034	0.0169	0.0026	6.62
		50	2.46	28.66	0.21	0.030	0.0253	0.00246	10.54
		100	3.03	14.33	0.12	0.012	0.0215	0.00213	10.44
		200	0.66	1.66	0	0	0	0	0
	24 h	0	1.879	39.33	0.28	0.034	0.0199	0.0025	7.88
		50	2.45	40	0.22	0.039	0.0196	0.003	6.65
		100	2.21	20.66	0.16	0.014	0.0239	0.0017	14.47
		200	2.33	6.33	0.09	0.013	0.0203	0.0010	19.11
LSD 5 %	-	-	1.34	10.59	0.05	0.005	0.0048	0.0006	8.0855

MGT: Mean Germination Time, *GI*: Germination Index, *FShM*: Fresh Shoot Mass, *FRM*: Fresh Root Mass, *DShMW*: Dry Shoot Mass, *DRM*: Dry Root Mass, *Sh/R*: Shoot/Root ratio

and hydro-prime in 50 mM salinity condition (Table 4). Seed priming is one of the simple, low risk and cost approaches used to cope with the adverse effect of salinity in agricultural lands. The privilege of seed priming or pretreatment in unfavorable conditions have been studied in several crops, instance hot pepper (Khan et al., 2009), tomato (Ebrahimi et al., 2014), pepper (Aloui et al., 2014), lettuce (Nasri et al., 2011), pea (*Pisum sativum* L.) (Naz et al., 2014), and maize (Abraha & Yohannes, 2013). Soil salinity has adverse effects on agriculture productivity. Therefore, agronomic and genetic solutions to enhancing salt tolerance are urgently required. We concluded that applying easy and low-cost techniques such as priming can remarkably increase the germination seed in salinity condition.

4 CONCLUSIONS

Priming is a technique that is capable to improve the performance of seeds in salinity stress conditions. Under saline condition, 24 h 2 % $Ca2CO_3$ had the highest germination percentage (43.3 %) in 50 mM, while 12 h treatment with 0.5 % $Na2SO_3$ (33.3 %) had high germination percentage in 100 mM levels of saline conditions. Also, the highest germination rate index was observed in 0.5 % Na_2SO_3 with 12 h treatment time (4.05 and 3.95 respectively) in 50 and 100 mM levels of saline conditions. There was no geminated seed at 24 h priming by 0.5 and 1 percentage of KNO_3, while priming with 1 % of KNO_3 at 12 h showed good performance in terms of shoot mass trait under saline condition. The result of various priming on studied traits revealed the importance of the type of priming compound and priming duration. The result implied that the best treatments in terms of *GP* were 12 h hydropriming, 24 h Ca_2CO_3_2 %, 12 h Na_2SO_3_0.5 %, 24 h Ca_2CO_3_1 %, in control, 50 mM salinity, 100 mM salinity, and 200 mM salinity conditions, respectively. We suggested performing the same studies with the suitable material at the precise concentration on similar species to determine and understand the reliability and efficiency of the approaches. Also, supplementary research should concentrate on molecular, metabolic, and physiological stimulate with priming agents in salt stress. Moreover, future studies need to assess germination and early seedling growth at the field condition.

5 ACKNOWLEDGMENTS

This research was conducted in a research project (No. 99.3821) at University of Hormozgan. The authors are thankful to Minab Higher Education Center, University of Hormozgan to provide laboratory facilities and equipment.

6 REFERENCES

Abdollahi, F., & Jafari, L. (2012). Effect of NaCl and KNO_3 priming on seed germination of canola (*Brassica Napus* L.) under salinity conditions. *International Journal of Agriculture, Research and Review*, *2*, 573–579.

Abraha, B., & Yohannes, G. (2013). The role of seed priming in improving seedlinggrowth of maize (*Zea mays* L.) under salt stress at field conditions. *Agricultural Sciences*, *4*(12), 666–672. https://doi.org/10.4236/as.2013.412089

Afkari Bajehbaj, A. (2010). The effects of NaCl priming on salt tolerance in sunflower germination and seedling grown under salinity conditions. *African Journal of Biotechnology*, *9*(12), 1764–1770. https://doi.org/10.5897/AJB10.1019

Afzal, I., Rehman, H. U., Naveed, M., & Basra, S. M. A. (2016). Recent advances in seed enhancements. In S. . Araujo & A. . Balestrazzi (Eds.), *New Challenges in Seed Biology - Basic and Translational Research Driving Seed Technology*. IntechOpen. https://doi.org/10.5772/64791

Aghdaei, M., Nemati, S. H., Samiei, L., & Sharifi, A. (2019). Effect of some priming methods on germination and vegetative parameters of pepino (*Solanum muricatum* Aiton). *EurAsian Journal of BioSciences*, *13*, 1919–1929.

Ahmadizadeh, M. (2013). Physiological and agro-morphological response to drought stress. *Middle East Journal of Scientific Research*, *13*(8).

Ahmadizadeh, M., Valizadeh, M., Zaefizadeh, M., & Shahbazi, H. (2011). Evaluation of interaction between genotype and environments in term of germination and seedling growth in durum wheat landraces. *Advances in Environmental Biology*, *5*(4).

Ahmadizadeh, M., Babaeian-Jelodar, N., Mohammadi-Nejad, G., Bagheri, N., & Singh, R. K. (2021). High-density linkage mapping for agronomic and physiological traits of rice (*Oryza sativa* L.) under reproductive-stage salt stress. *Journal of Genetics, 100*(2), 1–12. https://doi.org/10.1007/S12041-021-01301-6

Ahmadizadeh, M., Vispo, N. A., Calapit-Palao, C. D. O., Pangaan, I. D., Viña, C. Dela, & Singh, R. K. (2016). Reproductive stage salinity tolerance in rice: a complex trait to phenotype. *Indian Journal of Plant Physiology*, *21*(4), 528–536. https://doi.org/10.1007/s40502-016-0268-6

Ali, B., Al Wabel, N., & Blunden, G. (2005). Phytochemical, pharmacological and toxicological aspects of *Hibiscus sabdariffa* L.: a review. *Phytotherapy Research, PTR*, *19*(5), 369–375. https://doi.org/10.1002/PTR.1628

Ali, Q., Daud, M. K., Haider, M. Z., Ali, S., Rizwan, M., Aslam, N., … Zhu, S. J. (2017). Seed priming by sodium nitroprusside improves salt tolerance in wheat (*Triticum aestivum* L.) by enhancing physiological and biochemical parameters. *Plant Physiology and Biochemistry PPB*, *119*, 50–58. https://doi.org/10.1016/J.PLAPHY.2017.08.010

Al-Mudaris, M. A. (1998). Notes on various parameters re-

cording the speed of seed germination. Der Tropenlandwirt - *Journal of Agriculture in the Tropics and Subtropics*, *99*(2), 147–154.

Aloui, H., Souguir, M., Latique, S., & Hannachi, C. (2014). Germination and growth in control and primed seeds of pepper as affected by salt stress. *Cercetari Agronomice in Moldova*, *47*(3), 83–95. https://doi.org/10.2478/CERCE-2014-0029

Al-Tohafi, S.A., Hussain, A.S., Habeeb, H.A., & Azab, N.H. (2015). Response of growth and yield of roselle plant (*Hibiscuss abdariffa* L.) for adding (clean salt) and spraying with organic fertilizer (humic aljohara) in high salinity soil. *Kufa Journal for Agricultural Sciences*, *7*(1).

Amiri, M. B., Rezvani Moghaddam, P., Ehyai, H. R., Fallahi, J., & Aghhavani Shajari, A. (2010). Effect of osmotic and salinity stresses on germination and seedling growth indices of *Cynara scolymus* and *Echinacea purpurea*. *Environmental Stresses in Crop Sciences*, *3*(2), 165–176.

Ansari, O., Azadi, M. S., Sharif-Zadeh, F., & Younesi, E. (2013). Effect of hormone priming on germination characteristics and enzyme activity of mountain rye (*Secale montanum*) seeds under drought stress conditions. *Journal of Stress Physiology & Biochemistry*, *9*(3), 61–71.

Ashraf, M., & Foolad, M. R. (2005). Pre-sowing seed treatment—a shotgun approach to improve germination, plant growth, and crop yield under saline and non-saline conditions. *Advances in Agronomy*, *88*, 223–271. https://doi.org/10.1016/S0065-2113(05)88006-X

Askari Nejad, H. & Farahmand, S. (2012). Evaluating the potential of seed priming techniquesin improving germination and early seedling growth of *Aeluropus macrostachys* under salinity stress condition. *Annals of Biological Research, 3*, 5099-5105.

Atak, M., Kaya, M. D., Kaya, G., Cikili, Y., & Ciftci, C. Y. (2006). Effects of NaCl on the germination, seedling growth and water uptake of triticale. *Turkish Journal of Agriculture and Forestry*, *30*, 39–47.

Bahaeldeen, M. B., Sulaiman, A. A., & Adam, A. (2012). Roselle (*Hibiscus sabdariffa* L.) in Sudan, cultivation and their uses. *Bulletin of Environment, Pharmacology and Life Sciences*, *1*(6), 48–54.

Begcy, K., Sandhu, J. & Walia, H. (2018) Transient heat stress during early seed development primes germination and seedling establishment in rice. *Frontiers in Plant Science*, *9*, 1768. https://doi.org/10.3389/fpls.2018.01768

Bhanuprakash, K., & Yogeesha, H. S. (2016). Seed priming for abiotic stress tolerance: An overview. In R. N. K. . Srinivasa, K. S. . Shivashankara, & R. H. Laxman (Eds.), *Abiotic Stress Physiology of Horticultural Crops* (pp. 103–117). Springer, New Delhi. https://doi.org/10.1007/978-81-322-2725-0_6

Bruce, T. J. A., Matthes, M. C., Napier, J. A., & Pickett, J. A. (2007). Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science*, *173*(6), 603–608. https://doi.org/10.1016/J.PLANTSCI.2007.09.002

Chen, K., & Arora, R. (2011). Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in Spinach (*Spinacia oleracea*). *Plant Science : An International Journal of Experimental Plant Biology*, *180*(2), 212–220. https://doi.org/10.1016/J.PLANTSCI.2010.08.007

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., & Heinrich, M. (2014). *Hibiscus sabdariffa* L. a phytochemical and pharmacological review. *Food Chemistry*, *165*, 424-443. https://doi.org/10.1016/j.foodchem.2014.05.00

Dkhil, B. Ben, Issa, A., & Denden, M. (2014). Germination and seedling emergence of primed okra (*Abelmoschus esculentus* L.) seeds under salt stress and low temperature. *American Journal of Plant Physiology*, *9*(2), 38–45. https://doi.org/10.3923/AJPP.2014.38.45

Ebrahimi, R., Ahmadizadeh, M., & Rahbarian, P. (2014). Enhancing stand establishment of tomato cultivars under salt stress condition. *South Western Journal of Horticulture, Biology and Environment*, *5*(1), 19–42.

Elouaer, Mohamed Aymen, Hannachi, C. (2012). Seed priming to improve germination and seedling growth of safflower (*Carthamus tinctorius*) under salt stress. *EurAsian Journal of Biosciences*, *6*, 76–84. https://doi.org/10.5053/ejobios.2012.6.0.9

Eskandari, H. (2013). Effects of priming technique on seed germination properties, emergence and field performance of crops: a review. *International Journal of Agronomy and Plant Production* , *4*(3), 454–458.

Farnsworth, N. R., & Bunyapraphatsara, N. (1992). *Thai medicinal plants: recommended for primary health care system*. Medicinal Plant Information Center: Mahidol University, 409.

Farooq, M., Basra, S. M. A., Afzal, I., & Khaliq, A. (2006). Optimization of hydropriming techniques for rice seed invigoration. *Seed Science and Technology*, *34*(2), 507–512. https://doi.org/10.15258/SST.2006.34.2.25

Farooq, Muhammad, Barsa, S. M. A., & Wahid, A. (2006). Priming of field-sown rice seed enhances germination, seedling establishment, allometry and yield. *Plant Growth Regulation, 49*(2), 285–294. https://doi.org/10.1007/S10725-006-9138-Y

Farooq, Muhammad, Basra, S. M., Wahid, A., & Ahmad, N. (2010). Changes in nutrient-homeostasis and reserves metabolism during rice seed priming: Consequences for seedling emergence and growth. *Agricultural Sciences in China*, *9*(2), 191–198. https://doi.org/10.1016/S1671-2927(09)60083-3

Fazlali, R., Eradatmand Asli, D., & Moradi, P. (2013). The effect of seed priming by ascorbic acid on bioactive compounds of naked seed pumpkin (*Cucurbita pepo var. styriaca*) under salinity stress. *International Journal of Farming and Allied Sciences*, *2*(17), 587–590.

Feghhenabi, F., Hadi, H., Khodaverdiloo, H., & van Genuchten, M. T. (2020). Seed priming alleviated salinity stress during germination and emergence of wheat (*Triticum aestivum* L.). *Agricultural Water Management*, *231*, 106022. https://doi.org/10.1016/J.AGWAT.2020.106022

Fuller, M. P., Hamza, J. H., Rihan, H. Z., & Al-Issawi, M. (2012). Germination of primed seed under NaCl stress in wheat. *International Scholarly Research Network (ISRN) Botany*, 1–5. https://doi.org/10.5402/2012/167804

Galal, A. (2017). Physico-chemical changes in karkade (*Hibiscus sabdariffal* L.) seedlings responding to salt stress.

Acta Biologica Hungarica, *68*(1), 73-87. https://doi.org/10.1556/018.68.2017.1.7

Hasanuzzaman, M., & Fotopoulos, V. (2019). Priming and pretreatment of seeds and seedlings. (M. Hasanuzzaman & V. . Fotopoulos, Eds.), *Priming and Pretreatment of Seeds and Seedlings* (1st ed.). Springer Singapore. https://doi.org/10.1007/978-981-13-8625-1

Hussain, S., Khan, F., Hussain, H. A., & Nie, L. (2016). Physiological and biochemical mechanisms of seed priming-induced chilling tolerance in rice cultivars. *Frontiers in Plant Science*, *0*(FEB2016), 116. https://doi.org/10.3389/FPLS.2016.00116

Ibrahim, E. A. (2016). Seed priming to alleviate salinity stress in germinating seeds. *Journal of Plant Physiology*, *192*, 38–46. https://doi.org/10.1016/J.JPLPH.2015.12.011

Ibrahim, E. B., Abdalla, A. W. H., Ibrahim, E. A., & Naim, A. M. E. (2013). Variability in some Roselle (*Hibiscus sabdariffa* L.) genotypes for yield and its attributes. *International Journal of Agriculture and Forestry*, *3*(7), 261–266.

Iqbal, M., Ashraf, M., Jamil, A., & Ur-Rehman, S. (2006). Does seed priming induce changes in the levels of some endogenous plant hormones in hexaploid wheat plants under salt stress? *Journal of Integrative Plant Biology*, *48*(2), 181–189. https://doi.org/10.1111/J.1744-7909.2006.00181.X

Ismail, A. M., Heuer, S., Thomson, M. J., & Wissuwa, M. (2007). Genetic and genomic approaches to develop rice germplasm for problem soils. *Plant Molecular Biology*, *65*(4), 547–570. https://doi.org/10.1007/s11103-007-9215-2

Jime'nez-Arias, D., Borges, A. A., Luis, J. C., Valdés, F., Sandalio, L. M., & Pe'rez, J. A. (2015). Priming effect of menadione sodium bisulphite against salinity stress in Arabidopsis involves epigenetic changes in genes controlling proline metabolism. *Environmental and Experimental Botany*, *120*, 23–30. https://doi.org/10.1016/J.ENVEXPBOT.2015.07.003

Jisha, K. C., Vijayakumari, K., & Puthur, J. T. (2012). Seed priming for abiotic stress tolerance: an overview. *Acta Physiologiae Plantarum, 35*(5), 1381–1396. https://doi.org/10.1007/S11738-012-1186-5

Kadamanda, R. (2019). The salinity effect on leaf pigment content of roselle (*Hibiscus sabdariffa* L.) at different growth stages. *Journal of Food, Agriculture & Environment*, *17*(1), 36-39.

Katembe, W. J., Ungar, I. A., & Mitchel, J. P. (1998). Effect of salinity on germination and seedling growth of two Atriplex species (Chenopodiaceae). *Annals of Botany*, *82*(2), 167–175. https://doi.org/10.1006/ANBO.1998.0663

Kaveh, H., Nemati, H., Farsi, M., & Vatandoost Jartoodeh, S. (2111). How salinity affect germination and emergence of tomato lines. *Journal of Biological & Environmental Sciences*, *5*(15), 159–163.

Kaya, C., Ak, B. E., & Higgs, D. (2003). Response of salt-stressed strawberry plants to supplementary calcium nitrate and/or potassium nitrate. *Journal of Plant Nutrition*, *26*(3), 543–560. https://doi.org/10.1081/PLN-120017664

Kaya, M. D., Ipek, A., & Ozturk, A. (2003). Effects of different soil salinity levels on germination and seedling growth of safflower (*Carthamus tinctorius* L.). *Turkish Journal of Agriculture and Forestry*, *27*, 221–227.

Kerchev, P., van der Meer, T., Sujeeth, N., Verlee, A., Stevens, C.V., Van Breusegem, F., & Gechev, T. (2020). Molecular priming as an approach to induce tolerance against abiotic and oxidative stresses in crop plants. *Biotechnology Advances*, *40*, 107503. https://doi.org/10.1016/j.biotechadv.2019.107503

Khan, H. A., Ayub, C. M., Pervez, M. A., Bilal, R. M., Shahid, M. A., & Ziaf, K. (2009). Effect of seed priming with NaCl on salinity tolerance of hot pepper (*Capsicum annuum* L.) at seedling stage. *Soil & Environmental*, *28*(1), 81–87.

Khan, H. A., Pervez, M. A., Ayub, C. M., Ziaf, K., Bilal, R. M., Shahid, M. A., & Akhtar, M. A. (2009). Hormonal priming alleviates salt stress in hot Pepper (*Capsicum annuum* L.). *Soil and Environmental*, *28*(2), 130–135.

Khan, M. A., & Weber, D. J. (2006). Ecophysiology of high salinity tolerant plants *(Tasks for Vegetation Science)*. (M. A. Khan & D. J. Weber, Eds.), *Ecophysiology of High Salinity Tolerant Plants* (1st ed.). Springer Netherlands. https://doi.org/10.1007/1-4020-4018-0

Khodadad, M. (2011). An evaluation of safflower genotypes (*Carthamus tinctorius* L.), seed germination and seedling characters in salt stress conditions. *African Journal of Agricultural Research*, *6*(7), 1667–1672.

Lara, T. S., Marcel, J., Lira, S., Rodrigues, A. C., Rakocevic, M., Alvarenga, A. A., & Lira, J. M. S. (2014). potassium nitrate priming affects the activity of nitrate reductase and antioxidant enzymes in tomato germination. *Journal of Agricultural Science*, *6*(2), 72–80. https://doi.org/10.5539/jas.v6n2p72

Latef, A. A. H. A., Zaid, A., Alhmad, M. F. A., & Abdelfattah, K. E. (2020). The impact of priming with Al_2O_3 nanoparticles on growth, pigments, osmolytes, and antioxidant enzymes of egyptian Roselle (*Hibiscus sabdariffa* L.) cultivar. *Agronomy, 10*(5), 681. https://doi.org/10.3390/AGRONOMY10050681

Lin, J., Wang, Y., Sun, S., Mu, C., & Yan, X. (2017). Effects of arbuscular mycorrhizal fungi on the growth, photosynthesis and photosynthetic pigments of *Leymus chinensis* seedlings under salt-alkali stress and nitrogen deposition. *Science of the Total Environment*, *576,* 234–241. https://doi.org/10.1016/j.scitotenv.2016.10.091

Mahadevan, N., Shivali, P., & Kamboj, J. (2009). *Hibiscus sabdariffa* L. -an overview. *Natural Product Radiance*, *8*(1), 77–83.

Maher, S., Fraj, H., & Cherif, H. (2013). Effect of NaCl priming on seed germination of Tunisian fenugreek (*Trigonella foenum-graecum* L.) under salinity conditions. *Journal of Stress Physiology & Biochemistry*, *9*(2), 86–96.

Mahesh, H., Murali, M., Anup Chandra Pal, M., Melvin, P., & Sharada, M. (2017). Salicylic acid seed priming instigates defense mechanism by inducing PR-Proteins in *Solanum melongena* L. upon infection with *Verticillium dahliae* Kleb. *Plant Physiology and Biochemistry : PPB*, *117*, 12–23. https://doi.org/10.1016/J.PLAPHY.2017.05.012

Migahid, M. M., Elghobashy, R. M., Bidak, L. M., & Amin, A. W. (2019). Priming of *Silybum marianum* L. Gaertn seeds with H_2O_2 and magnetic field ameliorates seawater stress. *Heliyon*, *5*(6), e01886. https://doi.org/10.1016/J.HELIYON.2019.E01886

Moradi, A., & Younesi, O. (2009). Effects of osmo- and hydropriming on seed parameters of grain sorghum (*Sorghum bicolor* L.). *Australian Journal of Basic and Applied Sciences*, *3*(3), 1696–1700.

Muhie, S.H., Yildirim, E., Memis, N., & Demir, I. (2020a). Vermicompost priming stimulated germination and seedling emergence of onion seeds against abiotic stresses. *Seed Science and Technology*, *48*(2), 153-157. https://doi.org/10.15258/sst.2020.48.2.02

Muhie, S., Özdamar, C., Gökdaş, Z., Njie, E.S., Memiş, N., & Demir, İ. (2020b). Effect of solid matrix priming with seaweed extract on germination and seedling performance of onion seeds under abiotic stress conditions. *Black Sea Journal of Agriculture*, *3*(4), 233-238.

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*(1), 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Musa, M., & Lawal, A. A. (2015). Influence of priming concentration on the growth and yield of amaranth (*Amaranthus cruentus* L.) in sokoto semi-arid zone of Nigeria. *Journal of Plant Sciences*, *3*(1), 30. https://doi.org/10.11648/J.JPS.20150301.15

Nadjafi, F., Bannayan, M., Tabrizi, L., & Rastgoo, M. (2006). Seed germination and dormancy breaking techniques for *Ferula gummosa* and *Teucrium polium*. *Journal of Arid Environments*, *64*(3), 542–547. https://doi.org/10.1016/J.JARIDENV.2005.06.009

Nasri, N., Kaddour, R., Mahmoudi, H., Baatour, O., Bouraoui, N., & Lachaâl, M. (2011). The effect of osmopriming on germination, seedling growth and phosphatase activities of lettuce under saline condition. *African Journal of Biotechnology*, *10*(65), 14366–14372. https://doi.org/10.4314/ajb.v10i65

Nassar, K. H. (2010). *Effect of seed priming and microdose of organic fertilizer on yield and quality of Roselle*. University of Khartoum.

Naz, F., Gul, H., Hamayun, M., Sayyed, A., Khan, H., & Sherwani, S. (2014). Effect of NaCl stress on P. sativum germination and seedling growth with the influence ofseed priming with potassium (KCL and KOH). *American-Eurasian Journal of Agricultural & Environmental*, *14*(11), 1304–1311.

Neumann, P. (1997). Salinity resistance and plant growth revisited. *Plant, Cell & Environment*, *20*(9), 1193–1198. https://doi.org/10.1046/J.1365-3040.1997.D01-139.X

Neumann, P. M. (1995). Inhibition of root growth by salinity stress: Toxicity or an adaptive biophysical response? *Structure and Function of Roots*, 299–304. https://doi.org/10.1007/978-94-017-3101-0_39

Paparella, S., Araújo, S. S., Rossi, G., Wijayasinghe, M., Carbonera, D., & Balestrazzi, A. (2015). Seed priming: state of the art and new perspectives. *Plant Cell Reports, 34*(8), 1281–1293. https://doi.org/10.1007/S00299-015-1784-Y

Patade, V. Y., Bhargava, S., & Suprasanna, P. (2009). Halopriming imparts tolerance to salt and PEG induced drought stress in sugarcane. *Agriculture, Ecosystems & Environment*, *134*(1–2), 24–28. https://doi.org/10.1016/J.AGEE.2009.07.003

Qadir, I., Khan, Z. H., Khan, R. A., & Afzal, I. (2011). Evaluation the potential of seed priming techniques in improving germination and early seedling growth of various rangeland grasses. *Pakistan Journal of Botany*, *43*, 2797–2800.

Ratikanta, M. ., & Kalipada, P. (2013). vegetable seed priming: a low cost, simple and powerful techniques for farmers' livelihood. *International Journal of Bio-Resource and Stress Managemen*, *4*(4), 475–481.

Reed, R.C., Bradford, K.J. & Khanday, I. (2022). Seed germination and vigor: ensuring crop sustainability in a changing climate. *Heredity*, 1-10. https://doi.org/10.1038/s41437-022-00497-2

Riaz, G., & Chopra, R. (2018). A review on phytochemistry and therapeutic uses of *Hibiscus sabdariffa* L. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *102*, 575–586. https://doi.org/10.1016/J.BIOPHA.2018.03.023

Rouhi, H. R., Aboutalebian, M. A., & Sharif-zadeh, F. (2011). Effects of hydro and osmopriming on drought stress tolerance during germination in four grass species. *Intentional Journal of AgriScience* , *1*(2), 107–114.

Shaheen, H. L., Iqbal, M., Azeem, M., Shahbaz, M., & Shehzadi, M. (2015). K-priming positively modulates growth and nutrient status of salt-stressed cotton (*Gossypium hirsutum*) seedlings. *Archives of Agronomy and Soil Science*, *62*(6), 759–768. http://doi.org/10.1080/03650340.2015.1095292

Shahverdi, M., Omidi, H., & Tabatabaei, S. (2017). Determination of optimum duration and concentration of stevia (*Stevia rebaudiana* Bert.) seed priming with boric acid (H_3BO_3). *Türkiye Tarımsal Araştırmalar Dergisi*, *4*(1), 24–30. https://doi.org/10.19159/tutad.300701

Shannon, M. C., & Grieve, C. M. (1998). Tolerance of vegetable crops to salinity. *Scientia Horticulturae*, *78*(1–4), 5–38. https://doi.org/10.1016/S0304-4238(98)00189-7

Sheteiwy, M.S., Shao, H., Qi, W., Daly, P., Sharma, A., Shaghaleh, H., Hamoud, Y.A., El-Esawi, M.A., Pan, R., Wan, Q., & Lu, H. (2021). Seed priming and foliar application with jasmonic acid enhance salinity stress tolerance of soybean (*Glycine max* L.) seedlings. *Journal of the Science of Food and Agriculture*, *101*(5), 2027-2041. https://doi.org/10.1002/jsfa.10822

Sheyhakinia, S., Bamary, Z., Einali, A., & Valizadeh, J. (2020). The induction of salt stress tolerance by jasmonic acid treatment in roselle (*Hibiscus sabdariffa* L.) seedlings through enhancing antioxidant enzymes activity and metabolic changes. *Biologia, 75*(5), 681–692. https://doi.org/10.2478/S11756-020-00444-8

Shruthi, K., Balakrishna, P., & Sreeramu, B. S. (2018). the effects of seed treatments on germination and other seed quality attributes of rosella (*Hibiscus sabdariffa* Var. *sabdariffa* L.). *International Journal of Science, Environment*, *7*(1), 201–206.

Sivritepe, N., Sivritepe, H. O., & Eris, A. (2003). The effects of NaCl priming on salt tolerance in melon seedlings grown under saline conditions. *Scientia Horticulturae*, *97*(3–4), 229–237. https://doi.org/10.1016/S0304-4238(02)00198-X

Soeda, Y., Konings, M., Vorst, O., Houwelingen, A., Stoopen, G., Maliepaard, C., Geest, A. (2005). Gene expression programs during *Brassica oleracea* seed maturation, osmopriming, and germination are indicators of pro-

gression of the germination process and the stress tolerance level. *Plant Physiology*, *137*(1), 354–368. https://doi.org/10.1104/PP.104.051664

Subramanyam, K., Du Laing, G., & Van Damme, E. J. M. (2019). Sodium selenate treatment using a combination of seed priming and foliar spray alleviates salinity stress in Rice. *Frontiers in Plant Science*, *0*, 116. https://doi.org/10.3389/FPLS.2019.00116

Sukkhaeng, S., Promdang, S., & Doung-ngern, U. (2018). Fruit characters and physico-chemical properties of roselle (*Hibiscus sabdariffa* L.) in Thailand—A screening of 13 new genotypes. *Journal of Applied Research on Medicinal and Aromatic Plants*, *11*, 47–53. https://doi.org/10.1016/J.JARMAP.2018.10.001

Shruthi, K., Balakrishna, P., & Sreeramu, B.S. (2018). The effects of seed treatments on germination and other seed quality attributes of rosella. *International Journal of Science, Environment and Technology*, *7*, 201-206.

Tanou, G., Fotopoulos, V., & Molassiotis, A. (2012). Priming against environmental challenges and proteomics in plants: Update and agricultural perspectives. *Frontiers in Plant Science*, *0*(SEP), 216. https://doi.org/10.3389/FPLS.2012.00216

Taylor, A. G., & Harman, G. E. (1990). Concepts and technologies of selected seed treatments. *Annual Review of Phytopathology*, *28*, 321–339. https://doi.org/10.1146/ANNUREV.PY.28.090190.001541

Thiam, M., Champion, A., Diouf, D., & Ourèye SY, M. (2013). NaCl effects on in vitro germination and growth of some senegalese cowpea (*Vigna unguiculata* (L.) Walp cultivars. *International Scholarly Research Notices (ISRN) Biotechnology*, 1–11. https://doi.org/10.5402/2013/382417

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta, 218*(1), 1–14. https://doi.org/10.1007/S00425-003-1105-5

Werner, J. E., & Finkelstein, R. R. (1995). Arabidopsis mutants with reduced response to NaCl and osmotic stress. *Physiologia Plantarum*, *93*(4), 659–666. https://doi.org/10.1111/J.1399-3054.1995.TB05114.X

Wright, C., Van-Buren, L., Kroner, C., & Koning, M. M. (2007). Herbal medicines as diuretics: a review of the scientific evidence. *Journal of Ethnopharmacology*, *114*(1), 1–31. https://doi.org/10.1016/J.JEP.2007.07.023

doi:10.14720/aas.2022.118.2.1744 Original research article / izvirni znanstveni članek

Comparative study between fungicides and some chemical inducers for controlling root rot incidence of green bean (*Phaseolus vulgaris* L.) under field conditions

Nehal Samy EL-MOUGY [1], Nadia Gamel EL-GAMAL [1], Mohamed Saied Ali KALIL [1], Mokhtar Mohamed ABDEL-KADER [1, 2]

Received June 20, 2020; accepted March 24, 2022.
Delo je prispelo 20. junija 2020, sprejeto 24. marca 2022

Comparative study between fungicides and some chemical inducers for controlling root rot incidence of green bean (*Phaseolus vulgaris* L.) under field conditions

Abstract: Root rot disease caused by *Rhizoctonia solani* J.G. Kuhn and *Fusarium solani* (Mart.) Sacc. is a major problem restricting profitable farming of green bean (*Phaseolus vulgaris* L.). Under field conditions, some chemical inducers compared with chemical fungicides were evaluated for controlling bean root rot disease. Significant effect was observed for all applied treatments against disease incidence compared with control. Applied treatments of seed dressing plus foliar spray showed the highest reduction of root rot incidence followed by seed dressing then foliar spray. Salicylic acid as seed followed by foliar spray showed the highest suppressive effect against disease incidence followed by glutathione treatments. Furthermore, application of calcium silicate revealed higher effect against disease incidence compared with potassium and sodium silicate at both pre-, and post-emergence plant growth stages. Fungicidal treatments showed affect disease incidence in a lower extent compared with Plant Resistance Inducers (PRI). Treatment of Rizolex T50 followed by Topsin M70 was more effectively in controlling root rot than each fungicide alone. Such applied treatments could be useful for controlling root rot disease under field conditions

Key words: bean; fungicide alternatives; root rot; Rizolex T50; Topsin M70

Primerjalna raziskava fungicidov in kemičnih vzpodbujevalcev za nadzor koreninske gnilobe pri fižolu (*Phaseolus vulgaris* L.) v razmerah na prostem

Izvleček: Koreninska gniloba, ki jo povzročata glivi *Rhizoctonia solani* J.G. Kühn in *Fusarium solani* (Mart.) Sacc. je glavni omejujoči dejavnik za donosno pridelavo stročjega fižola(*Phaseolus vulgaris* L.). V razmerah poljskega poskusa so bili primerjani učinki kemičnih vzpodbujevalcev in kemičnih fungicidov v njihovi sposobnosti nadzora koreninske gnilobe. V primerjavi s kontrolo so bili opaženi značilni učinki vseh obravnavanj proti bolezni. Obravnavanja s kapsuliranimi semeni in foliarnimi pršili so pokazala največje zmanjšanje gnilobe, tem so sledila obravnavanja s kapsuliranimi semeni in nato obravnavanja s foliarnimi pršili. Salicilna kislina kot sredstvo obdelave semen in kot naknadno foliarno pršilo je imela največji učinek na zaviranje bolezni, temu je sledilo obravnavanje z glutationom. Nadalje je bila uporaba kalcijevega silikata bolj učinkovita pri zatiranju bolezni v primerjavi s kalijevim in natrijevim silikatom v obravnavanjih pred in po vzniku rastlin. Obravnavanja s fungicidi so pojav bolezni bolj zmanjšala kot tista z alternativnimi sredstvi. Obravnavanje s fungicidom Rizolex T50 in nato s fungicidom Topsinn M70 je bilo bolj učinkovito pri nadzoru gnilobe kot uporabi posameznih fungicidov. Našteta obravnavanja bi torej lahko bila koristna za nadzor fižolove gnilobe v poljskih razmerah.

Ključne besede: fižol; alternativni fungicidi; koreninska gniloba; Rizolex T50; Topsin M70

1 Plant Pathology Department, National Research Centre, Giza, Egypt
2 Corresponding author, e-mail: mokh_nrc@yahoo.com

1 INTRODUCTION

Soilborne plant pathogens are considered the main problems in agricultural production all-over the world that they affected seriously on plant stand causing great losses in produced yield. Therefore, growing plants are exposed to invasion by various soilborne pathogens during their different growth stages starts from seed sowing up to maturity. Bean (Phaseolus vulgaris L.) is one of food legume species that widely cultivated for domestic use, exportation, and it has considerable importance for human food especially in developing countries (Baudoin et al., 2001). Certain fungi could attack bean plants causing root rot, wilt and leaf spot diseases which greatly influenced plant stand and subsequently yield production. Root rot disease caused by particular soilborne pathogens have an effect on emerged seedlings and may be occurs earlier when seeds are attacked during their emergence causing pre-emergence infection leading to the need of re-sowing the missed hills or dead plants.

The most harmful soilborne fungi causing root rot disease of bean are Fusarium solani Sacc., Sclerotium rolfsii Sacc. and Rhizoctonia solani J.G. Kühn (Abdel-Kader, 1997; El-Mougy et al., 2007). A high buildup of root rot pathogen inoculums due to successive cultivation on the same land could leads to causes serious yield losses. Therefore, control of this disease is considered important especially in new reclaimed soil where green bean is wide prevalence crop in Egypt.

The present research focuses on comparing the use of fungicides and natural compounds that are capable to control root rot incidence. The objective of this study was evaluate some chemical fungicides and chemical inducers as alternatives to fungicide application to achieve effective management against the incidence of green bean root rot disease when used as seed dressing and/or foliar spray under natural field conditions.

2 MATERIALS AND METHODS

2.1 TESTED MATERIALS:

Green bean seeds (Phaseolus vulgaris 'Giza 3') kindly obtained from Vegetables Crop Research Department, Agricultural Research Centre, Giza, Egypt. The tested chemicals potassium silicate, calcium silicate, glutathione, salicylic acid and sodium silicate purchased from Al-Gamhoria Company Ltd. for chemicals and medicinal instruments, Cairo, Egypt. Meanwhile, the fungicides Rizolex-T 50 WP (20 % Tolclophos-methyl and 30 % Thiram) Sumi Agro Co. and Topsin M70 WSB (thiophanate-methyl 70 %) Martin's Co. purchased from local market.

2.2 FIELD EXPERIMENTS:

A field located at Al-Nubaria region, Beheira governorate, Egypt was chosen for this experiment. This field is well known by the authors to be characterized with semi homogeneous distribution with root rot pathogens mainly *Fusarium solani*, *Sclerotium rolfsii* and *Rhizoctonia solani* as naturally heavily infested soil. In addition, at prior growing season to the present study, samples of growing bean plants showing root rot disease symptoms were collected and subjected to isolation trails for the causal organisms.

The present field experiment was carried out for two successive growing seasons (March and September, 2019) to evaluate the efficacy of some chemical inducers and fungicides applied as seed dressing and/or foliar spray against root rot incidence. The experimental field contains plots (6 × 7 m), each included 12 rows with 35 holes. In all plots green bean seeds of ´Giza 3` were sown at the rate of three seed/hole. Seed dressing was carried out before sowing, meanwhile foliar spray was applied twice, the first was at the two true leaves age of emerged seedlings and the second after 15-day interval. All treated plants were sprayed using 20 l sprayer for each plot.

2.3 APPLICATIONS TO THE EXPERIMENTAL FIELD

For the two cultivation seasons the same proposed treatments were designed as follows:

a) Seed treatment at the rate of 3 g kg^{-1}
T1- potassium silicate
T2- calcium silicate
T3- glutathione
T4- salicylic acid
T5- sodium silicate
T6- Rizolex T50

b) Seed treatment at the rate of 3g l^{-1} + foliar spray at the rate of 3 g l^{-1}
T7- potassium silicate + potassium silicate
T8- calcium silicate + calcium silicate
T9- glutathione + glutathione
T10- salicylic acid + salicylic acid
T11- sodium silicate + sodium silicate
T12- Rizolex T50 (3 g l^{-1}) + Topsin M70 (3 g l^{-1})

c) Foliar spray at the rate of 3 g l^{-1}
T13- potassium silicate
T14- calcium silicate
T15- glutathione
T16- salicylic acid
T17- sodium silicate
T18- Topsin M70
T19- untreated control

2.4 DISEASE AND YIELD ASSESSMENT

Three plots as replicates were used for every specific treatment as well as untreated control. All plots were conducted in completely randomized block design. The traditional agricultural practices, that is, soil plowing, fertilization, irrigation, etc., were followed at all experimental plots. Monitoring and scouting for diseases incidence in all cultivated plots were preformed weekly (El-Mougy and Abdel-Kader et al., 2018). At all applied treatments and control as well the percent of pre-emergence root rot disease incidence was recorded after 15 days from sowing date as numbers of emerged seedlings referring to the numbers of sown seeds. Meanwhile, the percentage of post-emergence disease infection were recorded after 15, 30 and 45 days as numbers of diseases plants referring to the numbers of emerged seedlings. Percentage of healthy survivals was calculated as the numbers of survival plants referring to the numbers of sown seeds. Accumulated yield was determined as fresh pods (kg/plot) for each particular treatment at the end of growing season. The increase of obtained yield in relative to comparison treatment was also calculated.

2.5 STATISTICAL ANALYSIS

The obtained data subjected to analysis of variance using IBM SPSS software version 14.0. Mean separation performed using Duncan's Multiple Range Test at $p \leq 0.05$ by the MSTAT-C software

3 RESULTS

The fungi isolated from the bean plants showing root rot symptoms were identified as *Fusarium solani* and *Rhizoctonia solani.* The results showed that all applied treatments had announced effects against root rot incidence compared with the control (Table 1).

Applied treatments of a seed dressing + foliar spray showed lower root rot disease incidence followed by seed dressing alone, and then foliar spray alone. Moreover, the effects of the fungicide applications were lower than those of the chemical inducers and followed similar trend as the applied methods mentioned above. The results also indicate that the application of salicylic acid as a seed and/or foliar spray showed the highest reduction in root rot incidence followed by the glutathione treatments. The percentage of pre- and post-emergence root rot was recorded as 5.3 %, 8.0 % [T4]; 5.5 %, 6.5 % [T10] and 15.6 %, 21.0 % [T16] followed by 7.3 %, 12.6 % [T3]; 7.5 %, 9.6 % [T9] and 14.3 %, 27.0 % [T15] when compared with the 30.0 %, 57.3 % of the untreated control treatment [T19]. Moreover, the pre- and post-emergence potassium silicate [T1], [T7] applications showed greater disease reduction when compared with the calcium [T2[, [T8] and sodium silicate [T5], [11].

For the seed dressing, seed dressing + foliar spray, and foliar spray treatments disease reduction was calculated as 70.0 %, 73.8 % [T2]; 72.0 %, 80.8 % [T8] and 38.0 %, 46.5 % [14] in relation to the control treatment [T19], respectively. Meanwhile for the same applied treatments disease reductions of 61.3 %, 74.6 % [T1]; 60.0 %, 82.0 % [T7] and 21.6 %, 38.9 % [T13] were identified. Likewise, for treatments [T5], [T11], and [T17] the percentages of root rot disease reduction were 60.0 %, 69.4 %; 56.6 %, 75.0 %, 30.0, and 41.8 % at pre-, and post-emergence growth stages, in respective order.

The fungicidal treatment data showed that the combination of two fungicides applied as a seed dressing + foliar spray resulted in lower disease incidence when compared with the seed dressing and foliar spray, in descending order (Table 1). The percentage of root rot incidence for [T12] was 9.5 % and 10.6 % with disease reduction of 68.3 % and 81.5 % at the pre-, and post-emergence growth plant stages, respectively. Furthermore, for the [T6] treatment, disease incidence and reduction were recorded as 9.6 %, 14.6 %, and 68.0 %, 74.5 % for both plant growth stages in parallel, respectively. Meanwhile a higher root rot incidence of 27.3 %, 40.0 % and its lower reduction of 9.0 %, 30.1 % were recorded when Topsin M70 [18] was applied only as a foliar spray, although it significantly differed when compared with the untreated control [T19].

The results also revealed that applied treatments increased plant survival when compared with the untreated control (Table 1). The highest percentage of plant survival recorded with the seed dressing + foliar spray was followed by seed dressing then foliar spray treatments, respectively. The highest percentages of plant survival were 88.0 %, 81.9 %, and 81.5 % with the [T10], [T9], and [T8] treatments, followed by 86.6 %, 78.6 %, and 77.3 % with the [T4], [T3], and [T2] treat-

ments, and 63.3 %, 58.6 %, and 50.6 % with the [T16], [T15], and [T14] treatments. The other applied chemical inducer treatments ranged between 37.5 % with the [T13] treatment to 77.6 % with the [T7] treatment for plant survival. For the fungicide applications there was 79.8 %, 75.6 %, and 32.6 % survival with the [T12] Rizolex T50 WP + Topsin M70 WSB followed by [T6] Rizolex-T50 WP and [T18] Topsin M70 WSB, respectively which was significant when compared with 12.6% for the [T19] control treatment.

In contrast, the data indicated that the obtained decreases in root rot disease incidence due to the current applied treatments resulted in an increase in plant survival which was subsequently reflected in the accumulated product yield.

The green pod yields from the beans showed similar tendencies to the reduction in disease incidence identified in Table 1 and Fig. 1. The highest obtained yields recorded were 27.8, 26.2, and 24.6 kg/plot with an increase over the control calculated as 78.2 %, 67.9 %, and 57.6 % for the [T10], [T9], and [T8] treatments which included a seed dressing + foliar spray treatments, respectively. There were moderate yield increases of 70.5 %, 55.1 %, and 42.9 % for the [T4], [T3], and [T2] treatments which involved seed dressing. Percentages of 71.7 %, 50.0 %, and 36.5 % indicated lower increases in the produced yields and were recorded for the [T16], [T15], and [T14] treatments which involved foliar sprays. The rest of the applied chemical treatments revealed a yield increase between 12.8 % for [T13] with a foliar spray and 44.8 % for [T7] with a seed dressing + foliar spray.

Table 1: Average efficacy of some chemical inducers and fungicides against bean root rot incidence during two growing seasons under field conditions

	Root rot diseases incidence (%)				
	Pre-emergence**	Red.* (%)	Post-emergence**	Red.* (%)	Plant survivals (%)
			Seed dressing		
[T1] Potassium silicate	11.6 ± 0.8 cd	61.3	14.5 ± 1.9 de	74.6	73.8 ± 1.0 f
[T2] Calcium silicate	9.0 ± 0.5 e	70.0	15.0 ± 1.0 de	73.8	77.3 ± 1.1f
[T3] Glutathione	7.3 ± 0.5 f	75.6	12.6 ± 0.5 e	78.0	78.6 ± 2.0 f
[T4] Salicylic acid	5.3 ± 0.7 g	82.3	8.0 ± 1.5 fg	86.0	86.6 ± 1.2 g
[T5] Sodium silicate	12.0 ± 2.4 cd	60.0	17.5 ± 0.8 d	69.4	70.5 ± 1.7 f
[T6] Rizolex-T50	9.6 ± 0.8 e	68.0	14.6 ± 1.4 de	74.5	75.6 ± 1.9 f
			Seed dressing + Foliar spray		
[T7] Potassium silicate	12.0 ± 1.1 cd	60.0	10.3 ± 1.6 f	82.0	77.6 ± 1.1f
[T8] Calcium silicate	8.4 ± 0.8 ef	72.0	11.0 ± 1.7 e	80.8	81.5 ± 1.7 g
[T9] Glutathione	7.5 ± 0.8 f	75.0	9.6 ± 2.8 f	83.2	81.9 ± 1.7 g
[T10] Salicylic acid	5.5 ± 0.8 g	81.6	6.5 ± 2.6 g	88.6	88.0 ± 0.4 g
[T11] Sodium silicate	13.0 ± 1.1 cd	56.6	14.3 ± 2.3 de	75.0	72.6 ± 0.8 f
[T12] Rizolex-T50 + Topsin M70	9.5 ± 2.9 e	68.3	10.6 ± 1.0 f	81.5	79.8 ± 1.5 f
			Foliar spray		
[T13] Potassium silicate	23.5 ± 2.4 ab	21.6	35.0 ± 1.5 ab	38.9	37.5 ± 0.8 b
[T14] Calcium silicate	18.6 ± 2.3 b	38.0	30.6 ± 2.0 b	46.5	50.6 ± 2.0 d
[T15] Glutathione	14.3 ± 2.7 c	52.3	27.0 ± 1.3 bc	52.8	58.6 ± 1.0 d
[T16] Salicylic acid	15.6 ± 1.2 c	48.0	21.0 ± 0.6 c	63.3	63.3 ± 0.5 e
[T17] Sodium silicate	21.0 ± 0.6 ab	30.0	33.3 ± 3.3 ab	41.8	45.6 ± 1.2 c
[T18] Topsin M70	27.3 ± 0.4 a	9.0	40.0 ± 0.3 ab	30.1	32.6 ± 0.8 b
[T19] Control	30.0 ± 1.5 a	-	57.3 ± 1.3 a	-	12.6 ± 1.8 a

* Red. = Reduction
** Pre-emergence calculated after 15 days from seed sowing
*** post-emergence calculated after 45 days from seedlings emergence
Means ± standard deviations within a column followed by the same letter are not significantly different by Duncan multiple range test at $p < 0.05$

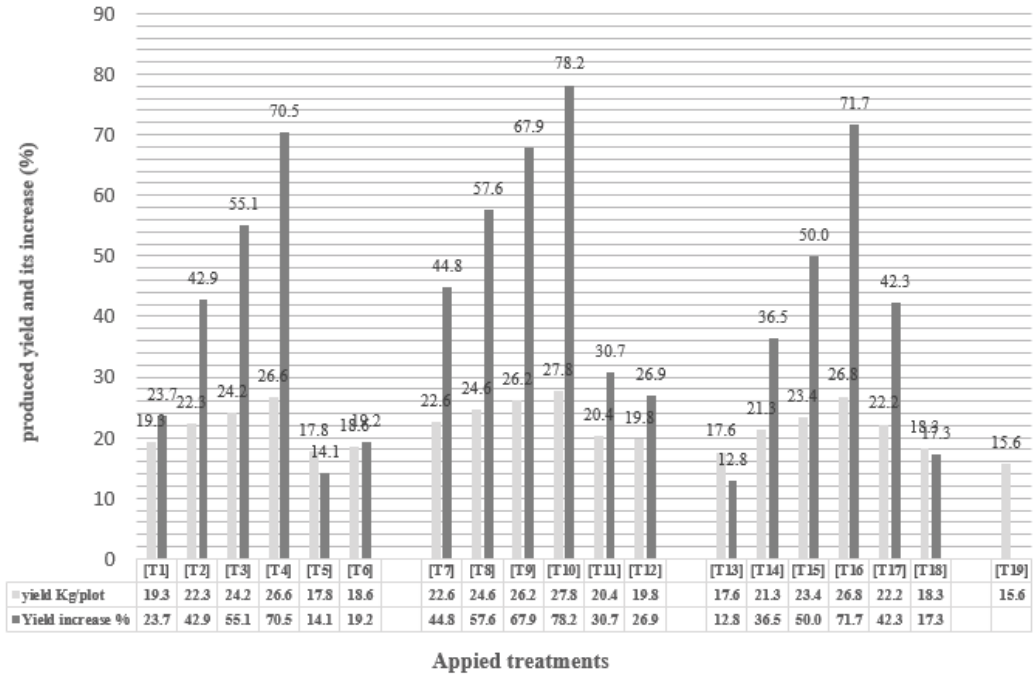

Fig. 1: Average accumulated yield of bean plants and its increase (%) in response to application of some chemical inducers and fungicides at two growing seasons under field conditions

For the fungicidal applications, yield was increased by 26.9 % for [T12] in response to the application of Rizolex T50 WP as a seed dressing followed by a foliar spray with Topsin M70 WSB. Meanwhile yield increases of 19.2 % and 17.3 % were recorded for [T6] and [T18] with the Rizolex T50 WP seed dressing and Topsin M70 WSB foliar spray, respectively.

4 DISCUSSION

In the current study the fungi isolated from the collected bean plants that showed root rot symptoms were identified as *Fusarium* sp. and *Rhizoctonia solani* Root rot disease of green caused by *F. solani* and *R. solani* has been previously reported (Abdel-Kader, 1997, El-Mougy et al., 2007, El-Mougy and Abdel-Kader, 2018). Additionally, common bean, cowpea, and faba bean, which are suitable pathogen hosts, are regularly grown in the study field, thus, it is assumed that there is an increasing population of the soilborne root rot pathogen as these crops are considered suitable hosts. This investigation aimed to evaluate the use of chemical inducers as fungicide alternatives when compared with chemical fungicides applied as seed dressing and/or foliar sprays to control the incidence of green bean root rot disease under field conditions. The obtained results revealed that the applied chemical inducers as well as the fungicidal treatments were all highly effective at reducing disease incidence and increasing yield. In this regard, salicylic acid treatments as a seed dressing and/or foliar spray had high efficacy against root rot incidence and yield increase. These results are in accordance with those of previous studies (El-Mougy et al., 2019). El-Mohamady et al. (2017) reported on the use of chemical inducers, such as chitosan (CH), salicylic acid (SA), and humic acid (HA) for the control of bean root rot caused by *Fusarium solani* and *Rhizoctonia solani* under both greenhouse and field conditions. They found that soaking bean seeds in CH 1.0 g/l^{-1} + SA 5% followed by foliar applications at half of this concentration, caused a superior reduction in both damping-off and root rot incidence when compared with their other applied treatments. In addition, Anderson (1988) reported that salicylic acid as a phenolic compound acts as a regulator key of the internal coding network in plants under either abiotic or biotic stress. It plays a major role in the plant resistance functions against pathogens as it promotes the production of pathogenesis-related proteins (PRPs). Furthermore, salicylic acid was accountable for the aggregation of phytoalexins in viable plant tissues. Mandel et al. (2009) stated that external or internal operators might ultimately affect the host plant physiology, leading to the fast and harmonic activation of defense-genes in plants which were susceptible to parasite infections. However, Jabnoun et al. (2015) reported that

systemic acquired resistance for controlling tomato fungal diseases could be induced by using salicylic acid and chitosan as chemical inducers. Likewise, several workers reported yield increases when chemical inducers or fungicides were applied (El-Mougy et al. 2007, El-Mohamady and Abd-El-Baky, 2008, Abd-El-Kareem et al., 2013, Abdel-Kader et. al., 2014, El-Mohamady et al., 2017, El-Mougy et al., 2019). In the present study the used chemical inducers, salicylic acid and potassium, calcium, sodium silicate had high activity against root rot incidence and yield increase as well as when applied as a seed dressing and/or foliar spray. Recently, the application of several chemical inducers received a large amount of attention due to various investigations into the control of plant diseases. Glutathione (GSH) as an antioxidant has a role in regulating plant tolerance to biotic stresses by repressing localized necrotic symptoms following viral infections. Utilizing the pharmacological and transgenic approaches confirmed the role of GSH for reducing disease symptoms in plants which were induced by pathogen infections. Furthermore, recent studies have shown that GSH also has a key role in restricting pathogen levels. In fact, it seems that GSH is a vital agent responsible for the elicitors involved in different types of plant disease resistance (Gullner et al., 2017). Furthermore, glutathione (GSH) was reported to be involved in the activation and regulation of the biosynthetic processes involved in plant defense (Bolter et al., 1993). Moreover, glutathione functions include several roles in biosynthetic pathways, detoxification, antioxidant biochemistry, and redox homeostasis (Noctor et al., 2012). In contrast, applications of silicon salts proved their activity against pathogenic fungal growth *in vitro* as well as plant disease incidence. Silicates were reported to have efficacy for reducing plant diseases in rice (Datnoff et al., 1997), strawberry (Kanto et al., 2006), wheat (Belanger et al., 2003), and cucumber (Menzies et al., 1992). Biggs et al. (1997) stated that PDA medium supplemented with calcium silicate inhibited 65% of the growth of *Monilinia fructicola* (G.Winter) Honey the causal agent of peach brown rot. Furthermore, it also inhibited mycelial growth for several phytopathogenic fungi grown on potassium silicate amended media in vitro (Bekker et al., 2006, 2009). Li et al. (2009) also reported an inhibitor effect for sodium silicate against the growth of *Fusarium sulphuureum* Schltdi. *in vitro*. Moreover, an in vivo foliar spray of potato plants with 100 and 200 mM sodium silicate was found to control tuber dry rot disease effectively. They concluded that sodium silicate has direct fungitoxic effects against the fungal pathogen. Furthermore, it was reported that soluble potassium silicate applied as a root and foliar spray caused reductions in disease incidence as well as an increase in growth and fruit quality for capsicum plants (Jayawardana et al., 2014). Ultimately, Shen et al. (2010) stated that it is probable that a reduction in disease incidence in plants treated with silicon sources under field conditions is not likely to be attributed to the fungistatic effects of silicon, but could act as a physical block which directly prevents pathogen penetration to plant tissues or indirectly by enhancing plant defense responses. It is suggested that silicon may act as the first protecting block in treated plants and could inhibit pathogen colonization and subsequent infection. Therefore, potassium silicate could be used as a fungicide since it has direct inhibitor effects on fungal growth and its ability to increase plant self-defense systems (Menzies et al., 1992) and strengthen plant cell walls, inhibiting disease infection (Epstein, 1999). The current investigation showed the lesser effects of contact and systemic fungicides for controlling bean root rot incidence when compared with chemical inducers, despite their superior activity over control treatments against disease occurrence. The fungicide Rizolex (Tolclofos - methyl) is an organophosphate ester chemical compound which has protective, curative and slightly systemic action and has high fungitoxicity to *Rhizoctonia solani* and *Sclerotium rolfsii* (Ohtsuki and Fujinami, 1982). Rizolex was reported to actively reduce the incidence of southern stem rot caused by *R. solani* and *S. rolfsii* and increase peanut yield (Csinos, 1985). Rizolex T50 is a seed treatment fungicide that delivers protection against a broad spectrum of soil borne and seed/seedling diseases, including *Rhizoctonia* damping-off and *Fusarium* (Hopkins 2013). Hamed (2008) reported that Rizolex completely inhibited the growth of *Rhzictonia solani* (100 %) at all concentrations (0.025, 0.05, 0.25, and 0.5 ppm) *in vitro*. Recently, El-Mohamady et al. (2017) concluded that using chemical inducers in comparison with the fungicide Rizolex had superior effects to RIS alone against bean damping-off, root rot diseases, and increasing the produced yields, and therefore may be considered as an eco-friendly applied method for the control of soil-borne plant pathogens. Topsin M70 (thiophanate-methyl) is a fungicidal substance which belongs to the agent group of benzimidazoles. It is a wide range systemic fungicide controlling a wide variety of plant pathogens (Hirschfeld et al. 2010). The primary effect of thiophanate-methyl is caused by the transformation product methyl-benzimidazole-2yl-carbamate, which binds to the fungal tubulin and disturbs the formation of the spindle apparatus during mitosis so that homologous chromosomes cannot divide, and cell growth will be inhibited. It is absorbed by the treated plant roots, leaves, and has a protective and curative action (Saber, et. al, 2011). A

combine use of the fungicides Topsin-M and Dimecron showed significant increase in phenolic content (Siddiqui et. al., 1999). Increase in phenolic and phenolic content produced as a result of stress may act as a protective compound against pathogenic fungi and insects (Friend, 1979). *In vitro* tests with Rizolex-T and Topsin-M at 200 ppm have completely inhibited the growth of *R. solani* and *F. oxysporum* Schlecht. emend. Snyder & Hansen the causal pathogens of root rot and wilt complex disease, which used soil culture medium in some surveyed nurseries (Abdel-Kader et. al., 2004). Moreover, the use of Topsin M70 as a foliar spray was effective against various plant diseases, such as citrus mold caused by *Penicillium italicum* Wehmer (Kanan and Al-Najar, 2009), *Fusarium mangiferae* Kanan and Al-Najar, 2009), *Fusarium mangiferae* Britz, Wingfield & Marasas on mango (Iqbal et al., 2010), *Phytophthora infestans* (Mont.) de Bary the causal agent of tomato late blight (Meya et al., 2014), faba bean chocolate spot caused by *Botrytis fabae* Sardina (Moustafa et al., 2015), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. the causal of die-back of grapevine (El-Habbaa et al., 2016) and *Fusarium oxysporum* f. sp. *capsici* the causal agent of wilt disease on chili pepper (Bashir et. al., 2018).

The present investigation demonstrates that the applied chemical inducers and fungicides were efficient at controlling green bean root rot disease incidence and increased the accumulated yield. Treatments of seed dressing followed by foliar spray showed higher effectivity on disease incidence and produced greater yields than with the seed dressing or foliar spray individually.

5 CONCLUSIONS

The results of the current study suggest that the combined application method of chemical inducers such as seed and/or foliar sprays was superior to single treatments for reducing root rot incidence on green bean plants and increasing accumulated pod yields. The commercial fungicides Rizolex T50 WP and Topsin M70 WSB showed similar trends for controlling disease incidence. Such treatments may be used commercially and could be said to have characteristics such as being eco-friendly, safe, cheap, and an easily applied alternative fungicide methods for use in natural field conditions.

6 REFERENCES

Abdel-Kader, M.M. (1997). Field application of *Trichoderma harzianum* as biocide for control of bean root rot disease. *Egyptian Journal of Phtopathology, 25*, 19-25.

Abdel-Kader, M.M., El-Bahr, M.K. & El-Mougy, N.S. (2004). Pathogenic Fungi and Soil Conditions Causing Root Rot and Wilt Disease Complex during Acclimatization of Tissue Culture-Derived Banana Plantlets. *Egyptian Journal of Phytopathology, 32*(1-2), 37-48. https://www.researchgate.net/publication/237702728

Abdel-Kader, M.M., El-Mougy, N.S., & Nadia Gad. (2014). Some Control Measures against Lupine Wilt Disease Incidence under Field Conditions. *International Journal of Engineering and Innovative Technology, 4*(2), 239-243. http://www.ijeit.com/Vol%204/Issue%202/IJEIT1412201408_41.pdf

Abd-El-Karem, F. Abdel-Kader, M.M., Fotouh, Y.O., Abd-Alla, A.M. El-Mougy, N.S., El- Mohamedy, R.S. & El-Gamal, N.G. (2013). Induction of Systemic Resistance in faba bean Against chocolate spot Diseases Severity Using Chemical Inducers under Field Conditions. *Journal of Applied Sciences Research, 9*(6), 4006-4014. http://www.aensiweb.com/old/jasr/jasr/2013/4006-4014.pdf

Anderson, A.J. (1988). Elicitors the hypersensitive response and phytoalexins. Pages 103-110 in: Physiology and Biochemistry of *Plant-Microbial Interactions*. American Society of Plant *Physiology*, Rockville, MD.

Bashir, M.R., Atiq, M., Sajid, M., Mohsan, M., Abbas, W., Alam, M.W. & Bashir, M. (2018). Antifungal exploitation of fungicides against *Fusarium oxysporum* f. sp. *capsici* causing *Fusarium* wilt of chilli pepper in Pakistan. *Environmental Science and Pollution Research, 25*(7), 6797-6801. https://doi.org/10.1007/s11356-017-1032-9

Baudoin, J.P., Camarena, F., Lobo, M., & Mergeai, G. (2001). Breeding Phaseolus for intercrop combinations in Andean highlands. In: Cooper, H.D., Spillane, C. and Hodgkin, T. eds. *Broadening the genetic bases of crop*. Oxford, UK: CABI Publishing, pp. 373-384. https://doi.org/10.1079/9780851994116.0373

Bekker, T.F., Kaiser, C., & Labuschagne, N. (2009). The antifungal activity of potassium silicate and the role of pH against selected plant pathogenic fungi *in vitro*. S *South African Journal of Plant and Soil 26*(1), 55-57. https://doi.org/10.1080/02571862.2009.10639934

Bekker, T.F., Kaiser, C., Van Der Merwe, R., & Labuschagne, N. (2006). *In vitro* inhibition of mycelial growth of several phytopathogenic fungi by soluble silicon. *South African Journal of Plant and Soil, 23*(3), 169-172. https://doi.org/10.1080/02571862.2006.10634750

Belanger, R.R., Benhamou, N., & Menzies, J.G. (2003). Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*). *Phytopathology, 93,* 402–12. https://doi.org/10.1094/PHYTO.2003.93.4.402

Bolter, C., Brammall, R.A., Cohen, R., & Lazarovits, G. (1993). Glutathione alterations in melon and tomato roots following treatment with chemicals which induce disease resistance to Fusarium wilt. *Physiological and Molecular Plant Pathology, 42*(5), 321-336. https://doi.org/10.1016/S0885-5765(05)80009-8

Csinos, A.S. (1985). Activity of Tolclofos - Methyl (Rizolex) on

Sclerotium rolfiii and *Rhizoctonia solani* in Peanut. *Peanut Science, 12,* 32-35. https://www.peanutscience.com/doi/pdf/10.3146/pnut.12.1.0008

Datnoff, L.E., Deren, C.W., & Snyder, G.H. (1997). Silicon fertilization for disease management of rice in Florida. *Crop Protection, 16*, 525–531. https://doi.org/10.1016/S0261-2194(97)00033-1

El-Habbaa, G.M., Mahdy, A.M.M., Mohamed, F.G., & El-Shaery, S.A. (2016). Biological and chemical control of grapevine die-back disease and their effect on defense related enzymes. *International Journal of Scientific & Engineering Research, 7*(3), 345-351. https://www.ijser.org/researchpaper/Biological-and-chemical-control-of-grapevine-die-back-disease-and-their-effect-on-defense-related-enzymes.pdf

El-Mohamedy, R.S.R., & Abd–El-Baky, M.M.H. (2008). Effect of seed treatment on control of root rots disease and improvement of growth and yield of pea plants. *Middle Eastern and Russian Journal of Plant Science and Biotechnology, 2*(2), 84-90. http://www.globalsciencebooks.info/Online/GSBOnline/images/0812/MERJPSB_2(1&2)/MERJPSB_2(2)84-90o.pdf

El-Mohamedy, R.S.R., Shafeek, M.R., Abd El-Samad, E.H., Salama, D.M., & Rizk, F. A. (2017). Field application of plant resistance inducers (PRIs) to control important root rot diseases and improvement growth and yield of green bean (*Phaseolus vulgaris* L.). *Australin Journal of Crop Science, 11*(5), 496-505. https://doi.org/10.21475/ajcs.17.11.05.p260

El-Mougy, N.S., & Abdel-Kader, M.M. (2018). Control of green bean root rot incidence using bioagent and antioxidants as seed dressing under natural field conditions. *BioScience Research, 15*(2), 1401-1408. https://www.isisn.org/BR15(2)2018/1401-1408-15(2)2018BR18-190.pdf

El-Mougy, N.S., El-Gamal, N.G., & Abdel-Kader, M.M. (2007). Control of wilt and root rot incidence in Phaseolus vulgaris L. by some volatile compounds. *Journal of Plant Protection Research, 47*(3), 255–265. http://www.plantprotection.pl/Control-of-wilt-and-root-rot-incidence-in-Phaseolus-vulgaris-L-by-some-plant-volatile,91109,0,2.html

El-Mougy, N.S., El-Mohamady, R.S., El-Gamal, N.G., & Abdel-Kader, MM (2019). Efficacy of some chemical resistance inducers agents and nitrogen-fixing *rhizobium* for suppressing root rot and wilt diseases incidence of *phaseolus vulgaris* L. under natural field conditions. *BioScience Research, 16*(1), 834-842. https://www.isisn.org/BR16(1)2019/834-842-16(1)2019BR18-566.pdf

Epstein, E. (1999). Silicon. *Annual Review of Plant Physiology and Plant Molecular Biology, 50*, 641-664. https://doi.org/10.1146/annurev.arplant.50.1.641

Friend, J. (1979). Phenolic substances and plant disease. *Recent Advances in Phytochemistry*, *12*, 557-588. https://doi.org/10.1007/978-1-4684-3372-2_17

Gullner G., Zechmann B., Künstler A., & Király L. (2017) The Signaling Roles of Glutathione in Plant Disease Resistance. In: Hossain M., Mostofa M., Diaz-Vivancos P., Burritt D., Fujita M., Tran LS. (eds) *Glutathione in Plant Growth, Development, and Stress Tolerance*. Springer, Cham. https://doi.org/10.1007/978-3-319-66682-2_15

Hamed, F.R. (2008). Effect of Rizolex on *Rhizoctonia solani* Kuhn isolates and biocontrol fungus *Trichoderma harzianum* Rifai *in vitro*. *Journal of Kerbala University, 6*(1), 218-223. https://www.iasj.net/iasj?func=fulltext&aId=50236

Hirschfeld, T., Ellner, F., Buschhaus, H., & Gossmann, M. (2010). New insight in the mode of action of thiophanate-methyl. Modern fungicides and antifungal compounds VI. 16th *International Reinhardsbrunn Symposium, Friedrichroda, Germany,* April 25-29, 2010 pp.417-421. https://www.cabdirect.org/cabdirect/abstract/20153047734

Hopkins, M. (2013). Valent's Rizolex Fungicide Seed Treatment Approved. https://www.croplife.com/crop-inputs/fungicides/valents-rizolex-fungicide-seed-treatment-approved/

Jayawardana, H.A.R.K., Weerahewa, H.L.D. & Saparamadu, M.D.J.S. (2014). Effect of root or foliar application of soluble silicon on plant growth, fruit quality and anthracnose development of capsicum. *Tropical Agricultural Research, 26*(1), 74–81. *https://doi.org/10.4038/tar.v26i1.8073*

Iqbal, Z., Pervez, M.A., Ahmed, S., Iftikhar, Y., Yasin, M., Nawaz, A., Ghazanfar, M.u., Dasti, A.A., & saleem, A. (2010). Determination of minimum inhibitory concentrations of fungicides against fungus *Fusarium mangiferae*. *Pakistan Journal of Botany, 42*(5), 3525-3532. http://www.pakbs.org/pjbot/PDFs/42(5)/PJB42(5)3525.pdf

Jabnoun, K.H., El-Mohamedy, R.S.R., Abdel-Kareem, F.I., Ben Abdallah, R.A., Gueddes-Chahed, M., & Daami-Remadi, M. (2015). Variation in chitosan and salicylic acid efficacy towards soil-borne and air-borne fungi and their suppressive effect of tomato wilt severity. *Journal of Plant Pathology and Microbiology, 6*(11), 1-10. https://doi.org/10.4172/2157-7471.1000325

Kanan, G.J.M., & Al-Najar, R.A.K. (2009). In vitro deferential sensitivity of *Pencillium italicum* causing postharvest rot to citrus fruits in Jordan to chemical fungicides and their combinations. *Journal of Plant Protection Research, 49*(1), 77-86. https://doi.org/10.2478/v10045-009-0010-8

Kanto, T., Miyoshi, A., Ogawa, T., Maekawa, K., & Aino, M. (2006). Suppressive effect of liquid potassium silicate on powdery mildew of strawberry in soil. *Journal of General Plant Pathology, 72*, 137–42. https://doi.org/10.1007/s10327-005-0270-8

Li, Y.C., Bi, Y., Ge, Y.H., Sun, X.J., & Wang, Y. (2009). Antifungal activity of sodium silicate on *Fusarium sulphureum* and its effect on dry rot of potato tubers. *Journal of food Science, 74*(5), 213-218. https://doi.org/10.1111/j.1750-3841.2009.01154.x

Mandel, M.J., Wollenberg, M.S., Stabb, E.V., Visick, K.L., & Ruby, E.G. (2009). A single regulatory gene is sufficient alter bacterial host range. *Nature, 458*, 215-218. https://doi.org/10.1038/nature07660

Menzies, J., Bowen, P., Ehret, D., & Glass, A.D.M. (1992). Foliar applications of potassium silicate reduce severity of powdery mildew on cucumber, muskmelon and zucchini squash. *Journal of American Society for Horticultural Science, 117*, 902-905. https://journals.ashs.org/jashs/view/journals/jashs/117/6/article-p902.xml

Menzies, J., Bowen, P., Ehret, D., & Glass, A.D.M. (1992). Fo-

liar applications of potassium silicate reduce severity of powdery mildew on cucumber, muskmelon, and zucchini squash. *Journal of American Society for Horticultural Science, 117*, 902–905. https://journals.ashs.org/jashs/view/journals/jashs/117/6/article-p902.xml

Meya, A.I., Mamiro, D.P., Kusolwa, P.M., Maerere, A.P., Sibuga, K.P., Erbaugh, M., Miller, S.A., & Mtui, H.D. (2014). Management of tomato late blight disease using reduced fungicide spray regimes in Morogoro, Tanzania. *Tanzania Journal of Agricultural Sciences, 13*(2), 8-17. http://www.suaire.suanet.ac.tz:8080/xmlui/handle/123456789/1303

Moustafa, M.S.H., El-Dakar, H.A.M., & Ismail, A.A. (2015). Adverse effect of rainfall on the efficacy of some fungicides used to control chocolate spot of faba bean (*Vicia faba*). *International Journal of Scientific & Engineering Research, 6*(12), 915-925. https://www.ijser.org/researchpaper/Adverse-Effect-of-Rainfall-on-the-Efficacy-of--some-Fungicides-Used-to-Control-Chocolate-Spot-of-Faba-Bean-Vicia-faba.pdf

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez, B., Garcia, A., Guillaume, G., & Foyer, C.H. (2012). Glutathione in plants: an integrated overview. *Plant, Cell and Environment, 35,* 454–484. https://doi.org/10.1111/j.1365-3040.2011.02400.x

Ohtsuki, S. and Fujinami, A. (1982). Rizolex (tolclofos-methyl). *Japan Pesticide Information, 41,* 21-25. http://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=PASCALAGROLINEINRA83X0220933

Saber, A.S., Mahran, A.H., & Abdel-Maksoud, M.A. (2011). Suppressive effect of Ginkgo biloba extract (EGb 761) on topsin induced ovarian toxicity and oxidative stress in albino rats. *Journal of Applied Pharmaceutical Science, 1*(4), 46-54. https://www.japsonline.com/abstract.php?article_id=59&sts=2

Shen, X., Zhou, Y., Duan, L., Li, Z., Eneji, A.E. & Li, J. (2010). Silicon effects on photosynthesis and antioxidant parameters of soybean seedlings under drought and ultraviolet-B radiation. *Journal of Plant Physiology 167*(15), 1248-1252. https://pubmed.ncbi.nlm.nih.gov/20713250/

doi:10.14720/aas.2022.118.2.1803 Original research article / izvirni znanstveni članek

Influence of different sources of nitrogen fertilizer and weed control on yield, yield components and some qualitative traits of chickpea (*Cicer arietinum* L.) cultivars under dryland conditions of Khorramabad

Sajad KORDI [1, 2], Tayebeh DANAYE-TOUS [3], Soheila DASTBORHAN [1]

Received July 30, 2020; accepted April 03, 2022.
Delo je prispelo 30. julija 2020, sprejeto 3. aprila 2022

Influence of different sources of nitrogen fertilizer and weed control on yield, yield components and some qualitative traits of chickpea (*Cicer arietinum* L.) cultivars under dryland conditions of Khorramabad

Abstract: A field experiment was conducted to evaluate yield, yield components, and some qualitative traits of chickpea (*Cicer arietinum* L.) cultivars under nitrogen fertilizers and weed control in dryland conditions of Khorramabad during the 2017 - 2018 growing season. Treatments were arranged in split-split-plot based on a randomized complete block design with three replications. The main factor included F1: control (without fertilizer); F2: bio-fertilizer (*Rhizobium*); F3: 100 % chemical fertilizer and F4: integration of bio-fertilizer + 50 % chemical fertilizer; sub-factor consisted of three cultivars of chickpea (Adel, Mansour, and Arman) and sub-sub-factor included weeds control (weeding) and weed infested (non-weeding). The results indicated that nitrogen fertilizers, especially the integration of bio-fertilizer + 50 % chemical fertilizer, had a positive effect on all studied traits. The highest number of pods per plant, grain yield, and biological yield were obtained from the Arman cultivar with the application of bio-fertilizer + 50 % chemical fertilizer and for the same cultivar under weed control conditions. The maximum number of pods per plant (28.2) and amount of grain protein content (25.3 %) were obtained by integrating of bio-fertilizer + 50% nitrogen chemical fertilizer and weeds control. In general, the Arman cultivar has priority over other cultivars for the grain yield under Khorramabad climate conditions, and integration of bio-fertilizer + 50 % chemical fertilizer could be considered as a means to reduce the consumption of chemical fertilizers for sustainable agriculture.

Key words: chickpea; grain protein; grain yield; hectoliter mass; *Rhizobium*; weed control

Vpliv različnih dušikovih gnojil in uravnavanja plevelov na pridelek, komponente pridelka in kakovostne lastnosti sort čičerke (*Cicer arietinum* L.) v sušnih razmerah Khorramabada

Izvleček: Za ovrednotenje pridelka, njegovih komponent in nekaterih kakovostnih lastnosti sort čičerke (*Cicer arietinum* L.) je bil izveden poljski poskus z gnojenjem z različnimi dušikovimi gnojili in načini zatiranja plevelov v sušnih razmerah Khorramabada v rastnih sezonah 2017 in 2018. Obravnavanja so bila izvedena kot popolen naključni bločni poskus z deljenkami s tremi ponovitvami. Glavna obravnavanja so bila: F1: kontrola (brez gnojil); F2: biognojila (*Rhizobium*); F3: 100 % mineralna gnojila in F4: integracija biognojil + 50 % mineralnih gnojil. Podobravnavanja so obsegala tri sorte čičerke (Adel, Mansour in Arman) in dva načina uravnavanja plevelov (zatiranje, kontrola). Rezultati so pokazali, da je imelo gnojenje z dušikovimi gnojili, še posebej hkratna uporaba biognojil z dodatkom 50 % mineralnih gnojil, pozitiven učinek na vse preučevane lastnosti. Največje število strokov na rastlino, največji pridelek zrnja in biološki pridelek so bili doseženi pri sorti Arman pri uporabi biognojil z dodatkom 50 % mineralnih gnojil in zatiranju plevelov. Podobno sta bila največje število strokov na rastlino (28,2) in največja vsebnost beljakovin v zrnju (25,3 %) dosežena pri hratni uporabi biognojil in 50 % mineralnih dušikovih gnojil in zatiranju plevelov. V splošnem se je sorta Arman izkazala v pridelku zrnja boljše kot ostale v podnebnih razmerah Khorramabada in hkratno uporabo biognojil z dodatkom 50 % mineralnih gnojil lahko smatramo kot primeren način gnojenja za zmanjševanje porabe mineralnih gnojil v trajnostnem kmetijstvu.

Ključne besede: čičerka; beljakovine v zrnju; pridelek zrnja, hektoliterska masa; *Rhizobium*, pletev

1 Department of Plant Ecophysiology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
2 Corresponding author, e-mail: sajad.kordi@gmail.com
3 Department of Agronomy and Plant Breeding, Faculty of Agriculture, Islamic Azad University, Khorramabad Branch, Iran

1 INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third main grain legume in the world, with an annual global production of 14.24 million tons from an area of 14.79 million ha (FAO, 2018). It is an essential component of the agricultural system in all over Iran, because this crop fits well in rotation patterns and can grow under low fertility and different soil and climate conditions. The main provinces producing chickpeas in Iran are Lorestan and Kermanshah (Mekuanint et al., 2018). The potential yield of chickpea cultivars is approximately 4 t ha^{-1}, while the average national yield is about 533 kg ha^{-1} (Khorsandi et al., 2016). The gap between actual and potential yields is mainly due to poor crop management such as imbalanced use of fertilizer, the lack of effective rhizobial strain, unavailability of high-quality seeds, and also damages caused by pests and diseases (Togay et al., 2008; Mekuanint et al., 2018). Moreover, Iran has nitrogen deficient soil and therefore, plants use a low amount of nitrogen which affects physiological processes and decreases photosynthesis activities, production of assimilate and biomass, and eventually yield (Ghilavizadeh et al., 2013).

Application of chemical fertilizers, especially macronutrients, can generally increase biomass production by 2-3 times (Elliott and Abbott, 2003), that is the reason why farmers are applying high amounts of chemical fertilizers, which are very costly and hazardous to the environment. Therefore, alternative sources of chemical fertilizers and the application of organic fertilizers (e.g., bio-fertilizers) are considered as options for sustainable agriculture to improve soil quality in modern agriculture (Chen et al., 2014; Meena et al., 2015). The utilization of bio-fertilizer (e.g., *Rhizobium* species) has become of paramount importance in the agriculture for their potential role in food safety, improving crop yield, and decreasing greenhouse gas emissions (Ghilavizadeh et al., 2013; Raei et al., 2015). The absence of compatible strains and low population of *Rhizobium* in the soil are essential limitations for nodule formation in chickpea (Kantar et al., 2010; Wolde-Meskel et al., 2018). Inoculation with effective strains at planting time is recommended if the population density of compatible rhizobia is less than 50 cells per gram of soil (Thies et al., 1991a, b; Wolde-Meskel et al., 2018). Previous studies showed that inoculation of chickpea seeds with *Rhizobium* could increase plant growth, grain yield, and biomass yield (Funga et al., 2016; Khaitov et al., 2016; Tena et al., 2016; Wolde-Meskel et al., 2018).

The weak ability of chickpea crops to compete with weeds is a vital issue in low input and organic farming systems (Melander, 1993). The critical period of weed interference in chickpea is 15 to 60 days after sowing in Iran, and the presence of weed at this time can cause severe loss of the yield (Mohammadi et al., 2005; Gupta et al., 2016). Hence, weed control needs to be undertaken during the initial periods of chickpea growth. Hand weeding is a well-proven effective method of weed control in chickpea fields in Iran. But implementation of this method is costly for farmers and can be used on small farms (Mohammadi et al., 2005). Mousavi (2010) reported that by twice weeding, the grain yield of chickpea was significantly increased (by 174 %) compared to weed infested treatment. The objective of this study was to determine the effects of bio-fertilizer and weed control and their interactions on yield, yield components, and some qualitative traits of chickpea in Khorramabad condition.

2 MATERIALS AND METHODS

2.1 LOCATION AND PLANT MATERIALS

This study was conducted in the Experimental Farm of the Pole Baba Hossein, Khorramabad, Iran (33°25′N, 48°19′E, and altitude 1,171 m), during the 2017 - 2018 growing season. The meteorological data during the experimental period are presented in Table 1. Physical and chemical characteristics of soil at the depth of 0-40 cm are shown in Table 2. Chickpea seeds were planted in early January 2018 in plots, consisting

Table 1: Khorramabad meteorological station monthly statistics in the experiment period

Month	Precipitation (mm)	Maximum temperature (°C)	Minimum temperature (°C)	Average temperature (°C)
Jan	50.1	23.5	- 4.3	7.6
Feb	68.7	21.6	- 4.3	8.3
Mar	62.7	23.5	1.1	11.7
Apr	103.7	30.3	3.3	15.2
May	151.7	30.0	5.6	17.2
Jun	12.1	37.0	11.8	24.6

of six 2-meter rows spaced 30 cm apart. The intra-row plant spacing was 10 cm. Hand weeding was done in weed control treatments during the growing season.

2.2 TREATMENTS AND EXPERIMENTAL DESIGN

The experiment was conducted as split-split-plot based on Randomized Complete Blocks Design (RCBD) with three replications. The main factor included F1: control (without application of fertilizer); F2: bio-fertilizer (*Rhizobium*); F3: 100 % nitrogen chemical fertilizer and F4: integration of bio-fertilizer + 50 % nitrogen chemical fertilizer. Sub-factor consisted of chickpea cultivars (Adel, Mansour, and Arman), and sub-sub-factor included weed control (weeding) and weed infestation (non-weeding).

2.3 FERTILIZER AND MICROBIAL INOCULA

Before cultivation, 100 kg ha^{-1} triple superphosphate was added to all plots according to the soil test. With the last plowing before planting, 50 and 25 kg N ha^{-1} as urea were added to 100 % chemical fertilizer and integration of bio-fertilizer + 50 % chemical fertilizer, respectively. The strain of the used *Rhizobium* bio-fertilizer was *Mesorhizobium* ciceri SWRI-3 which consisted of 10^8 colony forming units/ml (CFU ml^{-1}) inoculant and was purchased from Soil and Water Research Institute, Karaj, Iran. Before planting, the seeds were mixed entirely with bio-fertilizer and kept for half an hour in the shade to dry. The liquid bio-fertilizer (*Rhizobium*) was applied at the amount of 2 l ha^{-1}. The dried seeds were planted in early January.

2.4 TRAITS MEASUREMENT

The traits measured in this study included plant height, number of pods per plant, 100-grain mass, grain yield, biological yield, harvest index, hectoliter mass, and grain protein content. Five plants from each plot were selected randomly to determine the plant height and number of pods per plant. To measure the 100-grain mass, five samples containing 100 grains were randomly collected from each plot, and their mass was recorded. To measure the hectoliter mass, a container with known mass and volume was completely filled with the chickpea seeds (Singh and Goswami, 1996; Kordi and Ghanbari, 2019). After filling the container, excess seeds were removed by passing a flat stick across the top surface. The seeds were not compacted in any way. The container was weighed on a digital balance (Model GT2100, Germany) reading to 0.01 g. Hectoliter mass (ρb) was calculated by the ratio of seeds mass in the container (M_b) to its volume (V_b):

$$\rho b = \frac{M_b}{V_b}$$

The ρb was recorded from the average of 10 samples for each treatment.

To measure the grain yield, all plants in the one meter-length center of two rows located in the middle of each plot were taken, and grain yield was recorded with a portable balance and calculated based on 12 % seed moisture. To measure the dry biological yield, including aerial parts and roots, the samples were dried in an oven at 75 °C for 72 h and then weighed. The harvest index (*HI*) was accounted as follows:

$$HI = (\text{Grain yield} / \text{Biological yield}) \times 100$$

For determination of crude protein content, the nitrogen content of grains was obtained by the Kjeldahl method (digestion of organic matter with sulfuric acid in the presence of a catalyst; rendering the reaction product alkaline; distillation and titration of the liberated ammonia; and calculation of the nitrogen content) (Jensen, 1996). Crude protein content (*Cp*) of grain was determined as:

$$Cp = 6.25 \times C2$$

where *C2* is the total grain nitrogen concentration on a dry matter.

2.5 DATA ANALYSIS

SAS (version 9.1) and MSTAT-C statistical softwares were used for the analysis of variance (ANOVA)

Table 2: Physical and chemical analysis of soil before the experiment

Soil texture	Clay (%)	Silt (%)	Sand (%)	pH	EC (dS m^{-1})	Total N (%)	Available P (ppm)	Available K (ppm)
Clay loam	31.2	42.0	26.8	7.97	1.04	0.11	6.1	430

and comparisons of means, respectively. Duncan's multiple range test, at $p \leq 0.05$, was used to rank the differences among means. The graphs were drawn by Excel, and error bars were assigned based on standard error (SE).

3 RESULTS AND DISCUSSION

3.1 PLANT HEIGHT

The result of variance analysis showed that plant height was affected by fertilizer, cultivar, and weeding. The interaction effect of cultivar × weeding was significant on the mentioned trait (Table 3). In all studied cultivars, the plant height under weed infested treatment was lower than that under weed control conditions. This decrement was 12.8, 8.7, and 17 % in Adel, Mansour, and Arman cultivars, respectively. The highest plant height (66.0 cm) was obtained by the Arman cultivar under weed control conditions (Figure 1). It has been reported that weed competition has a negative effect on plant height in chickpea (Ratnam et al., 2011). Weeds compete with crops for essential nutrients, available water, and light used for photosynthesis (Merga and Alemu, 2019), and reduce crop yield. The results of previous experiments also indicated that hand weeding increased the plant height of chickpea (Rathod et al., 2017).

Among fertilizer treatments, the highest (59.4 cm) and the lowest (54.3 cm) plant height were related to the integration of bio-fertilizer + 50 % nitrogen chemical fertilizer and control (without fertilizer) treatments, respectively (Figure 2). Application of *Rhizobium* (F2), 100 % chemical fertilizer (F3), and integration of *Rhizobium* + 50 % nitrogen chemical fertilizer (F4) increased the plant height by 5.5, 5.9, and 9.2 %, respectively, compared to the control treatment (without fertilizer). These results are in line with the findings of Amany (2007) and Caliskan et al. (2008), who reported that plant height increased with the application of nitrogen fertilizer. Khan et al. (2017) stated that the application of *Rhizobium* increases the plant height of chickpea. The solubilizing ability of *Rhizobium* species may increase nitrogen availability in the soil, and the plants can uptake the required amount of nutrients (Khaitov and Abdiev, 2018).

3.2 NUMBER OF PODS PER PLANT

According to the results of variance analysis (Table 3), the number of pods per plant was affected by simple effects of fertilizer, cultivar, and weeding as well as the interaction effects of fertilizer × cultivar, fertilizer × weeding, and cultivar × weeding (Table 3). In all studied cultivars, maximum pods per plant were observed by applying of *Rhizobium* + 50 % nitrogen chemical fertilizer. On the other hand, the Arman cultivar had the highest pods per plant under all fertilization treat-

Table 3: Analysis of variance of grain yield, yield components and some qualitative traits of chickpea cultivars

		Mean squares							
Source of variation	df	Plant height	Pods per plant	100-grain mass	Grain yield	Biological yield	Harvest index	Hectoliter mass	Grain protein content
Replication	2	9.5 ^{ns}	13.8 ^{ns}	0.6 ^{ns}	691.8 ^{ns}	15477.4 ^{ns}	0.5 ^{ns}	0.00002 ^{ns}	0.5 ^{ns}
Fertilizer (F)	3	77.6**	85.8**	46.0 **	602344.3**	2911530.8**	35.85**	0.0006*	33.3**
Error 1	6	4.3	1.5	0.6	4618.3	6240.8	3.15	0.00007	0.3
Cultivar (C)	2	798.7**	86.3**	218.6**	419913.9**	5485802.1**	16.8**	0.0008**	6.6**
F × C	6	9.2 ^{ns}	5.0 *	3.7**	23171.4**	61900.3*	15.6**	0.00003 ^{ns}	0.4 ^{ns}
Error 2	16	4.4	1.7	0.7	2488.9	19679.5	1.4	0.00003	0.2
Weeding (W)	1	1111.9**	1106.5**	67.9**	4262226.7**	11947331.1**	531.4**	0.002**	10.7**
F × W	3	6.4 ^{ns}	30.5**	0.1 ^{ns}	2078.9 ^{ns}	32900.8 ^{ns}	3.1 ^{ns}	0.00003 ^{ns}	0.5*
C × W	2	53.2**	15.7 **	6.9*	47801.8**	120062.7*	15.3**	0.00002 ^{ns}	0.7*
F × C × W	6	7.2 ^{ns}	2.8 ^{ns}	0.5 ^{ns}	3261.5 ^{ns}	9939.7 ^{ns}	0.9 ^{ns}	0.000006 ^{ns}	0.1 ^{ns}
Error 3	24	3.0	1.9	1.5	4502.6	27361	2.6	0.00002	0.2
C.V (%)	---	3.0	6.5	3.7	4.9	4.0	4.9	0.6	1.7

*, ** and ns show significant difference at probability of 5 %, 1 % and no significant difference, respectively

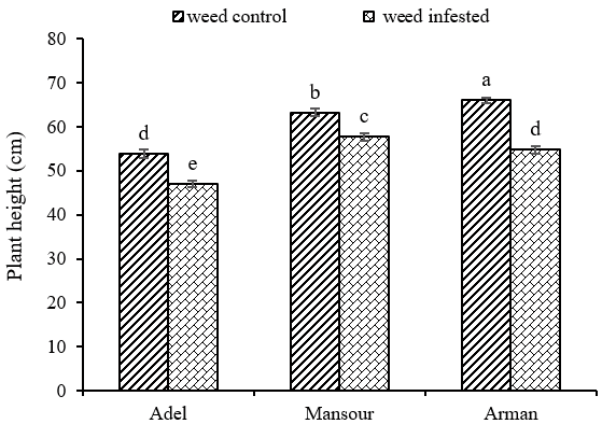

Figure 1: Plant height of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

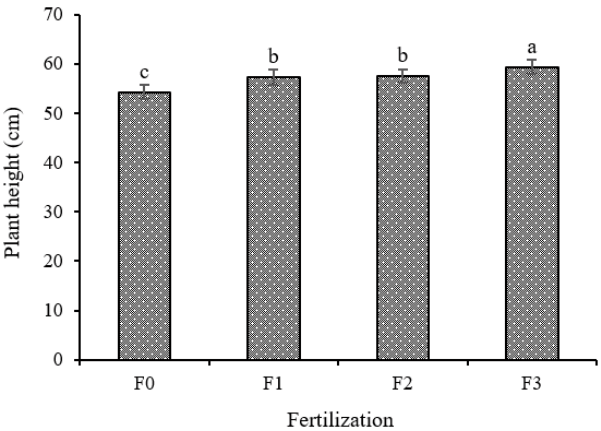

Figure 2: Plant height of chickpea under different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

ments. Adel cultivar without fertilization had the lowest number of pods per plant (16.3), whereas the highest pods number per plant (26.6) was obtained by the Arman cultivar with the application of *Rhizobium* + 50 % nitrogen fertilizer (Figure 3). The number of pods per plant generally depends on the cultivar (Ayaz et al., 2004). It is also affected by environmental factors and management practices (Knott, 1987). Yadav et al. (2011) reported that seed inoculation with *Rhizobium* enhanced nodulation, growth, and yield of legumes. Increasing the number of pods per plant under inoculation treatment can be due to the effect of *Rhizobium* on N, P, and K uptake, some enzyme activities, and root development (Wu, 2000). Many studies found positive effects of *Rhizobium* inoculation on the number of pods per plant in chickpea (Meena et al., 2013; Khaitov et al., 2016).

The result of mean comparisons of fertilizer × weeding showed that in all fertilizer treatments, especially the application of 100 % chemical fertilizer, weed control increased the pods number per plant compared to weed infested treatment. This increment was 43.2, 28.6, 69.9, and 40.8 % under F0, F1, F2, and F3 treat-

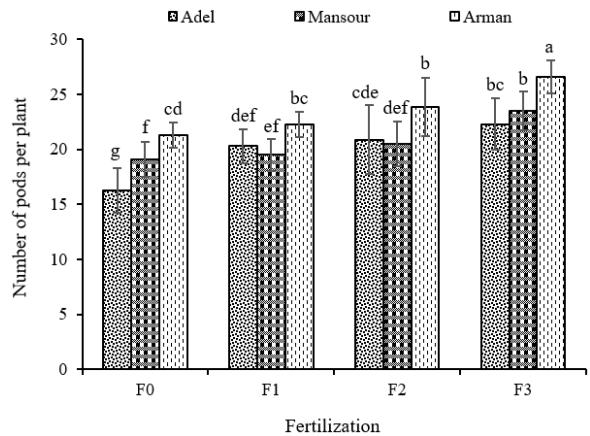

Figure 3: Number of pods per plant of chickpea cultivars under different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

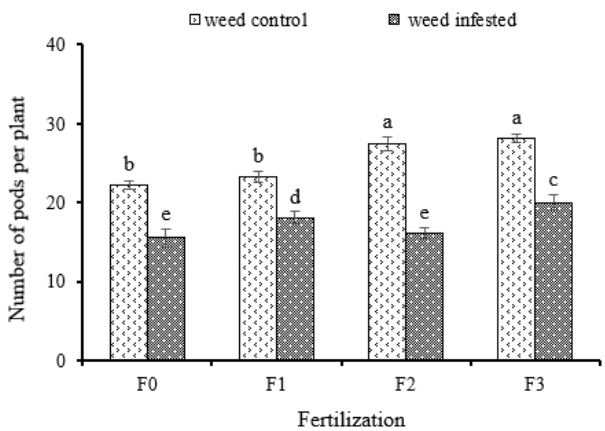

Figure 4: Number of pods per plant of chickpea under different nitrogen sources and weed control and weed infested conditions.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

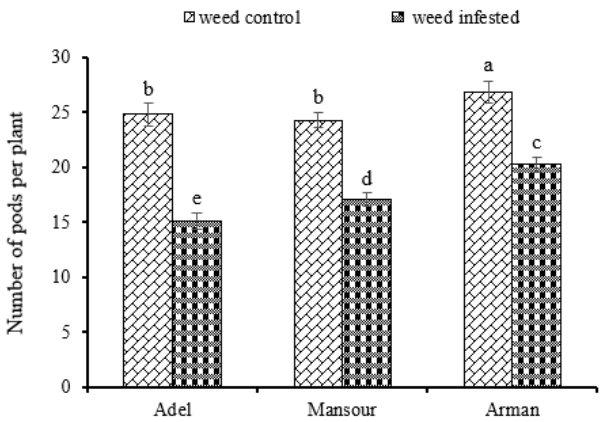

Figure 5: Number of pods per plant of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

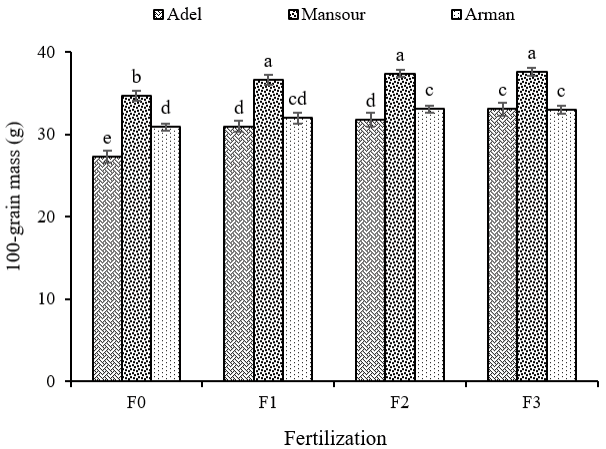

Figure 6: 100-grain mass of chickpea cultivars under different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

ments, respectively. Integration of *Rhizobium* + 50 % nitrogen chemical fertilizer combined with weed control produced the highest number of pods per plant (28.2), while the lowest number was related to weed infested treatment without fertilizer (15.5) (Figure 4). The number of pods per plant is one of the most important factors affecting the yield of pulse crops such as chickpea. The availability of nitrogen may reduce the weed competition pressure in the crops (Shafiq et al., 1994). Togay et al. (2008) reported that the plants from inoculated seeds with *Rhizobium* had a higher number of pods per plant compared to the control.

In all cultivars, weeding improved the number of pods per plant. However, the positive effect of weeds control on pods per plant in the Adel cultivar was higher than the other cultivars. The highest number of pods per plant (26.8) was obtained in the Arman cultivar under weed control conditions (Figure 5). The higher number of pods per plant in weed control conditions could be due to the lack of competition of weeds with chickpea plants in the field. Chickpea is sensitive to weed interference due to its slow growth rate and limited leaf development at the early stage of crop growth and establishment (Kaushik et al., 2014).

3.3 100-GRAIN MASS

Based on the results of variance analysis (Table 3), simple effects of fertilizer, cultivar, and weeding were significant on 100-grain mass. Also, the interaction effects of fertilizer × cultivar and cultivar × weeding were significant for this trait (Table 3). The mean comparisons of fertilizer × cultivar showed that in all fertilizer

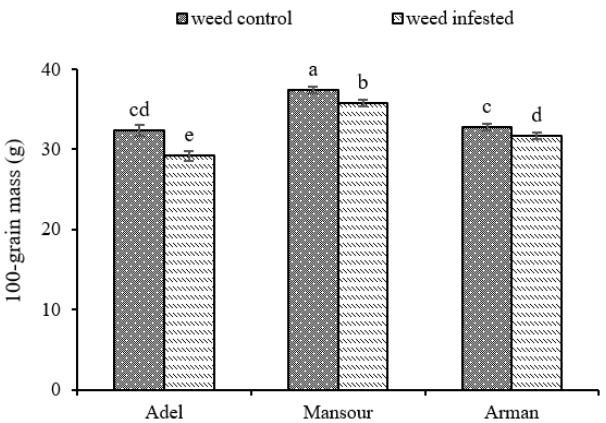

Figure 7: 100-grain mass of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

treatments, the maximum 100-grain mass belonged to the Mansour cultivar. In all three cultivars, the minimum 100-grain mass was related to control treatment (without fertilizer) (Figure 6). Increasing 100-grain mass under inoculation treatment can be due to the improved traits such as leaf area and photosynthetic pigments, which finally causes an increase in photosynthetic products (Nyoki and Nakidemi, 2016).

The 100-grain mass of chickpea under weed control conditions was higher than under weed infested treatment in all studied cultivars, especially the Adel cultivar. The highest (37.4 g) and lowest (29.2 g) 100-grain mass were achieved from the Mansour cultivar under

weed control conditions and the Adel cultivar under weed infested treatment, respectively (Figure 7). Most weeds exhibit faster initial growth than crops such as chickpea, thereby inhibiting crop growth, which might affect photosynthesis and crop yield (Tepe et al., 2011). It appears that some factors like nutrient deficiency and the level of plants competition over light and nutrient resources under weed infested treatment could be considered as reduction factors for production.

3.4 GRAIN YIELD

Fertilizer, cultivar, and weeding had significant effects on the grain yield of chickpea. The interaction effects of fertilizer × cultivar and cultivar × weeding were also significant for grain yield (Table 3). The result of mean comparisons showed that the application of nitrogen fertilizer, especially *Rhizobium* + 50 % nitrogen chemical fertilizer, increased grain yield in all three cultivars. The highest grain yield (1662.5 kg ha^{-1}) was related to the Arman cultivar with applying *Rhizobium* + 50 % nitrogen chemical fertilizer (Figure 8). Inoculation of chickpea seeds with *Mesorhizobium* ciceri strain resulted in a 23 % increase in grain yield compared to the control treatment (without fertilizer). In the present research, increment of the grain yield resulted from the application of different nitrogen sources in studied cultivars, especially the Arman cultivar, may be due to the more plant height and number of pods per plant in this condition (Figures 1, 2, 3). It seems that the positive effects of *Rhizobium* inoculation on chickpea can be a result of nitrogen supply for the crop (Togay et al., 2008). The effect of *Rhizobium* bacteria on plant growth is not only through nitrogen fixation, but it is also associated with the ability of the *Rhizobium* bacteria to synthesize phytohormones like auxin. Some phytohormones, including auxin, enhance root growth and development as well as promoting water and nutrients uptake (Werner and Newton, 2005). It has been reported that inoculation of chickpea seeds with *Rhizobium* improves grain yield by 9.6 - 27.9 % (Gupta and Namdeo, 1996). Increasing the nitrogen rate from 0 to 50 kg N ha^{-1} significantly improved the number of pods per plant, 1000-grain mass, grain yield, biological yield, and harvest index in chickpea (McKenzie and Hill, 1995). There is a negative correlation between soil mineral nitrogen content and the number or mass of rhizobia nodes, meaning that high mineral nitrogen reduces rhizobia activity (Flajšman et al., 2020).

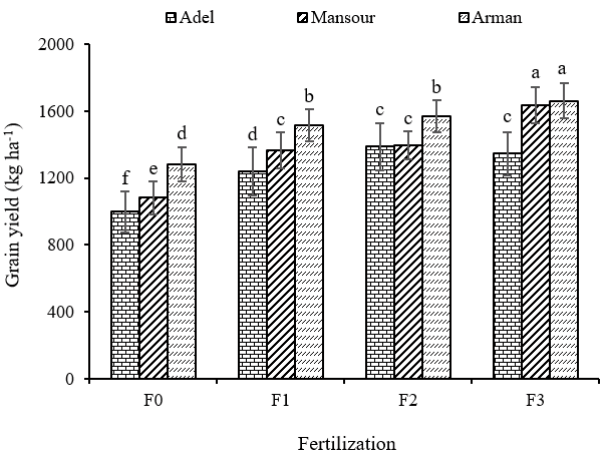

Figure 8: Grain yield of chickpea cultivars under different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

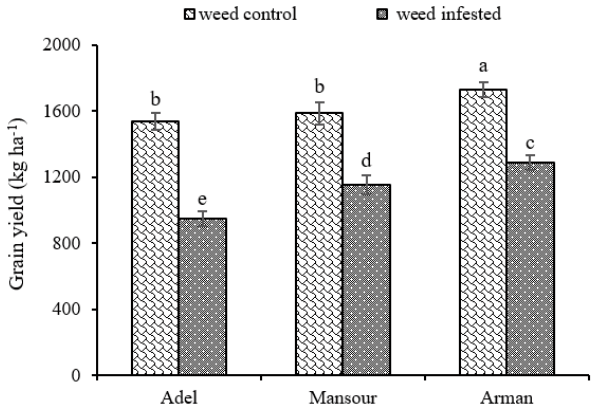

Figure 9: Grain yield of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

Mean comparisons indicated that in all studied cultivars, especially the Adel cultivar, weed control increased chickpea grain yield compared to weed infested treatment. The highest (1726.5 kg ha^{-1}) and the lowest (948 kg ha^{-1}) grain yields were achieved from the Arman cultivar with weed control and Adel cultivar under weed infested treatment, respectively. Weed control led to a 62.2, 37.4, and 34 % increase in grain yield of the Adel, Mansour and Arman cultivars, compared to weed infested treatment, respectively (Figure 9). This result indicates that poor weed management is one of the major grain yield limiting factors in chickpea. It has been reported that weed interference can decrease chickpea yield by more than 85 % (Ratnam et al., 2011).

3.5 BIOLOGICAL YIELD

The biological yield was significantly affected by simple effects of fertilizer, cultivar and weeding as well as interaction effects of fertilizer × cultivar and cultivar × weeding (Table 3). Similar to grain yield (Figure 8), the biological yield of chickpea cultivars increased with the application of nitrogen fertilizers. In all studied cultivars, applying nitrogen fertilizer (especially *Rhizobium* + 50 % nitrogen chemical fertilizer) enhanced biological yield compared to the control (Figure 10). The maximum (5104.1 kg ha^{-1}) and minimum (3139.2 kg ha^{-1}) biological yields were recorded for the Arman cultivar with the application of *Rhizobium* + 50 % nitrogen chemical fertilizer and the Adel cultivar without fertilizer (control), respectively (Figure 10). Nitrogen is one of the most essential nutrients with a considerable effect on plant growth and productivity (Tripathi et al., 2015). The production of phytohormones by *Rhizobium* species enhances root growth and development through improved water and nutrients uptake (Spaepen et al., 2009). According to Khaitov and Abdiev (2018), the combined application of bio-fertilizer and nitrogen fertilizer leads to a positive impact on basic metabolism, grain yield, and biomass. These results show that the integration of bio-fertilizer and nitrogen chemical fertilizer can be useful for crops production. Togay et al. (2008) found that inoculation of chickpea seeds with *Rhizobium* has significantly increased the plant height, number of branches per plant, and biological yield. Namvar et al. (2011) reported that the application of nitrogen increases the production of total dry matter in plants, which can be caused by increasing the plant height (Figure 2), number of branches per plant, and number of pods per plant (Figure 3) that eventually results in high grain and biological yields (Figures 8, 10).

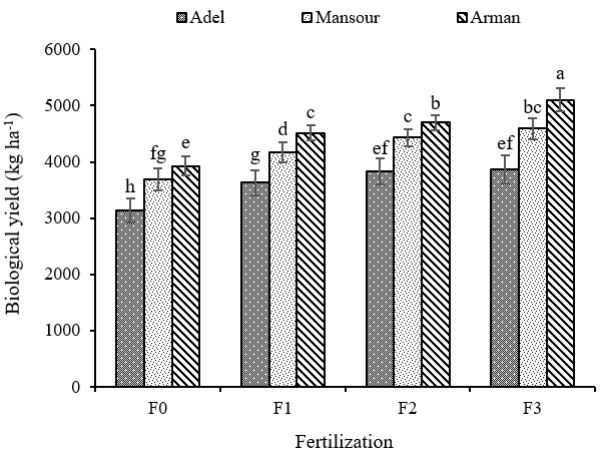

Figure 10: Biological yield of chickpea cultivars under different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

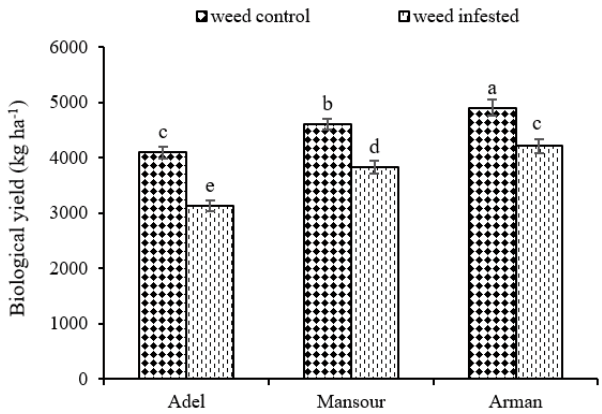

Figure 11: Biological yield of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

Weeds control increased biological yield compared to weed infested treatment in all three cultivars of chickpea. This increment was 31, 20.2, and 16.6 % in the Adel, Mansour, and Arman cultivars, respectively. The highest biological yield (4908.5 kg ha^{-1}) was obtained by the Arman cultivar under weed control conditions (Figure 11). Chickpea is highly susceptible to weed competition due to its slow growth rate and short stature at the early stage of crop growth and establishment (Singh et al., 2017). Therefore, under weed control conditions, soil moisture and nutrients are provided for the crop to increase biological yield (Khan et al., 2002).

3.6 HARVEST INDEX (*HI*)

The data presented in Table 3 showed that simple effects of fertilizer, cultivar, and weeding were significant on the harvest index. The interaction effects of fertilizer × cultivar and cultivar × weeding were also significant for the mentioned trait (Table 3). The application of different nitrogen sources improved the harvest index. The highest harvest index (35.8 and 35.4) was achieved by the Adel cultivar with the application of 100 % nitrogen chemical fertilizer and the Mansour cultivar with the application of *Rhizobium* + 50 % chemical nitrogen fertilizer, respectively (Figure 12). Malik et al. (2006) reported that inoculation of soybean seeds with *Rhizobium* has significantly increased the harvest index. On

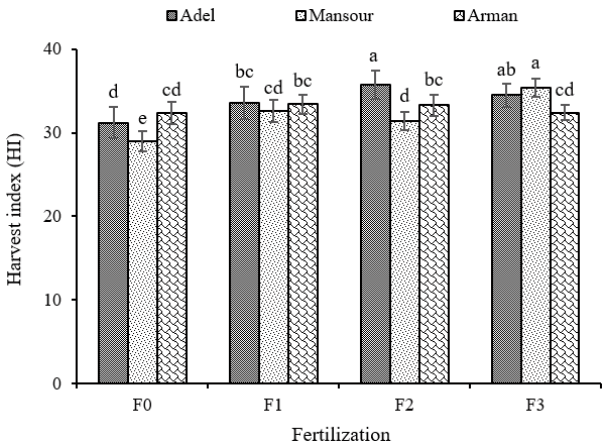

Figure 12: Harvest index response of chickpea cultivars to different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

the other hand, Flajšman et al. (2019) showed that the soybean harvest index was not influenced by bacteria seed inoculation. Weed control led to a 24.2, 14.7, and 15.4 % increase in harvest index in the Adel, Mansour, and Arman cultivars, respectively, compared to weed infested treatment. The highest harvest index (37.4) was obtained by the Adel cultivar under weed control conditions (Figure 13). Pooniya et al. (2009) found that weed management played an important role in improving the harvest index in the chickpea. According to the results of this research, it seems that weed control had a greater effect on grain yield than biological yield.

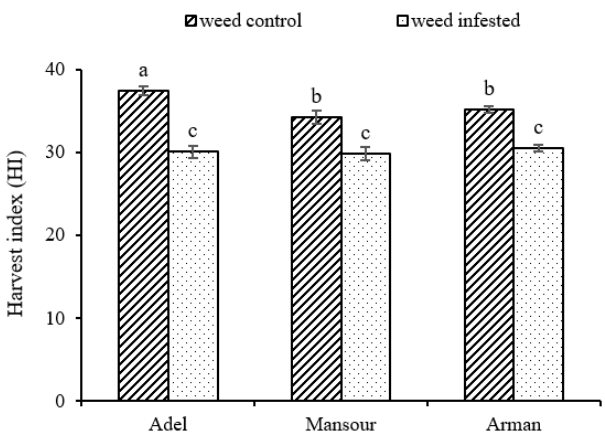

Figure 13: Harvest index of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

3.7 HECTOLITER MASS

According to the results of variance analysis (Table 3), the hectoliter mass was affected by fertilization, cultivar, and weeding. The interaction effects of the treatments were not significant for this trait (Table 3). The application of different sources of nitrogen improved hectoliter mass in the chickpea. Among the various fertilizer treatments, the highest (0.770 g cm^{-3}) and lowest (0.757 g cm^{-3}) hectoliter mass were achieved in the integration of *Rhizobium* + 50 % chemical fertilizer and without fertilization (control), respectively (Figure 14). These results are in agreement with the findings of Kordi and Ghanbari (2019). They reported that differ-

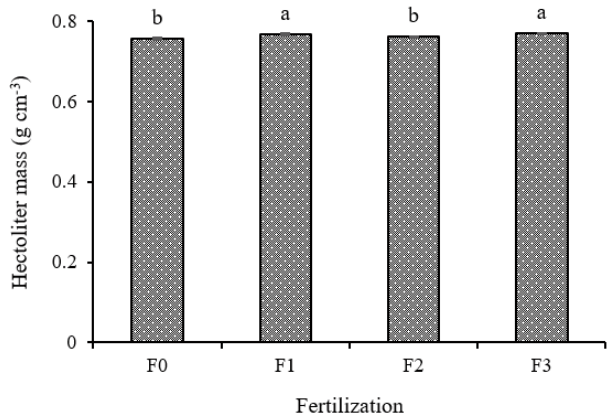

Figure 14: Hectoliter mass of chickpea under different nitrogen sources.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

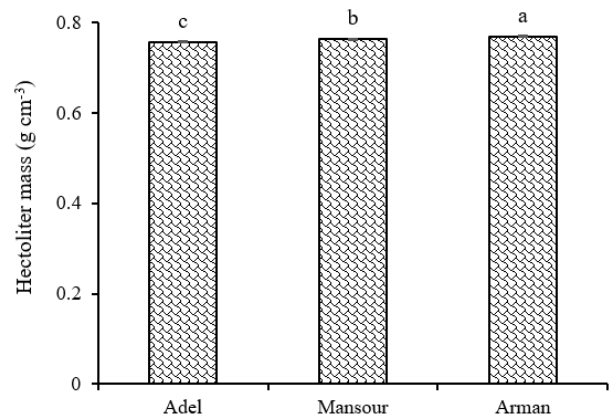

Figure 15: Hectoliter mass of different chickpea cultivars.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

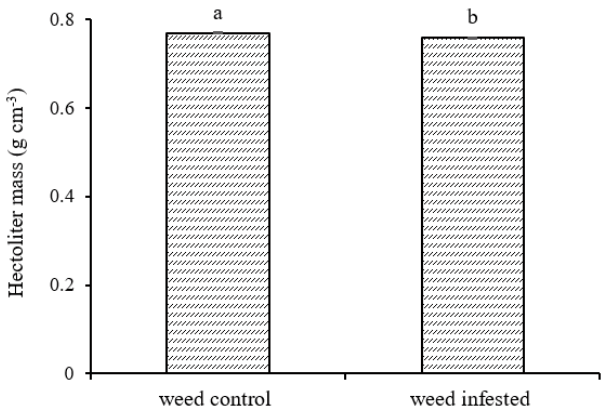

Figure 16: Hectoliter mass of chickpea under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

ent sources of nitrogen resulted in changes in the hectoliter mass of maize, and the highest and the lowest hectoliter mass appeared in the integration of bio-fertilizer + 75 % chemical fertilizer and without fertilization (control), respectively. Evaluation of different chickpea cultivars in terms of hectoliter mass showed that the maximum (0.770 g cm^{-3}) and minimum (0.758 g cm^{-3}) hectoliter mass were related to the Arman and Adel cultivars, respectively (Figure 15). The mean comparisons indicated that under weed control conditions, the hectoliter mass of chickpea was higher than that in weed infested treatment (Figure 16). The reduction of hectoliter mass under weed infested treatment can be due to the decreased traits such as 100-grain mass, leaf area, and photosynthetic pigments, which finally reduces assimilates production.

3.8 GRAIN PROTEIN CONTENT

The results showed that the simple effects of fertilizer, cultivar, and weeding were significant on grain protein content. The interaction effects of fertilizer × weeding and cultivar × weeding were also significant for this trait (Table 3). Integration of *Rhizobium* + 50 % chemical fertilizer had the highest grain protein content under weed control and weed infested conditions. However, maximum grain protein content was obtained by applying *Rhizobium* + 50 % chemical fertilizer under weed control conditions (Figure 17). The grain protein content is used as one of the most important parameters for measuring grain quality. Nitrogen is an integral part of the protein and has a vital role in the quality of crops due to its involvement in the synthesis of amino acids and proteins (Caliskan et al., 2008). Nitrogen deficiency is one of the limiting factors of yield in most of the crops (Liu et al., 2015). Adding nitrogen in any form (as a chemical fertilizer or bio-fertilizer) increases the grain protein content of crops. It has been reported that inoculation and nitrogen fertilization has resulted in a significant increase in grain protein content of chickpea (El-Hadi and Elsheikh, 1999). Kordi and Ghanbari (2019) reported that the highest and the lowest protein contents in maize grain were achieved from the integration of bio-fertilizer + 75 % chemical fertilizer and

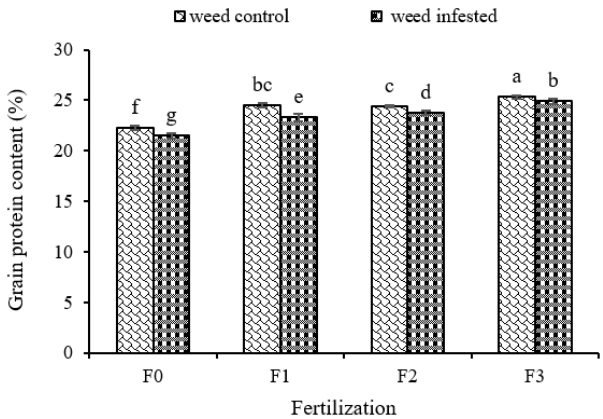

Figure 17: Grain protein content of chickpea under different nitrogen sources and weed control and weed infested conditions.
F0, F1, F2, F3: Control, bio-fertilizer, 100 % chemical fertilizer, and bio-fertilizer + 50 % chemical fertilizer, respectively.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

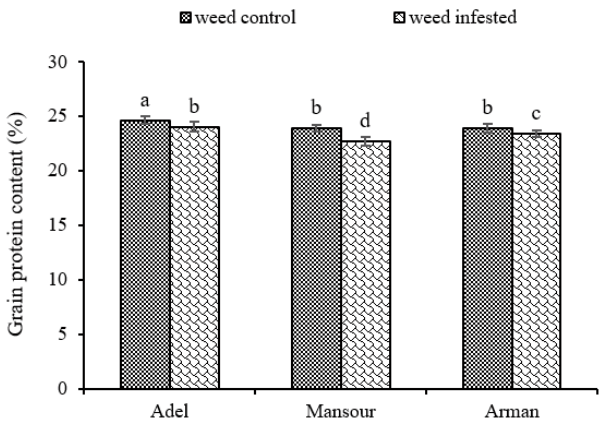

Figure 18: Grain protein content of chickpea cultivars under weed control and weed infested conditions.
Different letters indicate a significant difference at $p \leq 0.05$ (Duncan test)

without fertilization (control), respectively. Nitrogen-fixing bacteria activity increases the nitrogen fertilizer recovery by providing a part of the required nitrogen during the growing season and reducing the nitrogen loss within the soil (Jalilian et al., 2012). It is reported that *Rhizobium* has facilitated the uptake of nutrients in chickpea through the development of the root system (Rudresh et al., 2005).

The result of mean comparisons indicated that the Adel cultivar had higher grain protein content compared to the other studied cultivars in both weed control and weed infested conditions. The highest (24.6 %) and the lowest (22.7 %) grain proteins were achieved from the Adel cultivar under weed control conditions and the Mansour cultivar under weed infested treatment, respectively (Figure 18). The higher competitive ability of weeds compared to chickpea under weed infested treatment led to a significant reduction of available nitrogen and finally decreased the amount of protein in plants. Tanveer et al. (2015) reported that the grain protein content of chickpea decreased with increasing weed density.

4 CONCLUSIONS

The results obtained from this research clearly indicated that the application of nitrogen fertilizer, especially *Rhizobium* + 50 % chemical fertilizer, improved all the investigated parameters compared to the control treatment (without fertilizer). Thus, the integration of *Rhizobium* + 50% chemical fertilizer can be used as the most appropriate treatment for reducing the extensive use of chemical fertilizers in agriculture and paving the way for sustainable agricultures. Hand weeding had a positive and significant effect on yield, yield components, and some qualitative traits of chickpea cultivars. According to the results of this research, the Arman cultivar has priority over other cultivars for the grain yield under the climate conditions of Khorramabad.

5 ACKNOWLEDGEMENTS

This research was conducted as an internal research project in Islamic Azad University, Khorramabad Branch. All material and spiritual rights of the paper are preserved for Islamic Azad University, Khorramabad Branch.

6 REFERENCES

Amany, A.B. (2007). Effect of plant density and urea foliar application on yield and yield components of chickpea (*Cicer arietinum* L.). *Research Journal of Agriculture and Biological Sciences, 3*(4), 220-223.

Ayaz, S., McKenzie, B.A., Hill, G.D., McNeil, D.L. (2004). Variability in yield of four grain legume species in a subhumid temperate environment. II. Yield components. *The Journal of Agricultural Science, 142*(1), 21-28. https://doi.org/10.1017/S0021859604004113

Caliskan, S., Ozkaya, I., Caliskan, M.E., Arslan, M. (2008). The effect of nitrogen and iron fertilization on growth, yield, and fertilizer use efficiency of soybean in Mediterranean-type soil. *Field Crops Research, 108*(2), 126-132. https://doi.org/10.1016/j.fcr.2008.04.005

Chen, X., Cui, Z., Fan, M., Vitousek, P., Zhao, M., Ma, W., … Zhang, F. (2014). Producing more grain with lower environmental costs. *Nature, 514,* 486-489. https://doi.org/10.1038/nature13609

El Hadi, E.A., Elsheikh, E.A.E. (1999). Effect of *Rhizobium* inoculation and nitrogen fertilization on yield and protein content of six chickpea (*Cicer arietinum* L.) cultivars in marginal soils under irrigation. *Nutrient Cycling in Agroecosystems, 54*(1), 57-63. https://doi.org/10.1023/A:1009778727102

Elliott, D.E., Abbott, R.J. (2003). Nitrogen fertilizer use on rain-fed pasture in the Mt. Lofty Ranges, South Australia. 1. Pasture mass, composition and nutritive characteristics. *Australian Journal of Experimental Agriculture, 43*(6), 553-577. https://doi.org/10.1071/EA01131

FAO STAT. (2018). *Crops*. http://faostat3.fao.org/download/Q/QC/E. Accessed 7 February, 2018.

Flajšman, M., Šantavec, I., Kolmanič, A., Kocjan Ačko, D. (2019). Bacterial seed inoculation and row spacing affect the nutritional composition and agronomic performance of soybean. *International Journal of Plant Production,13,* 183-192. https://doi.org/10.1007/s42106-019-00046-8

Flajšman, M., Mihelič, R., Kolmanič, A., Kocjan Ačko, D. (2020). Influence of soil amended with zeolite and/or mineral N on agronomic performance and soil mineral N dynamics in a soybean–winter triticale crop rotation field experiment. *Cereal Research Communications, 48,* 239-246. https://doi.org/10.1007/s42976-020-00030-3

Funga, A., Ojiewo, C.O., Turoop, L., Mwangi, G.S. (2016). Symbiotic effectiveness of elite rhizobia strains nodulating Desi type chickpea (*Cicer arietinum* L.) varieties. *Journal of Plant Sciences, 4*(4), 88-94.

Ghilavizadeh, A., Darzi, M.T., Haj Seyed-Hadi, M. (2013). Effects of biofertilizer and plant density on essential oil content and yield traits of ajowan (*Carum copticum*). *Middle East Journal of Scientific Research, 14*(11), 1508-1512.

Gupta, S.C., Namdeo, S.L. (1996). Effect of *Rhizobium* strains on symbiotic traits and grain yield of chickpea. *Indian Journal of Pulses Research, 9*(1): 94-95.

Gupta, K.C., Gupta, A.K., Saxena, R. (2016). Weed manage-

ment in cowpea [*Vigna unguiculata* (L.) Wasp.] under rainfed conditions. *International Journal of Agricultural Science, 12*(2), 238-240. https://doi.org/10.15740/HAS/IJAS/12.2/238-240

Jalilian, J., Modarres-Sanavy, S.A.M., Saberali, S.F., Sadat-Asilan, K. (2012). Effects of the combination of beneficial microbes and nitrogen on sunflower seed yields and seed quality traits under different irrigation regimes. *Field Crops Research, 127,* 26-34. https://doi.org/10.1016/j.fcr.2011.11.001

Jensen, E.S. (1996). Grain yield, symbiotic N_2 fixation and interspecific competition for inorganic N in pea – barley intercrops. *Plant and Soil, 182,* 25-38. https://doi.org/10.1007/BF00010992

Kantar, F., Shivakumar, B.G., Arrese-Igor, C., Hafeez, F.Y., González, E.M., Imran, A., Larrainzar, E. (2010). Efficient biological nitrogen fixation under warming climates. In: S.S. Yadav, D.L. McNeil, R. Redden, S.A. Patil (Eds.), *Climate Change and Management of Cool Season Grain Legume Crops* (pp. 283-306). Springer, New York. https://doi.org/10.1007/978-90-481-3709-1_15

Kaushik, S.S., Rai, A.K., Sirothia, P., Sharma, A.K., Shukla, A.K. (2014). Growth, yield and economics of rain fed chickpea (*Cicer arietinum* L.) as influenced by integrated weed management. *Indian Journal of Natural Products and Resources, 5*(3), 282-285.

Khaitov, B., Kurbonov, A., Abdiev, A., Adilov, M. (2016). Effect of chickpea in association with *Rhizobium* to crop productivity and soil fertility. *Eurasian Journal of Soil Science, 5*(2), 105-112. https://doi.org/10.18393/ejss.2016.2.105-112

Khaitov, B., Abdiev, A. (2018). Performance of chickpea (*Cicer arietinum* L.) to biofertilizer and nitrogen application in arid condition. *Journal of Plant Nutrition, 41*(15), 1980-1987. https://doi.org/10.1080/01904167.2018.1484134

Khan, M.A., Gul, B., Weber, D.J. (2002). Improving seed germination of *Salicornia rubra* (Chenopodiaceae) under saline conditions using germination regulating chemicals. *Western North American Naturalist, 62*(1), 101-105.

Khan, N., Nawaz, F., Khan, A., Ul Haq, N., Ullah, S., Rehman Khalil, A.U., … Ali, M. (2017). Effect of farmyard manure and *Rhizobium* inoculation on growth of chickpea (*Cicer arietinum* L.) variety 'Karak-03'. *Pure and Applied Biology, 6*(1), 378-384. http://dx.doi.org/10.19045/bspab.2017.60037

Khorsandi, H., Valizadeh-Osalo, G., Sadeghzadeh-Ahari, D., Farayedi, Y. (2016). Study on effects of nitrogen starter and spray fertilizer application differences on chickpea genotype and variety yields and yield components in dryland condition. *Iranian Journal of Dryland Agriculture, 4*(2), 211-228.

Knott, C.M. (1987). A key for stages of development of the pea (*Pisum sativum* L.). *Annals of Applied Biology, 111*(1), 233-245. https://doi.org/10.1111/j.1744-7348.1987.tb01450.x

Kordi, S., Ghanbari, F. (2019). Evaluation of yield, yield components and some physiological and qualitative traits of corn affected by chemical and biological nitrogen fertilizers. *Acta Scientiarum Polonorum: Hortorum Cultus, 18*(1), 3-12. https://doi.org/10.24326/asphc.2019.1.1

Liu, K., Li, Y., Hu, H., Zhou, L., Xiaom, X., Yu, P. (2015). Estimating rice yield based on normalized difference vegetation index at heading stage of different nitrogen application rates in southeast of China. *Journal of Environmental and Agricultural Sciences, 2,* 13.

Malik, M.A., Cheema, M.A., Khan, H.Z., Wahid, M.A. (2006). Growth and yield response of soybean (*Glycine max* L.) to seed inoculation and varying phosphorus levels. *Journal of Agricultural Research, 44*(1), 47-53.

McKenzie, B.A., Hill, G.D. (1995). Growth and yield of two chickpea (*Cicer arietinum* L.) varieties in Canterbury, New Zealand. *New Zealand Journal of Crop and Horticultural Science, 23*(4), 467-474. https://doi.org/10.1080/01140671.1995.9513925

Meena, M.R., Dawson, J., Prasad, M. (2013). Effect of biofertilizers and phosphorus on growth and yield of chickpea (*Cicer arietinum* L.). *Bioinfolet, 10,* 235-237.

Meena, R.S., Meena, V.S., Meena, S.K., Verma, J.P. (2015). The needs of healthy soils for a healthy world. *Journal of Cleaner Production, 102,* 560-561. https://doi.org/10.1016/j.jclepro.2015.04.045

Mekuanint, Y., Tsehaye, Y., Egziabher, Y.G. (2018). Response of two chickpea (*Cicer arietinum* L.) varieties to rates of blended fertilizer and row spacing at Tselemti district, Northern Ethiopia. *Advances in Agriculture, 2018,* 1-8. https://doi.org/10.1155/2018/5085163

Melander, B. (1993). Modelling the effects of *Elysmus repens* L. (Gould). Competition on yield of cereals, peas and oilseed rape. *Weed Research, 33,* 99-108. https://doi.org/10.1111/j.1365-3180.1994.tb01977.x

Merga, B., Alemu, N. (2019). Integrated weed management in chickpea (*Cicer arietinum* L.). *Cogent Food & Agriculture, 5*(1), 1620152. https://doi.org/10.1080/23311932.2019.1620152

Mohammadi, G., Javanshir, A., Rahimzadeh-Khooie, F., Mohammadi, S.A., Zehtab-Salmasi, S. (2005). Critical period of weed interference in chickpea. *Weed Research, 45*(1), 57-63. https://doi.org/10.1111/j.1365-3180.2004.00431.x

Mousavi, S.K. (2010). Chemical weed control in autumn sowing of chickpea (*Cicer aretinum* L.) at Lorestan province. *Iranian Journal of Pulses Research, 1*(2), 131-142.

Namvar, A., Seyed-Sharifi, R., Sedghi, M., Asghari-Zakaria, R., Khandan, T., Eskandarpour, B. (2011). Study on the effects of organic and inorganic nitrogen fertilizer on yield, yield components, and nodulation state of chickpea (*Cicer arietinum* L.). *Communications in Soil Science and Plant Analysis, 42*(9), 1097-1109. https://doi.org/10.1080/00103624.2011.562587

Nyoki, D., Ndakidemi, P.A. (2016). Effects of rhizobia inoculation, phosphorus and potassium on chlorophyll concentration of soybean grown under maize intercropping system. *International Journal of Plant & Soil Science, 13*(6), 1-10. https://doi.org/10.9734/IJPSS/2016/30710

Pooniya, V., Rai, B., Jat, R.K. (2009). Yield and yield attributes of chickpea (*Cicer arietinum* L.) as influenced by various row spacings and weed control. *Indian Journal of Weed Science, 41*(3 & 4), 222-223.

Raei, Y., Kordi, S., Ghanbari, F., Shayan, A.A., Shahkarami, G., Fatahi, S. (2015). The effect of *Azospirilium* bacteria

and salicylic acid effects on drought stress tolerance in *Ocimum basilicum* L. medicinal plant. *Advances in Bioresearch, 6,* 44-53.

Rathod, P.S., Patil, D.H., Dodamani, B.M. (2017). Integrated weed management in chickpea (*Cicer arietinum* L.) under rainfed conditions of Karnataka, India. *Legume Research, 40*(3), 580-585. https://doi.org/10.18805/lr.v0iOF.9611

Ratnam, M., Rao, A.S., Reddy, T.Y. (2011). Integrated weed management in chickpea (*Cicer arietinum* L.). *Indian Journal of Weed Science, 43*(1 & 2), 70-72.

Rudresh, D.L., Shivaprakash, M.K., Prasad, R.D. (2005). Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoerma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer arietinum* L.). *Applied Soil Ecology, 28*(2), 139-146. https://doi.org/10.1016/j.apsoil.2004.07.005

Shafiq, M., Hassan, A., Ahmad, N., Rashid, A. (1994). Crop yields and nutrient uptake by rain-fed wheat and mungbean as affected by tillage, fertilization, and weeding. *Journal of Plant Nutrition, 17*(4), 561-577. https://doi.org/10.1080/01904169409364750

Singh, K.K., Goswami, T.K. (1996). Physical properties of cumin seed. *Journal of Agricultural Engineering Research, 64*(2), 93-98. https://doi.org/10.1006/jaer.1996.0049

Singh, B., Somanagouda, G., Das, R.C., Lal, G. (2017). Effects of integrated weed management practices on nutrient uptake by weeds and chickpea (*Cicer arietinum* L.). *International Journal of Current Microbiology and Applied Sciences, 6*(3), 2338-2343. https://doi.org/10.20546/ijcmas.2017.603.267

Spaepen, S., Vanderleyden, J., Okon, Y. (2009). Plant growth-promoting actions of rhizobacteria. *Advances in Botanical Research, 51,* 283-320. https://doi.org/10.1016/S0065-2296(09)51007-5

Tanveer, A., Javaid, M.M., Irfan, M., Khaliq, A., Yaseen, M. (2015). Yield losses in chickpea with varying densities of dragon spurge (*Euphorbia dracunculoides*). *Weed Science, 63*(2), 522-528. https://doi.org/10.1614/WS-D-13-00049.1

Tena, W., Wolde-Meskel, E., Walley, F. (2016). Response of chickpea (*Cicer arietinum* L.) to inoculation with native and exotic *Mesorhizobium* strains in Southern Ethiopia. *African Journal of Biotechnology, 15*(35), 1920-1929. https://doi.org/10.5897/AJB2015.15060

Tepe, I., Erman, M. Yergin, R. Bükün, B. (2011). Critical period of weed control in chickpea under non-irrigated conditions. *Turkish J of Agriculture and Forestry, 35*(5), 525-534.

Thies, J.E., Singleton, P.W., Bohlool, B.B. (1991a). Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Applied and Environmental Microbiology, 57*(1), 19-28. https://doi.org/10.1128/aem.57.1.19-28.1991

Thies, J.E., Singleton, P.W., Bohlool, B.B. (1991b). Modelling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil. *Applied and Environmental Microbiology, 57*(1): 29-37. https://doi.org/10.1128/aem.57.1.29-37.1991

Togay, N., Togay, Y., Cimrin, K.M., Turan, M. (2008). Effect of *Rhizobium* inoculation, sulfur and phosphorus applications on yield, yield components and nutrient uptake in chickpea (*Cicer aretinum* L.). *African Journal of Biotechnology, 7*(6), 776-782.

Tripathi, L.K., Thomas, T., Singh, V.J., Gampala, S., Kumar, R. (2015). Effect of nitrogen and phosphorus application on soil nutrient balance in chickpea (*Cicer arietinum* L.) cultivation. *Green Farming, 6*(2), 319-322.

Werner, D., Newton, W.E. (2005). *Nitrogen fixation in agriculture, forestry, ecology, and environment*. New York, Springer. https://doi.org/10.1007/1-4020-3544-6

Wolde-Meskel, E., Heerwaarden, J.V., Abdulkadir, B., Kassa, S., Aliyi, I., Degefu, T., … Giller, K.E. (2018). Additive yield response of chickpea (*Cicer arietinum* L.) to *rhizobium* inoculation and phosphorus fertilizer across smallholder farms in Ethiopia. *Agriculture, Ecosystems & Environment, 261,* 144-152. https://doi.org/10.1016/j.agee.2018.01.035

Wu Fei-Bo. (2000). Effects of inoculation with nitrogen-fixing organisms on N, P and K Uptake, some enzyme activities and lint yield in Sea Island Cotton (*Gossypium barbadense*). *Acta Phytophysiologica Sinica, 26*(4): 273-279.

Yadav, J., Verma, J.P., Rajak, V.K., Tiwari, K.N. (2011). Selection of effective indigenous *Rhizobium* strain for seed inoculation of chickpea (*Cicer aritenium* L.) production. *Bacteriology Journal, 1*(1), 24-30. https://doi.org/10.3923/bj.2011.24.30

doi:10.14720/aas.2022.118.2.2601 Original research article / izvirni znanstveni članek

The effect of some additives on the rheology of dough and quality of bread

Xhabir ABDULLAHI [1, 2], Gafur XHABIRI [1], Erhan SULEJMANI [1], Faton SELIMI [1]

Received March 09, 2022; accepted April 22, 2022.
Delo je prispelo 9. marca 2022, sprejeto 22. aprila 2022

The effect of some additives on the rheology of dough and quality of bread

Abstract: The technology of production of baking products today can not be imagined without the use of food additives. In this research it was aimed to investigate the use of some additives in wheat flour type 500 for bread production. The formulations and additives used in this study are: without additives for M0, emulsifiers (E 472e) for M1, calcium phosphate (E341 ii) for M2, L-ascorbic acid (E300) for M3 and Damil additive complex (antifouling E170 - 0.06 %; emulsifier E472e -0.08 %; antioxidant E300 -0.01 %; fungal a-amylase - 0.01 %) for M4 formulation. The results showed that the use of additives positively affects some rheological qualities such as water absorption capacity, stability and energy of the dough. M4 bread had a higher specific volume than all breads with 5.14 cm ^{3} g^{-1}, while M1 and M3 breads were similar. From the total points accumulated for the sensory qualities the M4 bread with a total of 88.8 points accumulated had the best qualities with volume, external appearance and very good crust and crumb taste. It is therefore recommended to use the Damil additive complex in bread production.

Key words: additives; wheat flour; rheological characteristics; specific volume of bread

Učinki nekaterih dodatkov na reološke lastnosti testa in kakovost kruha

Izvleček: Tehnologije proizvodnje pekovskih izdelkov si ne moremo predstavljati brez uporabe aditivov za živila. V članku so predstavljeni rezultati raziskave, kakšna je uporabnost dodatkov pšenični moki tip 500 za proizvodnjo kruha. V tej študiji smo uporabili naslednje recepture in dodatke: M0 brez dodatkov, M1 emulgator (E 472e), M2 kalcijev fosfat (E341 ii), M3 L-askorbinsko kislino (E300) in M4 dodatek Damil. Rezultati so pokazali, da uporaba dodatkov pozitivno vpliva na nekatere reološke lastnosti, kot so sposobnost vpijanja vode, stabilnost in energija testa. Kruh M4 je imel večji specifični volumen od vseh kruhov s 5,14 cm^3 g^{-1}, kruha M1 in M3 pa sta bila podobna. Od skupnih točk za senzorične lastnosti je imel kruh M4 s skupno 88,8 zbranimi točkami najboljše lastnosti za volumen, zunanji videz, zelo dobro skorjo in prijeten okus. Zato je pri proizvodnji kruha priporočljiva uporaba kompleksa aditivov Damil.

Ključne besede: aditivi; pšenična moka; reološke lastnosti; specifična prostornina kruha

1 Department of Food Technology, University of Tetova, Tetovo, North Macedonia
2 Corresponding author, e-mail: xhabir.abdullahi@unite.edu.mk

1 INTRODUCTION

Modern technology of production of baking products has enabled the use of ingredients of suitable quality and food additives with different functional properties. The use of food additives in the baking industry has facilitated the control of the technological process, has enabled the extension of shelf life and the maintenance of freshness for a longer period of time (Grujić, 2005). Baking technology plays an important role in the food industry and has increased interest in the use of these products by consumers (Eddy et al., 2007). However today it is impossible to imagine the production of baking products without additives. The addition of additives aims to give bakers the tolerance and flexibility required during the stages of the baking process (Hrušková & Novotná, 2003). It is common practice to use various additives such as emulsifiers, oxidants and enzymes to improve the quality of bread (Nanditha & Prabhasankar, 2009)

However, the technological quality of the flour is one of the main factors when considering which improver should be used. In order for dough improvers to work better, they are in most cases composed of oxidizing agents (Biebaut, 1991; Morita et al., 1960). It has been proven that oxidizing agents such as L-ascorbic acid (E300) will increase the level of water absorption in flour and the strength of the dough from the oxidized sulfhydryl groups (-SH) to the disulfide bond (SS).

Various empirical methods based on classical extensograph instruments, alveograph, farinograph and mixograph are currently used to obtain data on the rheological properties and baking properties of flour (Uthayakumaran et al., 2002; Dobraszczyk & Morgenstern, 2003; Tronsmo et al., 2003; Chiotelli et al., 2004).

Oxidation generally affects the strength and extensibility of the dough. Its effect can be clearly demonstrated by extension tests measured with extensographs (Šimurina et al., 2002). During the baking process to achieve a high quality bread, local producers used as usual additives in order to increase the energy of the dough, to make the dough more elastic, to increase the volume of the bread, to improve the sensory values and to other purposes.

The purpose of this research was to study the rheological and sensory qualities of bread produced from mixtures of type 500 flour with various additives such as: emulsifiers (E472e), calcium phosphate (E341), L-ascorbic acid (E300) and Damil additive complex (wheat flour, emulsifier E472e -0.08%; antifouling E170 - 0.06%; antioxidant E300 -0.01%; fungal a-amylase - 0.01%).

2 MATERIALS AND METHODS

2.1 MATERIALS

Wheat flour type 500 was used for the production of bread, which was taken from the flour factory "Kokra e Art"- Tetovo, where physico-chemical properties were analyzed. The flour is supplemented with the following mixture of additives: emulsifier (E472e), calcium phosphate (E341 ii), L-ascorbic acid and additive complex "Damil" (wheat flour, antifouling E170 - 0.06 %; emulsifier E472e -0.08 %; antioxidant E300 -0.01 %; fungal a-amylase - 0.01 %) in quantities depending on the use of the additive as shown in Table 1.

Since our country does not meet the demands of consumers for food or bread, we are dependent on wheat imports. With the mixture of imported and local wheat, the technological value of the flour also changes, which also affects the rheological properties of the dough and the quality of the bread. Therefore, due to the variable technological quality of flour, the use of additives has become important to standardize the flour in terms of rheological properties, to increase the volume of bread to improve sensory values and for other purposes.

This experimental design is based on our knowledge from previous research of various authors such as Baratto et al. (2015), Sana & Sinani (2017) and (Hor-

Table 1: Flour samples design with flour type 500 and additives

Samples	Flour T-500 (%)	Emulsifier (E472e) (%)	Calcium phosphate (E341 ii) (%)	L-ascorbic acid (%)	Damil additive mixture (%)
M0	100	-	-	-	-
M1	100	0.3	-	-	-
M2	100	-	0.01	-	-
M3	100	-	-	0.02	-
M4	100	-	-	-	0.02

vat et al., 2007). Considering that previous studies are mainly based on the use of additives separately, the novelty in our study is the use of the complex additive "Damil".

Bread samples are prepared and baked in the bread production company "Deni" -Skopje, based on the amount of flour mixtures of 300 g, additives from 0.02 to 0.30 % according to the table above. The amount of water is according to the absorption of water in the farinograph, while the amount of salt and yeast is 1.80% and 2.80%, respectively. The production of bread is carried out in a standardized way with the direct method where all the ingredients are added to the mixer. The kneading lasted 5 min at medium speed and then separated and given their shape. The dough divided into pieces was placed in the fermentation chamber for 90 minutes at a temperature of 30 °C with 75 % relative humidity and was baked for 25 min at 180 °C in an electric oven. After the breads come out of the oven, they are cooled for 2 hours at room temperature and sent for further evaluation of the quality of the bread.

2.2 METHODS

Evaluation of physico-chemical properties of flour such as: protein content, moisture, ash and wet gluten of flour were performed with the Infratec 1241- FOSS. The device is based on NIR (Near Infra Red) technology and is designed to determine the basic chemical parameters of cereals and flour.

The analysis of the rheological properties of the formulated mixtures of flour and additives was performed in the laboratory of the enterprise "Kokrra e Art" -Tetovo. To determine the rheological characteristics of the mixtures were used 300 g of flour, salt, yeast and additive. The rheological properties of the dough are determined with Farinograph Brabender according to the standard methods of ISO 5530-1 were the instrument measures the dough stability and degree of softening (Dapčević Hadnađev et al., 2011). Extensograph standard methods ISO 5530-2 where used for determination of the physical properties of the dough. The extensibility, resistance and energy of the dough were determined from the curve of the extensogram (Xhabiri & Sinani, 2011; Freund et al., 2006).

Determination of moisture, ash, and energy value of bread was performed by standard methods ISO6492: 1999 (E), ISO5984: 2002, SOP628, SOP200). The sensory qualities of bread such as volume, appearance, aroma and taste of crust and crumb were also analyzed by a 15 member experienced sensory assessors. All the features of the analyzed breads were evaluated with 1-5 points, then the points obtained were multiplied by the coefficient of importance for each feature and the total points were obtained. The specific volume, Vsp ($cm^3\ g^{-1}$) of bread was defined as the ratio of volume and mass of bread, where the mass was determined two hours after baking and cooling, while the volume of bread was determined by the method of removal of grains of millet (Kaluđerski & Filipović, 1998).

Statistical analysis was performed using SPSS 16 software. The multiple comparison test and the level of significance of the differences between the treatments were taken into account ($p < 0.05$). All experiments were performed in three replications and the mean values were given together with the standard deviations. Datas were also subjected to statistical analysis (Duncan test - multivariate analysis, at significance level $p < 0.05$).

3 RESULTS AND DISCUSSION

3.1 CHEMICAL COMPOSITION OF FLOUR

The results of physico-chemical composition of flour used in this study are given in Table 2.

Based on the analysis, the moisture content in type 500 flour is 14.00 ± 0.50% which indicates that the moisture content in type 500 flour is within the maximum allowable limit (Official Gazette of Republic of Macedonia, 2014). The protein content in type 500 flour was 11.80 ± 0.10% and is approximate to the results of (Abdullahi et.al., 2016). Gluten has an important role in the quality of flour and affects water absorption, viscosity, elongation, elasticity, resistance to deformation, gas holding capacity and hardening properties of dough (Lazarido et al., 2007, Wieser, 2007). The content of wet gluten in t 500 flour was 28.90 ± 0.20 %, indicating that the flour is suitable for bread production. The ash content in type 500 flour was 0.55 ± 0.08 %, which indicates that only the endosperm part was obtained during processing.

Table 2: Composition of flour

Physico-chemical parameters (%)	(Mean ± SD)
Moisture	1 4.00 ± 0.50
Protein	11.80 ± 0.10
Ash	0.60 ± 0.08
Wet gluten	28.90 ± 0.2 0

3.2 RHEOLOGICAL PARAMETERS OF THE DOUGH

Farinograph data of type 500 wheat flour with mixtures of additives are presented in Table 3. The results showed the dependence of the mixing of additives with type 500 wheat flour however they were also influenced by the additives that were used. As an important parameter that has the greatest practical value is the absorption of water, which is important in the evaluation of flour (Dapčević Hadnađev et al., 2011). Water absorption was highest in sample M3 with 57.20 ± 1.04 %, while the lowest in sample M1 with 53.90 ± 0.80 % and these differences are significant. From the data of table 3 it can be seen that the control dough M0 has no significant differences in (p <0.05) with M1 and M4, but expresses significant differences with M2 and M3.

The dough development time was much longer in the control dough M0, compared to the formulations with mixture of additives, therefore we have a significant difference ($p < 0.05$). This indicates that the dough development time in type 500 wheat flour without additive increases with increasing proteolytic degradation of proteins (Dua et al., 2009). Regarding the stability of the dough, there is a positive effect of additives in improving the stability of the dough, where samples M3 and M2 had a significant ($p < 0.05$) higher dough stability than the control dough. A positive effect of the addition of additives on the M2 sample was observed at the degree of softening, which had a better rate of 43 ± 1.06 FU. The degree was the same as that of the control M0, and both had a significant difference ($p < 0.05$) from M1, M3 and M4 samples. Although many authors emphasize that gluten is the main ingredient of the dough that affects the rheological qualities (Torbica et al., 2007) as well as increases the volume of baked products (Rakita, 2017). The addition of additives to the flour did not change the qualitative number observed from all samples.

Data from extensograph analyzes are presented in Table 4 which shows that the use of some additives has influenced extensographic parameters. The lowest dough extensibility had M3 dough, while the other doughs had higher extensibility than M0 control dough. Martin et al. (2003) investigated the effect of pentosanase and oxidases on glutenin dough and macropolymer characteristics and reported higher dough extensibility. M1 and M2 dough had lower resistance than M0 control dough, while M3 and M4 dough had higher resistance. The results obtained correspond to those of Ghanbari & Farmani (2013).

The data show that the dough M1 had the same energy as the control dough M0, while in other doughs M2, M3, M4 the energy increased. The results obtained are comparable to those of Horvat et al (2007).

The ideal ratio for bread production should be 1.5-2.5 and in most dough mixtures it is within the allowed limits while the M3 sample had a higher ratio that reaches up to 3.46.

Table 3: Rheological properties of farinograph doughs

Farinograph parameters	M0	M 1	M 2	M 3	M 4
Water absorption (%)	54.50 ± 0.32^{a}	53.90 ± 0.80^{a}	56.80 ± 0.24^{b}	57.20 ± 1.04^{b}	54.80 ± 0.73^{a}
Dough development (min)	3.10 ± 0.16^{b}	1.30 ± 0.04^{a}	1.25 ± 0.02^{a}	1.70 ± 0.04^{a}	1.28 ± 0.02^{a}
Dough stability (min)	0.90 ± 0.16^{a}	1.05 ± 0.01^{a}	4.56 ± 0.07^{b}	5.10 ± 0.08^{b}	1.09 ± 0.07^{a}
Degree of softening (FU)	43.00 ± 1.41^{a}	52.00 ± 2.16^{b}	43.00 ± 1.06^{a}	77.00 ± 1.41^{c}	54.00 ± 1.34^{b}
Quality number	71.00 ± 1.01^{c}	67.00 ± 1.63^{b}	75.00 ± 0.25^{c}	80.00 ± 1.63^{d}	64.00 ± 0.75^{a}

Different letters in the same order differ significantly, Duncan $p < 0.05$

Table 4: Rheological qualities of doughs with exstensograf

Extensograph parameters	M0	M 1	M 2	M 3	M 4
Extensibility (mm)	153.00 ± 2.88^{b}	165.00 ± 8.96^{bc}	170.00 ± 8.88^{c}	135.00 ± 9.64^{a}	156.00 ± 5.51^{bc}
Resistance (EU)	307.00 ± 18.61^{a}	272.00 ± 16.37^{a}	269.00 ± 8.51^{a}	546.00 ± 47.62^{b}	344.00 ± 39.51^{a}
Energy (cm ^{2})	85.60 ± 3.05^{a}	85.30 ± 12.58^{a}	88.30 ± 7.64^{a}	116.00 ± 15.51^{b}	102.00 ± 3.78^{ab}
Relation R / E	2.03 ± 0.23^{a}	1.63 ± 0.15^{a}	1.56 ± 0.15^{a}	3.46 ± 0.71^{b}	2.23 ± 0.31^{a}

Different letters in the same order differ significantly, Duncan p <0.05

3.3 SPECIFIC VOLUME OF BREAD

The specific volume of M4 bread was higher than M0, while the specific volume of M2 bread was lower than the specific volume of M0 ($p < 0.05$). Similar results have been found by Ribotta et al (2010).

3.4 SENSEORY PROPERTIES OF THE BREAD TYPES

The quality of bread depends on the quality of the protein in the flour (Lasztity, 2002) therefore high protein content has good effect on bread volume and performance (Pomeranz, 1988).

Based on the organoleptic analyzes performed on the quality of bread mixes with wheat flour type 500 and some additives, it was observed significant improvements in bread mixes compared to control bread M0. Khan et al (2011) had similar results. Improvements are particularly noticeable in the appearance, taste of the crust and crumb. Pomeranz (1988) confirms that all breads made with type-500 flour, with or without additives, have their own characteristic taste and aroma.

Better volume was in M4 and M3 bread rated with 4.8, while other breads have similar points to the control bread M0. This is also confirmed by the results obtained from the extensogram for the energy of the dough. M4 bread has better appearance while others have similar points to M0 control bread. Considering

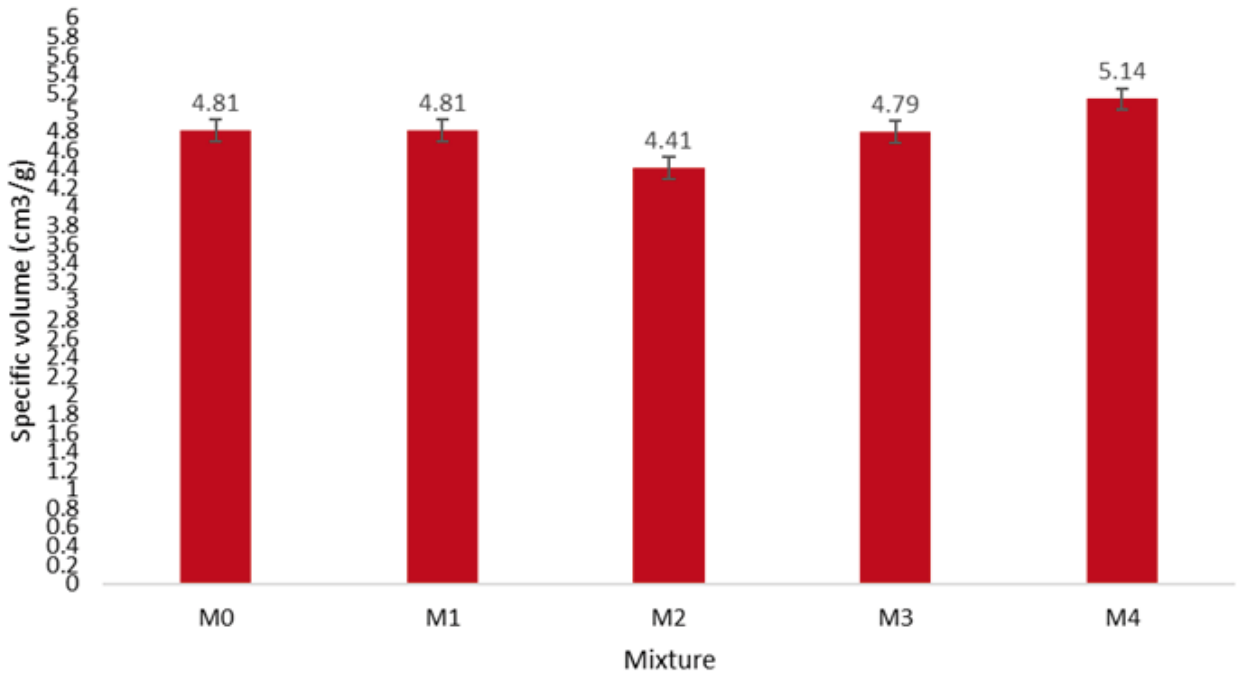

Figure 1: Specific volume ($cm^3\ g^{-1}$) of bread types

Table 5: Sensory properties of the bread samples

Bread samples	Volume (k = 4)	Exterior (k = 3)	Appearance of the crumb (k = 5)	Aroma of the crust and crumb (k = 3)	Taste of the crust and crumb (k = 5)	Total
M0	4.3	4. 3	4.6	4.1	4. 3	86.9
M1	4.2	4.4	4.5	4.0	4. 5	87.0
M2	4.3	4.3	4.5	4.1	4.5	87.3
M3	4.7	4.4	4.2	4.3	4.3	87.4
M4	4.7	4.5	4.4	4.0	4.5	88.8

k-coefficient of importance

the appearance of the pores created and their size, most breads including control bread M0 have similar points, while bread M3 has fewer points.

The aroma of crust and crumb was generally almost identical to M0 control bread, but M3 bread had a slightly higher. The taste was very similar to all breads, including the control bread. All bread with additives had more accumulated points than control especially the M4 bread had highest points, which corresponds to the findings of Grujić et al (2009).

4 CONCLUSION

The use of additives in wheat flour t-500 for bread production improved some rheological properties such as: water absorption capacity, dough stability and dough energy. M4 bread had shown much higher specific volume than M0 control bread, while M1 and M3 bread had similar specific volume. According to the sensory profile (volume, external appearance as well as better aroma and taste of crust and crumb) the additive containing bread had higher points. Therefore, the use of Damil additive complex (wheat flour, antifouling E170 - 0.06 %; emulsifier E472e -0.08 %; antioxidant E300 -0.01 %; fungal a-amylase - 0.01 %) for the production of bread with type 500 flour may be recomended.

5 ETHICS

The research does not involve human or animal subjects.

6 CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

7 ACKNOWLEDGMENT

We thank the Flour Industry "Kokra e Art" in Tetovo for providing the flour and rheological measurments.

8 REFERENCES

Abdullahi, XH., Idrizi, Xh., Sulejmani, E., Jusufi, E., Abdulahi, G. (2016). Qualitative indicators of some wheat cultivars that affect the flour quality in Pollog region. *Albanian Journal of Agriculture Science, Special edition,* 9-14.

Baratto, C. M., Becker, N. B., Gelinski, J., and Silveira, S. M. (2015). Influence of enzymes and ascorbic acid on dough rheology and wheat bread quality. *African Journal of Biotechnology, 14*(46), 3124-3130. https://doi.org/10.5897/AJB2015.14931

Biebaut, D. (1991). Flour improvers and raising agents. In: *Food Additive User's*, page 242-256. Handbook Blackie Academic & Professional (Ed.), London. https://doi.org/10.1007/978-1-4757-5247-2_13

Chiotelli, E., Rolee, A., Le Meste, M. (2004). Rheological properties of soft wheat flour doughs: effect of salt and triglicerides. *Cereal Chemistry, 81*,459. https://doi.org/10.1094/CCHEM.2004.81.4.459

Dapčević-Hadnađev, T., Pojić, M., Hadnađev, M., Torbica, T. (2011). The Role of Empirical Rheology in Flour Quality Control, Part of the book: *Wide Spectra of Quality Control*, Intech Open. https://doi.org/10.5772/24148

Dobraszczyk, B. J., Morgenstern, M. P. (2003). Rheology and the breadmaking process. *Journal of Cereal Science, 38*, 229. https://doi.org/10.1016/S0733-5210(03)00059-6

Dua, S., Lukow O.M., Humphreys, G., Adams, K. (2009). Effect of extraction methods and wheat cultivars on gluten functionality. *Open Food Science Journal, 3*, 84-92. https://doi.org/10.2174/1874256400903010084

Eddy, O.N., Udofia, G.P., Eyo, D. (2007). Sensory evaluation of wheat/cassava composite bread and effect of label information on acceptance and preference. *African Journal of Biotechnology, 6*(20), 2415-2418. https://doi.org/10.5897/AJB2007.000-2379

Freund, W., & Kim, M.Y. (2006). In: *Future of Flour–A Compendium of Flour Improvement*, L. Popper, W. Schäfer&W. Freund, (Ed.), Verlag Agrimedia, Clenze, Germany, p. 101.

Grujić, S. (2005). *Prehrambeni aditivi – funkcionalna svojstva i primjena (Food additives - functional properties and application)*, Faculty of Technology, Banja Luka, p. 207.

Ghanbari, M. & Farmani, J. (2013). Influence of hydrocolloids on dough properties and quality of Barbari:An Iranian leavened flat bread. *Journal of Agricultural Science and Technology, 15*, 545-555.

Grujić, S., Savanović, D., Odžaković, B., Šavija, O., Popara, D. (2009). Uticaj odabranih sastojaka i prehrambenih aditiva na poboljšanje senzornih svojstava peciva (Influence of selected ingredients and food additives on the improvement of sensory properties of pastries) *Glasnik hemičara, tehnologa i ekologa, 1*, 159-165.

Horvat, D., Magdić, D., Drezner, G., Šimiae, G., Dvojkoviae, K., Bročanac, M., & Lukinac, J. (2007). The effect of improver on dough rheology and bread properties. *In 4th International Congress FLOUR–BREAD, 07*, 37–42.

Hrušková, M. & Novotná, D. (2003). Effect of ascorbic acid on the rheological properties of wheat fermented dough. *Czech Journal Food Science*, *21*, 137–144. https://doi.org/10.17221/3490-CJFS

Kaluđerski, G. & Filipović, N. (1998). Metode ispitivanja kvaliteta žita, brašna i gotovih proizvoda (*Methods of testing the quality of cereals, flour and finished products*) Matica srpska, Novi Sad. p, 267-274.

Khan, A. N., Anwaar, A. A., Bhatti, M.S., Randhawa, M. A., Ahmad, A., and Yousaf, A. A. (2011). Effect of additives on

the shelf life extension of chapatti. *Food Sciense and Technology Researsh, 17*(3), 203–208. https://doi.org/10.3136/fstr.17.203

Lasztity, R. (2002). Prediction of wheat quality-succes and doubts. Periodica politechnica, *Serbian Chemical Engineering, 46*, p. 39-49.

Lazarido, A., Duta, D., Papageorgiou, M., Belc, N., Biliaderis, C. G. (2007). Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of Food Engineering, 79*, 1033–1047. https://doi.org/10.1016/j.jfoodeng.2006.03.032

Martin, C.P., Valera, R., Martinez-Anaya, M.A. (2003). Effectof pentosanase and oxidases on the characteristics of doughs and theglutenin macropolymer (GMP). *Journal of Agricultural and Food Chemistry, 51*(16), 4673–4679. https://doi: 10.1021/jf0257695

Morita, N.K., Nakata, Z.H. and Toyosawa, L. (1996). Effect of á–glucosyl rutin as improvers for wheat dough and breadmaking. *Cereal Chemistry, 73,* 99-104.

Official Gazette of Republic of Macedonia, Regulation on the minimum conditions for trading, the quality and types of flour, the manner and methods of sampling, as well as the methods for analysis of the quality of the flour, *24*(2014).

Pomeranz, Y. (1988). Composition and functionality of wheat Flour components. In Wheat:*Chemisty and Technology. 2*, ed. Pomeranz Y. American Association of Cereals Chemists, St Paul, MN, USA, 219-370.

Rakita, S. (2017). *Ph.D. Thesis, Faculty of Tehnology, University of Novi Sad, Serbia*, *49*(1), 1-27.

Ribotta, D. P., Pérez, T. G., Añón, C. M., León, E. A. (2010). Optimization of additive combination for improved soy–wheat bread quality. *Food Bioprocess Technology, 3*, 395 – 405. https://doi.org/10.1007/s11947-008-0080-z

Sana, M. & Sinani, A. (2017). The Usage of Edible Additives with Different Gluten Quality in Baking Products. *UBT International Conference*, p 160. https://doi.org/10.33107/ubt-ic.2017.160

Šimurina, O., Mastilivć, J., Psodorov, Đ., Filipčev, B. (2002). Uloga aditiva i mešavina aditiva u pekarskoj proizvodnji, *Žito-Hleb, 29,*105-116.

Torbica, A., Antov, M., Mastilović, J., Knežević, D. (2007). The influence of changes in gluten complex structure on technological quality of wheat (*Triticum aestivum* L). *Food Research International, 40*(8), 1038-1045. https://doi.org/10.1016/j.foodres.2007.05.009

Tronsmo, K. M., Magnus, E.M., Baardseth, P., Schofield, J. D., Aamondt, A., Faergestad, E. M. (2003). Comparison of small and large deformation rheological properties of wheat dough and gluten. *Cereal Chemistry, 80*, 587. https://doi.org/10.1094/CCHEM.2003.80.5.587

Uthayakumaran, S., Newberry, M., Phan-Tien, N., Tanner, R. (2002). Small and large strain rheology of wheat gluten. *Rheologica Acta, 41*, 162. https://doi.org/10.1007/s003970200015

Wieser, H. (2007). Chemistry of gluten proteins. *Food Microbiology*, *24*(2), 115-119. https://doi.org/10.1016/j.fm.2006.07.004

doi:10.14720/aas.2022.118.2.2246 Original research article / izvirni znanstveni članek

Sublethal effects of some insecticides on the functional response of *Aenasius bambawalei* Hayat, 2009 (Hymenoptera: Encyrtidae)

Zeinab RAFATIAN [1], Nooshin ZANDI-SOHANI [1, 2], Fatemeh YARAHMADI [1]

Received June 17, 2021; accepted April 23, 2022.
Delo je prispelo 17. junija 2021, sprejeto 23. aprila 2022

Sublethal effects of some insecticides on the functional response of *Aenasius bambawalei* Hayat, 2009 (Hymenoptera: Encyrtidae)

Abstract: *Aenasius bambawalei* Hayat, 2009 is one of the most effective natural enemies of *Phenacoccus solenopsis* Tinsley, 1898. The sublethal effects of dimethoate, imidacloprid, and thiodicarb on the functional response of *A. bambawalei* to different densities of third instar nymphs of *P. solenopsis* were evaluated under laboratory conditions. Young females were exposed to the insecticides and then introduced to the host densities of 2, 4, 8, 16, 32, and 64 for 24 h. The results revealed a type III functional response in control and insecticide treatments. The handling time and maximum attack rates of *A. bambawalei* females were adversely affected by insecticides. The longest handling time and the lowest value of maximum attack rate were observed in thiodicarb treatment, 5.03 h and 4.76, respectively. Therefore, for the simultaneous application of biological and chemical control of *P. solenopsis*, the influence of insecticides on the functional response behavior of natural enemies must be evaluated.

Key words: parasitoid; biological control; chemical control; IPM

Subletalni učinki nekaterih insekticidov na funkcionalen odziv vrste *Aenasius bambawalei* Hayat, 2009 (Hymenoptera: Encyrtidae)

Izvleček: Vrsta *Aenasius bambawalei* Hayat, 2009 je najučinkovitejši naravni sovražnik škodljivca *Phenacoccus solenopsis* Tinsley, 1898. Subletalni učinki dimetoata, imidakloprida in tiodikarba na funkcionalni odziv vrste *A. bambawalei* na različne gostote nimf tretjega štadija škodljivca *P. solenopsis* so bili ovrednoteni v laboratorijskih razmerah. Mlade samice so bile izpostavljene insekticidom in nato prinešene gostitelju v gostotah 2, 4, 8, 16, 32, in 64 za 24 h. Rezultati so pokazali funkcionalni odziv tipa III pri kontroli in obravnavanjih z insekticidi. Na čas obravnavanja in največji napad samic vrste *A. bambawalei* so negativno vplivala obravnavanja z insekticidi. Najdaljši čas obravnavanja (5,03 h) in najmanjša vrednost maksimalnega napada (4,76) sta bila opažena pri obravnavanju s tiodikarbom. Zaradi tega moramo pri hkratnem biološkem in kemijskem uravnavanju škodljivca *P. solenopsis* predhodno ovrednostiti vpliv insekticidov na funkcionalni odziv naravnega sovražnika.

Ključne besede: parazitoid; biološki nadzor; kemijski nadzor; IPM

1 Department of Plant Protection, Faculty of Agriculture, Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Ahvaz, Iran
2 Corresponding author, e-mail: nzandisohani@yahoo.com; zandi@asnrukh.ac.ir

1 INTRODUCTION

Extensive application of synthetic insecticides has caused unsuitable effects on ecosystems such as insect resistance, pest outbreaks, pesticide residues in soil and products, and undesirable effects on non-target organisms (Ambrose et al., 2010). On the other hand, in an integrated pest management (IPM) program, success mainly depends on the simultaneous use of chemical compounds and biological control agents and insecticides are essential elements for pest population suppression (Abedi et al., 2012). Thus, in such systems, the compatibility of insecticides with biocontrol agents is the main concern for IPM managers (Martinou & Stavrinides, 2015).

Parasitoid wasps can regulate the pest population and prevent the pest outbreak (Hentz et al., 1998). One of the major interactions between parasitoids and pests that can influence pest suppression is functional response (Holling, 1959). In parasitoids, functional response refers to the relationship between the number of hosts attack by a female parasitoid during a given time interval as a function of host density (Solomon, 1949). According to Holling (1959, 1966), there are three types of functional responses. In type I, the parasitoid has a constant search rate overall densities and the result is a linear response until parasitoid satiation (Hassell, 1978). In type II, the parasitoid response to the pest density is curvilinear and in higher densities changes to plateau. Type II response integrates parasitoid handling time, which defines as the time parasitoids spend for overcoming and parasitizing a host. Moreover, parasitoid cleaning and resting behavior before starting to search for a new host are included. Type III shows a sigmoid curve that rises to a plateau when the parasitoid feels satiation. The sigmoid form is due to a slow increase in the attack rate of the parasitoid in higher densities of the host (Holling, 1959, Hassell, 1978).

Cotton mealybug, *Phenacoccus soleopsis* Tinsley (Hemiptera: Pseudococcidae), has been reported as a serious pest on cotton in the United States for the first time (Fuchs et al., 1991) and then from India and Pakistan (Hodgson et al., 2008, Sahito et al., 2011). It was also described as a pest of *Hibiscus rosa-sinensis* L. in Nigeria and Iran (Akintola & Ande, 2008, Joodaki et al., 2018). *P. soleopsis* is highly polyphagous and more than 202 plant species are attacked across the world including ornamentals, fruits, weeds, and field crops (Kumar et al., 2009, Fand & Suroshe, 2015). Several chemical insecticides are used for managing mealybug infestation; however, due to the wax covers the whole body and the cryptic habit of the pest, the efficiency of the method is limited (Fand & Suroshe, 2015). The pest is attacked by 23 species of predators and 7 species of parasitoids in Iran and all of the reported parasitoids belong to the family Encyrtidae (Mossadegh et al., 2015). Among them, only the solitary parasitoid, *Aenasius bambawalei* Hayat is effective in suppressing the pest population and has a key role in its natural parasitism (Fand & Suroshe, 2015).

Pesticide exposure is one of the several factors which can influence the functional response of Natural enemies (Martinou et al., 2015). The effect of sublethal concentrations of insecticides on different parasitoids such as *Diaeretiella rapae* (McIntoch, 1855) (Hym.: Braconidae) (Rezaei et al., 2014), *Habrobraon hebetor* Say, 1836 (Hym.: Braconidae) (Abedi et al., 2012, Mahdavi et al., 2013, Rashidi et al., 2018), *Dolichogenidea tasmanica* (Cameron, 1912) (Hym.: Braconidae) (Paull et al., 2014), and *Eretmocerus mundus* Mercet, 1931 (Sohrabi et al., 2014) have been reported in previous studies. Nevertheless, there is no available information on the sublethal effects of insecticides on *A. bambawalei*. In the current study, the sublethal effects of dimethoate, imidacloprid, and thiodicarb on the functional response of the parasitoid wasp, *A. bamabawalei*, were investigated on *P. solenopsis*.

2 MATERIALS AND METHODS

2.1 INSECT REARING

Different life stages of *P. solenopsis* were collected from twigs of *Hibiscus rosa-sinensis* L. available at the campus of Agricultural Sciences and Natural Resources University of Khuzestan. Young potato, *Solanum tuberosum* L., sprouts (0.5-1.5 cm length) were used as a laboratory host of the mealybugs and the transfer was carried out using a fine brush. Then, the potato sprouts were kept in a container (24 × 10 × 16 cm) covered with fine mesh. The newly established colony was used in the experiments.

To create the colony of *A. bambawalei*, the parasitized nymphs of *P. solenopsis* were collected from the same *H. rosa-sinensis* twigs. A separate container was used to keep the mummies until the adults' emergence. While the adult parasitoids appeared in the containers they were moved by an aspirator to new containers containing 3rd instar nymphs of *P. solenopsis*. Both colonies were maintained in the incubators at 27 ± 2 ^{o}C, 65 ± 5 % R. H., and 14 l: 10 D h, and all the experiments were carried out in the mentioned conditions.

2.2 INSECTICIDES

Dimethoate, imidacloprid, and thiodicarb were tested in the experiments. Table1 shows more information about insecticides.

2.3 FUNCTIONAL RESPONSE BIOASSAY

For these experiments, the sublethal concentrations of 1, 0.5, and 25 ppm of dimethoate, imidacloprid, and thiodicarb were used, respectively. The cylindrical plastic containers (15 cm high and 7 cm diameter) were considered as exposure cages and impregnated to the insecticidal solution for 30 seconds. Distilled water was used as control. Then, they were allowed to dry for 24 h. After this time, 50 newly emerged mated female wasps (less than one day old) were released in each exposure cage for 24 h. Then, randomly selected females were transferred to the new containers consist of different densities of 2, 4, 8, 16, 32, and 64 third instar nymphs of *P. solenpsis*. The containers were transferred to the incubators with the above-mentioned conditions for 24 h. Then the females were removed and the containers were kept in the incubator until the appearance of mummies. The number of parasitized nymphs was recorded. Ten replications were considered for this experiment.

2.4 STATISTICAL ANALYSIS

Logistic regression analysis of the proportion of host-parasitoid (N_a/N_0) as a function of host density (N_0) was used to determine the functional response type of *A. bamabawalei* as recommended by Juliano (2001).This was done by fitting the below polynomial function:

$$\frac{N_a}{N_0} = \frac{\exp(P_0 + P_1N_0 + P_2N_0^2 + P_3N_0^3)}{1+\exp(P_0 + P_1N_0 + P_2N_0^2 + P_3N_0^3)} \quad (1)$$

Where N_a is the number of parasitized hosts, N_0 is the initial number of hosts offered, and P_0, P_1, P_2, and P_3 are intercept, linear, quadratic, and cubic parameters, respectively. These parameters were calculated using the method of maximum likelihood (PROC CATMOD, SAS Institute, 2001). If the linear parameter (P_1) is negative, the type of functional response is II, whereas a positive linear parameter reveals a type III functional response (Juliano, 2001).

In the second step, a nonlinear least-squares regression (PROC NLIN; SAS Institute Inc., 2001) was used to calculate the functional response parameters (T_h and either a for type II functional response or b, c, and d for type III functional response) using Roger's random parasitoid equation (Rogers, 1972; Juliano, 2001). The parameters were estimated according to the following equations:

$$N_a = N_0\{1 - \exp[a(T_hN_a - T)]\} \quad (2)$$

where N_a is the number of parasitized hosts, N_0, the initial number of hosts, is the instantaneous searching efficiency (attack rate), T is the total amount of time available for searching (24h), and is the handling time.

For modeling the type III functional response, the searching efficiency (a) in equation (2) was substituted with equation (3) with a function of host density.

$$a = \frac{(d+bN_0)}{(1+CN_0)} \quad (3)$$

Where a, b, c, and d are constants and must be estimated. In cases where both d and c were not significantly different from 0; the case observed in this study, led to $a = bN_0$ which was inserted into Equation (2). This yielded the following formula (Hassell, 1978):

$$N_a = N_0[1 - \exp(-bTN_0/1 + bT_hN_0^2)] \quad (4)$$

Since thedata from the above experiment fitted the type III functional response, the functional response parameters were obtained using (3) and (4).

The maximum attack rate (T/T_h) which indicates the maximum number of hosts that can be parasitized by an individual parasitoid during 24 h, was estimated using calculated T_h (Hassell, 2000).

Table 1: Information about experimental insecticides

Insecticide	Active ingredient	Company	Formulation	Dosage
Confidor®	Imidacloprid	Aria Chimi	35 % SC	0.5 ppm
Roxion®	Dimethoate	KimiyaGohareKhak	40 % EC	1 ppm
Larvin®	Thiodicarb	Moshkfam Fars	80 % DF	25 ppm

3 RESULTS

The logistic regression analysis of the proportion of *P. solenopsis* parasitized by *A. babmawalei* indicated Type III functional response for this parasitoid in control and all insecticidal treatments. The linear coefficient, P_1, was positive and the quadratic coefficient, P_2, was negative in all treatments (Table 2).

Therefore, the proportion of host parasitized was density-dependent, which shows a type III functional response (Figure 1).

Roger's random parasitoid equation was used for data analysis in control and other treatments. According to the results of nonlinear least square regression parameters, *c* and *d* were not significantly different from zero; therefore, they were removed from the model and a reduced model was used (Equation 4). Estimated *b* values for the control, dimethoate, imidacloprid, and thiodicarb were 0.00620, 0.00304, 0.00344, and 0.00267, and estimated handling times in these treatments were 2.87, 4.92, 3.70, and 5.03 h, respectively (Table 3). Comparing the asymptotic 95 % confidence of *b* values in Table 3 indicated that imidacloprid had no significant effect on the value of the *b* of *A. bambawalei*; however, it was significantly reduced in dimethoate and thiodicarb treatments. Nevertheless, handling time (T_h) significantly increased in all insecticide treatments. The values of r^2, coefficient of determination, revealed that Rogers's random parasitoid equation properly explained the functional response of *A. bambawalei* in all treatments (Table 3).

The maximum number of 3rd instar nymphs that could be parasitized by *A. bambawalei* (T/T_h) decreased from 8.35 in control to 6.49, 4.88, and 4.76 in imidacloprid, dimethoate, and thidicarb, respectively (Table 3).

4 DISCUSSION

Pesticides are prevalent elements in integrated pest management programs and the study of their behavioral effects such as functional response on natural enemies can be helpful for the success of IPM. According to the results of the current study, the functional response of *A. babmawalei* in control and insecticide treatments was of type III. Parasitoids with type III functional response are density-dependent and can increase their search rate on higher densities of the host. Although a previous overview on functional response indicated that type II is more common in parasitoids (Fernandez-arhex & Corley, 2003), several studies indicate type III in parasitoid wasps such as *Eretmocerus mundus* Mercet on *Bemisia tabaci* Gennadius, 1889 (Sohrabi et al., 2014), *Aphidius colemani* Viereck, 1912 on *Aphis gossypii* Glover, 1877 (Van Steenis & El-Whawass, 1995) and *Praon volucre* (Haliday, 1833) on *Myzus persicae* (Sulzer, 1776) (Tazerouni et al., 2016). According to an earlier study, type II functional response was

Table 2: Maximum likelihood estimates from logisitic regression analysis of the proportion of third instar nymphs of *Phenococcus solenopsis* parasitized by *Aenasius bambawalei* as a function of initial host density

Treatment	Parameters	Estimate	SE	P value
Control	P_0 (Constant)	-0.4730	0.3778	0.2105
	P_1 (Linear)	0.0528	0.0578	0.3614
	P_2 (Quadratic)	-0.00344	0.00220	0.1189
	P_3 (Cubic)	-0.000035	0.000022	0.1055
Dimetoat	P_0 (Constant)	-1.6313	0.4598	0.0004
	P_1 (Linear)	0.0853	0.0689	0.2160
	P_2 (Quadratic)	-0.00388	0.00261	0.1366
	P_3 (Cubic)	0.000036	0.000026	0.1619
Imidacloprid	P_0 (Constant)	-1.1003	0.4118	0.0075
	P_1 (Linear)	0.0659	0.0625	0.2919
	P_2 (Quadratic)	-0.00350	0.00238	0.1408
	P_3 (Cubic)	0.000034	0.000024	0.1458
Thiodicarb	P_0 (Constant)	-1.4500	0.4536	0.0014
	P_1 (Linear)	0.0488	0.0689	0.4787
	P_2 (Quadratic)	-0.00249	0.00262	0.3421
	P_3 (Cubic)	0.000022	0.000026	0.3894

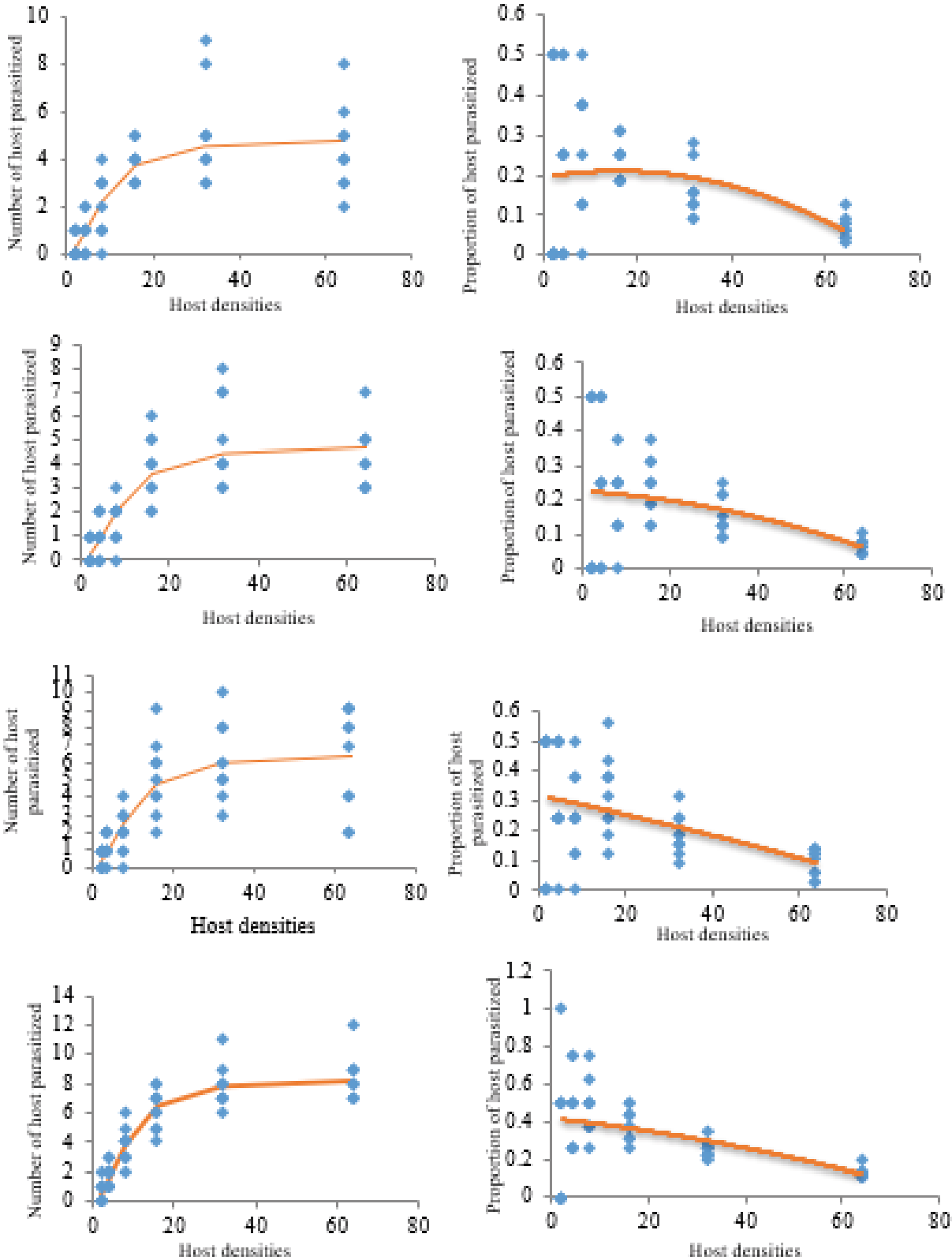

Figure 1: Functional response of *A. bambawalei* to different densities of 3rd instar nymphs of *P. solenopsis* in different insecticide treatments. From the top: dimethoate, thiodicarb, imidacloprid, and control treatments. Symbols are observed data and lines were predicted by the model (Equation 2)

Table 3: Parameters (± SE) estimated by random parasitoid equation indicating functional response of *A. bambawalei* to different treatments

Treatments	b	T_h	T/T_h	r^2
Control	0.00620 ± 0.00103	2.87 ± 0.0980	8.35	0.96
	(0.00484- 0.00825)	(2.6781-3.0704)		
Dimethoate	0.00304 ± 0.000881	4.92 ± 0.3197	4.88	0.96
	(0.00128-0.00480)	(4.2774-5.7773)		
Imidacloprid	0.00344 ± 0.00106	3.70 ± 0.2602	6.49	0.86
	(0.00131-0.00556)	(3.1747-4.2163)		
Thiodicarb	0.00267 ± 0.000740	5.03 ± 0.3203	4.76	0.88
	(0.00119-0.00415)	(4.3940-5.6765)		

The values in parentheses are 95 % confidence intervals; b: constant; T_h: handling time; T/T_h maximum attack rate

recorded for *A. bambawalei* to different densities of *P. solenopsis* at selected temperatures which is varied from the results of our study (Joodaki et al., 2018). Several factors including difference in experimental conditions, parasitoid strain, host plant cultivar, host species, the age of parasitoid and host, physiological state of the host may be responsible for the change of functional response type in parasitoid wasp (Sagarra et al., 2001, Ambrose et al., 2010, Asadi et al., 2012, Pasandideh et al., 2015, Tazerouni et al., 2016, Joodaki et al., 2018). In this study, the type of functional response did not change depending on different insecticide treatments. There is no available information about other studies reporting the effects of insecticides on the functional response of *A. bambawalei*. On the other hand, *Habrobracon hebetor* Say, 1836 showed type III functional response to different densities of *Anagasta kuheniella* Zeller, 1879 in control and all insecticide treatments (Mahdavi et al., 2013). In another study, the type of functional response of *Diaretiella rapae* (McIntosh) did not change when it was exposed to primicarb and thiamethoxam (Rezaei et al., 2014). However, Sohrabi et al. (2014) reported an alteration in type III functional response of *Eretmocerus mundus* Mercet in control treatment as well as imidacloprid to type II in buprofezin treatment. Moreover, the sublethal concentrations of primicarb, cypermethrin, and dimethoate changed the functional response of *D. rapae* from type II to type III (De-Jiu et al., 1991).

Handling time (the time needed to subdue and consume the prey item) and attack rate are important parameters used to assess the parasitoid functional response (Juliano, 2001). In this study, all insecticides had a significant negative effect on the handling time of parasitoids; however, the influence of thiodicarb was even higher than the other insecticides and had the most adverse effect on the host-finding of the parasitoid. The difference in handling time in insecticide treatments may be due to their various mode of action, which influence the neural system of parasitoids. The longer handling time in parasitoids exposed to insecticides could be related to the commotion of their neural system (Rezaei et al., 2014). An increase in handling time under insecticide treatments has been reported in different parasitoids such as *H. hebetor* when exposed to spinosad (Dastgersi, 2009), and cypermethrin (Abedi et al., 2012).

Among all insecticide treatments, dimethoate and thiodicarb had the most, and imidacloprid had the least negative effect on the value of maximum attack rate of the wasp. The parasitism rate of *A. bambawalei* in imidacloprid treatment was 1.3 times more than dimethoate and thiodicarb; however, in control, it was distinctly higher than insecticide treatments. Insecticides can cause a repellent effect on parasitoids or decrease their host finding ability due to boosting disturbance and reducing the olfactometric abilities, which may result in a reduction in parasitism rate (Decourtye et al., 2004).

5 CONCLUSIONS

The results confirmed the negative impact of insecticides on the functional response of parasitoids and probably the success of biological control programs. Hence, for the simultaneous application of biological and chemical control in integrated pest management programs, the influence of insecticides on the different behavior of natural enemies such as functional response has to be evaluated. Such information is essential to estimate the appropriate time to release natural enemies in the case of using insecticides.

6 ACKNOWLEDEMENTS

The authors would like to thank Agricultural Sciences and Natural Resources University of Khuzestan, Iran, for the financial support of this research project.

7 REFERENCES

Abedi, Z., Saber, M., Gharekhani, G., Mehrvar, A., Mahdavi, V. (2012). Effects of azadirachtin, cypermethrin, methoxyfenozide and pyridalil on functional response of *Habrobracon hebetor* Say (Hym.:Braconidae). *Journal of Plant Protection Research, 52*(3), 353-358. https://doi.org/10.2478/v10045-012-0058-8

Asadi, R., Talebi, A.A., Khalghani, J., Fathipour, Y., Moharramipour, S., AskariSiahooei, M. (2012). Age-specific functional response of *Psyllaephagus zdeneki* (Hymenoptera: Encyrtidae), parasitoid of *Euphyllurapakistanica* (Hemiptera: Psyllidae). *Journal of Crop Protection, 1*(1), 1-15.

Akintola, A. J. & Ande, A. T. (2008). First record of *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) on *Hibiscus rosa-sinensis* in Nigeria. *Agricultural Journal (Medwell Journals, Pakistan) 3*(1), 1-3.

Ambrose, D. P., Rajan, S. J., Raja, J. M. (2010). Impacts of Synergy-505 on the functional response and behavior of the reduviid bug, *Rhynocoris marginatus*. *Journal of Insect Science, 10*(1), 187. https://doi.org/10.1673/031.010.18701

Dastjerdi, H. R., Hejazi, M. J., Ganbalani, G. N., Saber, M. (2009). Sublethal effects of some conventional and biorational insecticides on ectoparasitoid, *Habrobracon hebetor* Say (Hymenoptera: Braconidae). *Journal of Entomology, 6*(2), 82-89. https://doi.org/10.3923/je.2009.82.89

De-Jiu, G., Ming-En, Y., Ren-Huan, H., Zhehuai, L. (1991). The effects of sublethal doses of insecticides on the foraging behavior of parasitoid, *Diaeretiella rapae* (Hym.: Braconidae). *Acta EcologicaSinica, 4,* 1-4. https://doi.org/10.1007/BF02374300

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., Pham-Delègue, M.H.)2004(. Imidacloprid impairs memory and brain metabolism in the honeybee (*Apismellifera* L.). *PesticideBiochemistry and Physiology, 78*(2), 83-92. https://doi.org/10.1016/j.pestbp.2003.10.001

Fand, B. B. & Suroshe, S. S. (2015). The invasive mealybug *Phenacoccus solenopsis* Tinsley, a threat to tropical and subtropical agricultural and horticultural production systems–a review. *Crop Protection, 69,* 34-43. https://doi.org/10.1016/j.cropro.2014.12.001

Fuchs, T. W., Stewart, J. W., Minzenmayer, R., Rose, M. (1991). First record of *Phenacoccus solenopsis* Tinsley in cultivated cotton in the United States. *Southwestern Entomologist, 16*(3), 215-221.

Hassell, M. P. (1978). *The dynamics of arthropod predator-prey systems*. Princeton University Press.

Hassel, M. (2000). *The spatial and temporal dynamics of host-parasitoid interactions*. OUP Oxford.

Hentz, M. G., Ellsworth, P. C., Naranjo, S. E., Watson, T. F. (1998). Development, longevity, and fecundity of *Chelonus*sp. nr. *curvimaculatus* (Hymenoptera: Braconidae), an egg–larval parasitoid of pink bollworm (Lepidoptera: Gelechiidae). *Environmenal Entomology, 27*(2), 443-449. https://doi.org/10.1093/ee/27.2.443

Holling, C. S. (1959). Some characteristics of simple types of predation and parasitism. *The Canadian Entomologist, 91*(7), 385-398. https://doi.org/10.4039/Ent91385-7

Holling, C. S. (1966). The functional response of invertebrate predators to prey density. *The Memoris of Entomological Society of Canada, 98*(S48), 5-86. https://doi.org/10.4039/entm9848fv

Hodgson, C., Abbas, G., Arif, M. J., Saeed, S., Karar, H. (2008). *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Coccoidea: Pseudococcidae), an invasive mealybug damaging cotton in Pakistan and India, with a discussion on seasonal morphological variation. *Zootaxa, 1913*(1), 1-35. https://doi.org/10.11646/zootaxa.1913.1.1

Joodaki, R., Zandi-Sohani, N., Zarghami, S., Yarahmadi, F. (2018). Temperature-dependent functional response of *Aenasius bambawalei* (Hymenoptera: Encyrtidae) to different population densities of the cotton mealybug *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae). *European Journal of Entomology, 115*, 326-331. https://doi.org/10.14411/eje.2018.032

Juliano, S. A. (2001). Nonlinear curve fitting: Predation and functional response curves. In S. M. Cheiner & J. Gurven (Eds.), *Design and Analysis of Ecological Experiments, 2nd Edition*, (pp.178-196), Chapman and Hall, London.

Kumar, R., Kranthi, K. R., Monga, D., Jat, S. L. (2009). Natural parasitization of *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) on cotton by *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae). *Journal of Biological Control, 23*(4), 457-460.

Mahdavi, V., Saber, M., Rafiee-Dastjerdi, H., Mehrvar, A., Hassanpour, M. (2013). Efficacy of pesticides on the functional response on larval ectoparasitoid, *Habrobracon hebetor* Say (Hymenoptera: Braconidae). *Archives of Phytopathology and Plant Protection, 46*(7), 841-848. https://doi.org/10.1080/03235408.2012.753182

Martinou, A. F., Stavrinides, M. C. (2015). Effects of sublethal concentrations of insecticides on the functional response of two mirid generalist predators. *PloS one, 10*(12). https://doi.org/10.1371/journal.pone.0144413

Mossadegh, M. S., Vafaei, S., Farsi, A., Zarghami, S., Esfandiari, M., Dehkordi, F.S… Seyfollahi, F. (2015).*Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Pseudococcidae), its natural enemies and host plants in Iran. In Sh. Manzari (Ed.), *Proceedings of the 1st Iranian International Congress of Entomology* (pp. 159-167). Tehran, Iran.

Paull, C. A., Schellhorn, N. A., Austin, A. D. (2014). Response to host density by the parasitoid *Dolichogenidea tasmanica* (Hymenoptera: Braconidae) and the influence of grapevine variety. *Bulletin of Entomological Research, 104*(1), 79-87. https://doi.org/10.1017/S0007485313000527

Pasandideh,A., Talebi, A. A., Hajiqanbar, H., Tazerouni, Z. (2015). Host stage preference and age-specific functional response of *Praon volucre* (Hymenoptera: Braconidae, Aphidiinae) a parasitoid of *Acyrthosiphon pisum* (Hemiptera: Aphididae). *Journal of Crop Protection, 4*(4), 563-575.

Rashidi, F., Nouri-Ganbalani, G., Imani, S. (2018). Sublethal effects of some insecticides on functional response of *Habrobracon hebetor* (Hymneoptera: Braconidae) when reared on two lepidopteran hosts. *Journal of Economic Entomology, 111*(3), 1104-1111. https://doi.org/10.1093/jee/toy069

Rezaei, N., Kocheyli, F., Mossadegh, M. S., Talebi Jahromi, K., Kavousi, A. (2014). Effect of sublethal doses of thiamethoxam and pirimicarb on functional response of *Diaeretiella rapae* (Hymenoptera: Braconidae), parasitoid of *Lipaphis erysimi* (Hemiptera: Aphididae). *Journal of Crop Protection, 3*(4), 467-477.

Rogers, D. (1972). Random search and insect population models. *Journal of Animal Ecology,* 369-383.

Sagarra, L.A., Vincent, C., Stewart, R.K. (2001). Body size as

an indicator of parasitoid quality in male and female *Anagyrus kamali* (Hymenoptera: Encyrtidae). *Bulletin of Entomological Research, 91*(5), 363-367. https://doi.org/10.1079/BER2001121

Sahito, H.A., Abro, G.H., Syed, T.S., Lanjar, A.G., Mal, B., Khajjak, A.S. (2011). Seasonal population abundance of cotton mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) and its natural enemies on various hosts. *Pakistan Journal of Entomology Karachi, 26(*1), 25-40.

Sohrabi, F., Shishehbor, P., Saber, M., Mosaddegh, M. S. (2014). Effects of buprofezin and imidacloprid on the functional response of *Eretmocerus mundus* Mercet. *Plant Protection Science, 50*(3), 145-150. https://doi.org/10.17221/64/2012-PPS

Tazerouni, Z., Talebi, A. A., Fathipour, Y., Soufbaf, M. (2016). Age-specific functional response of *Aphidius matricariae* and *Praon volucre* (Hymenoptera: Braconidae) on *Myzus persicae* (Hemiptera: Aphididae). *Neotropical Entomology, 45*(6), 642-651. https://doi.org/10.1007/s13744-016-0413-y

Van Steenis, M. J. & El-Khawass, K. A. M. H. (1995). Behaviour of *Aphidius colemani* searching for *Aphis gossypii*: functional response and reaction to previously searched aphid colonies. *Biocontrol Science and Technology, 5*(3), 339-348. https://doi.org/10.1080/09583159550039783

doi:10.14720/aas.2022.118.2.1557 Original research article / izvirni znanstveni članek

Morphological, biochemical, and nutritional value of prickly and smooth fruit spinach

Reza ABOLGHASEMI [1, 2], Maryam HAGHIGHI [3], Nematollah ETEMADI [3]

Received March 11, 2020; accepted May 10, 2022.
Delo je prispelo 11. marca 2020, sprejeto 10. maja 2022

Morphological, biochemical, and nutritional value of prickly and smooth fruit spinach

Abstract: This study aimed to investigate the morphological (qualitative and quantitative traits) and biochemical characteristics (such as leaf pigments and total antioxidant capacity, vitamin E and C content, total soluble carbohydrate, total amino acid content, nitrate concentration, nitrate reductase assay, oxalic acid content, Ca and Fe content) in spinach. The selected accessions in this study were prickly ('Varamin Prickly') and smooth ('Monatol') fruits of spinach selected among 44 accessions. This experiment was carried out in spring, arranged as a complete randomized block with three replicates and 18 observations. Results showed no significant differences between the two accessions for most qualitative and quantitative morphological traits. In contrast, biochemical characteristics showed significant differences between the two accessions. Both accessions had high yields, but the dry biomass of 'Varamin Prickly' accession was more than 'Monatol' (smooth fruit). The results indicated that the fruit type does not appear to cause variations in morphological traits, and differences in accessions could be due to genetic sources and environmental distribution. The prickly fruit accession showed a significant superiority for most qualitative nutraceutical traits, including DPPH, flavonoid, phenol, carbohydrate, amino acid, fiber, and Fe content compared to smooth fruit accession. Finally, it was found that prickly fruit accession is very suitable for mechanized harvesting and human diet due to its appropriate plant, leaf, petiole, and qualitative nutraceutical traits and can be used for breeding purposes and cultivation fields.

Key words: Iranian spinach accession; prickly fruit; smooth fruit; nutraceutical traits; *Spinacia oleracea*

Morfološka, biokemična in hranilna vrednost špinače z gladkimi in bodečimi plodovi

Izvleček: Namen raziskave je bil preučiti morfološke (kakovostne in količinske) in biokemijske lastnosti (listna barvila, celokupno antioksidacijsko sposobnost, vsebnost vitaminov E in C, celokupnih topnih ogljikovih hidratov, celokupnih amino kislin, nitrata, oksalne kisline, Ca in Fe, preiskus nitrate reduktaze) v špinači. Iz 44 akcesij so bile izbrane sorte z bodečimi ('Varamin Prickly') im gladkimi ('Monatol') plodovi. Poskus je bil izveden spomladi kot popolni naključni bločni poskus s tremi ponovitvami in 18 opazovanji. Rezultati so pokazali, da v večini kakovostnih in količinskih morfoloških lastnostih obeh tipov ni bilo značilnih razlik. Nasprotno so se vbiokemičnih lastnostih obeh tipov pokazale značilne razlike. Oba tipa akcesij sta imela velike pridelke, a je bila biomasa 'Varamin Prickly' večja kot pri akcesijah sorte Monatol. Rezultati so pokazali, da tip plodov ne povzroča raznolikosti v morfoloških lastnostih in, da bi razlike med akcesijami lahko bile genetskega ali okoljskega izvora. Akcesije z bodečimi plodovi so bile značilno superiorne v večini kakovostnih hranilnih lastnosti kot so DPPH, vsebnost flavonoidov, fenolov, ogljikovih hidratov, amino kislin, vlaknin in Fe v primerjavi z akcesijami z gladkimi plodovi. Povzamemo lahko, da bi bile akcesije z bodečimi plodovi zelo primerne v bodočih programih žlahtnenja in pridelave na polju zaradi primernosti za strojno spravilo in prehrano ljudi zaradi primernih lastnosti habitusa, listov, listnih pecljev rastlin in ugodnih hranilnih lastnosti.

Ključne besede: akcesije iranske špinače; bodeči plodovi; gladki plodovi; hranilne lastnosti; *Spinacia oleracea*

1 Ph.D. student, Department of Horticulture, College of Agriculture, Isfahan University of Technology, Isfahan, Iran
2 Corresponding author, e-mail: r.abolghasemi@ag.iut.ac.ir
3 Associate Professor, Department of Horticulture, College of Agriculture, Isfahan University of Technology, Isfahan, Iran

1 INTRODUCTION

Spinach (*Spinacia oleracea* L.) is one of the critical and commercial vegetables planted worldwide for fresh and processed consumption (Morelock & Correll, 2008). As one of the origins of spinach, Iran has ranked the seventh producer globally (Shi et al., 2016). This vegetable is a fast-growing, cold season, and nutritious that is highly recommended in the human diet. Spinach has high levels of iron, dietary fiber, vitamins, antioxidants, and several phytochemical components. On the other hand, some compounds, such as nitrate and oxalate, can be high accumulated in leaves that have potential health hazards to humans (Koh et al., 2012). Therefore, selecting and introducing low-capacity nitrate and oxalate varieties is an essential aim in spinach breeding programs (Koh et al., 2012).

Pprevious studies were devoted to the genetic structure associated with the morphological traits of spinach (Arif et al., 2013; Sabaghnia et al., 2014). High variation yield was reported among 54 Iranian spinach accessions (Sabaghnia et al., 2014). Nevertheless, variation in the phenotypic characteristics of plants such as leaf type, fruit type (seed type), and even leaf color can affect plant growth and biochemical traits. Like, leaf color affects morphological and biochemical characteristics of *Sassafras tsumu* (Hemsl.) Hemsl. Jiang et al., 2016). Phenotypic differences were also reported in *Vachellia nilotica* subsp. *indica* (Benth.) Kyal. & Boatwr. due to having two types of smooth and prickly seeds. (Gorain et al., 2014). Sunflowers from different seeds differ in biochemical properties such as oil and protein content (Balalic et al., 2012).

Fruit types, leaf morphology, leaf color (green vs. purple), and day to flowering are spinach's most critical phenotypic characteristics for breeding classification and commercial purposes (Shi et al., 2016). Wrinkled leaves are marketable in the USA, whereas flat leaves are favorable in Iran (Avsar, 2011). Moreover, fruit forms used in mechanization and handy cultivation are different (Wu et al., 2015). There are two types of taxonomic varieties of spinach (*S. oleracea* var. *spinosa* Moench.) with prickly and (*S. oleracea* var. *inermis* Peterm.) with smooth fruits (Mei et al., 2010). Spinach cultivars, with prickly fruits are not suitable for mechanized cultivation. So, it has been suggested that round and smooth fruits, is suited for mechanized spinach cultivation (Morelock & Correll, 2008). Some countries have solved the prickly problem of spinach fruit by using fruit coating (Shi et al., 2016). It is reported that most European varieties of spinach fruits are smooth (Meng et al., 2017). Conversely, most prickly spinach fruits belong to the Asian region consisting of Korea, Japan, Iraq, and Iran. There is a remarkable diversity among the fruits of Asian spinach cultivars due to the vast territory and different climates (Meng et al., 2017; Shi et al., 2016).

The researchers reported that prickly fruit spinach varieties had narrow and small leaves with long petioles that are generally resistant to low temperatures but sensitive to high temperatures (Meng et al., 2017; Mei et al., 2010). On the other hand, thick and wrinkled leaves with short petioles are generally observed in smooth fruit varieties (Mei et al., 2010). It is reported that smooth fruit varieties of spinach are tolerant to high temperatures and are sensitive to low temperatures (Mei et al., 2010). So, the appearance of spinach fruit is also an essential feature for spinach classification (Wu et al., 2015; Liu et al., 2004).

Besides, the type of fruit may be affected by spinach quality. Some relationships were observed between the morphological and biochemical characteristics of spinach (Mei et al., 2010). Wu et al. (2015) investigated a wide variety of morphological traits of two smooth and prickly fruits spinach varieties. He reported significant differences in the growth habits of two fruits types, and morphological and biochemical classification of spinach fruits are critical to distinguish among them (Wu et al., 2015). Therefore, the qualitative assessment of prickly and smooth fruits of spinach, such as antioxidants, fiber, iron content, and accumulation of nitrate and oxalate, provided helpful information on cultivar selection (Wu et al., 2015).

Recently, due to the extensive cultivation of spinach, there has been a vast demand for high-quality seed material for farmers (Jafari & Jalali, 2015). There is a lack of information on the differences between smooth and prickly fruit spinach for the future breeding program. Therefore, this study seems necessary as primitive information for broadcasting future research. The present experiment was conducted to investigate and compare the morphological and biochemical characteristics of the prickly and smooth fruit accessions and the relation of fruit morphology with spinach's biochemical and nutraceutical characteristics.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND FIELD EXPERIMENT DESIGN

Iranian spinach accession 'Varamin Prickly' (prickly fruit) and 'Monatol' accession (smooth fruit), in text later indicated as seeds, were selected from 44 spinach accessions provided by the Seed and Plant Improvement Institute and the Gene Bank of Iran (SPII)

and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) gene bank (IPK, 2018) based on the previous author's study (Abolghasemi et al., 2019). The selection was based on spinach's yield and mechanized planting characteristics, such as leaf features, plant height, petiole length, fresh mass and yield, dry mass, days to flowering, and percentage of the female plant.

In addition, these two accessions had different seed type that was selected for this study. The experiment was designed based on a randomized complete block design (RCBD) with 3 replications and 18 observations. The seeds were sown in a field located at the Isfahan University of Technology, Isfahan, Iran, in March 2018 (spring sowing). The conditions of daylight and temperature during spring are presented in Fig 1. The soil was sandy-clay, and manure fertilizer mixed with soil 40 t ha^{-1} pre-planting. The plot size was 2 m^2. After seed germination and plant growth, 18 observations (plant bush) in each plot were selected. It should be noted that this study was carried out in the growth chamber for a more precise evaluation and better comparison of morphological and biochemical characteristics of these two accessions (smooth and prickly seed). Finally, the results of our research in controlled conditions confirmed our field cultivation results (data was not presented). Therefore, due to the similarity of the two studies, the field study results are presented in this manuscript.

2.2 MORPHOLOGICAL CHARACTERIZATION

To investigate morphological features, 35-50 days after planting (due to non-homogeneous growth of spinach), 18 plants were selected from each plot. Then four leaves were selected from four directions of plants to measure the desired parameters. In the first step, the qualitative and visual characteristics of spinach accessions were evaluated based on the descriptor of the International Plant Genetic Resources Institute (IPGRI) presented in Table 1. (Arif et al., 2013; Jafari & Jalali 2017).

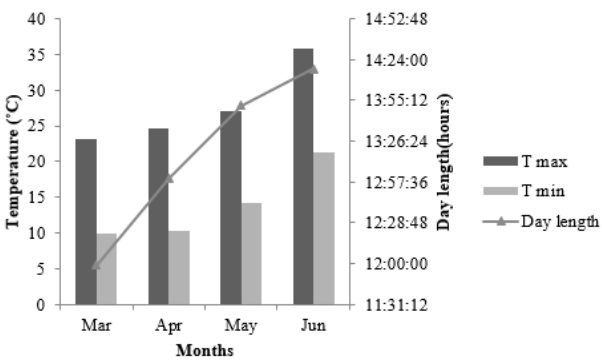

Fig 1: Changes in temperature and day length in spinach spring cultivation

Quantitative and morphological characteristics studied in this study included: 1000-grain mass, germination percentage was calculated using the formula when 50 percent of the seeds were germinated (Ikic et al., 2012):

Germination percentage = (number of germinated seeds / total number of seeds) × 100 when 50 percent of the seeds were germinated, leaf length (cm), leaf width (cm), petiole length (cm), petiole diameter (mm), leaf area (mm^2), plant height (cm), fresh and dry mass (g), yield (kg ha^{-1}), leaf numbers, male and female plants percent, days to flowering. The dry mass of the shoot was measured after putting the bush in an oven at 70 °C for 48 hours (Arif et al., 2013; Jafari & Jalali, 2017).

2.3 BIOCHEMICAL ANALYSIS

Leaf pigments**:** Some fresh leaf tissue (5 g) was mixed with 80 % acetone, then filtered and balanced to 10 ml, and its absorption was measured in 663, 647, and 470 nm with a spectrophotometer (U-2100, JASCO, Japan) (Lichtenthaler, 1987).

Chlorophyll a = (19.3 × A663 - 0.86 × A647) Volume / 100 Mass

Chlorophyll b = (19.3 × A647 - 3.6 × A663) Volume /100 Mass

Total chlorophyll = Chlorophyll a + Chlorophyll b

Carotenoids = 100(A470) - 3.27(mg g^{-1} Chl. a) - 104 (mg g^{-1} Chl. b)/227

Total antioxidant capacity**:** The total antioxidant capacity of spinach was measured by the 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) method (Prasad et al., 2008). Fresh tissue (0.1 g) was mixed in 1.0 ml MeOH and shacked for 2 hours, then centrifuged at 12000 g for 30 min. The supernatant (0.5 ml) was added to the 2.8 ml DPPH solution (0.1 mM) and incubated for 30 mat room temperature. Absorbance was read at 517 nm. The control sample was 2.8 ml DPPH, with the addition of 0.5 ml MeOH. The scavenging activity was determined by the following equation (Stojichevich et al., 2008):

Inhibition % = (A control – A sample / A control) × 100

A: Absorption at 517 nm

Total flavonoid content**:** 0.30 ml of the extract was mixed with 0.50 ml of $NaNO_2$ (5 %). After that,

$AlCl_3.6H_2O$ (10 %), 0.4 ml NaOH, and 0.20 ml were added. Then, its absorption was performed at 510 nm with a spectrophotometer (U-2100, JASCO, Japan) (Krizek et al., 1998).

Total phenolic content**:** The Folin–Ciocalteu method measured total phenolic content. We were using a spectrophotometer (U-2100, JASCO, Japan) at 765 nm. The standard curve was plotted with the Gallic acid solution (Raven, 2003).

Vitamin E content**:** 0.2 ml of alcoholic extract mixed with 5.0 ml toluene, 1.0 ml of ferric chloride, and 3.5 ml of 2, 2-Bipyridine. The mixture was diluted with 10 ml ethanol (95 %), and after 2 min, absorbance was recorded at 525 nm. The standard curve was plotted with vitamin E (Wang & Galletta, 2002).

Vitamin C content**:** Fresh leaf (1.0 g) mixed with 50 ml meta-phosphoric acid (6 %), keeps in the dark for 45 min then centrifuged at 6000 g for 15 min, then 1.0 ml of extract was added to 9.0 ml dichlorophenol indophenol solution (DCPIP) (0.025 %). The next absorbance was read at 515 nm. The standard curve was plotted with different vitamin C concentrations (Djioua et al., 2009).

Total soluble carbohydrate content**:** Total soluble carbohydrate content is measured by the anthrone method (Mc-Cready et al., 1950). For this purpose, 2.0 ml of anthrone was added to the alcoholic extract of the samples. Then, samples were boiled for 2 minutes in the water bath. After cooling and creating the color phase, the absorbance was read at 625 nm. The standard curve was plotted with the glucose standard (Mc-Cready et al., 1950).

Total amino acid content**:** The ninhydrin method was used to measure free amino acids. The ethanol extract of powdered samples was mixed with 1.0 ml of ninhydrin; after boiling and cooling, absorption was read in 575 nm wavelength by spectrophotometer (U-2100, JASCO, Japan). Calibrated curves were plotted with alanine: $y = 0.0198x - 0.0025$($r^2 = 0.997$) (Shih-wen et al., 2006).

Crude fiber content**:** Dried leaves (1.0 g) boiled with 200 ml of H_2SO_4 for 30 min. The extract was filtered, washed with boiling water, the residue boiled with 200 ml NaOH for 30 min then filtered, the solids solid part removed from the filter and placed in a tarred crucible for drying and ashing. It has placed tarred crucible with residue in an oven set at 100 ˚C for 8 hours. Then weighted the crucible with fiber residue and calculated the residue mass by subtracting the empty crucible mass from the crucible and sample mass. Then, the crucible was placed with the dried residue in the oven (550 ˚C) for 4 hours for ashing. Finally, we weighted the crucible with the ash residue in the analytical balance and calculated the ash mass by subtracting the empty crucible mass from the crucible and ash mass. The percentage of crude fiber was calculated using the following equation:

% Crude fiber content = ((dried residue mass – ash mass) / sample mass) × 100

Nitrate concentration**:** Nitrate concentration was evaluated by Narayana & Sunil (2009). Briefly, water extraction (0.1 g fresh leaf mixed in deionized water) was placed in a boiling water bath for 30 min and centrifuged at 4000 g for 30 min. Then, extraction was mixed with 0.8 ml salicylic acid (5 %) in concentrated sulfuric acid (95 %) and cooled at room temperature. Then, 19 ml of sodium hydroxide (NaOH, 2N) was added, and the absorbance at 410 nm was determined with a spectrophotometer (U-2100, JASCO, Japan). The standard curve was plotted with different KNO_3 concentration using following standard formula get from standard curve; $y = 0.053x + 0.035$ ($r^2 = 0.997$).

Nitrate reductase assay**:** The leaf sample (100 mg) was suspended in 5.0 ml of phosphate buffer (0.1 M), KNO_3 (0.02M), and propanol (5 %). The solution was kept in the dark water bath (37 °C) for 30 min. The solution was treated with 1.0 ml of sulfanilamide (1 %) and N-1-naphthyl-ethylenediamine (0.02 %). After 15 min, the absorbance was measured at 540 nm with a spectrophotometer (U-2100, JASCO, Japan). The standard curve was plotted with the KNO_2 solution: $y = 0.0049x + 0.0092$ ($r^2 = 0.988$) (Narayana & Sunil, 2009).

Oxalic acid content**:** The dry sample (0.5 g) was mixed with 30 ml HCl (0.25N) and put in a water bath for 15 min. Then, the supernatant was filtered and mixed with 5.0 ml sulfuric acid (2 N) and 2.0 ml potassium permanganate (0.003 M). After 10 minutes, the absorbance was detected at 528 nm. $y = 0.9126x - 0.0705$ ($r^2 = 0.9327$) and standard oxalic acid solution (1 mg ml^{-1}) was prepared with distilled water. (AOAC, 1970).

Ca and Fe content**:** The concentration of Ca and Fe shoot was measured by atomic absorption (Perkin Elmer, 3030, Netherland) after digesting for 12 h with 2.5 ml HCl (36 %) (Nolte, 2003).

2.4 ANALYSIS OF DATA

The experiment was arranged in RCBD with three replicates. The morphological and biochemical obtained data were analyzed using analysis of variance (ANOVA) by SAS 9.4 comparison of means was performed using the least significant difference (LSD) test at a 0.05 level of probability. The correlation between morphological and biochemical traits was also tested

using the least significant difference (LSD) test at $p \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 QUALITATIVE TRAITS

The qualitative characteristics of two accessions based on the spinach descriptor are shown in Table 1. Usually, smooth seeds of spinach are generally more favorable in the world. The 'Monatol' accession has a smooth seed (presented in Fig 2, C). On the other hand, prickly seed accession (Fig 2, A) is less desirable; they are challenging to plant (Asadi & Hasandokht, 2007). It has been reported that the best seed type for mechanized planting of spinach was a smooth and round seed (Morelock & Correll, 2008). In this study, both accessions had a gray background in seed color (Table 1).

It has been reported that accessions with petiole standing, leaf sheath standing, wrinkled, or slightly wrinkled, are suitable for mechanical harvesting (Mei et al., 2010). Accordingly, 'Varamin Prickly' (prickly seed) accession is more suitable for mechanical harvesting than 'Monatol' (smooth seed) accession because of petiole and leaf form (Table 1). Prickly seed accession has a feature of leaf standing, petiole standing, and low wrinkle, green leaf color appropriate, which can be used in breeding programs, mechanical harvesting, and genetic modification (Table 1). In the USA and other western countries, wrinkled leaves of spinach are more marketable than flat leaves (Kuwahara et al., 2014). In confirmation of this report, smooth seed accession had wrinkle leaves (Fig 2, C, and D).

The smooth seed accession has wrinkled dark green leaves and is more suitable for storage because the ventilation is better but hardly washable (Fig 2, D). Usually, spinach cultivars with lower leaf wrinkles have less nitrate content, and there is a direct correlation between leaf wrinkle and nitrate content (Arshi, 2000). So, no-wrinkle leaves as a desirable attribute are interested in researchers. While in Iran, the most favorable spinach has a large, fleshy, thick, low leaf wrinkle and juicy leaf (Fig 2, B), and according to Table 1, the endemic accession with prickly seed has these features. In Iran, the optimal form of spinach leaf is round, and overseas desirable triangular shapes have been reported (Kunicki et al., 2010). The seed type did not affect leaf shape (Table 1). Leaf color is critical in leafy vegetables since green pigments are desirable and marketable for fresh and frozen spinach (Eftekhari et al., 2010). In this study, both accessions had a complimentary green color, although the foreign accession with smooth seed

Table 1: Qualitative morphological features of prickly and smooth spinach accessions (Varamin Prickly and Monatol) according to spinach descriptor (Arif et al., 2013; Jafari and Jalali 2017)

Seed type	Seed color	Petiole attitude	Wrinkles of leaf	Leaf thickness	Leaf Sheath attitude	Leaf shape	Leaf color	Leaf Sheath	Lobation of leaf tip	The shape of leaf tip	Wave margin of leaf
Prickly seed	Gray-yellow	Erect	Very Low	Thick	Erect	Broad oval	Dark green	Concave	Bend	Circular	Yes
Smooth seed	Gray-yellow	Horizontal	High	Very thick	Horizontal	Broad oval	Very dark green	Concave	Upward	Circular	No

was darker in color (Table 1, Fig 2). The leaf shape, leaf sheath, and leaf tip shape were similar in both accessions. In general, although the type of seed did not affect most of the quality characteristics of spinach in this study, the prickly seed type of spinach was much more desirable for mechanical harvesting than the smooth seed due to the attitude of the petiole and leaf sheath (Table 1). Researchers have reported that prickly seed spinach has better growth in appearance characteristics (Wu et al., 2015; Amoli, 2012) that can be observed in mechanical harvesting for the prickly seed (Table 1).

3.2 QUANTITATIVE TRAITS

Analysis of variance showed a significant difference between the two accessions in dry mass, 1000-grain mass, germination percentage, male and female plants at 1 % probability level, and 5 % for petiole length (data was not presented). Mean comparison in Table 2 showed no significant difference in leaf number, plant height, leaf length and width, petiole diameter, yield, fresh mass, leaf area, and day to flowering between prickly and smooth seed accessions. There is not much similarity between the investigations that can be reported in each region according to different spinach weather and growth conditions. In this study, petiole length was more than 100 % higher in the prickly seed accession (Iranian accession) than in the smooth accession, indicating that the plant's form was more stable in the prickly seed than the smooth seed accession and more suitable for mechanical harvesting (Table 2). Confirming this, it has been reported that plant shape and petiole length are crucial for mechanical harvesting (Shi et al., 2016). Dry mass was the highest in prickly seed accession (Table 2). So, the highest amount of dry mass (14.77 g) was observed in 'Varamin Prickly' accession (Table 2). yield, fresh and dry mass of 'Varamin Prickly' was more suitable (Asadi & Hasandokht, 2007; Eftekhari et al., 2010; Jafari & Jalali, 2015). One of the desirable traits for spinach processing and packaging is dry mass, which directly relates to the smoothness of the leaf spinach (Arshi, 2000; Eftekhari et al., 2010). It can also be stated that the different water content in the tissues of these two accessions leads to the difference in dry mass. In Asadi & Hasandokht's (2007) study, the highest dry mass in 'Qom' accession was reported with prickly seed. The mass of 1000-grains of prickly seed accession was 16.81 g, which had bigger seeds than the smooth seed accession 48.3 % more than the 1000-grain mass of smooth seed accession (Table 2). According to our results, Eftekhari et al. (2010) reported that the 1000-grain mass of prickly seed accessions was higher than that of smooth seed accession. Prickly seeds appear larger in appearance than smooth ones. The percentage of seed germination of smooth seed accession was higher than the prickly seed accession (showed a 52.3 % increase in germination). There was a significant difference between the two accessions (Table 2), which may be due to more water absorption of smaller seeds during germination. Although prickly seeds are high in mass, it is hard to say that prickly seeds have a much larger surface area than smooth seeds. It is believed that the small seed varieties have higher water absorption capacity and better establishment due to the higher surface area to volume ratio (Zaferaniye, 2015). Observations on the germination process of spinach seeds have shown that genotypes with better germination had better vegetative growth and yield. (Jaliliyan, 2009; Zaferaniye, 2015). However, this study's performance was not statistically significant (Table 2). Spinach is a leafy vegetable, so longer vegetative growth is desirable. It is reported that the late-flowering varieties are a priority to increase the spinach production period (Jaliliyan, 2009). In this regard, this study classified prickly and smooth seed accessions as spring late flowering (near to 70 days) (Table 2). The researchers reported that the late-flowering accessions were mostly economically desirable (Asadi & Hasandokht, 2007). Therefore, spinach with better vegetative growth, yield, and appearance is more favorable to farmers. It should

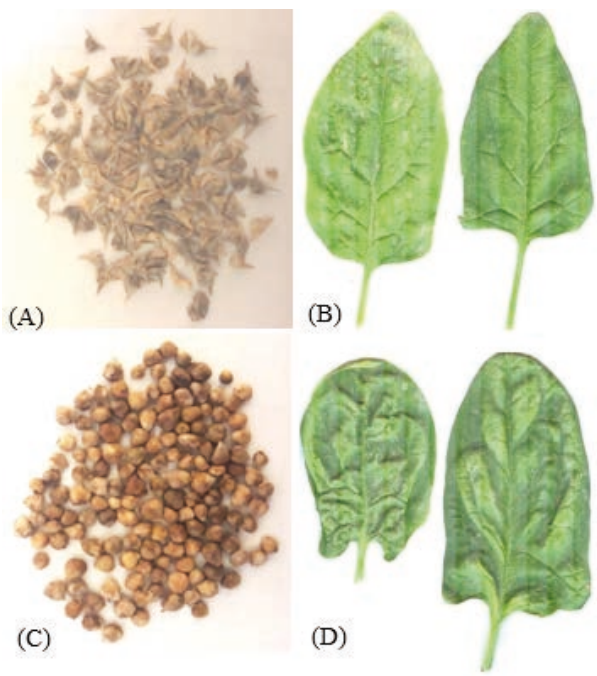

Fig 2: Comparison of 'Varamin Prickly' spinach seed (A); leaf (B) and 'Monatol' spinach seed (C); leaf (D)

Table 2: Morphological traits of prickly and smooth seed spinach accessions

Seed type	Leaf number	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Petiole diameter -(mm)	Yield (kg h^{-1})	Fresh mass (g)	Dry mass (g)	Leaf area (mm^2)	1000-grain mass (g)	Germination percentage (%)	Days to flowering	Male plant (%)	Female plant (%)
Prickly seed	15.66^a	25.00^a	13.73^a	7.33^a	12.66^a	4.35^a	30867^a	60.02^a	14.77^a	48015^a	16.81^a	64.71^b	69.66^a	51.86^a	49.59^b
Smooth seed	20.20^a	19.66^a	12.00^a	9.06^a	6.20^b	4.75^a	42056^a	81.77^a	8.63^b	60085^a	11.33^b	98.58^a	69.00^a	3.82^b	95.96^a
Standard Error	28.40	8.48	5.24	1.28	2.84	0.26	68595	259.2	1.14	57668	0.04	13.2	3.16	0.44	11.2

Different letters within the same column indicate significant differences of each type at $p \leq 0.05$ by the LSD test

be noted that the result of two traits of yield and day to the flowering of spinach in two different seasons (spring and autumn) can be different because these two traits are affected by the type of growing season (Asadi & Hasandokht, 2007). In this study, both Iranian and foreign accessions (the prickly and smooth seed) were similar in day to flowering and yield (Table 2)). In confirmation of various reports of spinach being dioecious , both of the accessions were male or female in this study as well (Morelock & Corell, 2008). As can be seen in table 2, the smooth seed accession had significantly more female plants than the Iranian prickly seed accession. So smooth seed accession showed 93.5 % more female plants (Table 2). According to studies, spring spinach cultivation is valuable if the plant can flower later with female flowers (Zaferaniye, 2015). Jaliliyan (2009) recommends planting female varieties for economic yield. As a result, the smooth seed accession in this study confirms this statement. It has been reported that prickly seeds in winter and spring conditions usually have better growth than smooth seeds (Wu et al., 2015). It can be concluded that prickly seed accession was similar to the smooth seed accession in many traits in spring cultivation.

According to the spinach descriptor coding (Table 5), prickly seed accession showed higher valuable traits (a score of 33) in yield. Smooth seed accession has more scores in the mechanical harvesting traits (rated 31), indicating the superiority of mechanical harvesting. According to qualitative characteristics (Table 1), these results are related to more standing leaves and petiole in the Iranian accession, than in the foreign accession. Our results confirm previous reports on favorable vegetative and mechanical harvesting characteristics for prickly seed accessions (Asadi & Hasandokht, 2007; Eftekhari et al., 2010; Jafari & Jalali, 2015). In the category of breeding traits, smooth seed accession (with a score of 13) was better than the Iranian accession (with a score of 12), which indicates that the foreign accession is better for breeding traits (Table 5). However, it seems that prickly seed accession should also be considered in terms of breeding characteristics due to near score according to the descriptor scale to smooth seed accession (Table 5).

3.3 BIOCHEMICAL TRAITS

Currently, many domestic and wild spinach accessions in the country may have more favorable biochemical characteristics than foreign accessions. Identifying these genotypes and crossing them improved cultivars with good biochemical characteristics (Sabaghnia et

al., 2014). Results of the analysis of variance showed that there were differences between DPPH, flavonoids, amino acid, and iron content in a 1 % probability level and 5 % probability level in phenol, carbohydrate, and fiber content. No significant differences were observed in some biochemical traits such as photosynthetic pigments, vitamins E, C, nitrate and nitrate reductase activity, and the amount of oxalic acid. (data was not presented).

There were significant differences between DPPH, flavonoids, and phenolic compounds they are increased in prickly Iranian accession (69.38 %, 0.76 %, and 79.55 mg 100 g^{-1} fresh mass, respectively, Table 3). Phenol and antioxidant properties of every region depend on many parameters such as climate, soil, altitude, and different species of plants (Mirzaei et al., 2010). Phenolic and antioxidant compounds of Iranian accessions 'Saleh-abad' and 'Langrood' had the highest amount of antioxidant compounds, and both of them had prickly seeds (Yosefi et al., 2010). Accordingly, the antioxidant capacity of prickly seed accession was 6.5 % higher than smooth seed accession (Table 3). Plants are one of the important sources of antioxidants compounds that can protect cells from oxidative damage. Secondary plant metabolites such as total phenols and flavonoids derived from plants have strong potential for free radical sweeping in different parts of the plant, such as spinach leaves. Iranian spinach is rich in phenolic compounds with antioxidant properties (Chan et al., 2009).

Spinach leaves contain active components of flavonoids, high antioxidants, and wide pharmacological and biochemical applications, including anti-allergic, anti-viral, and anti-cancer (Lamnitski et al., 2003; Bergman et al., 2001). In the present study, the content of flavonoids in prickly seed accession was 67.1 % higher than in smooth seed varieties (Table 3). Flavonoids are one of the most widespread and diverse natural compounds that can absorb free radicals like other phenolic compounds. In oxidative stresses, phenolic compounds, especially flavonoids, can interact with membrane phospholipids through hydrogen bonding to the polar heads of phospholipids, thereby contributing to membrane integrity (Mirzaei et al., 2010). Under non-stress conditions, there were differences in antioxidant, flavonoid, and total phenolic activity in different spinach varieties (Barbarin et al., 2005).

Soluble carbohydrates are nutrient chemicals valuable to humans and a source of plant energy. Carbohydrates of spinach are very important (Bavec et al., 2010). The highest amount of carbohydrate was observed in prickly seed accession with 0.64 mg g^{-1} fresh mass of spinach (Table 3). Hagen et al. (2009) reported that the carbohydrate content of leafy vegetables varied among the different accessions under the same growth conditions. The content of soluble sugars has been affected by pre-harvest growth temperature (Steindal et al., 2015).

Amino acids are involved in the structure of spinach protein, and amino acids make up about 30 % of all spinach dry matter (Lisiewska et al., 2011). Few articles have been published investigating factors affecting spinach amino acid content (Trejo-Tellez et al., 2005). The level of amino acids in spinach has been correlated with the amount of nitrogen content (Trejo-Tellez et al., 2005). Although nitrogen was not used as fertilizer in the present study, amino acid content was higher in Iranian prickly seed accession (Table 3) (66.6 %) than in other cultivars (Table 3).

The studied accessions had significant differences in total fiber content. Prickly Iranian accession showed the highest fiber content (2.03 %) (Table 3). One of the causes of vegetable consumption is fiber. In this opinion, the studied Iranian spinach has superiority over the foreign accession in fiber content was more suggested for consumption as a diet food (Erfani et al., 2006). In confirming Iranian spinach accession showed 27.6 % more crude fiber content (Table 3).

Spinach is one of the most significant nitrate accumulators because it has a very efficient absorption system and an inefficient nitrate recovery system (Cantliffe, 2005). According to the results of the analysis of variance, nitrate accumulation and nitrate reductase activity in prickly and smooth seed accessions were not significant (data was not presented). It has been reported that there is a difference in nitrate accumulation only in smooth and wrinkled leaves (Arshi, 2000). Although the difference in leaf wrinkling between the two accessions was obvious, their leaves were not sufficiently different in this trait. Probably, nitrate accumulation was not statistically significant (Table 3). Nitrate accumulation in vegetables is also reported to be affected by cultivars and even specific genotypic differences such as ploidy levels (Alamian et al., 2014).

The studied accessions had significant differences in Fe content (Fig 3). Zaferaniye's (2015) study reported that the Iranian accessions have higher iron content than the foreign accessions. In the present study, the Iranian spinach accession had higher Fe content, which was 62.1 % more than the foreign accession (Fig 3). Also, the concentration of iron in the Iranian and foreign accessions of spinach varied from 30 to 50 mg g^{-1}, which was consistent with the amount of iron in this study (Fig 3). Prickly accession was superior in morphological traits in most studies (Asadi & Hasandokht, 2007), and the present study also showed the superiority of nutraceutical traits in prickly seed accession. In the nutraceutical category, the superiority of the

Table 3: Biochemical traits of prickly and smooth seed spinach accessions

Seed type	Chlorophyll (mg g F M-1)	Carotenoid (mg g F M-1)	DPPH (%)	Flavonoid (%)	Phenol (mg/100 g F M-1)	Vit E (mg 100 g F M-1)	Vit C (mg 100 g F M-1)	Carbohydrate (mg g F M-1)	Amino acid (µg g F M-1)	Fiber (%)	Nitrate (mg NO_3 g-1 F M)	Nitrate Reductase (µgr NO_2^- g F M-1 h)	Oxalic Acid (mg g-1FM)
Prickly seed	7.27a	1.65a	69.38a	0.76a	79.55a	1.04a	20.54a	0.64a	0.05a	2.03a	106.2a	29.11a	2.09a
Smooth seed	6.1a	1.43a	65.11b	0.25b	78.75b	0.76a	19.49a	0.36b	0.03b	1.59b	107.2a	38.03a	3.02a
Standard Error	0.63	0.025	2.25	0.003	29.8	0.025	1.52	0.007	0.005	0.041	465.3	240.8	0.31

Different letters within the same column indicate significant differences of each type at $p \leq 0.05$ by the LSD test

prickly seed accession over the smooth seed accession was quite evident (Table 5). Therefore, it is concluded that prickly seed accession had significant antioxidants, flavonoids, total phenols, carbohydrates, amino acids, fibers, and iron content, which was higher than the foreign accession of smooth seed. Following the results of other researchers, Iranian accessions, including 'Varamin Prickly' have a significant superiority in terms of nutritional and functional traits compared to imported foreign accessions, which can be emphasized in the selection and improvement of Iranian spinach accessions (Erfani et al., 2006; Zaferaniye, 2015).

3.4 CORRELATION AMONG THE MORPHOLOGICAL AND BIOCHEMICAL TRAITS

Morphological characteristics of leaves in spring conditions were impressed, as a positive correlation was observed between leaf length and leaf width (r = 0.79*) (Table 4). Similar to our results, Eftekhari et al. (2010) reported a significant correlation between leaf length and width in spinach accessions. Plant height was positively but not significantly correlated with petiole length (r = 0.74), indicating that increasing petiole length was desirable for mechanized harvesting (Table 4). Leaf number is one of the most important yield components in spinach (Jafari & Jalali, 2015), and as shown in table 4, this trait had a significant correlation with yield (r = 0.85*). Also, a significant positive relationship was observed between fresh and dry mass (r = 0.86*). There was also a positive relationship between iron content, leaf area (r = 0.79*) and dry mass (r = 0.89**). A good correlation was observed between female plants and the amount of antioxidants' activity (r = 0.81*), flavonoids (r = 0.96**) and total phenols content (r = 0.61) (Table 4). Also, there was a significant correlation between the percentage of the female plant

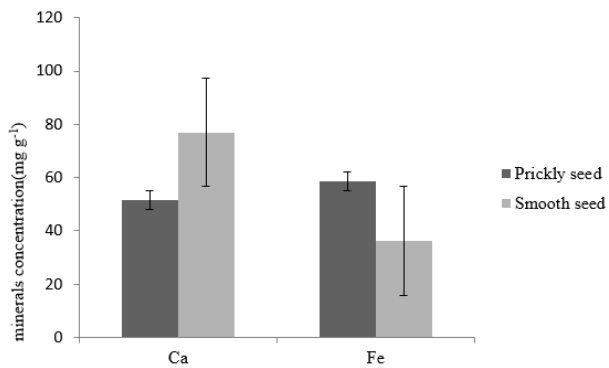

Fig 3: The mineral concentration of prickly and smooth seed spinach accessions

Table 4: Correlation coefficients 30 morphological and biochemical traits studied on prickly and smooth seed spinach accessions

Traits	L.N	P.H	L.L	L.W	P.L	P.D	Y	F.W	D.W	L.A	S.W	G.P	D.F	M	F	Ch	Car	DPPH	Flav	Ph	Vit E	Vit C	Carb	A.A	Fib	Nit	NitRD	OA	Ca
L.N	1																												
P.H	-0.52	1																											
L.L	-0.83*	0.70	1																										
L.W	0.72	-0.24	0.79*	1																									
P.L	-0.75	0.74	0.70	-0.55	1																								
P.D	0.29	0.07	0.22	0.17	-0.34	1																							
Y	0.85*	0.38	0.14	0.52	-0.44	0.44	1																						
F.W	0.79	0.40	0.26	0.42	-0.39	0.55	0.98**	1																					
D.W	0.64	-0.09	-0.10	0.68	-0.74*	0.72	0.85*	0.86*	1																				
L.A	0.46	-0.57	-0.28	0.48	-0.79*	0.64	0.38	0.43	0.76	1																			
S.W	0.58	-0.49	-0.48	0.77	-0.94**	0.48	0.58	0.56	0.85*	0.91*	1																		
G.P	0.64	-0.57	-0.60	0.82*	-0.98**	0.38	0.46	0.43	0.77	0.87*	0.98**	1																	
D.F	0.53	-0.15	-0.41	-0.06	0.13	-0.08	0.68	0.50	0.54	0.39	0.69	-0.25	1																
M	-0.58	0.61	0.55	-0.74	0.94**	-0.39	-0.45	-0.43	-0.76	-0.91**	-0.88*	-0.98**	0.28	1															
F	0.63	-0.57	-0.57	0.78*	-0.96**	0.41	0.50	0.48	0.79*	0.89*	0.69	0.98**	0.79*	-0.99**	1														
Ch	-0.76	-0.02	0.39	-0.76	0.69	-0.64	-0.57	-0.54	-0.78	-0.47	-0.63	-0.60	-0.23	0.53	0.61	1													
Car	-0.74	0.01	0.37	-0.71	0.64	-0.64	-0.34	-0.34	-0.64	-0.43	-0.52	-0.25	-0.17	0.46	0.51	0.9*	1												
DPPH	-0.45	0.16	0.12	0.55	0.72	-0.79	-0.74	-0.77	0.95*	0.65	0.66	-0.76	0.22	0.79	0.81*	0.74	0.57	1											
Flav	-0.68	0.54	0.59	0.54	0.48	-0.44	0.43	0.41	0.78*	0.56	0.66	-0.69	0.23	0.56	0.96**	0.65	0.65	0.76	1										
Ph	-0.25	-0.59	-0.21	-0.29	0.11	-0.67	0.46	0.49	0.5	0.03	0.08	-0.03	0.11	0.04	0.61	0.73	0.77	0.40	0.13	1									
Vit E	-0.73	0.24	0.32	-0.60	0.73	-0.83*	-0.47	-0.50	-0.83*	-0.77	0.70	-0.72	-0.12	0.30	0.74	0.89*	0.82	0.88*	0.76	0.56	1								
Vit C	0.28	-0.04	-0.29	-0.11	0.26	-0.27	-0.74	-0.77	-0.61	-0.51	0.53	-0.39	0.66	0.48	0.47	0.04	-0.20	0.62	0.31	-0.12	0.19	1							
Carb	-0.36	0.09	0.20	-0.76	0.39	-0.55	-0.64	-0.66	-0.85*	-0.71	0.82	-0.81*	0.54	0.57	0.77	0.63	0.69	0.73	0.85*	0.42	0.67	0.39	1						
A.A	-0.62	0.2	0.43	-0.87**	0.55	-0.54	-0.53	-0.52	-0.82*	-0.69	0.43	-0.86*	0.25	0.49	0.81*	0.78	0.84*	0.72	0.91*	0.47	0.78	0.18	0.94**	1					
Fi	-0.42	0.65	0.85	0.37	0.66	-0.11	-0.30	-0.21	-0.57	-0.66	0.56	0.51	-0.04	-0.78*	0.90*	0.59	0.49	0.57	0.89*	-0.08	0.60	0.17	0.59	0.72	1				
Nit	0.35	-0.41	-0.05	-0.26	0.63	0.51	-0.51	-0.41	-0.08	0.31	-0.007	0.008	0.44	0.33	0.21	-0.12	-0.28	-0.11	-0.07	-0.13	-0.41	-0.41	0.11	-0.01	0.05	1			
NitRD	0.002	-0.43	-0.46	0.42	0.41	-0.55	-0.04	-0.12	-0.04	0.14	0.31	0.43	-0.48	0.40	0.35	0.26	0.16	0.16	-0.38	0.57	0.29	-0.11	-0.31	-0.26	-0.46	-0.48	1		
OA	0.02	-0.18	-0.18	0.47	-0.54	-0.01	0.70	0.64	0.58	0.49	0.68	0.60	-0.50	0.65	0.66	-0.16	0.11	-0.55	-0.51	0.27	-0.18	0.85*	-0.44	-0.32	-0.55	-0.50	0.44	1	
Ca	0.16	-0.31	-0.49	0.50	-0.48	-0.41	0.41	0.29	0.26	0.21	0.49	0.49	-0.20	-0.50	0.53	-0.05	0.21	-0.22	-0.38	0.47	0.03	-0.52	-0.18	-0.16	-0.62	-0.67	0.60	0.87*	1
Fe	0.75	-0.26	-0.40	0.63	-0.33	0.45	0.59	0.57	0.89**	0.79*	0.65	0.51	-0.04	-0.79*	0.83*	0.50	-0.31	-0.44	-0.20	-0.50	0.38	0.28	-0.74	0.54	0.62	-0.64	-0.14	0.34	0.14

**indicates significant correlations at the level of $p < 0.01$; * indicates significant correlation at the level of $p < 0.05$. L.N: Leaf number; P.H: Plant height;L.L: Leaf length; L.W: Leaf width: P.L: Petiole length; P.D: Petiole diameter, Y: Yield; F.M: Fresh mass; D.M: Dry mass; L.A: Leaf area; S.W: 1000-grain mass; G.P: Germination percentage; D.F: Day to flowering; M: Male; F: Female; Ch: Chlorophyll; Car: Carotenoids; Flav: Flavonoids; Ph: Phenols; Carb: Carbohydrates; A.A: Amino acids; Fi: Fiber; Nit: Nitrate; NitRD: Nitrate reductase; OA: Oxalic acid

Table 5: Descriptive statistics of the measured traits in smooth and prickly seed of spinach accessions (Sabaghnia et al., 2014; Jafari and Jalali, 2015)

Seed type	Nutraceutical attributes															Total grade
	Chlorophylls	Carotenoids	DPPH	Flavonoids	Phenols	Vit E	Vit C	Carbohydrates	Amino acids	Fiber	Nitrate	Nitrate reductase	Oxalic acid	Ca	Fe	
Prickly seed	3	2	4	4	3	3	4	4	4	5	2	1	3	3	4	48
Smooth seed	2	1	3	2	3	2	3	3	2	2	1	2	2	4	2	34
	Yield and mechanical harvesting attributes															
	Leaf number	Plant height	Leaf length	Leaf width	Petiole length	Petiole diameter			Yield	Fresh mass	Dry mass	Leaf area	Germination			
Prickly seed	3	4	4	1	3	2			3	3	4	3	3			33
Smooth seed	4	2	2	2	1	2			4	4	1	4	5			31
	Breeding features															
	Seed type		Seed weight		Day to flowering				Male		Female					
Prickly seed	2		4		2				2		2					12
Smooth seed	1		2		2				4		4					13

† (1 = Low point; 2 = Medium point; 3 = High point; 4 = very high point)

with antioxidant properties and leaf iron content (Table 4). In confirmation of our results, Asadi & Hasandokht (2007) reported that female plants of spinach had more leafy, yield, and antioxidant properties.

A significant correlation was observed between chlorophyll and carotenoid (r = 0.90*). Carotenoid has been reported to have a protective effect on chlorophyll (Macfarland & Burchett, 2001).

A positive correlation was observed between nitrate and petiole length (r = 0.63). However, this relationship was not significant but consistent with other researchers' reports about the direct relationship between nitrate accumulation and petiole length (Asadi & Hasandokht, 2007; Jafari & Jalali, 2015).

The correlation in Table 4 shows that vitamin C has a negative relationship with nitrate (r = -0.41) and a positive relationship with oxalic acid (r = 0.85*). Increasing nitrate reduces vitamin C in spinach leaves. No significant negative relationship was observed between oxalic acid and nitrate (r = -0.50). According to studies, different results have been reported on nitrate and oxalate relation; (Kaminishi & Kita, 2006) reported a negative relationship between nitrate and oxalate in spinach. Koh et al. (2012) and Zhang et al. (2005) reported a positive correlation between these traits. These reports may indicate a complex pathway involved in nitrate and oxalate metabolism.

Fiber content was positively correlated with female plant percentage (r = 0.90*) and negatively with male plant percentage (r = -0.78). It seems that in addition to photosynthetic pigments (Asadi & Hasandokht, 2007), the fiber content will also increase in the female bushes.

4 CONCLUSION

It can be concluded that although the foreign smooth seed accession was better in breeding characteristics such as 1000-grain mass, germination percentage, leaf thickness, and female plant %, 'Varamin Prickly' (prickly seed) accession was significantly superior to nutraceutical traits such as antioxidant activity, total phenols, fibers, and iron content. Therefore, to enhance the quality traits in new spinach varieties, 'Varamin Prickly' (prickly seed accession) is suitable for transferring these traits and breeding purposes. These features may help obtain more information on the qualitative characteristics of Iranian spinach accessions or clarify the factors that influence the nutraceutical properties of Iranian accessions. Therefore, desirable Iranian accessions for nutraceutical and morphological traits can be used in breeding programs for spinach cultivar production.

5 AUTHOR CONTRIBUTION STATEMENTS

Each named author has contributed to conducting the underlying research and drafting this manuscropt.

6 ACKNOWLEDGMENTS

We thank Isfahan University of technology and the Iran National Science Foundation for supporting this work.

7 COMPLIANCE WITH ETHICAL STANDARDS (E.G., CONFLICT OF INTEREST)

To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

8 REFERENCE

Abolghasemi, R., Haghighi, M., Etemadi, N., Soorni, A., & Jafari, P. (2019). Screening of some native and foreign accessions of spinach for spring culture in Isfahan. *Iran Agricultural Research, 38*(1), 87-99.

Alamian, M., Eftekhari, S. A., Heidari, M., & Alamzadeh Ansari, N. (2014). Evaluation of nitrate accumulation and nitrate reductase activity in different vegetative growth of selected Iranian land races of Spinach (*Spinacia oleracea* L.). *Journal of Crop Production*, *3*, 25-36. (In Persian).

AOAC. (1970). Official methods of analysis. In W. Horwitz (Eds.), *Association of official analythical chemists*, 12th edn. Washington DC, USA.

Arif, M., Jatoi, S. A., Rafique, T., & Ghafoor, A. (2013). Genetic divergence in indigenous spinach genetic resources for agronomic performance and implication of multivariate analyses for future selection criteria science. *Development and Technology*, *32*, 7-15.

Arshi, Y. (2000). *Genetic improvement of vegetable crops*. Mashhad Jahad Daneshgahi, pp. 725.

Asadi, H., & Hasandokht, M. J. (2007). Study of genetic diversity on Iranian spinach genotypes. *Journal of Agricultural Science, 38*, 257-265. (In persian).

Avsar, B. (2011). *Genetic diversity of Turkish spinach cultivars*. A master dissertation graduate school of engineering and sciences, Izmir Institute of Technology, Turkey, pp. 256.

Balalić, I., Miklič, V., Jocić, S., Marinković, R., Cvejić, S., Hladni, N., & Miladinović, D. (2012). Evaluation of NS sunflower hybrids in small-plot trials via hybrid location interaction. *Ratarstvo I Povrtarstvo*, *49*, 270-281. https://doi.org/10.5937/ratpov49-1772

Barbarin, A. M., Williams, J., Bettman, G., Hauber, D., & Ratnayaka, H. (2005). *Gas exchange and antioxvdant properties of two spinach cultivars of contrasting morphology*.

102nd Annual International Conference of the American Society for Horticultural Science, Las Vegas, Vendal.

Bavec, M., Turinek, M., Grobelnik-Mlakar, S., Slatnar, A., & Bavec, F. (2010). Influence of industrial and alternative farming systems on contents of sugars, organic acids, total phenolic content, and the antioxidant activity of red beet (*Beta vulgaris* L. ssp. *vulgaris* 'Rote Kugel'). *Journal of Agricultural and Food Chemistry*, *58*(22), 11825-11831. https://doi.org/10.1021/jf103085p

Bergquist, S. A., Gertsson, U. E., Nordmark, L. Y., & Olsson, M. E. (2007). Ascorbic acid, carotenoids, and visual quality of baby spinach as affected by shade netting and postharvest storage. *Journal of Agricultural and Food Chemistry*, *55*(21), 8444-8451. https://doi.org/10.1021/jf070396z

Cantliffe, D. J. (2005). Nitrate accumulation in table beets and spinach as affected by nitrogen, phosphorous and potassium nutrition and light intensity. *Agronomy Journal, 65*, 563-565. https://doi.org/10.2134/agronj1973.00021962006500040012x

Chan, S. W., Lee, C. Y., Yap, C. F., Wan Aida, W. M., & Ho, C. W. (2009). Optimization of extraction conditions for phenolic compounds from Limaupurut (*Citrus hystrix*) peels. *International Food Research Journal*, *16*, 203-213.

Djioua, T., Charles, F., Lopez-Lauri, F., Filgueiras, H., Coudret, A., Freire, Jr. M., Ducamp-Collin, M. N., & Sallanon, H. (2009). Improving the storage of minimally processed mangoes (*Mangifera indica* L.) by hot water treatments. *Postharvest Biology and Technology*, *52*, 221-226. https://doi.org/10.1016/j.postharvbio.2008.10.006

Eftekhari, S. A. A., Hasandokht, M. R., Fatahi-Moghadam, M. R., & Kashi, A. (2010). Genetic diversity of some Iranian spinach (*Spinacia oleracea* L.) landraces using morphological traits. *Iranian Journal of Horticultural Sciences*, *41*, 83-93. (In persian).

Erfani, F., Hasandokht, M. R., Barzegar, M., & Jabari, A. (2006). Determination and comparison of some nutrient in seven Iranian spinach cultivar. *Iranian Journal of Food Science*, *3*(2), 27-33.

Gorain, M., Sharma, N., Sharma, A., Kumar Meena, A., Singh, S., Srivastava, K., & and Ahmed, S. I. (2014). Spatial prototype and phenotypes of variation in populations of prickly acacia in semi-arid regions in India. *International Journal of Agricultural Science Research*, *3*(7), 112-120.

Hagen, S. F., Borge, G. I. A., Solhaug, K. A., & Bengtsson, G. B. (2009). Effect of cold storage and harvest date on bioactive compounds in curly kale (*Brassica oleracea* L. var. *acephala*). *Postharvest Biology and Technology*, *51*(1), 36-42. https://doi.org/10.1016/j.postharvbio.2008.04.001

Ikic, I., Maričević, M., Tomasović, S., Gunjača, J., Satović, Z., Sarčević, H. (2012). The effect of germination temperature on seed dormancy in Croatian-grown winter wheats. *Euphytica, 3*(1), 25-34. https://doi.org/10.1007/s10681-012-0735-8

IPK. (2018). Institute of Plant Genetics and Crop Plant Research. *GBIS*. http://gbis.ipk-gatersleben.de/gbis_i/home.jsf.

Jafari, P., & Jalali, A. H. (2017). The effect of planting date on yield and nitrate accumulation in spinach (*Spinacia oleracea* L.) In Isfahan weather conditions. *Journal of Horticultural Science*, *31*(3), 533-542. (In persian).

Jafari, P., & Jalali, A. H. (2015). Yield and nitrate accumulation comparison in five Iranian spinach landraces in Isfahan province. *Journal of Crop Production*, *13*, 217-225. (In persian).

Jaliliyan, J. (2009). *Effect of plant density and supplemental irrigation on yield, yield components and protein content of four spinach cultivars in dry land conditions*. Master of Science Degree in Agriculture, West Islamabad University.

Jiang, A. P., Liu, J., & Jiang, J. M. (2016). Relationships of leaf color and pigment and nutrient elements in senescing leaves of *Sassafras tsumu*. *For Research*, *29*, 362-368. (In Chinese).

Kaminishi, A., & Kita, N. (2006). Seasonal change of nitrate and oxalate concentration in relation to the growth rate of spinach cultivars. *Horticultural Science*, *41*, 1589-1595. https://doi.org/10.21273/HORTSCI.41.7.1589

Koh, E., Charoenprasert, S., & Mitchell, A. E. (2012). Effect of organic and conventional cropping systems on ascorbic acid, vitamin C, flavonoids, nitrate, and oxalate in 27 varieties of spinach (*Spinacia oleracea* L.). *Journal of Agricultural and Food Chemistry*, *60*, 3144-3150. https://doi.org/10.1021/jf300051f

Krizek, D. T., Britz, S. J., & Mirecki, R. M. (1998). Inhibitory effect of ambient levels of solar UV-A and UV-B radiation on growth of CV. New red fire lettuce. *Physiology Plant*, *103*, 1-7. https://doi.org/10.1034/j.1399-3054.1998.1030101.x

Kunicki, E., Grabowska, A., Sękara, A., & Wojciechowska, R. J. F. H. (2010). The effect of cultivar type, time of cultivation, and biostimulant treatment on the yield of spinach (*Spinacia oleracea* L.). *Folia Horticulturae*, *22*(2), 9-13. https://doi.org/10.2478/fhort-2013-0153

Kuwahara, K., Suzuki, R., Ito, Y., Mikami, T., & Onodera, Y. (2014). An analysis of genetic differentiation and geographical variation of spinach germplasm using SSR markers. *Plant Genetic Resources*, *12*(2), 185-190. https://doi.org/10.1017/S1479262113000464

Lamnitski, L., Bergman, M., Nyska, A., Ben-Shaul, V., & Grossman, S. (2003). Composition, efficacy and safety of spinach extracts. *Nutrition and Cancer*, *46*, 222-31. https://doi.org/10.1207/S15327914NC4602_16

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods in Enzymology*, *1*(44), 350-382. https://doi.org/10.1016/0076-6879(87)48036-1

Lisiewska, Z., Kmiecik, W., Gebczynski, P., & Sobczynska, L. (2011). Amino acid profile of raw and as eaten products of spinach (*Spinacia oleracea* L.). *Food Chemistry*, *126*(2), 460-465. https://doi.org/10.1016/j.foodchem.2010.11.015

Liu, C. J., Lin, Q., & He, J. X. (2004). Methods and terminology of study on seed morphology from China. *Acta Botanica Sinica*, *24*, 178-188.

Mc-Cready, R. M., Silviera, V., & Owens, H. S. (1950). Determination of starch and amylase in vegetables. *Analytical Chemistry*, *22*, 1156-1158. https://doi.org/10.1021/ac60045a016

Mei, Y., Hou, L. P., Cui, Y. L., Chen, H. L., & Meng, S. C. (2010).

Optimization and establishment of AFLP analyzing system in spinach (*Spinacia oleracea* L.). *Acta Agriculturae Boreali Sinica*, *25*, 111-115.

Meng, S., Liu, C., Xu, X. I., Song, S., Song, SH., Zhang, Z. H., & Liu, L. (2017). Comparison of morphological features of fruits and seeds for identifying two taxonomic varieties of *Spinacia oleracea* L. *Canadian Journal of Plant Science, 98*(2), 318-331. https://doi.org/10.1139/CJPS-2017-0119

Mirzaei, A., Akbartabar, M., Sadeghi, H., & Sharifi, B. (2010). The antioxidant activities and total phenolic of *Artemisia martima, Achillea millefoliumand, Matricaria recutica*. *Journal Armaghan Danesh, 15*(1), 243-252.

Morelock, T. E., & Correll, J. C. (2008). Spinach. In J. Prohens & F. Nuez (Eds.), *Vegetables I*: *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *and Cucurbitaceae* (pp. 189-218). Springer, New York.

Narayana, B., & Sunil, K. (2009). A spectrophotometric method for the determination of nitrite and nitrate. *Eurasian Journal of Analytical Chemistry*, *4*(2), 204-214.

Nolte, J. (2003). *ICP emission spectroscopy*. Weinheim: Wiley-VCH Verlag GmbH and Co. KgaA, pp. 206-207.

Prasad, P. V. V., Pisipati, S. R., Mutava, R. N., & Tuinstra, M. R. (2008). Sensitivity of grain sorghum to high temperature stress during reproductive development. *Crop Science*, *48*, 1911-1917. https://doi.org/10.2135/cropsci2008.01.0036

Raven, J. A. (2003). Cyceing silicon: the role of accumulation in plants. *New Phytologist*, *3*, 419-421. https://doi.org/10.1046/j.1469-8137.2003.00778.x

Sabaghnia, N., Asadi-Gharneh, H. A., & Janmohammadi, M. (2014). Genetic diversity of spinach (*Spinacia oleracea* L.) landraces collected in Iran using some morphological traits. *Acta Agriculturae Slovenica*, *103*(1), 101-111. https://doi.org/10.14720/aas.2014.103.1.11

Shi, A., Mou, B., Evans, M., Clark, J. R., Motes, D., Correll, J., Xiong, H., Qin, J., Chitwood, J., & Weng, Y. (2016). Association mapping of leaf traits in spinach (*Spinacia oleracea* L.). *Plant Breeding*, *135*, 399-404. https://doi.org/10.1111/pbr.12369

Shih-wen, S., Yi-cheng, L., Yih-ming, W., & Min-jane, C. (2006). Efficiency improvements on ninhydrin method for amino acid quantification. *Journal of Food Composition and Analysis*, *19*, 112-117. https://doi.org/10.1016/j.jfca.2005.04.006

Steindal, A. L. H., Rodven, R., Hansen, E., & Molmann, J. (2015). Effects of photoperiod, growth temperature and cold acclimatization on glucosinolates, sugars and fatty acids in kale. *Food Chemistry*, *174*, 44-51. https://doi.org/10.1016/j.foodchem.2014.10.129

Stojičevič, S. S., Stanisavljević, I. V., Veličković, D. T., Veljković, V. B., & Lazić, M. L. (2008). Comparative of the antioxidant and antimicrobial activities of *Sempervivum marmoreum* L. extracts obtained by various extraction techniques. *Journal of the Serbian Chemical Society, 73*(6), 597-607. https://doi.org/10.2298/JSC0806597S

Trejo-Tellez, L. I., Gomez-Merino, F. C., Rodriguea-Mendoza, M. N., & Alcantar-Gonzalez, G. (2005). Foliar fertilization with urea on the portioning of nitrogen in spinach. *Journal of Agricultural Science and Technology*, *23*, 495-503.

Wang, S. Y., & Galletta, G. J. (2002). Compositional change in *Colletotrichum* (Anthracnose) infected strawberry fruit. *Acta Horticulturae*, *567*, 815-819. https://doi.org/10.17660/ActaHortic.1997.439.135

Wu, Y. N., Mei, Y., Cui, Y. L., Chen, H. L., & Meng, S. C. (2015). Morphological traits analysis of spinach (*Spinacia oleracea* L.) germplasm resources. *Journal of Henan Agricultural Science, 44*, 119-124.

Yosefi, Z., Tabaraki, R. H., Asadi Gharneh, A., & Mehrabi, A. A. (2010). Variation in antioxidant activity, total phenolic, and nitrate in spinach. *International Journal of Vegetable Science, 16*(3), 233-242. https://doi.org/10.1080/19315260903577278

Zaferaniye, M. (2015). *Evaluate vegetative and reproductive characteristics of indigenous and foreign rarities*. International conference on applied research in agriculture, Mashhad, Iran, pp. 1-17.

Zhang, Y., Lin, X., Zhang, Y., Zheng, S. J., & Du, S. (2005). Effects of nitrogen levels and nitrate/ ammonium ratios on oxalate concentrations of different forms in edible parts of spinach. *Journal of Plant Nutrition, 28*, 2011-2025. https://doi.org/10.1080/01904160500311086

doi:10.14720/aas.2022.118.2.2404 Original research article / izvirni znanstveni članek

Studies of the impact of environmental conditions and varietal features of sweet cherry on the accumulation of vitamin C in fruits by using the regression analysis method

Iryna IVANOVA [1], Marina SERDYUK [2], Vira MALKINA [3], Tetiana TYMOSHCHUK [4, 5], Marharyta VOROVKA [6], Ivan MRYNSKYI [7], Anastasiia ADAMOVYCH [8]

Received November 02, 2021; accepted April 03, 2022.
Delo je prispelo 2. novembra 2021, sprejeto 3. aprila 2022

Studies of the impact of environmental conditions and varietal features of sweet cherry on the accumulation of vitamin C in fruits by using the regression analysis method

Abstract: The accumulation of vitamin C in sweet cherry fruits depends on the variety and environmental conditions. The aim of our research was to substantiate the rate of impact of weather factors as well as of varietal features on vitamin C accumulation in sweet cherry fruits. The varieties 'Kazka' and 'Zabuta', 'Kordia' and 'Mirazh' were chosen as the best ones from among 33 varieties of early, medium and late term of ripening (7.31–10.67 mg 100 g^{-1}) according to the average content of vitamin C in sweet cherry fruits. The studies found that the environmental conditions of the research years had the largest impact on the vitamin C content in the fruits of late and early ripening varieties, and in the fruits of medium ripening variety the vitamin C amount depended on the varietal features. The practicability of forecasting of vitamin C content in sweet cherry fruits on the average indices for a group of early and late maturity varieties, but not separately for every pomological variety, has been proven. For the medium ripening variety this index can be forecasted within each pomological variety. The models of dependence of vitamins C accumulation on the impact of meteorological parameters were evaluated on the basis of the principle components analysis and the least square method.

Key words: antioxidants; variety; terms of fruit ripening; vitamin C; weather conditions; principle components analysis

Preučevanje vpliva vremenskih dejavnikov in lastnosti sort na vsebnost vitamin C v plodovih češenj z metodo regresijske analize

Izvleček: Količina vitamin C v plodovih češenj je odvisna od sorte in okoljskih razmer. Namen te raziskave je bil ovrednotiti vpliv vremenskih dejavnikov in lastnosti sort na količino vitamina C v plodovih češenj. Izmed 33 zgodnjih, srednjih in poznih sort so bile izbrane najboljše sorte kot so 'Kazka','Zabuta', 'Kordia' in 'Mirazh' glede na poprečno vsebnost vitamin C (7,31–10,67 mg 100 g^{-1}) v plodovih. Raziskava je odkrila, da so imele na vsebnost vitamina C v plodovih zgodnjih in poznih sort češenj največji vpliv vremenske razmere posamezne rastne sezone, pri srednje dozorevajočih sortah pa so imele največji vpliv na vsebnost vitamina lastnosti sort. Dokazana je bila možnost napovedovanja vsebnosti vitamina C v plodovih zgodnjih in poznih sort na osnovi povprečnih indeksov, vendar ne za vsako sorto posebej. Za srednje dozorevajoče sorte bi lahko uporabili ta indeks za vsako sorto posebej. Model za ugotavljanje odvisnosti kopičenja vitamina C v odvisnosti od vremenskih dejavnikov je bil ovrednoten na osnovi analize glavnih komponent in metode najmanjšega kvadrata.

Ključne besede: antioksidanti; sorte; čas dozorevanja; vitamin C; vremenske razmere; analiza glavnih komponent

1 Department of Horticulture, Viticulture and Biochemistry, Faculty of Agrotechnology and Ecology, Dmytro Motornyi Tavria State Agrotechnological University, Melitopol, Ukraine
2 Department of Food Technology and Hotel and Restaurant Business, Faculty of Agrotechnology and Ecology, Dmytro Motornyi Tavria State Agrotechnological University, Melitopol, Ukraine
3 Department of Higher Mathematics and Physics, Faculty of Energy and Computer Technology, Dmytro Motornyi Tavria State Agrotechnological University, Melitopol, Ukraine
4 Department of Plant Protection, Faculty of Agronomy, Polissia National University, Zhytomyr, Ukraine
5 Corresponding author, e-mail: tat-niktim@ukr.net
6 Department of Pedagogy and Pedagogical Mastership, Bogdan Khmelnitsky Melitopol State Pedagogical University, Melitopol, Ukraine
7 Department of Botany and Plant Protection, Faculty of Agronomy, Kherson State Agrarian and Economic University, Kherson, Ukraine
8 Department of Social Sciences and Humanities, Dmytro Motornyi Tavria State Agrotechnological University, Melitopol, Ukraine

1 INTRODUCTION

Sensible nutrition of people is one of the main priorities of the state policy of most countries. The fruits are considered as an essential element of good nutrition, as they are the source of a vitamin complex. Vitamins belong to a group of indispensable nutrients of organic nature, which facilitate metabolism. They are scarcely synthesized in an organism, so they should be in taken with food. The amount of vitamin C in fruits (Bastos et al., 2015; Hayaloglu & Demir, 2015) is an important characteristic of fruits for their consumption in raw condition, for processing and storing (Vasylyshyna, 2018). One of the directions of solving the defined problem is to provide the population with fruits with a high amount of biologically active substances which are essential for people (Dhandevi & Jeewon, 2015).

The sweet cherry fruits (*Prunus avium* L.) belong to the food stuff which contains digestible sugars, phenolic acids with a predominant amount of anthocyanins, mineral substances and vitamins (Leong & Oey, 2012; Pissard et al., 2016; Ivanova et al., 2021a, 2021b, 2021c). Biologically active substances represented by phyto-nutrients and antioxidants provide antioxidant, anti-cancerogenic and anti-phlogogenic action on human organism (Ballistreri et al., 2013; Popescu et al., 2014; Legua et al., 2017). Due to this, sweet cherry fruits reveal some preventive effect against cardiovascular diseases, diabetes, cancer, which is connected with an oxidative stress (He et al., 2007; Faniadis et al., 2008; Schmitz-Eiberger & Blanke, 2012). Sweet cherry fruits are characterized by a high amount of dietary medicative substances, which promote to organism functioning. Sweet cherry fruits contain water soluble (C, B) and fat soluble (A, E, K) vitamins (Antognoni et al., 2020). Vitamins take part in oxidation-reductions, respiration, nucleic acids formation and amino-acids exchange, protein secretion as well as improve carbohydrate digestion. Besides, they control cholesterol metabolism, prevent the accumulation of harmful free radicals in body tissues, improve the resistance to infectious diseases as well as to unfavorable environmental factors which cause overheating, excessive heat loss, hypoxia and improve person's performance (Bastos et al., 2015). Vitamin C or L-ascorbic acid is one of the most important phyto-nutrients, which determines the biological value of sweet cherry fruits. Vitamin C belongs to a group of water soluble vitamins (Antognoni et al., 2020). Vitamin C amount in sweet cherry fruits equals 7.26–10.78 mg 100 g^{-1} on the average. In living organisms, the ascorbic acid is an antioxidant, as it protects the organism against the oxidative stress and is a co-factor in essential vital enzyme reactions (Nowak et al., 2018; Antognoni et al., 2020). The immunity protection and the maintaining of psychical processes in a proper condition are the most important functions of vitamin C. Vitamin C is one of the regulators of reductive-oxidative processes in living cells. Vitamin C scarcity results in metabolic disorder in the whole organism (Prior, 2003). For a human an every-day need for vitamin C equals 50–100 mg. Vitamin C scarcity in a human diet can cause hypoavitaminosis and avitaminosis C, as this vitamin is not synthesized in an organism (Acero et al., 2019). Fresh fruits are the main source of vitamin C. This fact testifies to the expediency of prolonging the term of fruits consuming. Thus, it is important to retain this valuable nutritive constituent under sweet cherry fruits keeping and processing (Correia et al., 2017). Palatability traits and biochemical composition of sweet cherry fruits depend on the genetic traits of the variety (Serrano et al., 2005; Faniadis et al., 2008; Correia et al., 2020). The researchers have produced the varieties of stone fruits which meet modern requirements, but there are still some problems which are very topical (Correia et al., 2017; Grandi et al., 2017). The chemical composition of the fruits of any harvest, except varietal features, depend on the meteorological conditions of the growing period as well as of the zone of fruit growing (Hayaloglu & Demir, 2015; Luna-Vázquez et al., 2016).

The researchers of the subtropical regions of Brazil estimated the chemical composition, identificated the biologically active compounds and estimated the antioxidant activity of berries and fruits including sweet cherries. It was established that the amount of ascorbic acid in the fruits of the subtropical regions of Brazil is much higher than in the zone with moderate climate (Rios de Souza et al., 2014). The formation of the biochemical composition of sweet cherry fruits as well as of their consumption value depend on the temperature, light intensity, fruit ripeness (Martini et al., 2017; Acero et al., 2019). The dependence of vitamin C amount on the meteorological indicators within the period from blooming to stone fruit ripening was studied by many scientists. A higher concentration of vitamin C in fruits was registered in the years with sufficient water availability (Lakatos et al., 2010, 2014). The level of ascorbic acid content in fruits is formed genetically. But the weather factors during the growing period have great impact on the accumulation of vitamin C fund (Bieniek et al., 2011). The greatest amount of vitamin C is formed during the years with moderately warm and wet growing period. A sufficient fruits lightning is a very important factor which affects the process of ascorbic acid synthesis (Kevers et al., 2011). On the basis of literature sources it can be stated that there is a strong correlation between vitamin C amount and the environmental conditions of the region of fruit growing. Under conditions of climate changes, stress abiotic factors have negative impact on the formation of vitamin C fund in sweet cherry

fruits under conditions of a Southern Steppe sub-zone of Ukraine. Therefore, there is a necessity to study the peculiarities of vitamin C accumulation in sweet cherry fruits of different terms of ripening under the influence of stress weather factors in order to single out the most suitable varieties for fruit storing and processing.

The aim of the study was to develop a model for vitamin C content forecasting in the fruits of early, medium and late terms of ripening depending on the environmental conditions. The received mathematic model is the basis for the forecasting of a test parameter of fruits quality in the regions with similar environmental conditions. The task of the research was to recommend the fruits of early, medium and late maturity varieties with a high amount of vitamin C for consuming fresh as well for fruit storing and processing.

To achieve the aim, it is necessary to:

- analyze the environmental conditions during the period of phenological stages of sweet cherry fruits growing and developing;
- estimate the vitamin C amount in fruits on the stage of their economic maturity and choose the best varieties;
- study the correlations between the accumulation of vitamin C and weather factors and to develop the mathematical models of their dependence;
- estimate the rate of impact of each weather factor on the formation of vitamin C fund in test varieties of three groups.

2 MATERIALS AND METHODS

The research was conducted during 2008–2019 in sweet cherry fruiteries in the Southern Steppe sub-zone of Ukraine. The region is characterized by insufficient water availability as to the amount of rainfalls. The climate is Atlantic-continental, dry with a high temperature regime. The dry winds are of Northeastern direction. According to the complex of climatic parameters, the test region is favorable for sweet cherry fruits growing (Table 1). The data of Melitopol meteorological station of the South of Ukraine (46° 49'N, 35° 22'E) were used for the calculation of the model of forecasting of vitamin C amount in sweet cherry fruits

The research areas have black southern loamy soil, which was formed on the loesses. The agrochemical characteristic of soil is given in Table 2.

The technology of sweet cherry growing in the experiment was standard for a given region. The scheme of trees planting in 2001 was – 5 × 3 m. The space between rows on the fruit plantation was kept under autumn fallow. All sweet cherry varieties on ‚Magalebska' cherry rootstock were divided into three groups according to the term of ripening (early, medium and late): early term of ripening 7 varieties, medium term of ripening 13 varieties, late term of ripening 13 varieties. The fruits of an early ripening term were harvested in the third decade of June and in the first decade of July. The fruits of a medium ripening term were harvested in the second decade of July. The fruits of a late ripening term were harvested in the third decade of July. To study the vitamin C amount, 100 fruits were chosen from 6 trees of each sweet cherry variety of the same age on the stage of heavy bearing with average intensity. There was a threefold frequency of variants in the experiment. The fruits of each variety were picked by hand from four different sides of the tree crown on

Table 1: Meteorological conditions of Southern Steppe sub-zone of Ukraine

Readings	Value
Average annual air temperatures, °C	9.1–9.9
Average monthly air temperatures in the warmest months, C	20.5–23.1
Sum of active temperatures higher than 10 C from April to October, C	3316
Average amount of rainfalls per year, mm	475
Average annual relative air humidity, %	73
Average annual air velocity, m s^{-1}	3
Hydrothermic coefficient	0.22–0.77

Table 2: Agrochemical characteristics of topsoil of tested soil

			Nutrient content, mg g^{-1} 100 of soil		
Depth of arable layer, cm	Humus content, %	pH_{kCl}	N	P_2O_5	K_2O
40.0	1.38	6.9	27.0	90.0	154.0

the stage of economic maturity. After harvesting the fruits, they were weighed and counted (Serdyuk et al., 2020). The sweet cherry fruits were transported to the laboratory for 2–3 hours after their harvesting to estimate the tested parameter. During the period of sweet cherry fruits harvesting, their economic maturity was estimated by visual and organoleptic examination. The fruit pulp was rather firm, color and flavor were typical for each pomological variety. The sweet cherry fruits were harvested with a fruit-stalk. The fruits transportation and storing were conducted under condition of preserving of the external appearance and flavor typical for a variety.

The restoring of Tilman's reagent (2.6-dichlorphenol-indophenol) was taken as the basis of the technique on the evaluation of a mass fraction of vitamin C. The amount of ascorbic acid in the extracts (Serdyuk et al., 2020) was evaluated in terms of the amount of the reagent which was used for titration.

The model of dependence of vitamin C amount in sweet cherry fruits on the weather factors was developed according to the schedule (Ivanova et al., 2021a, 2021b):

1. Evaluation of a mass fraction of ascorbic acid (AA).

2. Analysis of the weather factor during the years of the research.

3. Choosing the weather factor which show the correlation with vitamin C amount in fruits.

4. Developing the regression model of dependence of vitamin C amount in the fruits of sweet cherry varieties on the weather factor.

5. Evaluation and ranging of the rate of impact of each weather factor on the tested parameter of fruits quality.

The statistical analysis was made using modern computer technologies DataMining – software environment RStudio.

3 RESULTS AND DISCUSSION

For the sweet cherry fruits of three groups of ripening the average content of vitamin C equaled 8.17 mg 100 g^{-1} (Tables 3–5). The accumulation of vitamin C in the fruits of seven cultivars grown in Turkey was analyzed by Demir T. The average content of vitamin C in sweet cherry fruits varied between 4 and 7 g kg^{-1} of fresh mass (Demir, 2013). The ascorbate levels for 22 sweet cherry cultivars grown in southern of Italy was considerably lower – 0.034–0.260 g kg^{-1} of fresh mass (Matteo et al., 2016). The rate of accumulation of vitamin C fund in sweet cherry fruits of an early term of ripening equaled 7.10 mg 100 g^{-1}, which is by 13.1 % lower as compared with the average index for three groups.

In a group of an early term of ripening the varieties, 'Kazka' (7.36 ± 1.40 %) and 'Zabuta' (7.31 ± 1.49 %) had the largest vitamin C content on the average during the years of studies. In the fruits of 'Bigaro Burlat' variety vitamin C content was the lowest (6.84 ± 1.22 %). The fruits of 'Bigaro Burlat' (the early term ripening group) had the lowest content of vitamin C in 2018 – 5.02 mg 100 g^{-1}, which is for 26.6 % lower than the average varietal parameters. The fruits of 'Merchant' variety had a maximal accumulation of vitamin C (11.29 mg 100 g^{-1}) in 2019, which is for 63.6 % higher as compared with the average varietal parameters. The highest content of vitamin C during the years of research was in 'Kazka' and 'Zabuta' varieties, and the lowest – in 'Bigaro Burlat' variety (Table 3).

The average content of vitamin C in sweet cherry

Table 3: The accumulation of vitamin C in sweet cherry fruits of early term ripening varieties, mg 100 g^{-1} (2008–2019), $\overline{X} \pm S\overline{X}$, n = 5

Variety	Vitamin C content, %			Variation according to years, Vp, %
	average	min	max	
'Bigaro Burlat'	6.84 ± 1.22	5.02	9.83	17.9
'Zabuta'	7.31 ± 1.49	5.16	10.27	20.4
'Kazka'	7.36 ± 1.40	6.08	10.12	19.1
'Merchant'	6.90 ± 1.84	5.18	11.29	26.7
'Rubinova Rannia'	6.92 ± 1.28	5.12	9.03	18.5
'Sweet Erliz'	7.26 ± 1.68	5.17	11.00	23.1
'Valeriy Chkalov'	7.13 ± 1.54	5.19	10.12	21.5
Average value	7.10 ± 1.46	5.26	10.23	21.02
LSD_{05}	0.579	–	–	–

Table 4: Vitamin C content in sweet cherry fruits of medium term ripening varieties, mg 100 g^{-1} (2008–2019), $\overline{x} \pm s\overline{x}$, n=5

Variety	Vitamin C content, %			Variation according to years, Vp, %
	average	min	max	
'Chervneva Rannia'	5.95 ± 1.06	4.12	7.67	17.9
'Dachnytsia'	6.32 ± 1.11	5.01	7.88	17.5
'Dilema'	10.94 ± 2.20	8.19	14.51	20.1
'Kordia'	10.63 ± 1.81	8.01	13.85	17.1
'Oktavia'	10.11 ± 1.74	7.40	12.08	17.2
'Oktavia'	9.25 ± 2.25	5.12	12.47	24.3
'Orion'	10.46 ±1.83	7.51	12.82	17.8
'Pervystok'	9.05 ± 1.59	6.34	11.18	17.5
'Prostir'	7.73 ± 1.19	5.19	9.27	15.4
'Talisman'	10.48 ± 2.46	7.23	14.11	23.4
'Temp'	8.07 ± 1.49	5.89	9.85	18.5
'Uliublenytsia Turovtseva'	9.02 ± 1.60	6.11	11.91	17.7
'Vynka'	8.08 ± 1.51	6.23	10.14	18.7
Average value	8.93 ± 2.27	6.33	11.36	18.7
LSD_{05}	0.645	–	–	–

fruits of two varieties (medium and late terms of ripening) equals 8.93 ± 2.27 % and 8.48 ± 1.74 % respectively (Table 4, 5). The rate of formation of vitamin C fund in sweet cherry fruits of medium term of ripening exceeds the average varietal parameters by 3.8 % and in the fruits of late term of ripening by 9.3 %. It has been established that the fruits of a medium term of ripening had the maximal content of vitamin C.

In a medium term ripening group a minimal amount of vitamin C had the fruits of 'Dachnytsia',

Table 5: Vitamin C content in sweet cherry fruits of late ripening varieties, mg 100 g^{-1} (2008–2019), $\overline{x} \pm s\overline{x}$, n = 5

Variety	Vitamin C content, %			Variation according to years, Vp, %
	average	min	max	
'Anons'	8.20 ± 1.59	5.71	11.81	19.3
'Karina'	8.33 ± 1.48	5.78	10.28	17.7
'Kolhoznytsia'	7.85 ± 1.24	5.79	10.92	15.8
'Kosmichna'	8.95 ± 1.60	6.69	12.03	17.9
'Krupnoplidna'	7.74 ± 1.16	5.79	10.23	14.9
'Meotyda'	8.03 ± 1.45	5.61	10.72	18.1
'Mirazh'	10.67 ± 1.49	8.28	14.14	14.0
'Prazdnichna'	10.25 ± 2.02	7.61	13.08	19.7
'Regina'	7.29 ± 1.01	6.03	10.54	13.8
'Surpryz'	8.10 ± 1.47	5.65	11.01	18.2
'Temporion'	7.72 ± 1.44	5.01	9.76	18.7
'Udivitelna'	7.58 ± 1.31	5.41	9.23	17.3
'Zodiak'	9.60 ± 1.46	7.79	11.19	15.2
Average value	8.93 ± 2.27	6.33	11.36	18.7
LSD_{05}	0.645	–	–	–

'Temp' and 'Chervneva Rannia' varieties of 2008. Vitamin C content was lower than the average varietal parameter by 20.7 %, 27.0 % and 30.7 % respectively. A maximal amount of vitamin C was registered in the fruits of 'Talisman' (14.11 %) and 'Dilema' (14.51 %) of 2010 that is by 34.6 and 32.6 % higher than the average varietal parameter. The highest accumulation of an average content of vitamin C was registered in the fruits of 'Dilema' (10.94 ± 2.20 %) and 'Kordia' (10.63 ± 1.81 %) varieties.

In a late term ripening group a minimal amount of vitamin C had the fruits of 'Temporion' variety (5.01 %) of 2008 (Table 5). The content of vitamin C was by 9.8 % lower than the average varietal parameter. The fruits of 'Mirazh' (14.14 %) and 'Prazdnichna' (13.08 %) of 2014 had a maximal amount of vitamin C that is by 34.9 and 27.6 % higher as compared with an average varietal parameter. A maximal accumulation of an average vitamin C content was registered in the fruits of 'Mirazh' (10.67 ± 1.49 %) and 'Prazdnichna' (10.25 ± 2.02 %). Especially valuable are the varieties whose fruits are characterized by a high and stable amount of vitamin C content (Leong & Oey, 2012).

Their variation parameter Vp can be used as an indicator of stability – of a variety in reference to the meteorological conditions of different years of fruits growing. The variation of sampling can be considered insignificant or low under the Vp lower than 10 %, average – under 10 – 20 % and high under 20 % and more. Therefore, the varieties whose fruits have a high and stable vitamin C content are of special value.

The variability of vitamin C content during the years of research in the sweet cherry fruits of an early and medium terms of ripening was in the range from 15,4 to 26,7 %. For a group of early ripening varieties ('Valeriy Chkalov', 'Svit Earliz', 'Merchant' and 'Zabuta') the variation parameter equaled 20.4–26.7 %, for a group of medium terms ripening varieties ('Talisman', 'Dilema', 'Oktavia') Vp equaled 20.1–24.3 %. It testifies to a maximal impact of weather factors on vitamin C content in the sweet cherry fruits of these groups. The variation parameter for 'Prostir' and 'Bigaro Burlat' varieties equals 15.4 % and 17.9 % respectively that testifies to their resistance to stress factors.

The Vp range for the sweet cherry fruits of the late term of ripening varied within the average values 13.8–19.7 %. The lowest values of the variation parameter were registered in 'Regina' and 'Mirazh' varieties (Vp = 13.8–14.0 %). The highest vitamin C stability in a group of an early term of ripening is in 'Bigaro Burlat' variety (Vp = 17.9 %). The varieties 'Kazka' and 'Zabuta' have the highest rate of vitamin C accumulation (7.36 ± 1.40 %) and (7.31 ± 1.49 %) respectively. The most perspective from the point of view of fruits storing and processing are 'Kordia' (Vp = 17.1 %) of the medium term and 'Mirazh' (Vp = 14.0 %) of the late term of ripening. These varieties are characterized by a high and nearly high content of vitamin C as well as by minimal variability as compared with other varieties. The received results of the research have been confirmed by literature data (Bieniek et al., 2011). The chemical composition analysis of fruit revealed significant differences both between the cultivars and between the years of the research.

Environmental conditions, Factor A (Table 6) had the greatest impact on vitamin C content in the fruits of

Table 6: The results of two factors dispersion analysis under the accumulation of vitamin C in sweet cherry fruits of different terms of ripening

Source of variation	Sum of squares	Degree of freedom	Dispersion	F_{fact}	$F_{table095}$	Impact, %
Group 1, early ripening sweet cherry varieties						
Factor A (year)	448.5	11	40.7	321.5	1.8	80.2
Factor B (cultivar)	9.9	6	1.6	13.0	2.2	1.7
Interaction AB	79.6	66	1.2	9.5	1.4	1.2
Group 2, medium term sweet cherry varieties						
Factor A (year)	998.05	11	90.7	577.7	1.8	39.1
Factor B (cultivar)	1266.13	12	105.5	671.9	1.8	49.6
Interaction AB	237.68	132	1.8	11.4	1.3	9.3
Group 3, late ripening sweet cherry varieties						
Factor A (year)	640.38	11	58.2	244.3	1.8	43.5
Factor B (cultivar)	513.89	12	42.8	179.7	1.8	34.9
Interaction AB	241.40	132	1.8	7.67	1.3	16.4

early and late terms of ripening. The rate of impact of the environmental conditions of the years of research (Factor A) for early ripening varieties equaled 80.2 %, for late ripening varieties only 43.5 %. The rate of impact on varietal features (Factor B) was significantly lower for these two groups of varieties in terms of ripeness rate. For early ripening varieties, it equaled 1.7 %, for late ripening varieties 34.9 %. It was established that the formation of vitamin C fund in the sweet cherry fruits of medium term of ripening depended on the varietal features (Factor B, 49.6 %). The impact of environmental conditions of the research years (Factor A) for this group of varieties equaled 39.1 %.

The research shows the practicability of forecasting of vitamin C accumulation in fruits of early and late ripening sweet cherry varieties in terms of average variety values but not for each pomological variety. The forecasting of accumulation of vitamin C fund in the fruits of medium ripening varieties is expedient to make in terms of average varietal parameters and rate of content for each pomological variety.

To establish the correlation relationships between vitamin C content in fruits of early (Y_1), medium (Y_2), late (Y_3) terms of ripening and the climatic factors, the following analysis was made. On the basis of the established matching coefficients of correlation r_{Y1Xi}, r_{Y2Xi}, r_{Y3Xi} were chosen the most significant factors. The significance of these correlation coefficients was established by checking a statistical hypothesis H_0: $p = 0$ (where p - is a correlation coefficient of general population) under the alternative hypothesis H_1: $p \neq 0$ under the reliability level α = 0,05. Student's criteria was used to check the statistical hypothesis. It was established that significant correlation coefficients, under the significance level of 0.05 and the number of degrees of freedom k = 10, were within an interval [–0.55; 0.55].

Further experiments were conducted according to the schedule:

First step. On the basis of the principle components analysis we build a set of the principal components PC_i $(i = 1 \ldots n)$ represented by a liniar combination of weather factors:

$$PC_i = \sum_{j=1}^{m} p_{ij} X_j,\ i = 1 \ldots n \qquad (1)$$

where X_j – weather factors parameters, $j = 1..m$

PC_i - principal components, $i = 1 \ldots n$

p_{ij} - coefficients, $i = 1 \ldots n$, $j = 1..m$.

We choose first five principal components (PC_i, $i = 1..5$), which provide more than 93,5 % of cumulative proportion of variance.

Second step. The development of the regression models of dependence of vitamin C value on the weather factors for each group of varieties from the principal components was as follows:

$$\hat{Y} = b_0 + \sum_{i=1}^{5} b_i \cdot PC_i \qquad (2)$$

where $\hat{Y}$ – vitamin C content, mg 100 g^{-1}

PC_i - principal components, $i = 1 \ldots 5$

b_i - regression coefficients, $i = 1 \ldots 5$.

The regression equation for early ripening varieties is as follows:

$$\hat{Y}_1 = 7.10762 - 0.41631PC_1 + 0.13542PC_2 + 0.61543PC_3 + 0.13960PC_4 - 0.25401PC_5$$

The regression equation for medium ripening varieties is as follows:

$$\hat{Y}_2 = 8.9078 - 0.4252PC_1 + 0.1872PC_2 - 0.0064PC_3 + 0.7698PC_4 + 0.1628PC_5$$

The regression equation for late ripening varieties is as follows:

$$\hat{Y}_3 = 8.4881 - 0.3533PC_1 + 0.3044PC_2 - 0.0150PC_3 - 0.1113PC_4 - 0.0869PC_5$$

The value of the determination coefficient (R-squared) for early ripening varieties equals 0.9239, for medium –ripening, 0.7273, for late ripening varieties, 0.7069, it indicates that there is a strong impact of independent variables on the dependent variables.

The p-value is < 0.05 for F-statistic value for all regression models, it testifies to the adequacy of the models according to Fisher's criterion under the level of significance -0.05.

Third step. We proceed from the principal components to the initial factors by applying formula 1. We carry on the standardization process of variable models. We receive a regression model which characterizes the dependence of vitamin C value (for $\hat{Y}_1, \hat{Y}_2, \hat{Y}_3$) from weather factors.

$$\hat{Y} = \sum_{j=1}^{n} \tilde{a}_j \cdot \tilde{X}_j \qquad (3)$$

where $\tilde{X}_j = \frac{X_j - \overline{X}_j}{\sigma_{X_j}}$ – values of weather

and environmental factors in a standardized form $j = 1 \ldots n$

$\bar{X}_j$ - arithmetic mean of factors X_j, $j = 1 \ldots n$

σ_{X_j} - standard deviation of factors X_j, $j = 1 \ldots n$

$\tilde{a}_j$ - model coefficients, $j = 1 \ldots n$

$\hat{Y}$ - vitamin C content, mg 100 g^{-1}.

Table 7 presents the coefficients of model (3) for vitamin C content in sweet cherry fruits of early ($\hat{Y}_1$), medium ($\hat{Y}_2$) and late ($\hat{Y}_3$) terms of ripening.

The coefficients $\Delta_i, (i = 1..12)$ were estimated by formula for each factor on the basis of the developed models:

$$\Delta_i = \left| \frac{\tilde{a}_i \cdot r_{yx_i}}{R^2} \right| \quad (4)$$

where $\tilde{a}_i$ - coefficients of a regression model (3)

r_{yx_i} - matching coefficients correlation

R^2 – determination coefficients.

Twelve parameters of the climatic factors (X_i) which in a particular growing period could have a significant impact on the ascorbic acid content in the sweet cherry fruits of early (Y_1), medium (Y_2) and late (Y_3) ripening varieties were chosen (Table 8).

Table 7: Coefficients of a regression model in standardized factors

	$\tilde{a}_1$	$\tilde{a}_2$	$\tilde{a}_3$	$\tilde{a}_4$	$\tilde{a}_5$	$\tilde{a}_6$
$\hat{Y}_1$	-0,529	-0,131	-0,207	-0,067	-0,234	-0,278
$\hat{Y}_2$	-0,420	-0,164	-0,225	-0,082	-0,167	-0,263
$\hat{Y}_3$	-0,360	-0,444	-0,478	0,070	-0,309	0,120
	$\tilde{a}_7$	$\tilde{a}_8$	$\tilde{a}_9$	$\tilde{a}_{10}$	$\tilde{a}_{11}$	$\tilde{a}_{12}$
$\hat{Y}_1$	-0,382	0,061	-0,021	-0,227	-0,183	0,101
$\hat{Y}_2$	-0,320	0,129	0,056	-0,184	-0,090	0,056
$\hat{Y}_3$	-0,166	0,477	0,147	0,004	0,380	0,064

These parameters are the following: air humidity; average monthly air humidity in May (X_2); average monthly precipitation amount in May (X_1) and in June (X_{11}); average number of days with precipitation amount more than 1 mm in May (X_5), June (X_6) and July (X_7); average minimal relative air humidity in May (X_3) and in June (X_4); the amount of precipitation during the period after blooming (X_{10}); hydrothermal coefficient (X_{12}). The parameters (X_8), the difference between the average maximal and minimal temperatures in May, and in June (X_9) were chosen from among the temperature air indicators (°C).

A complex of weather factors which have average and strong linear correlation dependence in terms of vitamin C content has been established for each group of varieties.

The factors of impact which are expedient to study from the point of view of the importance and the logicality of the experiment, despite the insignificance of their correlation coefficients, have been found.

The coefficients Δ_i estimate the rate of each factor in a total dispersion of vitamin C amount in sweet cherry fruits. On the basis of the estimated indices $\Delta_i, i = 1..12$ we rank all the factors in terms of their impact from the most significant (rank 1) to the factor which has the lowest impact (rank 12). Table 7 represents the values of index $\Delta_i, \%$ and the rank of factors.

For the experimental groups of sweet cherry varieties of three terms of ripening, Δ_i varies in the range of 0.16 – 30.64 % (Table 7). According to the estimated indices $\Delta_i, (i = 1..12)$ all the factors were divided into the ranks.

An average monthly amount of precipitation in May (X_1) had a maximal impact on vitamin C content in sweet cherry fruits of early and late ripening varieties. An average precipitation amount in June (X_{11}) had impact on the formation of vitamin C fund in sweet cherry fruits of late term of ripening. This parameter got the first rank and varied in the range of Δ_i indices from 14.92 % to 30.64 %. Air humidity indices had a significant impact on vitamin C content in sweet cherry fruits.

The parameters of the second rank are average monthly relative air humidity in May (X_2), late term of ripening, total number of days with precipitation amount more than 1 mm in June (X_6), medium term of ripening, total number of days with precipitation amount more than 1 mm in July (X_7), early term of ripening. The significance of the rate of impact of these

factors, Δ_{X6}, Δ_{X7}, Δ_{X2}, was in the range of 11.57–18.57 %.

Weather factors which have a significant impact on vitamin C formation in sweet cherry fruits of three groups belong to the parameters of the third rank, they are: average minimal air humidity in May, % (X_3), a total number of days with precipitation amount more than 1mm in June (X_6). The rate of impact equaled Δ_{X7} = 18.57 % for the varieties of an early term of ripening, and for the varieties of medium and late terms of ripening the rate of impact equaled Δ_{X3} 11.38 % and 14.81 % respectively. The factors of the 1–7th ranks with the rates of impact Δ_i (6.09–30.64 %) had a maximal impact on vitamin C content for early ripening varieties of sweet cherry.

Table 8: The table of the coefficients of matching correlation $(r_{Y_j X_i})$, indices of the impact rate (Δ_i) and their ranks, which characterize the impact of factors (X_i,) on vitamin C content in the sweet cherry fruits of three terms of ripening

Relative factors term (X_i)	Factors	Matching coefficients of correlation ($r_{Y_j X_i}$), indices of the impact rate of factors (Δ_i) and indices of the factors ranks for the varieties groups								
		early ripening			medium ripening			late ripening		
		$r_{y_1 x_i}$	Δ_i	rank	$r_{y_2 x_i}$	Δ_i	rank	$r_{y_3 x_i}$	Δ_i	rank
X_1	Average monthly amount of precipitation in May, mm	0.889	30.64	1	0.651	23.86	1	0.535	13.45	4
X_2	Average monthly relative air humidity in May, %	0.712	6.09	7	0.575	8.22	7	0.479*	14.87	2
X_3	Average minimal relative air humidity in May, %	0.733	9.90	4	0.579	11.38	3	0.443*	14.81	3
X_4	Average minimal relative air humidity in June, %	0.201*	0.88	11	0.348*	2.49	10	0.476*	2.34	11
X_5	Total number of days with precipitation amount more than 1 mm in May, per day	0.531*	8.09	5	0.702	10.25	5	0.431*	9.29	5
X_6	Total number of days with precipitation amount more than 1 mm in June, per day	0.604	10.97	3	0.503*	11.57	2	0.719	6.03	8
X_7	Total number of days with precipitation amount more than 1 mm in July, per day	0.745	18.57	2	0.368*	10.28	4	0.634	7.37	7
X_8	Difference between average maximal and minimal temperatures in May, °C	–0.52*	2.07	9	–0.29*	3.35	9	–0.24*	8.24	6
X_9	Difference between average maximal and minimal temperatures in June, °C	–0.27*	0.36	12	–0.27*	1.35	12	–0.541	5.56	9
X_{10}	Amount of precipitation in blooming period, mm	0.498*	7.37	6	0.631	10.13	6	0.528	0.16	12
X_{11}	Average monthly amount of precipitation in June, mm	0.159*	1.90	10	0.683	5.38	8	0.563	14.92	1
X_{12}	Hydrothermical coefficient	0.480*	3.15	8	0.452	1.74	11	0.661	2.97	10

*some important factors which must be studied in the experiment as expedient and logical ones, though their correlation coefficients are not significant

Some additional impact on vitamin C content had the following parameters: average minimal relative air humidity in June (X_4); the difference between average maximal and minimal temperatures in May (X_8); the difference between average maximal and minimal temperatures in June (X_9); average monthly precipitation amount in June (X_{11}); hydrothermical coefficient (X_{12}).

The overall value of index Δ_i for factors X_{12}, X_8 and X_{11}, X_4, X_9 equaled 8.36 %.

Factors of 1–8 ranks had a maximal impact on vitamin C content for medium ripening varieties. The range of Δ_i for them was in the range of 5.38–23.86 %. A less significant impact on the formation of vitamin C content in sweet cherry fruits of a medium term of ripening had the climatic parameters like: average minimal relative air humidity in June (X_4), the difference between average maximal and minimal temperatures in May (X_8); the difference between average maximal and minimal temperatures in June (X_9); hydrothermical coefficient (X_{11}). The overall value of index Δ_i for factors X_4, X_8, X_9, X_{11}, which belong to ranks 9–12 within the given group of sweet cherry varieties, equaled 8.93 %.

All factors in a group of late ripening sweet cherry varieties (Δ_i – 5.56–14.92 %) had a largest impact on the formation of vitamin C content. The factors of 10–12 ranks (X_{10}, X_{11}, X_{12}) had a less significant impact on vitamin C content in sweet cherry fruits of a given group. The overall value of index Δ_i for them equaled 5.47 %.

The analysis of the rate of impact of weather factors on vitamin C content in sweet cherry fruits of three terms of ripening testifies to the fact that the most significant climatic parameters are: humidity indices in May and June (the last months of fruits formation), the average monthly precipitation amount in May (X_1) for early ripening and medium ripening varieties, and the precipitation depth in June (X_{11}) for late ripening varieties respectively.

4 CONCLUSIONS

1. The varieties 'Kazka' (7.36 mg 100 g^{-1}), 'Zabuta' (7.31 mg 100 g^{-1}) have been chosen in terms of vitamin C content for the group of an early term of ripening. 'Bugaro Burlat' variety (with a minimal variability parameter Vp = 17.9 %) has been chosen in terms of minimal variability parameter during the years of research.

2. A medium ripening variety 'Kordia' (10.63 mg 100 g^{-1} under Vp = 17.1 %) and late ripening variety 'Mirazh' (10.67 mg 100 g^{-1} under Vp = 14.0 %) were chosen as the most perspective from the point of view of fruit storing and processing in terms of vitamin C content. The variation parameters of vitamin C content in sweet cherry fruits of three terms of ripening ranged from 13,8% to 26,7%.

3. The environmental conditions of the years of research (Factor A) – 43.5 % and 80.2 % respectively, had a dominating impact on vitamin C content in the fruits of late and early ripening varieties.

4. The impact of varietal features (Factor B) was dominating (49.6 %) in terms of vitamin C content in the sweet cherry fruits of medium term of ripening.

5. The models of dependence of vitamin C content on the impact of climatic conditions for three groups of varieties were developed by using the method of the principle components and the method of least squares.

6. The analysis of the rate of impact of each meteorological parameter on vitamin C content in sweet cherry fruits was made by using the developed regression models. The range of impact of the meteorological parameters in the formation of vitamin C fund has been established and their maximal values (Δ_i 14.92 % to 30.6 %) for the groups of three-term ripening varieties have been estimated.

5 REFERENCES

Acero, N., Gradillas, A., Beltran, M., García, A., Muñoz Mingarro, D. (2019). Comparison of phenolic compounds profile and antioxidant properties of different sweet cherry (*Prunus avium* L.) varieties. *Food Chemistry*, *279*, 260–271. https://doi.org/10.1016/j.foodchem.2018.12.008

Antognoni, F., Potente, G., Mandrioli, R., Angeloni, C., Freschi, M., Malaguti, M., Hrelia, S., Lugli, S., Gennari, F., Muzzi, E., & Tartarini, S. (2020). Fruit quality characterization of new sweet cherry cultivars as a good source of bioactive phenolic compounds with antioxidant and neuroprotective potential. *Antioxidants (Basel, Switzerland)*, *9*(8), 677. https://doi.org/10.3390/antiox9080677

Ballistreri, G., Continella, A., Gentile, A., Amenta, M., Fabroni, S., Rapisarda, P. (2013). Fruit quality and bioactive compounds relevant to human health of sweet cherry (*Prunus avium* L.) cultivars grown in Italy. *Food Chemistry*, *140*, 630–638. https://doi.org/10.1016/j.foodchem.2012.11.024

Bastos, C.A.A., Barros, L., Dueñas, M., Calhelha, R. C., Queiroz, M.J.R.P., Santos-Buelga, C., Ferreira, I.C.F.R. (2015). Chemical characterization and bioactive properties of *Prunus avium* L.: the widely studied fruits and the unexplored stems. *Food Chemistry*, *173*, 1045–1053. https://doi.org/10.1016/j.foodchem.2014.10.145

Bieniek, A., Kawecki, Z., Kopytowski, J., Zielenkiewicz, J. (2011). Yielding and fruit quality of Lithuanian sweet cherry cultivars grown under the climatic and soil con-

ditions of Warmia. *Folia Horticulturae*, *23*(2), 101–106. https://doi.org/10.2478/v10245-011-0015-4

Correia, S., Schouten, R., Silva, A. P., Gonçalves, B. (2017). Factors affecting quality and health promoting compounds during growth and postharvest life of sweet cherry (*Prunus avium* L.) *Frontiers in Plant Science*, *8*, 2166. https://doi.org/10.3389/fpls.2017.02166

Correia, S., Aires, A., Queirós, F., Carvalho, R., Schouten, R., Silva, A.P., Gonçalves, B. (2020). Climate conditions and spray treatments induce shifts in health promoting compounds in cherry (*Prunus avium* L.) fruits. *Scientia Horticulturae*, *263*, 109–147. https://doi.org/10.1016/j.scienta.2019.109147

Demir, T. (2013). Determination of carotenoid, organic acid and sugar content in some sweet cherry cultivars grown in Sakarya, Turkey. *Journal of Food, Agriculture and Environment, 11*, 73–75.

Dhandevi, P. E. M., & Jeewon, R. (2015). Fruit and vegetable intake: Benefits and progress of nutrition education interventions-narrative review article. *Iranian Journal of Public Health*, *44*, 1309.

Faniadis, D. Drogoudi, P. D., Vasilakakis, M. (2008). Effects of cultivar, orchard elevation, and storage on fruit quality characters of sweet cherry (*Prunus avium* L.). *Scientia Horticulturae, 125*, 301–304. https://doi.org/10.1016/j.scienta.2010.04.013

Grandi, M., Lugli, S., Correale, R. (2017). Fruit quality changes in postponed picking of new cherry cultivars. *Acta Horticulturae*, *1161*, 599–602. https://doi.org/10.17660/ActaHortic.2017.1161.95

Hayaloglu, A. A., & Demir, N. (2015). Physicochemical characteristics, antioxidant activity, organic acid and sugar contents of 12 sweet cherry (*Prunus avium* L.) cultivars grown in Turkey. *Journal of Food Science*, *80*(3), 564–570. https://doi.org/10.1111/1750-3841.12781

He, F. J., Nowson, C. A., Lucas, M., MacGregor, G. A. (2007). Increased consumption of fruit and vegetables is related to a reduced risk of coronary heart disease: meta-analysis of cohort studies. *Journal of Human Hypertension*, *21*, 717–728. https://doi.org/10.1038/sj.jhh.1002212

Ivanova, I., Serdyuk, M., Malkina, V., Priss, T., Herasko, T., Tymoshchuk, T. (2021a). Investigation into sugars accumulation in sweet cherry fruits under abiotic factors effects. *Agronomy Research*, *19*(2), 444–457. https://doi.org/10.15159/ar.21.004

Ivanova, I., Serdiuk, M., Malkina, V., Bandura, I., Kovalenko, I., Tymoshchuk, T., Tonkha, O., Tsyz, O., Mushtruk, M., Omelian, A. (2021b). The study of soluble solids content accumulation dynamics under the influence of weather factors in the fruits of cherries. *Potravinarstvo Slovak Journal of Food Sciences*, *15*, 350–359. https://doi.org/10.5219/1554

Ivanova, I., Serdyuk, M., Malkina, V., Tymoshchuk, T., Kotelnytska, A., Moisiienko, V. (2021c). The forecasting of polyphenolic substances in sweet cherry fruits under the impact of weather factors. *Agraarteadus*, *32*(2), 239–250. https://doi.org/10.15159/jas.21.27

Kevers, C., Pincemail, J., Tabat, J., Defrasgne, J. O., Dommes, J. (2011). Influence of cultsvator. Harvest time, storage conditions, and reelsng on the antioxidant capacity and phenolsc and ascorbic acid contents of apples and pears *Journal of agricultural and food chemistry, 59*(11), 6165–6171. https://doi.org/10.1021/jf201013k

Lakatos, L., Dussi, M. C., Szabo, Z. (2014). The influence of meteorological variables on sour cherry quality parameters. *Acta horticulturae*, *1020*, 287–292. https://doi.org/10.17660/ActaHortic.2014.1020.41

Lakatos, L., Szab, T., Sun, Z., Soltész, M. (2010). The role of meteorological variables of blossoming and ripening within the tendency of qualitative indexes of sour cherry. *International Journal of Horticultural Science*, 16(1), 7–10. https://doi.org/10.31421/IJHS/16/1/854

Legua, P., Domenech, A., Martínez, J.J., Sánchez-Rodríguez, L., Hernández, F., Carbonell-Barrachina, A.A., Melgarejo, P. (2017). Bioactive and volatile compounds in sweet cherry. *Journal of Food and Nutrition Research*, *5*(11), 844–851. https://doi.org/10.12691/jfnr-5-11-8

Leong, S. Y., & Oey, I. (2012). Effects of processing on anthocyanins, carotenoids and vitamin C in summer fruits and vegetables. *Food Chemistry, 133*, 1577–1587. https://doi.org/10.1016/j.foodchem.2012.02.052

Luna-Vázquez F. J., Ibarra-Alvarado, C., Rojas-Molina, A., Rojas-Molina, J. I., Yahia E. M., Rivera-Pastrana, D. M., Rojas-Molina, A., & Zavala-Sánchez, M. Á. (2016). Nutraceutical value of black cherry *Prunus serotina* Ehrh. fruits: antioxidant and antihypertensive properties. *Molecules, 18*(12), 14597–14612. https://doi.org/10.3390/molecules181214597

Martini, S., Conte, A., & Tagliazucchi D. (2017). Phenolic compounds profile and antioxidant properties of six sweet cherry (*Prunus avium*) cultivars. *Food Research International*, *97*, 15–26. https://doi.org/10.1016/j.foodres.2017.03.030

Matteo, A. D., Russo, R., Graziani, G., Ritieni, A., Vaio, C. D. (2016). Characterization of autochthonous sweet cherry cultivars (*Prunus avium* L.) of southern Italy for fruit quality, bioactive compounds and antioxidant activity. *Journal of the Science of Food and Agriculture*. *97*(9), 2782–2794. https://doi.org/10.1002/jsfa.8106

Nowak, D., Gośliński, M., Wojtowicz, E., Przygoński, K. (2018). Antioxidant properties and phenolic compounds of vitamin C-rich juices. *Journal of Food Science*, *83*(8), 2237–2246. https://doi.org/10.1111/1750-3841.14284

Pissard, A., Lateur, M., Baeten, V., Magein, H., Dupont, P., Tabart, J., Pincemail J., Kevers, C. (2016). Determination of total phenolic compound content and antioxidant activity in cherry species and cultivars. *Journal of Berry Research*, *6*, 81–91. https://doi.org/10.3233/JBR-150109

Popescu, S., Velciov, A.B., Riviş, A., Costescu, C., Petolescu, C. (2014). Antioxidant activity and phenolic content of sweet cherries (*Prunus avium* L.) from West and South-West of Romania. *Bulletin UASVM Food Science and Technology*, *71*(2), 215–216. https://doi.org/10.15835/buasvmcn-fst:10762

Prior, R. L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. *American Journal of Clinical Nutrition, 78*(3), 570–578. https://doi.org/10.1093/ajcn/78.3.570S

Rios de Souza, V., Pereira P. A. P., Teodoro da Silva, T.L., Carlos

de Oliveira Lima, L., Pio, R., Queiroz, F. (2014). Determination of the bioactive compounds, antioxidant activity and chemical composition of Brazilian blackberry, red raspberry, strawberry, blueberry and sweet cherry fruits. *Food Chemistry*, *156*, 362–368. http://dx.doi.org/10.1016/j.foodchem.2014.01.125

Schmitz-Eiberger, M. A. & Blanke, M. M. (2012). Bioactive components in forced sweet cherry fruit (*Prunus avium* L.), antioxidative capacity and allergenic potential as dependent on cultivation under cover. *Food Science and Technology*, *46*, 388–392. https://doi.org/10.1016/j.lwt.2011.12.015

Serdyuk, M. E., Priss O. P., Haprindashvili, N. A., Ivanova, I. Ye. (2020). Research methods of fruit, vegetable and berry products. (Ch. 1). Melitopol : Liuks. (In Ukraine).

Serrano, M., Guillén F., Martínez-Romero, D., Castillo S., Valero, D. (2005). Chemical constituents and antioxidant activity of sweet cherry at different ripening stages. *Journal Agricultural Food Chemistry*, *53*(7), 2741–2745. https://doi.org/10.1021/jf0479160

Vasylyshyna, O. (2018). The quality of sour cherry fruits (*Prunus cerasus* L.), treated with chitosan solution before storage. *Acta Agriculturae Slovenica*, *111*(3), 633–637. https://doi.org/10.14720/aas.2018.111.3.11

doi:10.14720/aas.2022.118.2.2541 Original research article / izvirni znanstveni članek

Genotypic variation in response to drought stress is associated with biochemical and transcriptional regulation of ureides metabolism in common bean (*Phaseolus vulgaris* L.)

Motlalepula PHOLO-TAIT [1, 2], Thuto KGETSE [3], Gaone Nthabeleng TSHEKO [3], Olerato Tsotlhe THEDI [3], Katso LETHOLA [1], Ebenezer Oteng MOTLAMME [1], Moagisi Innocent ITHUTENG [1] and Samodimo NGWAKO [4]

Received February 23, 2022; accepted May 29, 2022.
Delo je prispelo 23. februarja 2022, sprejeto 29. maja 2022

Genotypic variation in response to drought stress is associated with biochemical and transcriptional regulation of ureides metabolism in common bean (*Phaseolus vulgaris* L.)

Abstract: Ureidic legumes such as common bean (*Phaseoulus valgaris* L.) plants export nitrogen from the nodules to shoots and leaves as ureides during symbiotic biological nitrogen fixation. Common bean gene encoding allantoinase (allantoin amidohydrolase, EC 3.5.2.5), is a key enzyme that catalyses the hydrolysis of allantoin to allantoic acid. It plays a role in ureide generation for export and ureide catabolism to generate a nitrogen source in sinks tissues. As such, one of the adaptive mechanisms of plants to drought stress, is associated with ureides accumulation. To identify genetic variation of common bean in response to drought stress, changes in the expression of *ALLANTONAISE* (*PvALN*) gene and ureides content were examined in the leaf tissues of the three common bean genotypes (CAL96, DAB514 and DAB541) and one tepary bean genotype (*Phaseolus acutifolius* A.Gray). Amongst all the genotypes, the suggested drought susceptibility in DAB514 common bean genotype, was probably attributed to a repressed *PvALN* expression rate which were corroborated by an impaired ureides levels, and reduced plant growth. On contrary, drought stress induced an upregulated relative expression of *PvALN* coupled with an increase in allantoin and allantoate in DAB541 common bean genotype. In addition, the sustained plant growth in CAL96 was probably attributed to a steady amount of allantoin synthesized under drought stress. Taken together, DAB541 and CAL96 common bean genotypes are the promising genotypes with an induced upregulated transcriptional control of catabolism and/or biosynthesis of ureides, hence potential genotypes for selection and introduction under Botswana semi-arid conditions.

Key words: common bean; drought stress; ureides: allantonaise; allantoin; allantoate

Genetska spremenljivost odziva navadnega fižola (*Phaseolus vulgaris* L.) na sušni stres je povezana z biokemičnim in transkripcijskim uravnavanjem presnove ureidov

Izvleček: Ureidne stročnice kot je navadni fižol (*Phaseoulus valgaris* L.) transportirajo med simbiontsko vezavo dušik iz nodulov v liste kot ureide. Pri navadnem fižolu je pomemben gen, ki kodira alantoinazo (alantoin amidohidrolaza, EC 3.5.2.5), ključni encim, ki katalizira hidrolizo alantoina v alantoinsko kislino. Ta ima pomembno vlogo pri tvorbi ureidov za njihov eksport in razgradnjo kot vir dušika v tkivih ponora. Pri rastlinah je eden izmed prilagoditvenih mehanizmov na sušni stres povezan s kopičenjem ureidov. Za določitev genetske variabilnosti navadnega fižola na sušni stres so bile analizirane spremembe v izražanju gena za alantoinazo, *ALLANTONAISE* (*PvALN*) in vsebnosti ureidov v listnih tkivih pri treh genotipih navadnega (CAL96, DAB514 and DAB541) in enem genotipu ostrega fižola, *Phaseolus acutifolius* A.Gray. Med vsemi genotipi bi občutljivost genotipa DAB514 navadnega fižola verejtno lahko pripisali zavrtju izražanja gena *PvALN*, kar je bilo povezano z zmanjšano tvorbo ureidov in slabšo rastjo. V nasprotju je sušni stres vzpodbudil povečano izražanje tega gena, kar je bilo povezano s povečanjem vsebnosti alantoina in alantoata pri genotipu DAB541. Dodatno bi ohranjeno rast genotipa CAL96 lahko pripisali stalni količini allantoina, ki se sintetizira med sušnim stresom. Zaključimo lahko, da sta genotipa navadnega fižola DAB541 in CAL96 obetajoča, z vzpodbujeno povečano transkripcijsko kontrolo katabolizma in/ali biosinteze ureidov, ki bi lahko služila kot potencial za izbor in uvajanje ustreznih genotipov v sušnih razmerah Botswane.

Ključne besede: navadni fižol; sušni stres; ureidi; alantonaza; allantoin; alantoat

1 Department of Agricultural Research, Ministry of Agricultural Development and Food Security, Gaborone, Botswana
2 Corresponding author, e-mail: pholom@webmail.co.za
3 Department of Chemistry & Forensic Sciences, Botswana International University of Science and Technology, Palapye, Botswana
4 Faculty of Research and Graduate Studies, Botswana University of Agriculture and Natural Resources, Gaborone, Botswana

1 INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important grain legume in the human diet due to its high nutritional properties, such as proteins, vitamins and minerals (Broughton et al., 2003). One of the major benefits of common beans in agriculture is their capacity to symbiotically fix atmospheric nitrogen through associations with soil nitrogen-fixing rhizobia, thus reducing the need to use nitrogen fertilizers (Coleto et al., 2014). As such, common bean plants are not dependent on nitrogen fertilization for growth due to their ability to form symbioses with atmospheric di-nitrogen fixing bacteroid located in root nodules. Plant growth and productivity is dependent on the accessibility of the newly available fixed nitrogen from the root to the vegetative and reproductive plant tissues.

Ureide allantoin and its degradation derivate allantoate are a group of soil heterocyclic nitrogen compounds that play an essential role in the assimilation, metabolism, transport, and storage of nitrogen in higher plants (Smith & Atkins, 2002). They serve as the vehicle for storage and xylem transport of symbiotically fixed nitrogen from root to the shoot, and as such play a key role in nitrogen utilization in ureide-type legumes (Kohl et al., 1990; Smith & Atkins, 2002; Zrenner et al., 2006). Once delivered to sink tissues, allantoin is converted to allantoate, which in-turn can be broken down completely to glyoxylate, releasing four molecules of ammonia and two molecules of CO_2. Genes encoding allantoinase (allantoin amidohydrolase, EC 3.5.2.5), catalysis the first step in the degradation of the ureide allantoin and the synthesis of allantoate, the second most prominent ureide. It is therefore unique in this pathway such that it plays a role in ureide generation for export from the nodules as well as ureide catabolism to generate a nitrogen source in leaves and other nitrogen sinks (Muñoz et al., 2001; Watanabe et al., 2014; Werner et al., 2013).

Adaptive mechanisms of plants to abiotic stresses such as drought, include changes in the expression of genes involved, biosynthesis of compatible osmolytes and scavenging systems for reactive oxygen species (Han et al., 2014; Hasegawa et al., 2000). The inhibition of nitrogen utilization under drought stress, is proposed to be attributed to N-feedback regulation, in which ureides would be among the signaling molecules triggering the inhibition (Charlson et al., 2009; King & Purcell, 2005; Rachid Serraj, Vadez et al., 1999). The induction and activation of enzymes with a subsequent increased levels of intermediary metabolites, particularly ureides allantoin and allantoate play a vital role in plant responses and adaptation to abiotic stresses (Alamillo et al., 2010; Smith & Atkins, 2002) . In soybean, high ureides levels in shoots and leaves correlated with nitrogen fixation inhibition (Rachid Serraj, Vadez, et al., 1999). In *Arabidopsis thaliana* (L.) Heynh. mutant lacking *ALLANTONAISE (ALN)*, high levels of allantoin metabolites were reported due to an activated allantoin biosynthetic genes and/or repression of *ALN* expression rate. The response suggested that ureide metabolism and accumulation contribute to the abiotic stress response, which is regulated, at least in part, at the transcriptional level. In addition, this implied a possible elevated drought stress tolerance, possibly by reducing oxidative damage. (Irani & Todd, 2016).

The symbiotic nitrogen fixation showed to be extremely sensitive to drought stress and this effect could result in decreasing N accumulation and yield of legume crops (Serraj, 1999; Rachid Serraj, 2003). However, ureide-exporting legumes, such as common beans are more sensitive to drought stress due to rapid decline in nitrogen fixation compared to amidic ones (Purcell et al., 2004; R Serraj, 1999; Rachid Serraj, Vadez et al., 1999). On contrary, a variable degree of nitrogen fixation inhibition due to drought stress was found among the bean genotypes. An increase in both mRNA levels and *ALN* activity with a concomitant increase in roots, shoots and leaves ureide levels in common bean in response to drought was attributed to an elevated synthesis of allantoate (Alamillo et al., 2010). Remarkably, other studies demonstrated a positive correlation between suppressed nitrogen fixation and accumulation of ureides in stems and leaves of both sensitive and tolerant genotypes. Further variability was associated with the rise in allantoate level coupled with an increase in *ALLANTOINASE* gene expression and enzyme activity in the most sensitive genotype, which increased after inhibition of nitrogen fixation, suggesting that ureides originate in vegetative tissues as a response to water stress, probably mediated by the induction of allantoinase (Coleto et al., 2014).

The overreliance on erratic rain coupled with relatively poor soil quality has resulted in poor productivity of crops in Botswana, making the agricultural sector most vulnerable to climate change (FANRPAN, 2017). Crop diversification such as the use of drought-tolerant legumes with enhanced nitrogen fixation ability and improved utilization of the newly fixed nitrogen to enhance crop productivity crops has been hailed as one of the potential adaptive measures to mitigate climate change. As such, Botswana has considered the introduction of common bean into the cropping system as one of the climate smart agriculture approaches, combating poverty, environmental degradation, and improving soil health. This was further justified by its high nutri-

tive value and commercial benefits such as source of income for many rural household (Beebe et al., 2013; Molosiwa et al., 2019). However, information on the performance of the potential common bean genotypes for introduction, particularly nitrogen fixation and utilization capability and crop productivity under Botswana conditions remain elusive. Therefore, this study was conducted to identify the growth and genetic response of common bean genotypes under drought stress. Biochemical analysis of ureides-derived metabolites and transcriptional analysis of *Phaseoulus vulguris ALLANTOINASE* relative gene expression was conducted for the identification and selection of the best and promising common bean genotypes in terms of nitrogen fixation and utilization under drought stress.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND GROWTH CONDITIONS

Common bean genotypes were selected based on their superior stability, adaptability and yield performance in the previous studies conducted at Sebele and Pandamatenga respectively (Molosiwa et al., 2019). These includes three common bean (*Phaseolus vulgaris* L.) genotypes (DAB541; DAB514; CAL96) and GK011 tepary bean (*Phaseolus acutifolius* A.Gray.; GK011), the latter being reported in previous studies as a drought tolerant bean. The experiment was conducted in a growth cabinet, in a randomized block design, with six replications, under a 16 h light/8 h dark photoperiod at 25 °C and a light intensity of 100-150 µmol photons $m^{-2}s^{-1}$. Plants were exposed to water holding treatment three (3) weeks after emergence. Drought stress experiment consisted of two treatments, namely drought stress treatment by withholding water application with a serious drought stress (35-45 % water holding capacity) and the control by ensuring maximum water holding capacity by watering (70-80 %).

2.2 UREIDES ACCUMULATION: ALLANTOIN AND ALLANTOATE

The determination of ureides allantoin and allantoate were performed by differential analyses of glyoxylate derivative according to published protocol (Lescano, 2020). Ureides were extracted from leaf 15 mg liquid nitrogen grounded leaf tissue samples by boiling it in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 18,000 xg for 25 min at 4 °C to ensure the absence of debris and a clear supernatant containing ureides. Six biological replicates of 100 µl aliquots of each sample were collected in three separate tubes for the measurement of endogenous glyoxylate, allantoic acid-derived glyoxylate and allantoin-derived glyoxylate. Glyoxylate is converted into glycolic acid-phenylhydrazone and then oxidized by ferricyanide in the presence of concentrated acid and phenylhydrazine to give red-colored 1,5-diphenylformazan. The absorbance of supernatants was measured using a spectrophotometer at 535 nm.

2.3 TRANSCRIPTOMIC ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES

Total RNA was extracted using a Quick-RNA Miniprep Kit (Zymo Research Corporation, Irvine, CA, United States) as per the manufacturer's protocol and treated with *DNase1* (Zymo Research Corporation, Irvine, CA, United States). The quantity and quality of the isolated RNA were evaluated, respectively, using a NanoDrop ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific) and by 1 % electrophoresis agarose gels according to manufacturer's instructions. The quality of each cDNA and the RT-qPCR were checked per by using standard PCR reaction and the housekeeping gene *PvACTIN-2* primers (Díaz-Leal et al., 2012) and *PvALN* (Table 1). These primer pairs were designed using GeneScript qPCR primer design (https://www.genscript.com/tools/pcr-primers-designer/advanced). Luna Universal qPCR Master Mix (New England Biolab Inc., MA, USA) and primers were used to determine RNA expression. The qPCR reactions were performed using triple replicates of cDNA samples in 96-well plates and performed on the LineGene 9600 (Hangzhou Bioer Techonology), following SYBR Green/FAM detection. Reactions were prepared in a total volume of 20 µl according to Luna® Universal qPCR Master Mix Protocol (M3003; New England Biolab Inc., MA, USA) containing: 1x Luna Universal qPCR Master Mix, 10 µM of forward and reverse primer, 100 ng cDNA template and nuclease-free water. The PCR cycles consisted of 1 cycle of initial denaturing at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 40 s. The melting curve was obtained by applying increasing temperature from 60 to 90 °C. The relative fold change for *Phaseoulus vulgaris* ALLANTOINASE *(PvALN)* was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), and normalized against the housekeeping *PvACTIN-2* gene (Díaz-Leal et al., 2012).

Table 1: Primer pairs used to determine expression of genes

Gene	Forward Primers	Reverse Primer
ACTIN-2 (PvActin-2)	5'-TTGCTTTCAAGGAGGGGGTATGC-3'	5'-GGAGCTTGGAACCTTTCGGTGC-3'
ALLANOTONAISE (PvALN)	5'-ACAAGCATGATGCAGGTGCTGTGA-3'	5'-TGCCTCCACGACATCGCACA-3'

2.4 DATA ANALYSIS

The data collected were subjected to analysis of variance (ANOVA) using MINITAB computer software program, significant means were separated using pairwise Tukey comparison at $p < 0.05$.

3 RESULTS

3.1 PHYSIOLOGICAL BIOMASS RESPONSE

To determine the response of common bean to drought stress, fresh biomass was determined from above ground plant tissues after watering was withheld for 10 consecutive days. Biomass for the three genotypes including GK011, CAL96 and DAB541 did not differ under normal water growth conditions. However, a significant reduced biomass was observed in DAB514 common bean genotypes compared to GK011 tepary bean as well as compared to the two common bean genotypes, namely, CAL96 and DAB541. Comparing drought stressed plants to their relative control plants demonstrated no significant variation in biomass in GK011 tepary bean and DAB541 common bean genotype. However, drought stress induced a 38.6 % and 38.7 % significant reduction in biomass in CAL96 and DAB514 respectively. Further biomass comparisons were made between drought stressed plants in all the genotypes. The result indicated a 42.6 % reduction in

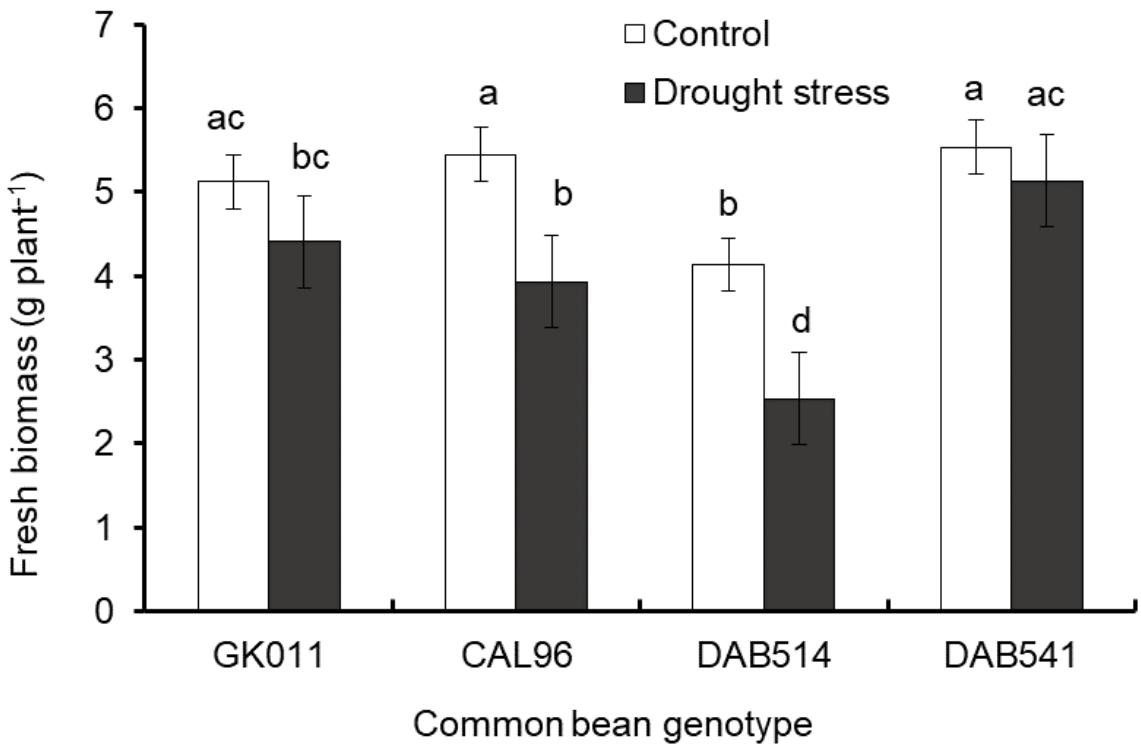

Figure 1: Biomass of common bean genotypes (*Phaseolus vulgaris* L.) in response to drought stress. Biomass was measured unbve ground plat tissues of three common bean genotypes (CAL97; DAB514; DAB541) and GK011 tepary bean. The standard error of mean of three independent flow cells is indicated by the error bars (n = 6). Bars with different lowercase letters indicate significant differences ($p < 0.05$)

biomass in drought stressed DAB514 plants relative to drought stressed GK011 tepary bean. In addition, a 35.6 % and a 50.6 % reduction in biomass in drought stressed DAB514 plants were observed in comparison to drought stressed CAL96 and DAB541 common bean plants respectively. Taken together, all comparisons demonstrated the existence of clear variation between DAB514 and DAB541 common bean genotypes in terms of biomass (Figure 1).

3.2 UREIDES ACCUMULATION IN RESPONSE TO DROUGHT STRESS

To investigate the production of ureides-derived metabolites in response to drought stress, levels of allantoin and allantoate were measured in the leaves of drought stressed plants and control plants (Figure 2). The results were visualized using heat map (Figure 3), generated with MINITAB analytical software (version 21.1). The result demonstrated a significant increase in allantoin metabolite in CAL96 (50.1 %), DAB514 (45.5 %) and DAB541 (47.1 %) common bean genotypes compared to the GK011 tepary bean under normal water conditions. A significant 60.0 % and 23.8 % increase in allantoin accumulation between the plants under normal condition and drought stress was detected for GK011 tepary bean and DAB541common bean genotypes respectively. In contrast, allantoin content was not significantly affected between the control plants and the drought stressed DAB514 and CAL96 common bean genotypes.

In respect to allantoate metabolite, the levels of allantoate were not significantly affected in CAL96 and DAB514 common bean genotypes compared to GK011 tepary bean under normal water growth conditions. The study further compared variation in allantoate levels for the drought stressed plants compared to their relative control plants. The result exhibited 24.6 %, 26.5 % and 47.8 % of reduced allantoate levels for GK011 tepary bean and two common bean genotypes, namely, CAL96 and DAB514 in drought stressed plants compared to the control plants. In contrast, DAB541 common bean genotype elicited a 31.0 % significantly increased levels of allantoate in the drought stressed plants relative to their control plants. Taken together, the response of DAB541 common bean genotype under water stress showed a similar trend for both allantoin and allantoate. Thus, water stress induced a significant increase in both allantoin and allantoate metabolite levels (Figure 2).

3.3 RELATIVE GENE EXPRESSION OF UREIDE METABOLISM

To assess the correlation between allantonaise ureide and changes in *ALLANTONAISE (PvALN)* relative gene expression, both metabolic accumulation of allantonaise and relative *PvALN* gene expression was performed on the leaves of genotypes. To assess whether the accumulation of ureides results from changes in the transcription of genes related to ureide metabolism, quantitative real time PCR was performed to determine the mRNA levels of genes coding for key enzymes in the synthesis of ureides, *ALLANTONAISE (ALN)*. Expression level of *PvALN* gene in the three replicates samples were normalized against the expression of *ACTIN-2* as the internal control. According to the pairwise Tukey comparison, the relative expression of *PvALN gene* in water-deficit plants compared to the control plants was significantly depressed for all the common beans genotypes, except for DAB541. GK011 tepary bean showed the highest 7.7-folds reduction in the relative expression of *PvALN*. This decrease was however insignificantly different from DAB514 and CAL96 common bean genotypes, which also showed a decreased *PvALN* expression rate by 3.2 and 5.4-folds respectively. Intriguingly, only DAB541 common bean genotype, showed an increase in the expression rate *PvALN* mRNA (1.2-folds) in the leaves of drought stressed plants relative to the control plants (Figure 4).

4 DISCUSSIONS

Legumes are agronomically and economically important in many cropping systems because of their ability to assimilate atmospheric nitrogen and maintaining soil fertility. These are highly desirable traits to consider in the improvement of legume productivity for sustainable agricultural practices (Serraj, 1999; Rachid Serraj, 2003, 2003; Rachid Serraj et al., 1999). Drought stress is one of the most important environmental factors that regulate plant growth and development and limit its production. Legumes exhibit reduction in nodulation and biological nitrogen fixation in response to drought stress (Pimratch et al., 2008). Accumulation of ureide compounds has been reported in several plant species under stress conditions, and a considerable number of research articles argue for a hindered rather than active ureide catabolism as the survival trait for plants subjected to periods of mild drought or salinity due to the

alternative prime stress signaling function of uric acid and allantoin.

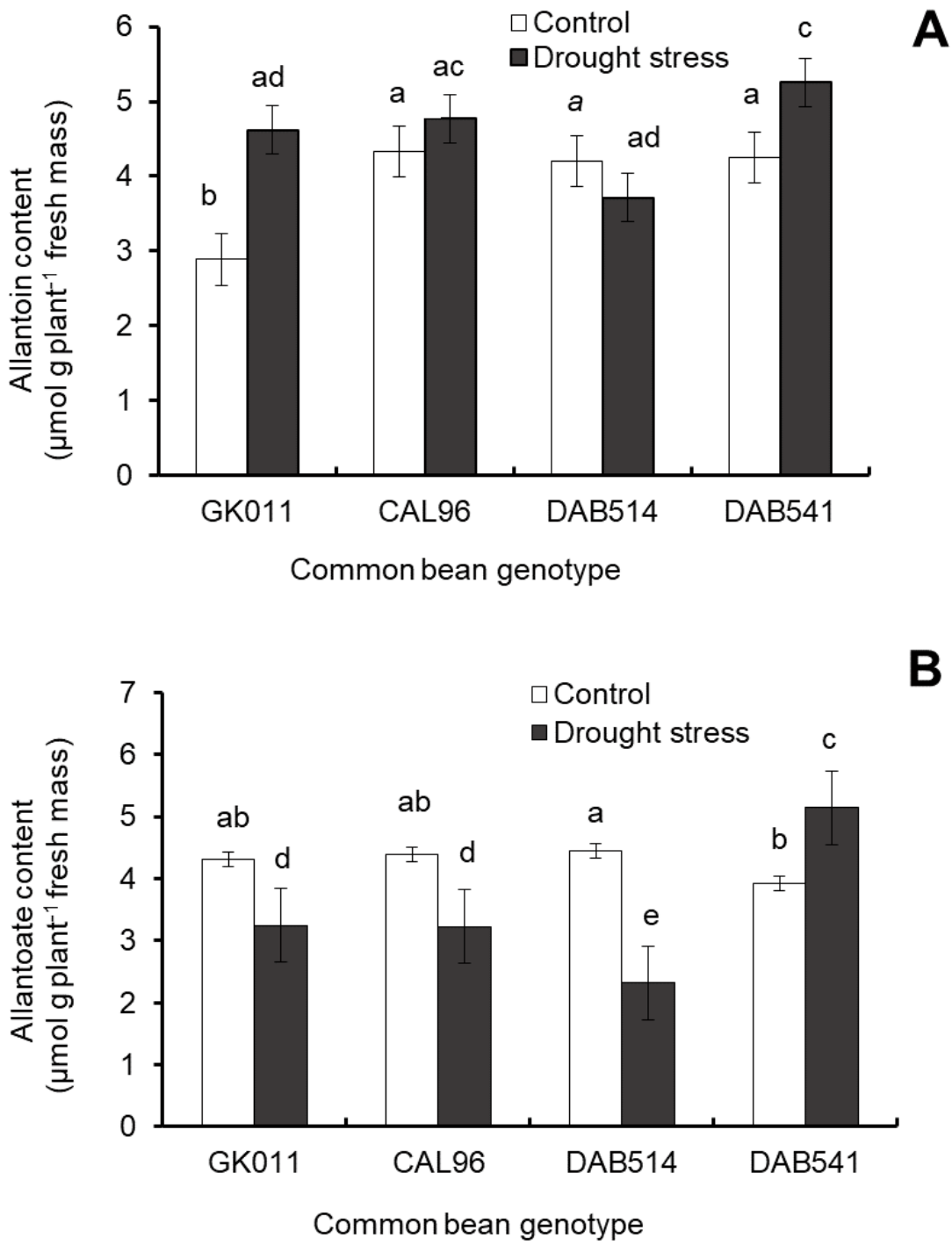

Figure 2: Ureides accumulation in response to drought stress. Ureides measurement consisted of allantoin (A) and allantoate (B) accumulation for pants under control (T0) and drought stress (T1). Ureides accumulation was measured on leaf tissues of three common bean genotypes (CAL97; DAB514; DAB541) and GK011 tepary bean. Bars with different letters are statistically different according to $p < 0.05$. The standard of mean of three independent flow cells is indicated by the error bars error (n = 6)

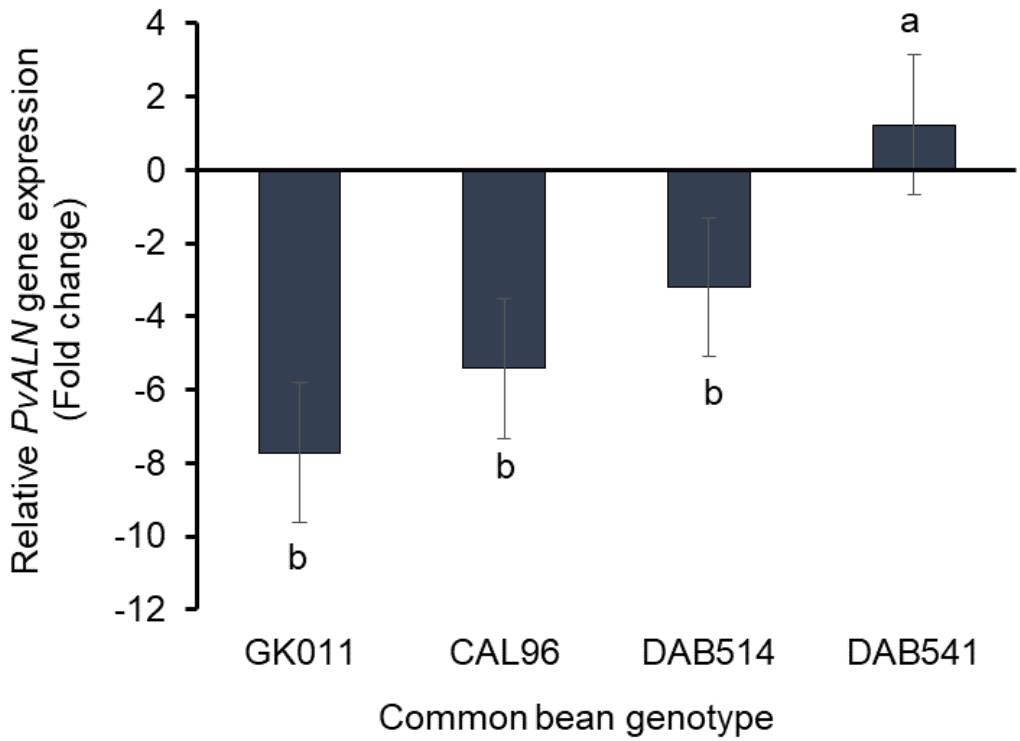

Figure 4: Relative *Phaseolus vulgaris ALLANTONAISE* (*PvALN*) gene expression in common beans in response to drought stress. Relative gene expression was measured on leaf tissues of three common bean genotypes (CAL97; DAB514; DAB541) and GK011 tepary bean. Bars with different letters are statistically different according to $p < 0.05$. The standard of mean of three independent flow cells is indicated by the error bars error (n = 3)

The current study evaluated the response of three common bean genotypes to drought stress at both biochemical and transcriptional level. Firstly, the response of common bean genotypes under normal growth conditions were tested against tepary bean (*Phaseolus acutifolius* A. Gray), a relatively higher drought-tolerant crop than common bean (*Phaseoulus vulgaris*) and serving as genetic resource for food and genetic enhancement of related legumes (Mwale et al., 2020). The insignificant growth rate in terms of biomass under normal growth condition was accompanied by a significant increase in ureide allantoin levels in CAL96, DAB514 and DAB541 common bean genotypes relative to GK011 tepary bean. On contrary, the allantoate content was not affected in CAL96 and DAB514 when compared to GK011 tepary bean. Taken together, the normal growth rate of common bean genotypes compared to GK011 tepary bean might have been sustained by an enhanced assimilation and metabolism of nitrogen, which is attributed increased levels of allantoin and a sustained level of allantoate. Taking into consideration the 16 hours day growth period in the current study, this results are consistent with *Arabidopsis thaliana* studies, which indicated that allantoin ureide degradation is important for the growth and development during vegetative growth under long-day conditions (Takagi et al., 2018).

The response of bean genotypes was further evaluated under drought stress by comparing plants under drought stress against their relative control ones. Intriguingly, all the common bean genotypes, including tepary bean revealed a similar trend of induced inhibited plant growth under drought stress. However, only DAB514 common bean genotype showed a significant reduced plant growth in drought stressed plants compared to their relative control plants. The impaired plant growth rate in DAB514 was positively associated with the reduction in both allantoin and allantoate levels, with a concomitant induced down-regulated *PvALN* relative gene expression. This response proposed an impaired ureides degradation at transcriptional level, which inevitably negatively affected assimilation and use of fixed N and eventually plant growth in DAB514 common bean genotype under drought stress. This finding is contrary to reports that indicated that DAB514 common bean genotype as a stable and high

yielding genotype under drought stress (Molosiwa et al., 2019). Our results implies that DAB514 common bean genotype is a drought-sensitive genotype possibly due to an impaired ureides metabolism at both chemical and transcriptional level with a substantial reduced plant growth. Though similar results were observed in terms of a suppressed expression of *PvALN* coupled with low levels of allantoate, the plant growth rate was not affected in water stressed CAL96 common bean genotype. The suppressed expression of *PvALN* in CAL96 common bean genotype might be responsible for an impaired rate of degradation of allantoin and the synthesis of allantoate, subsequently owing to a steady amount of allantoin synthesized under drought stress.

Water deficit also resulted in another notable increase in ureides allantoin and allantoate levels coupled with an induced upregulated relative expression of *PvALN* in DAB541 common bean genotype. This is in concert with studies on *Arabidopsis*, *Phaseolus vulgaris*, and Soybean which demonstrated an increase in shoot ureides under drought stress (Alamillo et al., 2010; Ladrera et al., 2007; Rachid Serraj, 2003; Vadez & Sinclair, 2001). This advocated for an increased transcriptional regulation of purine metabolism by *PvALN*, which in turn resulted in enhancing both the degradation of the ureide allantoin and the synthesis of allantoate (Alamillo et al., 2010; Coleto et al., 2014). This response suggested that ureide accumulation is a general response to drought stress and is regulated at the transcriptional level mainly through the induction of allantonaise degradation and the subsequent allantoate synthesis in DAB541 common bean genotype leaf tissues.

5 CONCLUSIONS

The current study evaluated the response of common bean genotypes to drought stress by assessing ureides metabolism at biochemical and transcriptional level coupled with the ultimate plant growth in terms of biomass. Overall results suggested a degree of genetical variation among common bean genotypes. The enhanced plant growth or maintained growth rate under drought stress in DAB541 and CAL96 common bean genotypes was probably due to an enhanced degradation of the ureide allantoin and the synthesis of allantoate metabolites. These findings suggested an enhanced ureide generation for export and ureide catabolism to generate a nitrogen source in leaves under drought. Therefore, the study concludes that DAB541 and CAL96 common bean genotype are potential genotypes for selection and introduction under Botswana semi-arid condition. Molecular reverse genetic studies can further be conducted to confirm ureides metabolism and crop performance of DAB541 and CAL96 common bean genotypes under drought stress.

6 ACKNOWLEDGEMENTS

This research was conducted at the Biotechnology and Genetics laboratory, Department of Agricultural Research for providing, who provided both infrastructure and financial capital for consumables and molecular reagents. Many thanks to Legumes and Oil Seed Unit for providing us with the seed materials.

7 REFERENCES

Alamillo, J. M., Luís Díaz-Leal, J., Victoria Sánchez-Moran, M. A., & Pineda, M. (2010). Molecular analysis of ureide accumulation under drought stress in *Phaseolus vulgaris* L. *Plant, Cell and Environment*, *33*, 1828–1837. https://doi.org/10.1111/j.1365-3040.2010.02187.x

Beebe, S. E., Rao, I. M., Blair, M. W., & Acosta-Gallegos, J. A. (2013). Phenotyping common beans for adaptation to drought. *Frontiers in Physiology*, *4 MAR*. https://doi.org/10.3389/fphys.2013.00035

Broughton, W. J., Hernández, G., Blair, M., Beebe, S., Gepts, P., & Vanderleyden, J. (2003). Beans (*Phaseolus* spp.) - Model food legumes. *Plant and Soil*, *252*(1), 55–128. https://doi.org/10.1023/A:1024146710611

Charlson, D. V., Korth, K. L., & Purcell, L. C. (2009). Allantoate amidohydrolase transcript expression is independent of drought tolerance in soybean. *Journal of Experimental Botany*, *60*(3), 847–851. https://doi.org/10.1093/jxb/ern332

Coleto, I., Pineda, M., Rodiñ, O. A. P., De Ron, A. ., & Alamillo, J. . (2014). Comparison of inhibition of N_2 fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance. *Annals of Botany*, *113*, 1071–1082. https://doi.org/10.1093/aob/mcu029

Díaz-Leal, J. L., Gálvez-Valdivieso, G., Fernández, J., Pineda, M., & Alamillo, J. M. (2012). Developmental effects on ureide levels are mediated by tissue-specific regulation of allantoinase in *Phaseolus vulgaris* L. *Journal of Experimental Botany*, *63*(11), 4095–4106. https://doi.org/10.1093/jxb/ers090

FANRPAN. (2017). FANRPAN Policy Brief CLIMATE-SMART AGRICULTURE IN BOTSWANA. In *Promoting a Conducive Policy Environment for a Food and Nutrition Secure Africa* (Issue 2017).

Irani, S., & Todd, C. D. (2016). Ureide metabolism under abiotic stress in *Arabidopsis thaliana*. *Journal of Plant Physiology*, *199*, 87–95. https://doi.org/10.1016/j.jplph.2016.05.011

King, C. ., & Purcell, L. C. (2005). Inhibition of N_2 fixation in soybean is associated with elevated ureides and ami-

no acids. *Plant Physiology*, *137*, 1389–1396. https://doi.org/10.1104/pp.104.056317

Kohl, D. H., Lin, J. J., Shearer, G., & Schubert, K. R. (1990). Activities of the pentose phosphate pathway and enzymes of proline metabolism in legume root nodules. *Plant Physiology*, *94*(3), 1258–1264. https://doi.org/10.1104/pp.94.3.1258

Ladrera, R., Marino, D., Larrainzar, E., González, E. M., & Arrese-Igor, C. (2007). Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean 1[OA]. *Plant Physiology, 145*, 539–546. https://doi.org/10.1104/pp.107.102491

Lescano, I. (2020). Determination of ureides content in plant tissues. *Bio-Protocol*, *10*(11), 1–9. https://doi.org/10.21769/bioprotoc.3642

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/METH.2001.1262

Molosiwa, O. O., Pharudi, J., Seketeme, S., Mashiqa, P., & Chirwa, R. (2019). Assessing yield stability and adaptability of Andean common bean genotypes in the semi-arid environment of Botswana. *African Journal of Agricultural Research*, *14*, 1593–1600. https://doi.org/10.5897/ajar2019.13988

Muñoz, A., Piedras, P., Aguilar, M., & Pineda, M. (2001). Urea is a product of ureidoglycolate degradation in chickpea. Purification and characterization of the ureidoglycolate urea-lyase. *Plant Physiology*, *125*(2), 828–834. https://doi.org/10.1104/pp.125.2.828

Mwale, S. E., Shimelis, H., Mafongoya, P., & Mashilo, J. (2020). Breeding tepary bean (*Phaseolus acutifolius*) for drought adaptation: A review. *Plant Breeding*, *139*, 821–833. https://doi.org/10.1111/pbr.12806

Pimratch, S., Jogloy, S., Vorasoot, N., Toomsan, B., Patanothai, A., & Holbrook, C. C. (2008). Relationship between biomass production and nitrogen fixation under drought-stress conditions in peanut genotypes with different levels of drought resistance. *Journal of Agronomy and Crop Science*, *194*(1), 15–25. https://doi.org/10.1111/J.1439-037X.2007.00286.X

Purcell, L. C., Serraj, R., SinclairT.R., & DeA. (2004). Soybean N_2 fixation estimates, ureide concentration, and yield responses to drought. *Crop Science*, *44*, 484–492. https://doi.org/10.2135/cropsci2004.4840

Serraj, R. (1999). Review article. Symbiotic N_2 fixation response to drought. *Journal of Experimental Botany*, *50*(331), 143–155. https://doi.org/10.1093/jexbot/50.331.143

Serraj, Rachid. (2003). Effects of drought stress on legume symbiotic nitrogen fixation: Physiological mechanisms. *Indian Journal of Experimental Biology*, *41*(10), 1136–1141.

Serraj, Rachid, Sinclair, T. R., & Purcell, L. C. (1999). Symbiotic N_2 fixation response to drought. *Journal of Experimental Botany*, *50*(331), 143–155. https://doi.org/10.1093/jxb/50.331.143

Serraj, Rachid, Vadez, V., Denison, R. F., & Sinclair, T. R. (1999). Involvement of ureides in nitrogen fixation inhibition in soybean. *Plant Physiology*, *119*(1), 289–296. https://doi.org/10.1104/pp.119.1.289

Smith, P. M. C., & Atkins, C. A. (2002). Purine biosynthesis. Big in cell division, even bigger in nitrogen assimilation. *Plant Physiology*, *128*(3), 793–802. https://doi.org/10.1104/pp.010912

Takagi, H., Watanabe, S., Tanaka, S., Matsuura, T., Mori, I. C., Hirayama, T., Shimada, H., & Sakamoto, A. (2018). Disruption of ureide degradation affects plant growth and development during and after transition from vegetative to reproductive stages. *BMC Plant Biology*, *18*(1), 1–16. https://doi.org/10.1186/s12870-018-1491-2

Vadez, V., & Sinclair, T. R. (2001). Leaf ureide degradation and N_2 fixation tolerance to water deficit in soybean. *Journal of Experimental Botany*, *52*(354), 153–159. https://doi.org/10.1093/jxb/52.354.153

Watanabe, S., Matsumoto, M., Hakomori, Y., Takagi, H., Shimada, H., & Sakamoto, A. (2013). *The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism*. https://doi.org/10.1111/pce.12218

Watanabe, S., Matsumoto, M., Hakomori, Y., Takagi, H., Shimada, H., & Sakamoto, A. (2014). The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism. *Plant, Cell and Environment*, *37*(4), 1022–1036. https://doi.org/10.1111/pce.12218

Werner, A. K., Medina-Escobar, N., Zulawski, M., Sparkes, I. A., Cao, F. Q., & Witte, C. P. (2013). The ureide-degrading reactions of purine ring catabolism employ three amidohydrolases and one aminohydrolase in arabidopsis, soybean, and rice. *Plant Physiology*, *163*(2), 672–681. https://doi.org/10.1104/pp.113.224261

Zrenner, R., Stitt, M., Sonnewald, U., & Boldt, R. (2006). Pyrimidine and purine biosynthesis and degradation in plants. *Annual Review of Plant Biology*, *57*, 805–836. https://doi.org/10.1146/annurev.arplant.57.032905.105421

doi:10.14720/aas.2022.118.2.2416 Original research article / izvirni znanstveni članek

Enhancement of shoot proliferation and evaluation of biotic elicitation effects on anatomical changes of pseudostem and anti-lipid peroxidation activity of *Curcuma mangga* Val.

Fariz ABRAHAM [1], Lai Keng CHAN [1, 2, 3], Gunawan INDRAYANTO [4, 5], Peng Lim BOEY [6, 7]

Received November 10, 2021; accepted June 06, 2022.
Delo je prispelo 10. novembra 2021, sprejeto 6. junija 2022

Enhancement of shoot proliferation and evaluation of biotic elicitation effects on anatomical changes of pseudo stem and anti-lipid peroxidation activity of *Curcuma mangga* Val.

Abstract: Mango turmeric (*Curcuma mangga* Val.) contains many bioactive compounds that are used for traditional treatment of various health problems and ailments. Slow propagation nature of *C. mangga* have resulted in short supply to meet the market demand. The longitudinally incised half shoot explants promote 100 % increased of shoot number compared with non-incised shoots with the formation of average 6.6 shoots/explant when they were cultured either vertically or horizontally on MS medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA. Biotic elicitation with 3.5 mg l^{-1} or 5.0 mg l^{-1} yeast extract or combination of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract did not promote shoot proliferation but exhibited anti-lipid peroxidation activity slightly lower than quercetin, a potent plant antioxidant flavonoid and butyl hydroxyl toluene (BHT), a commercial preservative agent which is used as a positive control. While absolute ethanol which served as a negative control did not show any anti-lipid peroxidation activity. Biotic elicitation of *C. mangga* plantlets using similar elicitors resulted in anatomical changes of its pseudostem with reduced number of thin lignified xylem cells and the presence of druse suspected to be oxalate crystals inside the cortex cells with delicate cell wall.

Key words: anti-lipid peroxidation activity; chitosan; mango turmeric; pseudostem; shoot proliferation; yeast extract

Pospeševanje tvorbe poganjkov in ovrednotenje elicitacijskih učinkov na anatomske spremembe navideznih stebel in proti maščobne peroksidacijske aktivnosti kurkume (*Curcuma mangga* Val.)

Izvleček: Kurkuma (*Curcuma mangga* Val.) vsebuje številne bioaktivne snovi, ki se uporabljajo pri tradicionalnem obravnavanju številnih zdravstvenih težav in obolenj. Počasen način njenega razmnoževanja povzroča njeno pomanjkanje glede na veliko povpraševanje na trgu. Do polovice vzdolžno zarezani stebelni izsečki so stoodstotno povečali število poganjkov v primerjavi z nezarezanimi s tvorbo povprečno 6,6 poganjkov na izseček, če so bili gojeni navpično ali vodoravno v MS gojišču, obogatenim z 2,0 mg l^{-1} BA in 0,5 mg l^{-1} NAA. Biotično vzpodbujanje s 3,5 mg l^{-1} ali 5,0 mg l^{-1} izvlečka kvasa v kombinaciji z 150 mg l^{-1} hitozana in 3,5 mg l^{-1} izvlečka kvasa ni pospešilo tvorbe poganjkov ampak je pokazalo malo manjšo antiperoksidacijsko aktivnost v primerjavi s kvercetinom, močnim rastlinskim flavonoidnim antioksidatom in butil hidroksi toluenom (BHT), komercialnim zaščitnim sredstvom, ki sta bila uporabljena kot pozitivna kontrola. Uporaba absolutnega etanola kot negativne kontrole ni pokazala nobene antiperoksidacijske aktivnosti. Biotično vzpodbujanje nastanka rastlinic korkume s podobnimi eliciatorji je povzročilo anatomske spremembe v nastajajočih navideznih steblih z zmanjšanjem števila tankih lignificiranih celic ksilema in prisotnostjo kritalnih kopuč, domnevno iz kalcijevega oksalata, v celicah primarne skorje, ki so imele zelo tanko celično steno.

Ključne besede: antiperoksidacijska aktivnost za maščobe; hitozan; kurkuma; navidezna stebla; tvorba poganjkov; izvlečki kvasa

1 Universiti Sains Malaysia, School of Biological Sciences, Penang, Malaysia
2 D'Arboretum, Bukit Rambai, Melaka, Malaysia
3 Corresponding author, e-mail: merrilynchan@gmail.com
4 Airlangga University, Faculty of Pharmacy, Surabaya, Indonesia
5 University of Surabaya, Faculty of Pharmacy, Surabaya, Indonesia
6 Universiti Sains Malaysia, School of Chemical Sciences, Penang, Malaysia
7 Sunrich Biotech Sdn. Bhd., Kuala Ketil, Kedah, Malaysia

1 INTRODUCTION

Mango turmeric (*Curcuma mangga* Val.), a member of Zingiberaceae family, is commonly used in Ayurvedic, traditional Chinese medicine (TCM) and alternative Malay medicines (Ramadanil et al., 2019). It has been used as food spices, food preservatives and treatment of various health problems and minor ailments such as fever, stomach-ache, general debility, and postpartum care (Subositi and Wahyono, 2019; Furmuly and Azemi, 2020). It has been proven to have many health beneficial activities due to some bioactive compounds or secondary metabolites such as the alkaloids, flavonoids, tannins, terpenoid and curcuminoid that are present mainly in the rhizomes (Muchtaromah et al., 2018; Yuandani et al., 2019; Awin et al., 2020; Fitriastuti et al., 2020; Maryam and Martiningsih, 2021).

Conventionally, *C. mangga* is propagated via its rhizome during the rainy season. However, *C. mangga* and many other *Curcuma* species have slow propagation rate and very prone to soil borne diseases and rhizome dormancy (Škorničková, 2007; Pikulthong et al., 2016; Soonthornkalump et al., 2020; Leong-Škorničková et al. 2021). This has resulted in insufficient supply of *C. mangga* to meet the market demand. *In vitro* culture techniques have been employed as alternative measures for improving the production of plantlets for some *Curcuma* species such as *C. longa* (El-Hawaz et al., 2015; Marchant et al., 2021), *C. aromatica* (Sharmin et al., 2013; Mohanty et al., 2015), *C. alismatifolia* (Li et al., 2021) and *C. zedoaria* (Sudipta et al., 2020). Addition of elicitors into culture medium had been reported to enhance the production of useful secondary metabolites but high amount of elicitation was found to cause cell or tissue damage of the cultured materials (Espinosa-Leal et al., 2018). Our previous study (Abraham et al., 2011) had reported that biotic elicitation using yeast extract and chitosan did not enhance shoot proliferation but increased the production of total phenolic compounds which resulted in severe abnormality of the *C. mangga in vitro* plantlets especially the pseudo stems. Hence, the present study was carried out with three main objectives. Firstly, to determine whether a shoot incision technique could enhance *in vitro* shoot proliferation and used it as an alternative means for mass production of *C. mangga* plantlets. Secondly, to determine whether yeast extract and chitosan that have been found to promote production of total phenolic compounds with good free radical scavenging activity could also enhance the anti-lipid peroxidation activity of *C. mangga*. Thirdly, to study the effect of biotic elicitation of yeast extract and chitosan on the anatomical structures of *C. mangga* pseudostem.

2 MATERIALS AND METHODS

2.1 ESTABLISHMENT OF *IN VITRO* PLANTLETS

Young buds of *C. mangga* of approximately 1.5 cm^3 in size, excised from the actively growing rhizomes during the raining season. They were washed with commercial detergent solution (Sunlight®, Unilever, Selangor, Malaysia) to remove all soil and organic matters and rinsed under running tap water for 40 min. The cleansed bud explants (1.5 cm^3) were then immersed in 70 % ethanol (Chemical Industries (M) Sdn. Bhd., Selangor, Malaysia) for 10 min followed by surface sterilization with 20 % Clorox®, a commercial bleach solution containing 5.3 % sodium hypochlorite (The Clorox Company, Oakland, CA), for 20 min. The surface-sterilized buds were then rinsed three times with sterile distilled water before being inoculated onto gelled MS (Murashige and Skoog, 1962) medium without any plant growth regulator (PGR). The cultures were incubated in a culture room regulated at 25 ± 2 °C with continuous illumination at average light intensity of 32.5 µmol m^{-2} s^{-1} for 6 weeks (Abraham, 2010).

2.2 EFFECT OF SHOOT INCISION ON SHOOT PROLIFERATION

The shoot explants of 1.0 cm length were obtained from the 6 weeks old *in vitro* plantlets. Each shoot explant was vertically cut into half or quarter while the non-incised whole shoot explants were used as control. These shoot explants were then cultured onto shoot proliferation medium, MS supplemented with 2 mg l^{-1} 6-benzylaminopurine (BAP) and 0.5 mg l^{-1} 1-naphthalene acetic acid (NAA) (Sigma-Aldrich (M) Sdn. Bhd. Subang Jaya, Malaysia) (Abraham, 2010). Three shoot explants were used for each experimental unit and ten experimental units were used for each explant type. The explants were placed vertically and horizontally on the proliferation medium. The number of shoots produced from each explant with different mode of placement was determined after 6 weeks of culture.

2.3 ANTI-LIPID PEROXIDATION ACTIVITY OF *CURCUMA MANGGA*

2.3.1 Sample extraction

Our previous study (Abraham et al., 2011) have indicated that plantlets cultured in proliferation medium added with 3.5 mg l^{-1} yeast extract, 5.0 mg l^{-1} yeast

extract, and plantlets cultured in proliferation medium supplemented with150 mg l^{-1} chitosan plus 3.5 mg l^{-1} yeast extract produced high total phenolic compounds with high free radical scavenging activity (RSA). Hence, these plantlets together with plantlets cultured in shoot proliferation medium (Control) were selected for their anti-lipid peroxidation activity. Two grams (g) of dried sample derived from each treatment condition was grounded into powder form using blender (Philips, Selangor, Malaysia). Each sample was placed into 250 ml conical flask and soaked with 100 ml methanol (Chemical Industries Sdn. Bhd., Selangor, Malaysia) at 40 °C for two hours and the soaking process was repeated three times. The methanol extracts from each sample were collected, combined, filtered, and evaporated using rotary evaporator machine (EYELA, N-N Series, Japan).

2.3.2 Anti-lipid peroxidation activity

Anti-lipid peroxidation activity was determined using modified Ferric thiocyannate (FTC) method (Osawa and Namiki, 1981). *C. mangga* sample extract (4 mg) was dissolved in 4 ml absolute ethanol (99.5 %) followed by addition of 8 ml 0.05M phosphate buffer (pH 7.0), 4.1 ml 2.5 % linoleic acid solution (Sigma, Ronkonkoma, NY) and 3.9 ml distilled water. The extract solutions were kept in aluminium foil wrapped vessels and incubated at 40 °C. Butyl hydroxyl toluene (BTH) (Sigma, Ronkonkoma, NY) was used as a positive control and 4 ml absolute ethanol served as a negative control. A volume of 0.1 ml extract solution was added to 9.7 ml 7.5 % ethanol followed by 0.1 ml 30 % ammonium thiocyannate solution and 0.1 ml 0.02 M ferric chloride (Sigma, Ronkonkoma, NY) in 3.5 % HCl (v/v). The reaction was incubated for three minutes under dark condition. Three replicates were prepared for each sample and three repetition of spectrophotometer reading were applied for each sample. The absorbance of sample was measured at 500 nm wavelength using UV-Vis spectrophotometer (Mettler Toledo, Columbus, Ohio). This procedure was repeated every 24 hours until both the positive and negative controls gave the maximum absorbance. The degree of lipid peroxidation was represented by percentage of oxidized lipid in tested samples at the day before the absorbance decreased.

2.4 HISTOLOGY STUDY OF BASAL PSEUDOSTEM

The basal pseudostems of the morphological abnormal *C. mangga* plantlets were selected for anatomical study. They were trimmed into approximately 0.5 cm^3 in size and were then immersed in FAA solution (40 % formaldehyde: acetic acid glacial: 95 % ethanol = 5: 5: 90) for fixation. During preparation of sections, the fixed tissues were passed through a series of alcohol solutions, starting with 50 % ethanol/tetra butyl alcohol (TBA) (ethanol 95 %: absolute TBA: water = 4: 1: 5) and finally treated with absolute ethanol/TBA (ethanol 95 %: absolute TBA = 2.5: 7.5). After which the tissues were immersed in mixture solution of TBA and liquid wax with ratio 1:1 at 60-62 °C for 24 hours. At the embedding stage, the tissues were immersed in liquid wax at 60-62 °C for 12 hours. After the wax solidified, the wax blocks containing the tissue samples were sliced into thin slices with 15 µm thickness using rotary microtome (Leica, Germany) for slide preparation. Double stained standard technique was used for the preparation of permanent slides. Each sliced section treated with a few drops of safranin after it was placed on the glass slide. This was followed by a few drops of 95 % ethanol to remove the excess safranin. After this, a few drops of Fast Green and 95 % ethanol were added respectively. Finally, one drop of xylene was added on the section. The sliced section was then covered with a glass cover slip and sealed with Shandon Mount (Shandon, USA) as a mounting agent. The prepared slides were observed under light microscope (Olympus BX-50, Japan) fitted with coloured video camera (JVC KF-55B, Japan) and image analysing system (analySIS docu version 3.1, Germany) for determination of cell size and cell morphology.

2.5 STATISTICAL ANALYSIS

For the effect of shoot incision and mode of placement on shoot proliferation, the experiment was conducted in complete randomized block design (CRBD). The data was analysed using two-way ANOVA and the best explant type was determined using Tukey's HSD test at $p \leq 0.05$. The anti-lipid peroxidation activity for all the selected samples was analysed using one-way ANOVA and the comparison of means was determined using to Tukey's HSD test at $p \leq 0.05$

3 RESULTS AND DISCUSSION

3.1 EFFECTS OF SHOOT INCISION ON SHOOT PROLIFERATION

Shoot proliferation is an essential step for mass production of *in vitro* plantlets which is normally car-

ried out by inducing multiple shoots formation using plant growth regulators. Many researchers have been using 6-benzylaminopurine (BAP) or benzyl adenine (BA) combination with naphthalene acetic acid (NAA) for multiple shoot induction in *Zingiber* species (Abbas et al., 2011; Zahid et al., 2021) and *Curcuma* species (Bejoy et al., 2012; Jala, 2012; Ferrari et al., 2016) with the formation of an average of 3 to 5 shoots per explants. Similar result was obtained in the present study whereby an average of 3.3 and 3.7 shoots per shoot explant were formed when the shoot explants were cultured horizontally and vertically respectively on MS medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA for induction of multiple shoot formation. When the shoot explants were cut longitudinally into half, the number of shoots formed per shoot explant was greatly enhanced with the formation of 6.6 shoots per shoot explant and the number of shots formed were not significantly different when the explants were placed horizontally or vertically. The quarterly cut shoot explants further enhanced the formation of multiple shoots with 7.6 and 8.4 shoots per shoot explant formed when the shoot explants were cultured horizontally and vertically respectively (Table 1). However, the multiple shoots derived from the quarterly cut shoot explants were small and became necrotic with copious release of phenolic compounds in the culture medium and eventually resulted in death of plantlets after two subculture cycles (6 weeks/cycle). Mode of inoculation either vertically or horizontally was statistically found to have no effect on promoting shoot proliferation in *C. mangga*. The half shoot explants grow into healthy multiple shoots when cultured on the shoot proliferation medium. Hence, half shoot explants were used for the subsequent studies.

Results obtained in the present study clearly demonstrated that shoot incision longitudinally could enhance 100 % increment of shoot production. The enhancement of shoot proliferation in longitudinally incised shoots was due to increased cut surface area that exposed to the culture medium for better absorption of nutrients and inhibit apical dominance and hence induce more lateral shoot formation (Mok and Ho, 2019). Longitudinally dissecting of shoot explants to promote high multiple shoot formation has become a common practice in propagation of banana (Ahmed Hasan et al., 2020). Hence, shoot incision can be used as an alternative means to promote *in vitro* shoot multiplication of *C. mangga* that resulted in more and faster production of plantlets.

3.2 ANTI-LIPID PEROXIDATION ACTIVITY OF BIOTIC ELICITED *CURCUMA MANGGA*

Our previous study (Abraham et al., 2011) had shown that *C. mangga* plantlets, cultured in proliferation medium supplemented with 3.5 mg l^{-1} yeast extract; 5.0 mg l^{-1} yeast extract or combination of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract, exhibited high free radical scavenging activity (RSA). These crude extracts together with the positive and negative controls were tested for their anti-lipid peroxidation activity using the ferric thiocyannate (FTC) assay. This method was used to measure the antioxidant activity of the studied samples toward auto peroxidation of the linoleic acid. BHT (Butyl Hydroxyl Toluene) (Sigma, USA) was used as a positive control and 99.5 % ethanol served as a negative control. Positive control is essential for comparing the biotic elicited *C. mangga* extracts with BHT, a most used antioxidant as preservative in foods containing fats, pharmaceuticals, petroleum products and it inhibits autoxidation of unsaturated organic compounds. The *C. mannga* extracts used in this study were dissolved in 99.5 % ethanol, hence 99.5 % ethanol was used as the negative control. The negative control is used to show that any positive effects of the tested samples are not due to the ethanol effect. The selected *C. mangga* extracts together with BHT (positive control) and 99.5 % ethanol (negative control) and quercetin, a potent plant antioxidants flavonoid, exhibited similar pattern of lipid peroxidation activity from day 0 to day

Table 1: Effect of shoot explant incision and inoculation mode on enhancing multiple shoot formation of *C. mangga*

Explants	Vertical		Horizontal	
	No. of shoots/explant ± *Se*	No. of shoots/shoot	No. of shoots/explant ± *Se*	No. of shoots/shoot
Whole	3.7 ± 0.2	$3.7^{c}{}_{c}$	3.3 ± 0.3	$3.3^{c}{}_{c}$
Half	3.3 ± 0.3	$6.6^{b}{}_{b}$	3.3 ± 0.3	$6.6^{b}{}_{b}$
Quarter	1.9 ± 0.2	$7.6^{a}{}_{a}$	2.1 ± 0.2	$8.4^{a}{}_{a}$

Mean values within the row followed by same superscript letter indicate not significantly different when the different explant types were vertically or horizontally placed on the culture medium. Mean values within the same column (for parameter No. of shoots/shoot explant) followed by different subscript alphabet indicate significantlydifferent of shoot numbers for different explant types (Tukey, HSD, $p \leq 0.05$)

six. However, the oxidation of lipid (linoleic acid) of 99.5 % ethanol (negative control) drastically increased started from day seven until day ten while others remain constant until day 10 (Figure 1).

Quercetin is a potent antioxidant flavonoid found in many plant species such as onions, grapes, berries, broccoli, and citrus (David et al., 2016). Even though the three extracts obtained from biotic elicited plantlets of *C. mangga* showed slightly lower anti-lipid peroxidation activity when compared with quercetin, it could be assumed that *C. mangga* could also a potent antioxidant. Their anti-lipid peroxidation activity was also found to be lower when compared to BHT, a commercial preservative agent and a synthetic antioxidant. BHT and quercetin showed similar lipid-peroxidation activity with percentage of oxidized lipid as 29.5 ± 0.1 % and 30.0 ± 0.1 % respectively. Crude extract derived from *C. mangga in vitro* plantlets cultured in proliferation medium supplemented with 3.5 or 5.0 mg l^{-1} yeast extract exhibited no difference in anti-lipid peroxidation activity. Plantlets cultured in proliferation medium supplemented with 3.5 mg l^{-1} yeast extract showed better anti-lipid peroxidation activity as compared to extract derived from *C. mangga* plantlets cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan plus 3.5 mg l^{-1} yeast extract. Plantlets cultured in proliferation medium without elicitation exhibit the least inhibition of lipid peroxidation (Table 2). It opened an interesting possibility to use *C. mangga* extracts as food preservative agents or topical medication for treatment of cold sore such as BHT. Besides using as food preservative agent, BHT has been used as medicine for the treatment of cold sore (Freeman et al., 1985).

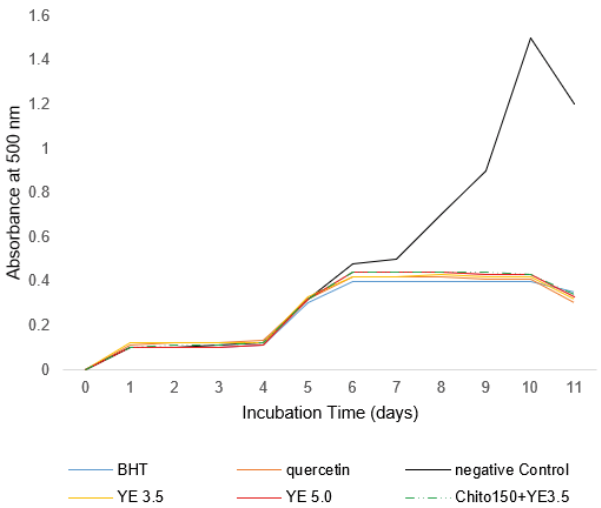

Figure 1: The kinetic of anti-lipid peroxidation of biotic elicited *C. mangga* extracts and controls [BHT (positive control); 95.5 % ethanol (negative control)]

3.3 EFFECT ON ANATOMY OF PSEUDOSTEM

Our previous study (Abraham et al., 2011) had shown that the morphology of the plantlets cultured in the proliferation medium without elicitation (Control) were normal. While those cultured in proliferation medium with the addition of yeast extracts (3.5 mg l^{-1} or 5.0 mg l^{-1} were slightly abnormal with retarded growth. Those plantlets cultured in proliferation medium with combination of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract were grossly abnormal with chlorosis and globular shaped shoots, fragile leaf petiole and experience severe growth retardation. Results obtained from the histological study of the pseudostem in the present study (Figure 2, Figure 3 & Table 3) clearly showed that these elicited plantlets did affect the anatomical structures of the basal pseudostem of *C. mangga* and could be linked directly with the morphological characteristics of *C. mangga* plantlets as reported in Abraham et al. (2011). The plantlets cultured in the shoot proliferation medium without elicitation (Control) were consisted of smooth spherical to oval shape cortex cells

Table 2: Percentage of oxidized lipid in tested samples at day 10 using ferric thiocyanate (FTC) assay

Sample	Oxidized lipid in sample (%) ± se
Absolute ethanol (Negative Control)	100 a
Quercetine	30.0 ± 0.1 e
BHT (Butyl Hydroxyl Toluene) (positive control)	29.5 ± 0.1 e
Extract from plantlets cultured without biotic elicitors	33.0 ± 0.2 b
Extract of 3.5 mg l^{-1} yeast-extract treated plantlets	31.6 ± 0.2 d
Extract of 5.0 mg l^{-1} yeast-extract treated plantlets	32.1 ± 0.1 cd
Extract of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast-extract treated plantlets	32.5 ± 0.1 c

Mean values within the column followed by same alphabets represent non-significantly different mean values based on Tukey, HSD at $p \leq 0.05$

and normal xylem cells. The cortex cells did not contain any druse, a crystal substance present in plant such as calcium oxalate crystal. The well distributed cortex cells without druse and well-formed lignified xylems (Figure 2 a & b) were able to supply sufficient nutrients to support healthy and normal plantlets. For the plantlets cultured in medium supplemented with 3.5 mg l^{-1} yeast extract, the xylems were also well-formed except the lignified layer of these xylem cells were not as thick as the ones observed in the control. Their cortex cells were found to contain druse, which could be seen as black dots inside the cells (Figure 2 c & d). The accumulation of druses was more obvious in plantlets that were cultured in proliferation medium supplemented with 5.0 mg l^{-1} yeast extract (Figure 2 e & f). The size of xylem cells of the control plantlets and that cultured in proliferation added with 3.5 mg l^{-1} yeast extract was not significantly different, with an average diameter of 32.9 ± 4.0 µm and 40.8 ± 5.1 µm respectively. They were double the size of the xylem cells present in the basal pseudostem of plantlets cultured in proliferation medium supplemented with 5.0 mg l-1 yeast extract (15.4 ± 0.8 µm). However, the number of xylem cells found in plantlets cultured with the presence of 3.5 mg l^{-1} yeast extract was only 22 when compared to that of control with 41 xylem cells. The plantlets cultured in medium supplemented with 5.0 mg l^{-1} yeast extract produced even much lesser number of not well-lignified xylems, with an average of only 9 xylem cells in each 4x optical magnification field (Table 3). The control plantlets developed bigger cortex cells (43.0 ± 3.0 µm) compared to plantlets cultured with the proliferation medium supplemented with 3.5 mg l^{-1} and 5.0 mg l^{-1} yeast extract with diameter of cortex cells as 29.9 ± 1.8 µm and 30.8 ± 1.8 µm respectively, which were not significantly different in size (Table 3). The smaller size of cortex cells with the presence of druse, and a smaller number of poor lignified xylem cells might not be able to provide sufficient nutrients derived from the culture medium to all parts of plantlets. Hence, it explained the retarded growth with abnormal characteristic of the plantlets that were cultured in proliferation medium supplemented with 3.5 mg l^{-1} and 5.0 mg l^{-1} yeast extract as reported in our previous study.

Most of the tissues of the plantlets cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract were fragile, and parts of the section were damaged after dehydration process (Figure 3 a & b). The size of some of the cortex could be measured and determined but not that of xylem cells because of cell damage. The number of the xylem cells (7 in each 4x optical magnification field) was roughly estimated from the location of the ruptured xylem cells. The size of the cortex cells found in the basal pseudostem of *C. mangga* plantlets cultured in the shoot proliferation medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract was found to be not significantly different with those plantlets cultured in proliferation medium added with yeast extract (Table 3). The small number and fragile xylem cells could greatly reduce the absorption of nutrients, and this was linked directly with the severe growth retardation and gross morphological abnormality of the plantlets cultured in proliferation medium supplemented with yeast extract and chitosan as reported in Abraham et al. (2011). Yeast extract was reported to induce a complex stress response resulted in activation of secondary metabolites production (Farjaminezhad and Garoosi, 2021; Kochan et al., 2017) but resulted in slow cell growth (Hedayati et al., 2021) and cell damage at high concentration (Sánchez-Sampedro et al., 2005).

In the present study, the formation of druse (crystal inclusion inside cortex cells) was detected in *C. mangga* plantlets cultured in proliferation medium elicited with yeast extract. The druse inside the cells of *C. mangga* plantlets were suspected to be oxalate crystal because oxalate crystals are the most common crystal inclusion of higher plants (Franceschi and Horner,

Table 3: Summary of cortex and xylem diameter and estimated number of xylem cells in *Curcuma mangga* plantlets cultured in proliferation medium supplemented with biotic elicitors

Elicitor treated peudostems	Diameter ± s.e. (µm)		Estimated No. of xylems/ 4x optical magnification field
	Cortex	Xylem	
Control	43.0 ± 3.0 a	32.9 ± 4.0 a	41
5.0 mg l^{-1} yeast extract	30.8 ± 1.8 b	15.4 ± 0.8 b	9
3.5 mg l^{-1} yeast extract	29.9 ± 1.8 b	40.8 ± 5.1 a	22
150 mg l^{-1} chitosan + 3.5 mg l^{-1} yeast extract	35.2 ± 3.9 b	ND	7

Mean values within the same column followed by different alphabet indicate significantly different values (Tukey, HSD, $p \leq 0.05$).
ND = Not determine

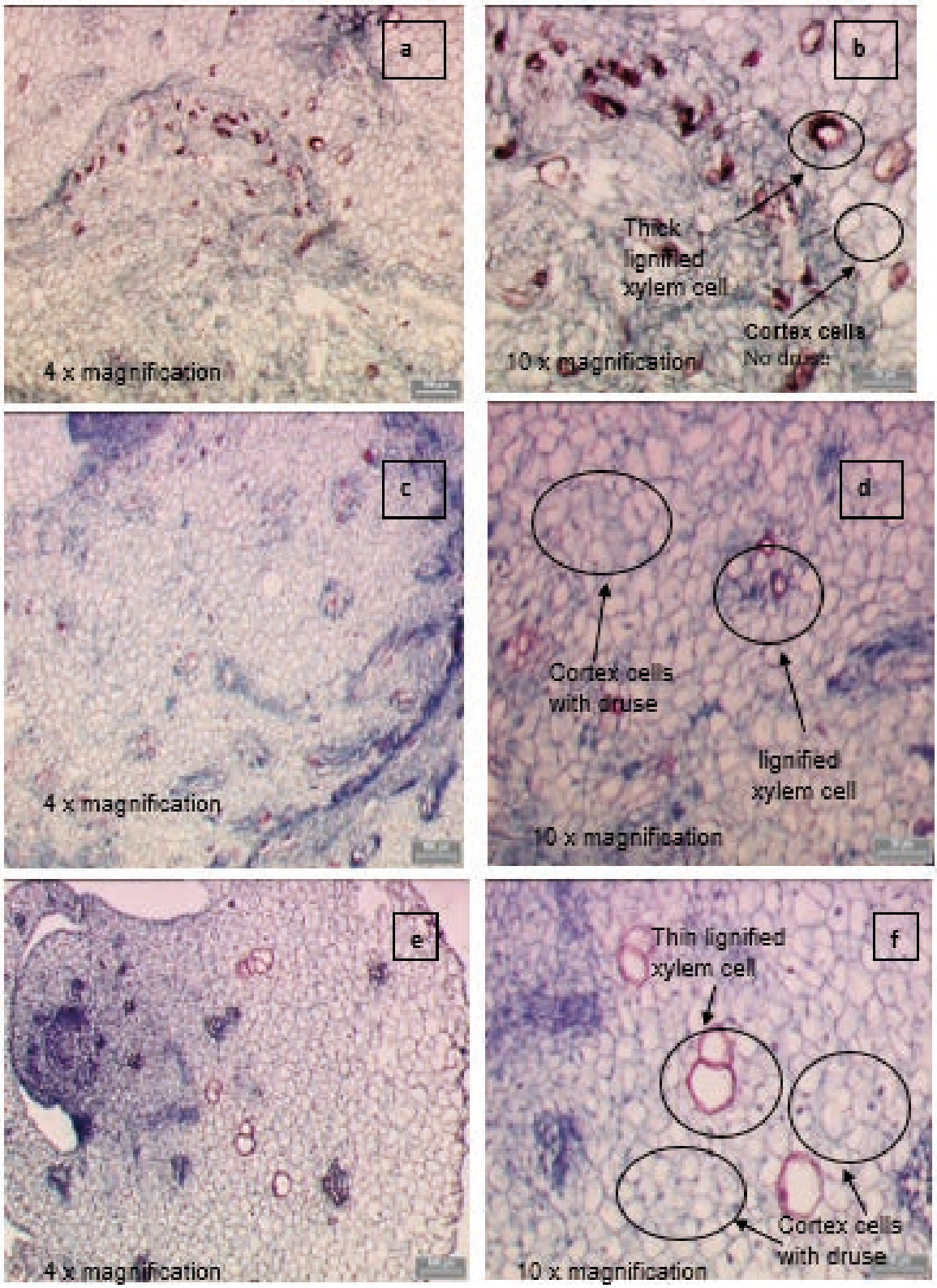

Figure 2: Histology images of basal pseudostem of *C. mangga* plantlets cultured in proliferation medium (Control) at 4 x optical magnification (a) and 10 x optical magnification (b); Proliferation medium supplemented with 3.5 mgL^{-1} YE at 4 x optical magnification (c) and 10x optical magnification (d); Proliferation medium supplemented with 5.0 $mg\ l^{-1}$ YE at 4 x optical magnification (e) and 10x optical magnification (f)

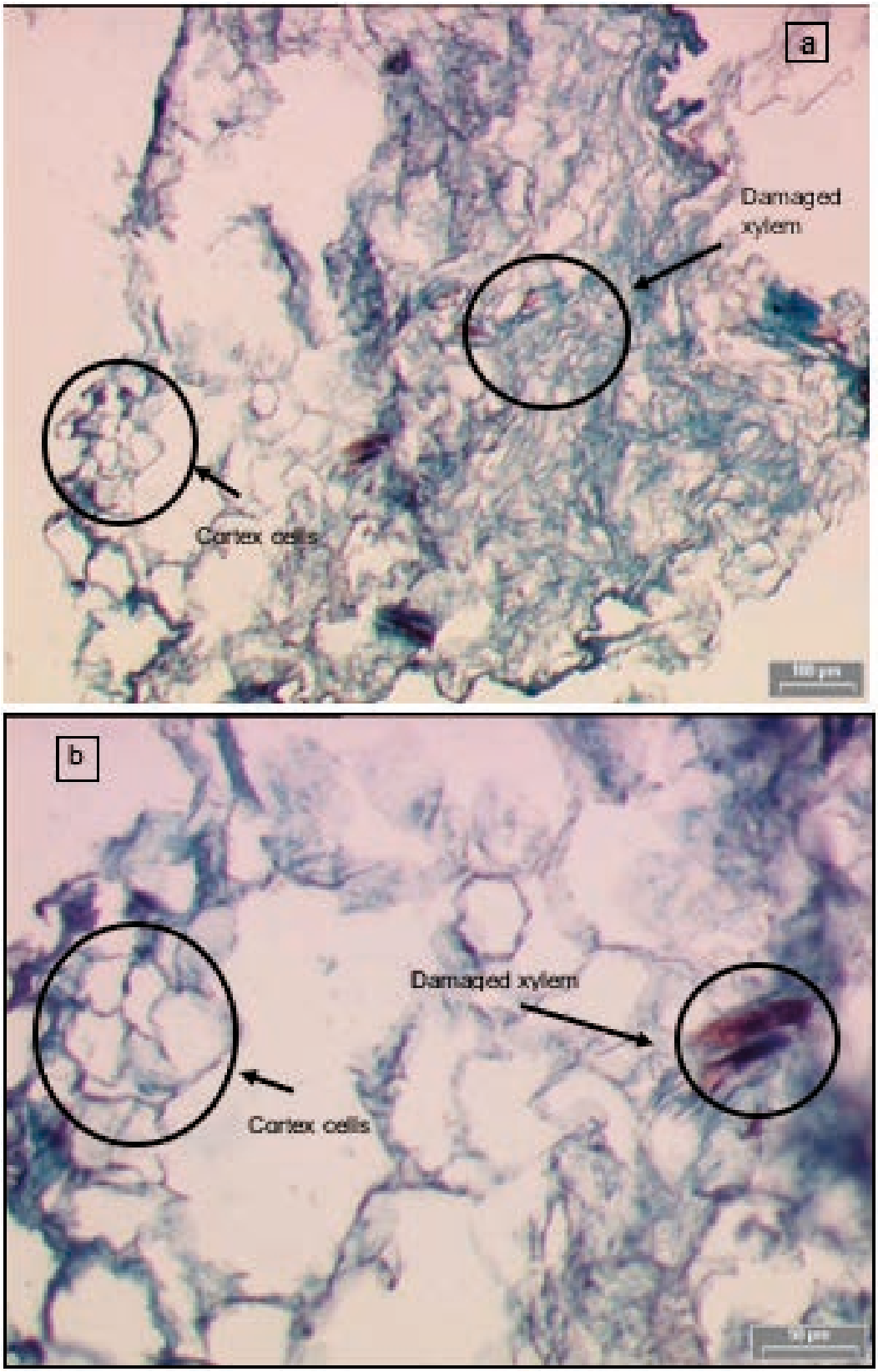

Figure 3: Histology images of basal pseudostem of *C. mangga* plantlets cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract at 4x optical magnification (a) and 10x optical magnification (b)

1980; Webb, 1999; Nakata, 2012). Even though oxalate crystals were often found in higher plant, the formation and function of oxalate crystals in plants were still unclear (Webb, 1999). It was proposed that oxalate crystal might be involved in ion balance, plant defence, tissue rigidity and support, detoxification, light accumulator, and reflector (Franceschi and Homer, 1980; Doege, 2003). The possible function of oxalate crystal as part of plant defence might be relevant in *C. mangga* study. The oxalate crystal could not be detected in plantlets cultured in medium without elicitor (control) (Figure 2 a & b). Most part of the tissue of *C. mangga* plantlets cultured in medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract were damaged during dehydration process of histology preparation. The damaged tissue indirectly indicate that the cell walls of those plantlets were more delicate than the cell walls of plantlets cultured in medium supplemented with yeast extract or the control. This finding was also supported the observation on the visual morphological characteristic of those plantlets which exhibited brittleness on the abnormal formed shoots cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan plus 3.5 mg l^{-1} yeast extract.

Chitosan has been used to study the defence mechanism of plants towards their fungal pathogens. Unlike yeast extract, chitosan is a compound with defined molecular structure (polycationic b-1,4-linked-d-glucosamine polymers) which resemble to cell wall component of fungi (Walker–Simmons et al., 1983). The interaction between oligosaccharins and receptors located in the plant membranes results in the production of many plants defence secondary metabolites and many phenolic and terpenoid compounds (Kim and Lee 2011; Pang et al 2021). It was found to induce the synthesis of pathogenesis-related (PR) proteins and several defence enzymes (such as phenylalanine ammonia lyase and peroxidase) (Riaz et al., 2014). The plant defence compounds, synthesized after chitosan elicitation, have relevant physiological activity, mainly as antioxidants (the polyphenols), might have resulted in cell damage as well.

4 CONCLUSIONS

Longitudinally half incised shoot explants and cultured vertically on shoot proliferation medium, MS supplemented with 2.0 mg l^{-1} benzyl adenine (BA) and 0.5 mg l^{-1} NAA, promote shoot proliferation by 100 %. This method can be used as an alternative technique for multiplication of the *in vitro* plantlets of *C. mangga* which normally propagate at a slow rate. Supplementation of 3.5 or 5.0 mg l^{-1} yeast extract, or combination of 3.5 mg l^{-1} yeast extract and 150 mg l^{-1} chitosan, into the shoot proliferation medium could be used as an alternative mode for enhancing anti-lipid peroxidation activity. However, biotic elicitation with 3.5 or 5.0 mg l^{-1} yeast extract, or combination of 3.5 mg l^{-1} yeast extract and 150 mg l^{-1} chitosan did not affect the cortex cell size but a reduction in xylem cell numbers of *C. mangga* pseudostem. Supplementation of 5.0 mg l^{-1} yeast extract reduced the size of the xylem cell by more than half as compared to the xylem cell of those cultured in proliferation supplemented with 3.5 mg l^{-1} yeast extract and the control. The addition of 3.5 mg l^{-1} yeast extract and 150 mg l^{-1} chitosan into the proliferation medium resulted in abnormal anatomy of their pseudostems with damage of the xylem cells.

5 REFERENCES

Abbas, M. S., Taha, H. S., Aly, U. I., El-Shabrawi, H. M., Gaber, E. S. I. (2011). In vitro propagation of ginger (*Zingiber officinale* Rosco). *Journal of Genetic Engineering and Biotechnology*, *9*(2), 165–172. https://doi.org/10.1016/j.jgeb.2011.11.002

Abraham, F. (2010). *Elicitation effect on the growth and biochemical activities of Curcuma mangga* Val. *(*Zingiberaceae*) in vitro plantlets*. Master of Science Thesis, Universiti Sains Malaysia, Penang, Malaysia.

Abraham, F., Bhatt, A., Chan, L.K., Indrayanto, G., Sulaiman, S. F. (2011). Effect of yeast extract and chitosan on shoot proliferation, morphology and antioxidant activity of *Curcuma mangga* in vitro plantlets. *African Journal of Biotechnology*, *10*(40), 7787–7795. https://doi.org/10.5897/ajb10.1261

Ahmed Hasan, S., Khasim, S.M., Ramudu, J. (2020). Development of Standard Protocols for In Vitro Regeneration of Some Selected Banana Cultivars (Musa spp.) from India. In: Khasim, S.M., Long, C., Thammasiri, K., Lutken, H. (eds) *Medicinal Plants: Biodiversity, Sustainable Utilization and Conservation*. Springer, Singapore. https://doi.org/10.1007/978-981-15-1636-8_45

Awin, T., Mediani, A., Mohd Faudzi, S. M., Maulidiani, Leong, S. W., Shaari, K., Abas, F. (2020). Identification of α-glucosidase inhibitory compounds from *Curcuma mangga* fractions. *International Journal of Food Properties*, *23*(1), 154–166. https://doi.org/10.1080/10942912.2020.1716792

Bejoy, M., Dan, M., Anish, N. P., Anjana, R. G., Radhika, B.J., Manesh, K. (2012). Micropropagation of Indian ginger (*Curcuma vamana* Sabu & Mangaly): A wild relative of tumeric. *Biotechnology*, *11*(6), 333–338. https://doi.org/10.3923/biotech.2012.333.338

David, A.V.A., Arulmoli, R., Parasuraman, S. (2016). Overviews of biological importance of quercetin: a bioactive flavonoid. *Pharmacognosy Reviews*, *10*, 84-89. https://doi.org/10.4103/0973-7847.194044

Doege, S. J. (2003). The role of natural calcium oxalate crystals in plant defense against chewing insects. Inquiry: *The University of Arkansas Undergraduate Research Journal*, *4*, 88–94.

El-Hawaz, R.F., Bridges, W.C., Adelberg, J.W. (2015). In vitro growth of *Curcuma longa* L. in response to five mineral elements and plant density in fed-batch culture systems. *PLOS One*, *10*(4), 1-13. http://doi10.1371/journal-pone.0118912

Espinosa-Leal, C.A., Puente-Garza, C.A., Garcia-Lara, S. (2018). *In vitro* plant tissue culture: means for production of biological active compounds. *Planta*, *248*, 1-18. https://doi.org/10.1007/s00425-018-2910-1

Farjaminezhad, R. and Garoosi, G. (2021). Improvement and prediction of secondary metabolites production under yeast extract elicitation of *Azadirachta indica* cell suspension culture using response surface methodology. *AMB Express, 11*, 1-16. https://doi.org/10.1186/s13568-021-01203-x

Ferrari, M. P. de S., Antoniazzi, D., Nascimento, A. B., Franz, L. F., Bezerra, C. S., Magalhães, H. M. (2016). Evaluation of new protocols to *Curcuma longa* micropropagation: A medicinal and ornamental specie. *Journal of Medicinal Plants Research*, *10*(25), 367–376. https://doi.org/10.5897/jmpr2016.6109

Fitriastuti, D., Julianto, T.S., Iman, A. W. N. (2020). Identification and heme polymerization inhibition activity (HPIA) assay of ethanolic extract and fraction of Temu Mangga (*Curcuma mangga* Val.) rhizome. *EKSAKTA: Journal of Sciences and Data Analysis*, *1*(1), 64–72. https://doi.org/10.20885/eksakta.vol1.iss1.art10

Franceschi, V.R. and Horner, H.T. (1980). Calcium oxalate crystals in plants. *The Botanical Review*, *46*, 361–427. https://doi.org/10.1007/BF02860532

Freeman DJ, Wenerstrom G, Spruance SL. (1985).Treatment of recurrent herpes simplex labialis with topical butylated hydroxytoluene. *Clinical Pharmacology & Therapeutics*, *38*(1), 56-9. https://doi.org/10.1038/clpt.1985.134

Furmuly, A. M., Azemi, N. (2020). A review on golden species of Zingiberaceae family: Genus *Curcuma*. *Journal of Critical Reviews*, *7*(16), 882–895. https://doi.org/10.31838/jcr.07.16.114

Hedayati, A., Hemmaty, S., Nourozi, E. , Amirsadeghi, A. (2021) Effect of yeast extract on the gene expression and tropane alkaloids production in *Atropa belladonna* L. hairy roots. *Russian Journal of Plant Physiology*, *68*, 102–109 (2021). https://doi.org/10.1134/S1021443721010088

Jala, A. (2012). Effects of NAA BA and sucrose on shoot induction and rapid micropropagation by trimming shoot of *Curcuma longa* L. *International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies*, *3*(2), 101–109. http://TuEngr.com/V03/101-109.pdf.

Leong-Škorničková, J., Soonthornkalump, S., Thongbai, W. (2021). Four new *Curcuma* species (Zingiberaceae) from Thailand. *Blumea*, *65*, 244-253. https://doi.org/10.3767/blumea.2021.65.03.09

Kim, Y.O. and Lee, E.J. (2011). Comparison of phenolic compounds and the effects of invasive and native species in East Asia: support for the novel weapons hypothesis. *Ecological Research*, *26*, 87–94. https://doi.org/10.1007/s11284-010-0762-7

Kochan, E., Szymczyk, P., Kuzma, L., Lipert, A., Szymanska, G. (2017). Yeast extract stimulates ginsenoside production in hairy root cultures of American ginseng cultivated in shake flasks and nutrient sprinkle bioreactors. *Molecules*, *22*, 1-15. https://doi.org/10.3390/molecules22060880

Li, Y.Y., Tian, Q.L., Yu, H.W., Lu, L.M. (2021). Progress towards a molecular-level understanding of *Curcuma alismatifolia*. *European Journal of Horticultural Science*, *86*(3), 328-334. https://doi.org/10.17660/eJHS.2021/86.3.12

Marchant, M.J., Molina, P., Montecinos, M., Guzman, L., Balada, C., Fassio., C., Castro, M. (2021). *In vitro* propagation of Easter Islaand *Curcuma longa* from rhizome explants using temporary immersion system. *Agronomy*, *2121*, 1-11. http://doi.org/10.3390/agronomy11112121

Maryam, S. and Martiningsih (2021). Antioxidant activity and total fenol content white saffron (*Curcuma mangga* Val). *IOP Conference Series: Materials Science and Engineering*, *1115*(1), 012081. https://doi.org/10.1088/1757-899x/1115/1/012081

Mohanty, S., Parida, R., Sandeep,I., Sahoo, S., Nayak, S. (2015). Evaluation of drug yielding potential of micropropagated *Curcuma aromatica*. *International Journal of Pharmacy and Pharmaceutical Sciences*, *7*(4), 71-76.

Mok., P.K. and Ho., W.S. (2019). Rapid in vitro propagation and efficient acclimatization of *Neolamarckia cadamba*. *Asian Journal of Plant Science*, *18*, 153-163. https://doi.org/10.3923/ajps.2019.153.163

Muchtaromah, B., Ahmad, M., Romaidi, Nazilah, L. A., Naja, N. A. (2018). Antibacterial activity of water and ethanol extract of *Allium sativum*, *Curcuma mangga*, and *Acorus calamus* combination. *Journal of Biological Researches*, *24*(1), 8–15. https://doi.org/10.23869/bphjbr.24.1.20182

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nakata, P.A. (2012). Plant calcium oxalate crystal formation, function, and its impact on human health. Frontiers of Biology, *7*, 254–266. https://doi.org/10.1007/s11515-012-1224-0

Osawa, T. and Namiki, M. (1981). A novel type of antioxidant isolated from leaf wax of Eucalyptus leaves. *Agricultural and Biological Chemistry*, *45*(3), 735–739. https://doi.org/10.1080/00021369.1981.10864583

Pang, Z., Chen, J., Wang, T., Gao, C., Li, Z. Guo, L., Xu, J., Cheng, Yi. (2021). Linking plant secondary metabolites and plant microbiomes: a review. *Frontiers in Plant Science*, *22*, 1-22. https://doi.org/10.3389/fpls.2021.621276

Pikulthong, V., Teerakathiti, T., Thamchaipenet, A., Peyachoknagul, S. (2016). Development of somatic embryos for genetic transformation in *Curcuma longa* L. and *Curcuma mangga* Valeton & Zijp. *Agriculture and Natural Resources*, *50*(4), 276-285. https://doi.org/10.1016/j.anres.2015.08.004

Ramadanil, Damry, Rusdi, Hamzah, B., Zubair, M. S. (2019). Traditional usages and phytochemical screenings of selected Zingiberaceae from central Sulawesi, Indonesia.

Pharmacognosy Journal, *11*(3), 505–510. https://doi.org/10.5530/pj.2019.11.80

Riaz, A., Riaz, A., Rattu, A.U.R., Tahir, M.I., Azeem, M. (2014). Phenylalanine ammonia-lyse (PAL) and peroxidase activity in brown rust infected tissues of Pakistani wheat cultivars. *Pakistan Journal of Botany*, *46*(3), 1101-1107. https://www.researchgate.net/publication/262896801

Sánchez-Sampedro, M. A., Fernández-Tárrago, J., Corchete, P. (2005). Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. *Journal of Biotechnology*, *119*(1), 60–69. https://doi.org/10.1016/j.jbiotec.2005.06.012

Sharmin, S.A., Alam, M.J., Sheikh, M.M.I., Zaman, R., Khalekuzzaman, M., Mondal, S.C., Haque, M.A., Alam, M.F., Alam, I. (2013). Micropropagation and antimicrobial activity of *Curcuma aromatica* Salisb., a threatened aromatic medicinal plant. *Turkish Journal of Biology*, *37*, 698-708. http://doi:10.3906/biy-1212-11

Škorničková, J. (2007). *Taxonomic Studies in Indian Curcuma* L. Ph.D Thesis, Charles University, Prague, Czech Republic.

Soonthornkalump, S., Ongsakul, A., Dolaji, A., Leong-Škorničková, J. (2020). *Curcuma papilionacea* (Zingiberaceae), an unusual new species from southern Thailand. *Phytotaxa*, *432*(1), 011-016. https://doi.org/10.11646/phytotaxa.432.1.2

Subositi, D. and Wahyono, S. (2019). Study of the genus curcuma in Indonesia used as traditional herbal medicines. *Biodiversitas*, *20*(5), 1356–1361. https://doi.org/10.13057/biodiv/d200527

Sudipta, J., Asit, R., Ambika, S., Suprava, S., Biswabhusan, D., Basudeba, K., Sanghamitra, N. (2020). Rapid plant regeneration in industrially important *Curcuma zedoaria* revealing genetic and biochemical fidelity of the regenerants. *3 Biotech, 10*, 17. https://doi.org/10.1007/s13205-019-2009-9

Walker-Simmons, M., Hadwiger, L., Ryan, C.A. (1983). Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. *Biochemical and Biophysical Research Communications*, *110*(1), 194-199. http://10.1016/0006-291x(83)91279-2

Webb, M. A. (1999). Cell-mediated crystallization of calcium oxalate in plants. *Plant Cell*, *11*(4), 751–761. https://doi.org/10.1105/tpc.11.4.751

Yuandani, Yuliasmi, S., Satria, D., Dongoran, R. F., Sinaga, M. S., Marpaung, N. H. A. (2019). Correlation between the phytochemical constituents of *Curcuma mangga* and its immunomodulatory effect. *Rasayan Journal of Chemistry*, *12*(1), 1–6. https://doi.org/10.31788/RJC.2019.1215050

Zahid, N. A., Jaafar, H. Z., Hakiman, M. (2021). Micropropagation of ginger (Zingiber officinale 'Bentong') and evaluation of its secondary metabolites and antioxidant activities compared with the conventionally propagated plant. *Plants*, *10*, 1–17. https://doi.org/10.3390/plants10040630

doi:10.14720/aas.2022.118.2.2538 Original research article / izvirni znanstveni članek

Effects of γ-radiation on chickpea (*Cicer arietinum*) varieties and their tolerance to salinity stress

Amal ABDOUN [1, 2], Laila MEKKI [1], Aladdin HAMWIEH [3] and Abdelfattah BADR [4]

Received February 01, 2022; accepted June 07, 2022.
Delo je prispelo 1. februarja 2022, sprejeto 7. junija 2022

Effects of γ-radiation on chickpea (*Cicer arietinum*) varieties and their tolerance to salinity stress

Abstract: Chickpea (*Cicer arietinum* L.) is a bisexual and self-pollinated legume. It improves the soil fertility through its natural ability to fix atmospheric nitrogen with its symbiotic bacteria. Salinity is one of the most important abiotic stress factors affecting plant growth. γ-radiation is a very effective tool for inducing mutations in many plants. This study evaluated the γ-radiation effect on germination, cell division and plant growth of first-generation plants. Seeds of seven chickpea varieties were irradiated with γ-radiation doses ranging between 50 Gy and 600 Gy. Non-significant differences in germination percentage were recorded for seeds exposed to 50 Gy, 100 Gy, and 200 Gy of γ-radiation in comparison to the corresponding controls except ILC 484. The mitotic index (MI) of root cells increased at the low doses of 50 Gy, 100 Gy and 200 Gy comparing and reduced at the higher doses in all chickpea varieties to the control. All doses of γ-radiation induced a variable range of chromosomal abnormalities; the most common were bridges, laggard chromosomes, stickiness at metaphase, chromosome breaks, micronuclei and binucleate cells. The 300 Gy to 600 Gy doses induced degradation of nuclear membranes. The salinity treatments at 25 mM NaCl and 60 mM NaCl reduced seedling's growth of all cultivars. The dose of 100 Gy alleviated the impact of salinity at a concentration of 25 mM NaCl for all varieties, except FLIP 84-188 and FLIP 97-263. The 60 mM NaCl treatment significantly reduced early growth of all cultivars and its effect was not alleviated by the γ-radiation.

Key words: chickpea; γ-radiation; germination; mitotic index; chromosomal aberrations

Učinki γ-sevanja na sorte čičerke (*Cicer arietinum* L.) in njihova toleranca na slanostni stres

Izvleček: Čičerka (*Cicer arietinum* L.) je obojespolna samoprašna stročnica. Zaradi sposobnosti vezave atmosferskega dušika s simbiontskimi bakterijami izboljšuje rodovitnost tal. Slanost je eden izmed najpomembnejših abiotskih stresnih dejavnikov, ki vpliva na rast rastlin. V raziskavi je bil ovrednoten vpliv γ-sevanja na kalitev, celične delitve in rast rastlin F1 generacije čičerke. Semena sedmih sort čičerke so bila obsevana z γ-žarki v jakosti od 50 Gy do 600 Gy. V primerjavi s kontrolo so bile zabeležene neznačilne razlike v odstotku kalitve pri semenih, ki so bila izpostavljena 50 Gy, 100 Gy in 200 Gy γ-sevanja, razen pri sorti ILC 484. V primerjavi s kontrolo se je pri vseh sortah povečal mitotski indeks (MI) celic rastnega vršička korenine, ki so bile obsevane z majhnimi dozami 50 Gy, 100 Gy in 200 Gy ter zmanjšal pri obsevanju z večjimi dozami. Vse uporabljene doze γ-sevanja so vzpodbudile različen obseg kromosomskih aberacij. Najbolj pogoste so bile mostički, zaostali kromosomi in zlepljeni kromosomi v metafazah ter zlomljeni kromosomi, mikronukleusi in dvojedrene celice po delitvi. Doze sevanja z jakostjo od 300 Gy do 600 Gy so vzpodbudile razpad jedrnih membran. Slanostna obravnavanja s 25 mM NaCl in 60 mM NaCl so zmanjšala rast sejank vseh sort. Doza obsevanja s 100 Gy je zmajšala učinek slanostnega stresa 25 mM NaCl pri večini sort, razen pri sortah FLIP 84-188 in FLIP 97-263. Obravnavanje z dozo 60 mM NaCl je značilno zmanjšalo zgodnjo rast pri vseh sortah in negativnega učinka ni bilo mogoče zmanjašati z γ-obsevanjem.

Ključne besede: čičerka; γ-sevanje; kalitev; mitotski indeks; kromosomske aberacije

1 Botany and Microbiology Department, Faculty of Science, Suez Canal University, Ismailia, Egypt
2 Corresponding author, e-mail: amal_abdoun@science.suez.edu.eg
3 International Center for Agriculture Research in Dry Land (ICARDA), Aleppo, Syria
4 Botany and Microbiology Department, Faculty of Science, Helwan University, Cairo, Egypt

1 INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an annual herbaceous self-pollinated legume (Ladizinsky & Adler, 1976). Its seeds are a significant source of proteins, carbohydrates, vitamins, minerals and unsaturated fatty acids (Jimenez-Lopez et al., 2020; Jukanti, Gaur, Gowda, & Chibbar, 2012). It is also important for sustainable agriculture since fixing atmospheric nitrogen via symbiotic bacteria provides rotational value to subsequent crops and improve the growth and yield of chickpea (Marques et al., 2020). The domesticated chickpea is divided into two major distinct chickpea types. One is the "microsperma" or 'desi' with small and dark colored seeds with reticulated surface and anthocyanin pigmented aerial parts and pink or purple flowers (Moreno & Cubero, 1978; Van der Maesen, 1972). The other is "macrosperma" or 'kabuli' with large seeds with beige seed coat and green aerial parts that lack anthocyanin pigmentation and with white flowers (Upadhyaya et al., 2008).

Soil salinity is considered to be one of the most common abiotic stresses controlling agricultural production around the world by threatening crop yield, and agricultural sustainability (Munns & Gilliham, 2015). It will become progressively more severe over time due to climatic changes, unsuitable irrigation and excessive fertilization (Sun et al., 2018). Salinity affects crops in two ways; by osmotic stress caused by high concentrations of salts in the soil, which make it harder for roots to absorb water, and by ion stress caused by an increased levels of soluble salts within the plant cells caused by exchangeable sodium (Na^+) during salinity stress (Munns et al., 2020). The impacts of salt stress on plants vary greatly depending on the type and dose of salt used, environmental factors, plant species, cultivars within a species, and plant development stages (Tabur, Avci, & Özmen, 2021). The osmotic stress induces formation of harmful free radicals, including reactive oxygen species (ROS) which causes oxidative damages and induces negative effects on the cell functional integrity (Gaafar, Hamouda, & Badr, 2016; Sharma, Jha, Dubey, & PessarakliM, 2012). Tolerance to salinity may consequently include variations in responses to these factors (Munns & Tester, 2008).

Gamma rays have been used frequently in mutation breeding of grain legumes (Abdelfattah Badr, El-Shazly, & Halawa, 2014; Chopra, 2005; El-Azab, Ahmed Soliman, Soliman, & Badr, 2018; Soliman, Elkelish, Souad, Alhaithloul, & Farooq, 2020). Many mutant crop varieties resistant to diseases, cold, salt and with desired qualities have been developed using γ-radiation (Chopra, 2005; Gnanamurthy, Mariyammal, Dhanavel, & Bharathi, 2012; Tshilenge-Lukanda, Kalonji-Mbuyi, Nkongolo, & Kizungu, 2013). Low frequency of γ-radiation may be beneficial, while the treatments with high doses can be harmful to germination, growth rate, vigor, pollen and ovule fertility (Singh, 2005). The γ-radiation has been used for mutation induction in chickpea (Amri-Tiliouine et al., 2018; Joshi-Saha, Reddy, Petwal, & Dwivedi, 2015; Wani, 2009). Assessment of LD50, lethality, injury, mitotic, and meiotic aberration frequency is required for determining sublethal doses for successful mutation breeding experiments (Bhat & Wani, 2017). At high doses, γ-radiation interact with several metabolites and cell components and can induce many cytogenetic mutations such as chromosomal rearrangements: chromatid and chromosome bridges, single and double fragments, micronuclei, and delayed chromosomes segregation (Abdelfattah Badr et al., 2014; El-Azab et al., 2018; Nazarenko & Izhboldin, 2017). Kamble and Patil (2014) reported the rate of cell division (as mitotic index) and induced qualitative and quantitative chromosomal aberration comprising chromosomes, clumping, polyploidy, ring formation, stickiness, chromatin bridges, laggards, multipolarity at anaphase in chickpea.

Shah, Mirza, Haq, and Atta (2008) tested the effect of γ-radiation doses ranging from 100 Gy to 1200 Gy in the first generation (M1) of four chickpea genotypes. The germination percentage (GP) reduced gradually with increasing γ-radiation doses from 400 Gy 1200 Gy. Brahmi et al. (2014) reported that the 150 Gy dose was determined as the optimum causing 50 % reduction in seed survival of local chickpea variety, but higher, more than 250 Gy doses caused a slow decline in germination rate; reaching values lower than 10 % for treatments of over 650 Gy. The shoot lengths of nine *Cicer* species, including three kabuli and four desi types as well as two annual wild species were inhibited with a 200 Gy of γ-radiation and growth curves gradually decreased at the 300 Gy and 400 Gy doses (Toker, Uzun, Canci, & Ceylan, 2005). Melki, Mhamdi, and Achouri (2011) investigated the impact of low doses of γ-radiation from radioactive cobalt on chickpea growth, protein content in leaves and grains harvested from irradiated seeds. The dose of 20 Gy γ-radiation enhanced plant growth by 146.35 % compared to plants grown from non-irradiated seeds. Sohrabi, Heidari, and Esmailpoor (2008) evaluated the effect of NaCl salinity at different levels (0, 3, 6 and 9 dS m^{-1}) on chickpea and reported reduction of plant growth, pod number, flowers, seed mass and seed number.

The objective of this study is to evaluate the potential of induced mutation with γ-radiation in chickpea varieties to alleviate the effects of salinity stress treat-

ments on germination, seedling's growth and cell division and chromosomes in the M1 chickpea.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Seven varieties of chickpea (FLIP 81-71, FLIP 84-188, FLIP 97-263, ILC 72, ILC 464, ILC 484 and ILC 2555) were obtained from International Center for Agricultural Research in Dry Areas (ICARDA), currently hosted by the Agricultural Research Center (Giza, Egypt), and used in this study.

2.2 EXPERIMENTAL SET-UP

Air-dried seeds of the chickpea varieties were irradiated with 50, 100, 200, 300, 400, 500 and 600 Gy of γ-radiation (dose rate 1.249 kGy h^{-1}). The irradiation was done at the Atomic Energy Center, Nasr City, Cairo, Egypt using irradiation device GSR D1 (Germany). The 50 % lethal irradiation dose (LD50) was determined by calculating the germination and survival percentage. Germination percentage (GP) of irradiated seeds for all doses and their controls was determined on the 7^{th} day of germination.

2.3 CYTOLOGICAL ANALYSIS

The chickpea seeds were germinated in Petri dishes and germinating roots (7 days) were fixed in freshly prepared Carnoy's fixative for 24 h and kept at 4 °C until used. The fixed roots were washed briefly with distilled water, hydrolyzed with 1N HCl for 8-10 min at 60 °C or for 20-25 min at room temperature. The hydrolyzed roots were washed briefly again with distilled water and stained with the basic fuchsin stain (Germany) for 15 min at 23 °C. The stained root tips were cut off and squashed in a drop of 45 % acetic acid, using coverslip. The slides were examined using the 40 × magnification of the light microscope (KRÜSS, Germany) and five slides were examined for each treatment. Photomicrographs of abnormal and control cells were taken with digital camera (Fujifilm FinePix JV100 12 MP Digital Camera, China).

The following data were measured and calculated for each treatment using the following equations

MI (%) = (Number of cells in mitosis / Number of all examined cells) × 100.

Abnormality type (%) = (Number of cells showing the specific abnormality type / Total number cells showing all abnormalities) × 100.

Total abnormalities (%) = (Total number of cells showing all abnormalities / Total number of all examined cells) × 100.

2.4 MORPHOLOGICAL ANALYSIS

For studying the effects of NaCl salinity and of γ-radiation doses and their combination, the treated and control seeds were sown in 30 cm wide plastic boxes containing 30 kg soil (EC = 0.6 ds cm^{-1}) with five replicates during the early winter season of 2018 -2019. Three treatments were applied, first: control plants were not treated by neither γ-radiation nor NaCl; second, the plants were treated with two concentrations of NaCl (25mM and 60mM NaCl); third, the plants were treated with γ-radiation doses (50, 100 and 200 Gy) and NaCl (25 mM NaCl or 60 mM NaCl) as combination treatments.

Seven vegetative growth parameters were measured after eight weeks from sowing: shoot length (cm), root length (cm), number of leaves per plant, shoot fresh biomass (g), root fresh biomass (g), shoot dry biomass (g) and root dry biomass (g).

2.5 STATISTICAL ANALYSIS

The data were statistically analyzed using the ANOVA by IBM SPSS Statistics 25 software. The significant difference between the treatments comparing to the control in the same variety was recorded at an alpha level of 0.05 according to the Least Significant Difference (LSD) test.

3 RESULTS

The seed GP calculations revealed no significant variations among varieties under normal condition. The highest GP of 100 % was recorded for 'ILC 484' and the lowest GP of (86.7 % (was recorded for ‹ FLIP 84-188'. In general, non-significant differences were recorded for seeds exposed to 50 Gy, 100 Gy, and 200 Gy doses of γ-radiation in comparison with the corresponding controls except 'ILC 484'. The GP values for the studied varieties slightly decreased at 300 Gy and decreased significantly at 400 Gy, 500 Gy and 600 Gy of γ-radiation (Figure 2). The maximum inhibitory effect on germination was recorded at 500 Gy for 'FLIP 81-71', 'ILC 72' (50 %)) and 'ILC 2555' (53.3 %) but the lowest value of

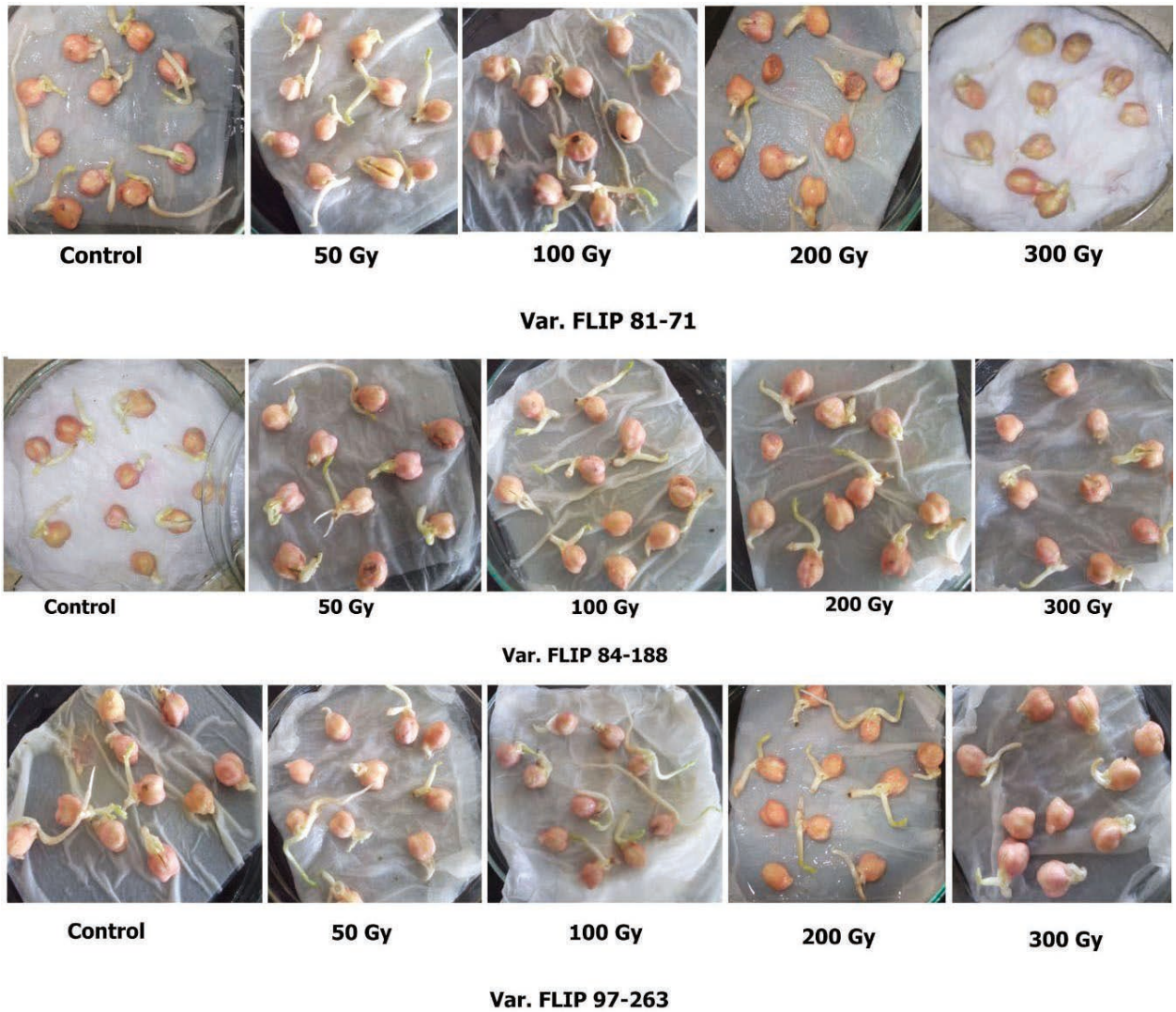

Figure 1: Photos illustrating the germinating seeds of the three chickpea varieties FLIP 81-71. FLIP 84-188, and FLIP 97-263 under control conditions and after exposure to 50, 100, 200, and 300Gy of γ-radiation

GP for the other four varieties was recorded at the maximum dose of 600 Gy of γ-radiation.

Cytological analysis of root meristematic cells, for all chickpea varieties, following seeds exposure to γ-radiation doses from 300 Gy to 600 Gy showed degradation of most nuclear membranes. Consequently, the cytological analyses were only made on the roots exposed to the γ-radiation doses of 50 Gy, 100 Gy and 200 Gy and the control (Figure 3). The MI and the chromosomal abnormalities of the treatments are presented in Table 1. The MI showed significant variation between the different doses of gamma irradiation compared to the control in 'FLIP 81-71', 'ILC 464' and 'ILC 484'. The lowest MI value (3.4) was scored in 'FLIP 97-263' at 50 Gy and 200 Gy while the highest MI value (7.3) was recorded in 'ILC 72' at 200 Gy. The results showed an increase in the MI with the increasing doses from 50 Gy to 200 Gy in all the studied chickpea varieties.

All the applied doses of γ-radiation induced a variable range of mitotic chromosomal abnormalities; bridges, laggard chromosomes, sticky metaphase, chromosome breaks, micronuclei and binucleated cells (Table 1). Five of the studied chickpea varieties (FLIP 84-188, FLIP97-263, ILC 464, ILC 484 and ILC 2555) showed significant difference in bridge percentage between the treatments. The appearance of laggard chromosome was more frequent in all treatments of all varieties except the 50 Gy of 'ILC 2555'. The only significant difference of laggard chromosome percentage was recorded in 'ILC 2555'. The highest value of laggard

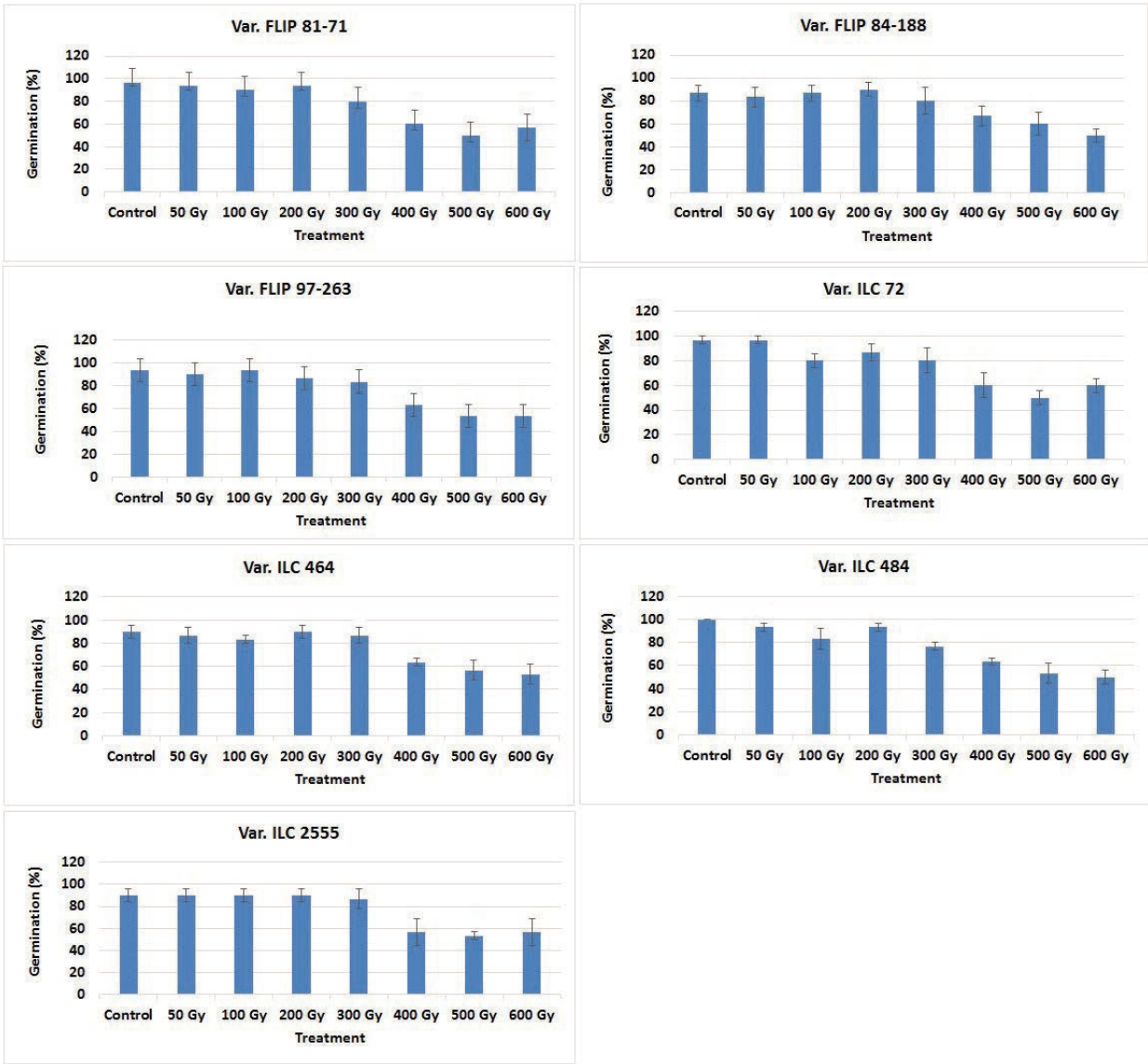

Figure 2: Germination of seven chickpea varieties under control conditions and after exposure to γ-radiation. The mean values ± standard errors are presented (n = 5)

chromosomes (74.5 %) was induced by 200 Gy in ILC 72, while the highest value of sticky metaphase (81.5 %) was induced by 200 Gy in ILC 464. In three varieties, ILC 72, ILC 484 and ILC 2555, a highly significant difference (p = 0.010, 0.000 and 0.001, respectively) in breaks was recorded. While 'FLIP 81-71' and 'ILC 464' showed significant difference (0.010, 0.002, respectively) in the presence of micronuclei, the significant difference of binucleated cells was recorded in 'FLIP 84-188' and 'FLIP 97-263'. The maximum values of the percentage of micronuclei and binucleated cells were 45.0 % and 27.3 %, respectively, induced by 50 Gy γ-radiation in 'ILC 464'. The total abnormalities increased with increased γ-radiation doses. The highest value of the total abnormalities induced by γ-radiation was 5.4%, which was recorded at 200 Gy dose in 'ILC 72' while the minimum value (2.0 %) was recorded at the 50 Gy dose in 'FLIP 81-71' and 'FLIP 97-263'. Highly significant difference appeared at the total abnormalities between all chickpea varieties (Table 1).

The shoots of all chickpea varieties grew above ground after 12 days of sowing. The seedlings treated with 600 Gy and 500 Gy died after three weeks of sowing while seedlings exposed to 400 Gy and 300 Gy died after five weeks of sowing.

The effect of the 60 mM NaCl treatment was sig-

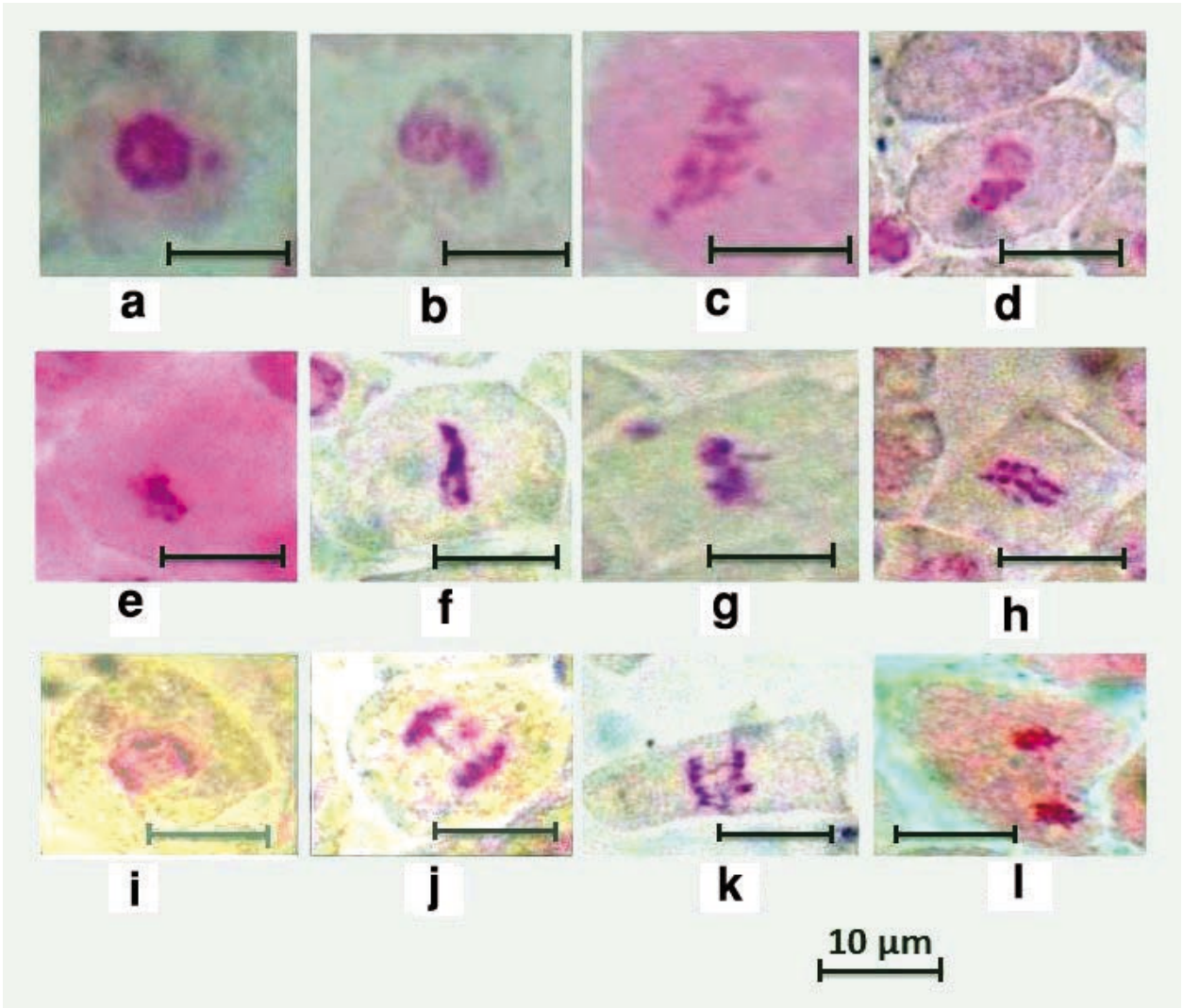

Figure 3: Photographs illustrating types of chromosomal abnormalities induced in the root meristems of seedlings of seven chickpea varieties exposed to three doses of γ-radiation (50 Gy,100 Gy and 200 Gy): a) micronucleus induced by 50 Gy; b) binucleated cell induced by 50 Gy; c) severe stickiness and disturbance at metaphase induced by 100 Gy; d) binucleated cell with sticky metaphase induced by 100 Gy;, e, f) stickiness at metaphase induced by 100 Gy; g) micronucleus, lag-chromosome and sticky metaphase induced by 200 Gy; h) multi-bridges with vagrant chromosome induced 50Gy; i) anaphase bridge induced by 200 Gy; j) anaphase bridge and lagging chromosomes induced by 50 Gy; k) anaphase multi-bridges induced by 50 Gy; l) telophase bridge break induced by 200 Gy.

nificantly higher than the 25 mM NaCl treatment in all studied varieties. In 'FLIP 81-71', measurements under the two salt treatments showed significant reductions in shoot and root traits. Combining low doses of γ-radiation with the low concentration of NaCl treatments alleviated the effect of salinity treatments particularly the shoot length, which scored the highest length in plants exposed to combination of 100 Gy of γ-radiation and 25 mM NaCl compared to the control value (Table 2). The data of 'ILC 72' illustrated that the combination of 100 Gy of *γ*-radiation and 25 mM NaCl treatment showed significant increase in shoot length (24.0 ± 0.58 cm) as compared with 25 mM salt treatment alone. On the other hand, the *γ*-radiation doses (50, 100 and 200 Gy) combined with 60 mM NaCl treatments showed non-significant increase in shoot length comparing with 60 mM salt treatment alone. The combination of γ-radiation doses of 50 Gy, 100 Gy and 200 Gy with the 25 mM NaCl, significantly increased root length (approx. 11 cm) compared to the control plants and plants exposed to 25 mM NaCl only of 'ILC 464'. The data of 'ILC 2555' showed root length reductions

Table 1: Cytological analysis of chickpea root meristem showing mitotic index (MI), specific and total chromosomal abnormalities after γ-radiation

Variety	Treatment	Total cells examined	MI (%)	LSD	Different abnormalities (%)												Total abnormalities (%)		
					Bridge	LSD	Lag. Chrom.	LSD	Sticky metaphase	LSD	Break	LSD	Micro nuclues	LSD	Binuclei	LSD	*Total*	One-way ANOVA		
																		LSD	F-value	p-value
FLIP 81-71	Control	2355	3.4 ± 0.8	-	25.0 ± 12.6	-	31.7 ± 15.9	-	43.3 ± 28.5	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	1.5 ± 0.1	-	10.5	0.004
	50 Gy	2963	3.6 ± 0.3	ns	18.8 ± 10.5	ns	17.4 ± 9.0	ns	28.2 ± 17.4	ns	3.7 ± 3.7	ns	20.8 ± 7.0	*	11.1 ± 11.1	ns	2.0 ± 0.2	ns		
	100 Gy	3655	6.0 ± 0.8	*	24.1 ± 3.5	ns	36.2 ± 5.1	ns	35.7 ± 2.5	ns	0.7 ± 0.7	ns	2.0 ± 1.1	ns	1.3 ± 1.3	ns	2.4 ± 0.3	*		
	200 Gy	3967	5.5 ± 0.3	*	2.9 ± 2.9	ns	31.8 ± 14.1	ns	62.9 ± 19.4	ns	0.8 ± 0.8	ns	1.2 ± 1.2	ns	0.4 ± 0.4	ns	3.3 ± 0.3	*		
	One-way ANOVA		0.034		0.297		0.704		0.636		0.566		0.010		0.483					
FLIP 84-188	Control	5385	5.0 ± 1.3	-	43.3 ± 5.1	-	43.3 ± 5.1	-	13.3 ± 10.2	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.7 ± 0.1	-	30.7	0.000
	50 Gy	3050	5.2 ± 0.3	ns	25.9 ± 2.0	*	49.9 ± 1.3	ns	7.3 ± 3.7	ns	0.0 ± 0.0[a]	ns	10.7 ± 4.3	ns	6.2 ± 1.7	*	2.1 ± 0.4	*		
	100 Gy	2900	5.3 ± 0.6	ns	7.8 ± 3.9	*	30.9 ± 15.5	ns	46.7 ± 26.7	ns	1.8 ± 0.9	ns	12.8 ± 6.4	*	0.0 ± 0.0	ns	2.9 ± 0.3	*		
	200 Gy	4100	6.8 ± 0.8	ns	10.9 ± 0.8	*	53.5 ± 3.5	ns	28.5 ± 2.3	ns	5.0 ± 2.5	*	1.0 ± 1.0	ns	1.0 ± 1.0	ns	4.8 ± 0.3	*		
	One-way ANOVA		0.484		0.000		0.308		0.292		0.087		0.106		0.007					
FLIP 97-263	Control	6155	3.6 ± 0.5	-	18.8 ± 10.5	-	69.9 ± 10.5	-	6.4 ± 3.2	-	5.0 ± 5.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.6 ± 0.1	-	7.3	0.011
	50 Gy	3566	3.4 ± 0.4	ns	29.2 ± 3.8	ns	46.9 ± 7.6	ns	6.0 ± 3.0	ns	0.0 ± 0.0	ns	10.6 ± 5.3	ns	7.4 ± 2.2	*	2.0 ± 0.2	*		
	100 Gy	3688	3.7 ± 0.4	ns	0.0 ± 0.0	*	56.0 ± 5.4	ns	30.9 ± 1.2	*	0.0 ± 0.0	ns	13.2 ± 6.6	ns	0.0 ± 0.0	ns	2.3 ± 0.5	*		
	200 Gy	2210	3.4 ± 0.1	ns	14.9 ± 1.2	ns	62.0 ± 6.0	ns	9.5 ± 1.5	ns	0.0 ± 0.0	ns	0.0 ± 0.0	ns	9.5 ± 1.5	*	2.5 ± 0.4	*		
	One-way ANOVA		0.923		0.037		0.262		0.000		0.441		0.117		0.001					

Continued on the next page

ILC 72	Control	5890	4.0 ± 0.9	-	16.7 ± 16.7	-	66.7 ± 16.7	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	16.7 ± 16.7	-	0.4 ± 0.1	-	36.9	0
	50 Gy	3000	5.0 ± 1.2	ns	17.7 ± 9.2	ns	53.3 ± 3.3	ns	11.8 ± 8.3	ns	0.0 ± 0.0	ns	13.3 ± 13.3	ns	3.8 ± 3.8	ns	3.2 ± 0.4	*		
	100 Gy	2800	6.7 ± 0.5	ns	19.8 ± 1.1	ns	47.2 ± 2.8	ns	13.0 ± 1.5	ns	7.6 ± 2.7	*	11.0 ± 3.1	ns	0.0 ± 0.0	ns	3.4 ± 0.3	*		
	200 Gy	4600	7.3 ± 1.5	ns	7.1 ± 0.8	ns	74.5 ± 1.9	ns	10.1 ± 1.7	ns	4.4 ± 0.2	ns	4.0 ± 0.1	ns	0.0 ± 0.0	ns	5.4 ± 0.5	*		
	One-way ANOVA		0.184		0.789		0.185		0.206		0.01		0.523		0.503					
ILC 464	Control	3441	3.7 ± 0.4	-	26.1 ± 3.9	-	21.1 ± 10.6	-	52.8 ± 12.1	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.3 ± 0.2	-	42.4	0
	50 Gy	3065	4.5 ± 0.3	ns	0.0 ± 0.0	*	18.5 ± 18.5	ns	9.2 ± 4.9	*	0.0 ± 0.0	ns	45.0 ± 11.7	*	27.3 ± 11.7	*	2.2 ± 0.3	*		
	100 Gy	3350	7.0 ± 0.3	*	17.7 ± 1.5	*	52.9 ± 10.6	ns	30.3 ± 8.5	ns	1.0 ±1.0	ns	3.2 ± 3.2	ns	10.5 ± 9.1	ns	4.4 ± 0.3	*		
	200 Gy	3910	4.9 ± 0.3[b]	*	0.0 ± 0.0	*	18.5 ± 1.0	ns	81.5 ± 1.0	*	0.0 ± 0.0	ns	0.0 ± 0.0	ns	0.0 0.0	ns	2.2 ± 0.2	*		
	One-way ANOVA		0.001		0.000		0.195		0.001		0.441		0.002		0.094					
ILC 484	Control	3529	3.9 ± 0.3	-	38.1 ± 14.3	-	38.9 ± 20.0	-	14.7 ± 9.8	-	0.0 ± 0.0	-	8.3 ± 8.3	-	0.0 ± 0.0	-	0.5 ± 0.1	-	20.8	0
	50 Gy	4200	6.4 ± 0.3	*	2.8 ± 2.8	*	40.4 ± 29.9	ns	5.7 ± 3.0	ns	0.0 ± 0.0	ns	25.6 ± 14.4	ns	25.6 ± 17.9	ns	2.8 ± 0.4	*		
	100 Gy	2800	5.4 ± 0.4	*	1.5 ± 1.5	*	55.7 ± 1.6	ns	13.0 ± 2.1	ns	11.7 ± 1.0	*	1.2 ± 1.2	ns	16.9 ± 3.5	ns	2.9 ± 0.4	*		
	200 Gy	3100	5.4 ± 0.2	*	20.3 ± 0.6	ns	40.8 ± 0.6	ns	14.5 ± 0.1	ns	10.6 ± 0.5	*	5.0 ± 0.2	ns	8.9 ± 0.4	ns	5.1 ± 0.6	*		
	One-way ANOVA		0.004		0.024		0.899		0.602		0.000		0.252		0.301					
ILC 2555	Control	6345	4.5 ± 0.2	-	38.8 ± 3.6	-	61.2 ± 3.6	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.5 ± 0.1	-	16.8	0.001
	50 Gy	3540	4.6 ± 0.3	ns	20.8 ± 11.0	ns	0.0 ± 0.0	*	26.7 ± 18.7	ns	0.0 ± 0.0	ns	41.7 ± 21.3	*	10.8 ± 5.8	*	2.6 ± 0.3	*		
	100 Gy	3050	4.5 ± 0.0	ns	9.6 ± 0.8	*	51.2 ± 1.2	ns	39.2 ± 1.8	*	0.0 ± 0.0	ns	0.0 ± 0.0	ns	0.0 ± 0.0	ns	2.8 ± 0.2	*		
	200 Gy	4150	4.7 ± 0.3	ns	9.4 ± 4.8	*	36.5 ± 9.6	*	41.7 ± 4.4	*	7.5 ± 1.8	*	0.0 ± 0.0	ns	4.9 ± 2.4	ns	3.9 ± 0.5	*		
	One-way ANOVA		0.825		0.033		0.00		0.054		0.001		0.057		0.12					

Mean values ± SE (n = 5), and p-values (ANOVA) are presented. ns = non-significant difference, * denotes significant difference against the control in the same variety at $p < 0.05$ according to LSD test. Abbreviations: MI, Mitotic Index, Lag. Chrom., Laggard chromosome, Sticky metaphase, Micronucleu

Table 2: Morphological measurements of chickpea plants following seed exposure to NaCl treatments alone and in combination with *γ*-radiation

Variety	Treatment	Shoot length (cm)	LSD	Root length (cm)	LSD	No. of leaves / plant	LSD	Fresh biomass (g) Shoot	LSD	Fresh biomass (g) Root	LSD	Dry biomass (g) Shoot	LSD	Dry biomass (g) Root	LSD
FLIP 81-71	Control	24.33 ± 0.92	-	8.77 ± 0.15	-	25.00 ± 3.22	-	1.33 ± 0.04	-	0.45 ± 0.03	-	0.31 ± 0.02	-	0.07 ± 0.01	-
	25 mM NaCl salt	19.33 ± 1.86	*	8.07 ± 0.87	ns	20.33 ± 3.84	ns	0.9 7 ± 0.11	*	0.49 ± 0.09	ns	0.28 ± 0.003	ns	0.06 ± 0.006	ns
	60 mM NaCl salt	13.83 ± 1.59	*	4.67 ± 0.58	*	6.00 ± 0.58	*	0.53 ± 0.088	*	0.30 ± 0.058	ns	0.21 ± 0.009	*	0.03 ± 0.003	*
	50 Gy+25 mM NaCl	23.00 ± 1.53	ns	8.50 ± 0.35	ns	16.67 ± 0.88	*	1.43 ± 0.075	ns	0.55 ± 0.029	ns	0.33 ± 0.012	ns	0.08 ± 0.003	ns
	100 Gy+25 mM NaCl	25.00 ± 1.53	ns	7.03 ± 0.61	*	23.00 ± 1.16	ns	1.30 ± 0.058	ns	0.38 ± 0.103	ns	0.36 ± 0.073	ns	0.03 ± 0.003	*
	200 Gy+25 mM NaCl	20.33 ± 0.88	ns	7.70 ± 0.49	ns	23.33 ± 0.88	ns	1.10 ± 0.058	*	0.26 ± 0.006	*	0.31 ± 0.039	ns	0.03 ± 0.000	*
	50 Gy+60 mM NaCl	13.33 ± 2.40	*	6.10 ± 0.31	*	7.67 ± 1.20	*	0.78 ± 0.012	*	0.36 ± 0.024	ns	0.28 ± 0.007	ns	0.03 ± 0.002	*
	100 Gy+60 mM NaCl	17.33 ± 1.20	*	5.50 ± 0.68	*	14.00 ± 1.16	*	1.05 ± 0.053	*	0.24 ± 0.023	*	0.33 ± 0.012	ns	0.03 ± 0.000	*
	200 Gy+60 mM NaCl	17.67 ± 0.33	*	4.93 ± 0.30	*	12.00 ± 11.16	*	0.92 ± 0.009	*	0.25 ± 0.006	*	0.28 ± 0.038	ns	0.03 ± 0.000	*
FLIP 84-188	Control	21.17± 0.17	-	10.83 ± 1.09	-	22.00 ± 3.06	-	1.38 ± 0.034	-	0.50 ± 0.024	-	0.22 ± 0.019	-	0.05 ± 0.003	-
	25 mM NaCl salt	19.17 ± 2.40	ns	6.50 ± 1.53	*	20.00 ± 5.03	ns	0.62 ± 0.100	*	0.32 ± 0.107	*	0.18 ± 0.015	ns	0.03 ± 0.013	ns
	60 mM NaCl salt	20.17 ± 0.93	ns	8.67 ± 0.44	*	9.67 ± 1.20	*	1.04 ± 0.028	ns	0.32 ± 0.023	*	0.21 ± 0.018	ns	0.03 ± 0.000	*
	50 Gy+25 mM NaCl	9.83 ± 0.93	*	4.87 ± 0.32	*	17.33 ± 1.45	ns	0.52 ± 0.108	*	0.14 ± 0.009	*	0.16 ± 0.022	*	0.01 ± 0.001	*
	100 Gy+25 mM NaCl	15.00 ± 2.89	*	6.50 ± 0.58	*	19.67 ± 1.20	ns	0.97 ± 0.274	*	0.31 ± 0.022	*	0.16 ± 0.023	*	0.02 ± 0.005	*
	200 Gy+25 mM NaCl	11.00 ± 1.16	*	5.20 ± 0.25	*	11.67 ± 0.88	*	0.62 ± 0.079	*	0.24 ± 0.007	*	0.12 ± 0.006	*	0.02 ± 0.001	*
	50 Gy+60 mM NaCl	12.00 ± 1.16	*	4.83 ± 0.15	*	7.00 ± 1.16	*	0.67 ± 0.064	*	0.19 ± 0.026	*	0.19 ± 0.024	ns	0.02 ± 0.001	*
	100 Gy+60 mM NaCl	12.67 ± 0.88	*	6.43 ± 0.41	*	8.33 ± 0.33	*	0.72 ± 0.051	*	0.29 ± 0.015	*	0.13 ± 0.009	*	0.03 ± 0.001	*
	200 Gy+60 mM NaCl	16.33 ± 0.88	*	9.40 ± 0.21	ns	9.33 ± 0.67	*	0.62 ± 0.129	*	0.35 ± 0.018	*	0.23 ± 0.034	ns	0.04 ± 0.003	ns
FLIP 97-263	Control	26.83 ± 1.92	-	5.90 ± 0.86	-	21.67 ± 3.283	-	2.10 ± 0.048	-	0.42 ± 0.052	-	0.64 ± 0.018	-	0.07 ± 0.009	-
	25 mM NaCl salt	24.67 ± 0.93	ns	4.93 ± 0.98	ns	18.67 ± 2.728	ns	0.65 ± 0.036	*	0.21 ± 0.064	*	0.33 ± 0.024	*	0.04 ± 0.007	*
	60 mM NaCl salt	19.67 ± 2.60	*	3.90 ± 0.31	*	12.33 ± 0.882	*	0.75 ± 0.124	*	0.24 ± 0.045	*	0.30 ± 0.027	*	0.04 ± 0.007	*
	50 Gy+25 mM NaCl	18.83 ± 0.60	*	4.27 ± 0.50	*	18.33 ± 3.756	ns	1.02 ± 0.089	*	0.30 ± 0.072	ns	0.45 ± 0.057	*	0.05 ± 0.003	ns
	100 Gy+25 mM NaCl	18.33 ± 1.67	*	8.07 ± 0.12	*	14.67 ± 1.764	*	1.24 ± 0.121	*	0.44 ± 0.035	ns	0.51± 0.035	*	0.07 ± 0.009	ns
	200 Gy+25 mM NaCl	16.67 ± 2.40	*	3.87 ± 0.34	*	13.33 ± 1.764	*	1.05 ± 0.187	*	0.2833 ± 0.022	*	0.48 ± 0.055	*	0.05 ± 0.002	ns
	50 Gy+60 mM NaCl	16.00 ± 1.53	*	3.60 ± 0.23	*	18.67 ± 0.882	ns	0.76 ± 0.067	*	0.26 ± 0.021	*	0.33 ± 0.013	*	0.05 ± 0.001	ns
	100 Gy+60 mM NaCl	12.33 ± 1.45	*	7.97 ± 0.24	*	13.00 ± 1.155	*	0.66 ± 0.023	*	0.43 ± 0.032	ns	0.33 ± 0.021	*	0.06 ± 0.006	ns
	200 Gy+60 mM NaCl	14.00 ± 1.53	*	5.17 ± 0.524	ns	7.33 ± 1.202	*	0.88 ± 0.209	*	0.3867 ± 0.039	ns	0.40 ± 0.073	*	0.06 ± 0.007	ns

Table 2 continued

ILC 72	Control	24.30 ± 2.00	-	3.67 ± 0.44	-	17.00 ± 1.00	-	0.68 ± 0.09	-	0.06 ± 0.003	-	0.30 ± 0.012[a]	-	0.011 ± 0.001	-
	25 mM NaCl salt	18.50 ± 2.08	*	4.50 ± 1.53	ns	15.33 ± 0.88	ns	0.77 ± 0.09	ns	0.17 ± 0.064	*	0.3± 0.015	ns	0.029 ± 0.011	*
	60 mM NaCl salt	14.33 ± 0.88	*	3.43 ± 0.35	ns	7.67 ± 0.88	*	0.74 ± 0.07	ns	0.10 ± 0.008	ns	0.32 ± 0.015	ns	0.017 ± 0.001	ns
	50 Gy+25 mM NaCl	18.33 ± 1.20	*	4.90 ± 0.21	ns	20.33 ± 1.45	ns	0.78 ± 0.04	ns	0.14 ± 0.019	*	0.30 ± 0.018	ns	0.023 ± 0.003	*
	100 Gy+25 mM NaCl	24.00 ± 0.58	ns	5.37 ± 0.59	ns	19.00 ± 3.61	ns	1.15 ± 0.11	*	0.07 ± 0.012	ns	0.46 ± 0.044	*	0.011 ± 0.002	ns
	200 Gy+25 mM NaCl	20.67 ± 1.20	ns	5.30 ± 0.47	ns	18.67 ± 0.88	ns	0.96 ± 0.09	*	0.10 ± 0.007	ns	0.39 ± 0.035	*	0.016 ± 0.001	ns
	50 Gy+60 mM NaCl	14.87 ± 0.59	*	4.07 ± 0.58	ns	12.00 ± 0.58	*	0.41± 0.01	ns	0.08 ± 0.009	ns	0.16 ± 0.006	*	0.014 ± 0.002	ns
	100 Gy+60 mM NaCl	13.57 ± 0.96	*	6.20 ± 0.46	*	10.33 ± 0.88	*	0.36 ± 0.03	ns	0.10 ± 0.009	ns	0.14 ± 0.015	*	0.017 ± 0.001	ns
	200 Gy+60 mM NaCl	13.43 ± 0.74	*	4.57 ± 0.52	ns	11.67 ± 0.33	*	0.38 ± 0.04	ns	0.09 ± 0.003	ns	0.15 ± 0.018	*	0.014 ± 0.001	ns
ILC 464	Control	28.73 ± 1.47	-	9.8 3 ± 0.73	-	22.67 ± 2.67	-	1.79 ± 0.243	-	0.43 ± 0.123	-	0.48 ± 0.066	-	0.06 ± 0.012	-
	25 mM NaCl salt	24.17 ± 0.44	*	9.00 ± 0.29	ns	16.33 ± 2.03	*	1.06 ± 0.045	*	0.31 ± 0.009	ns	0.29 ± 0.012	*	0.05 ± 0.001	ns
	60 mM NaCl salt	20.00 ± 1.16	*	6.33 ± 0.60	*	10.33 ± 1.45	*	0.98 ± 0.015	*	0.26 ± 0.015	*	0.26 ± 0.004	*	0.04 ± 0.003	*
	50 Gy+25 mM NaCl	26.00 ± 0.58	ns	11.17 ± 0.44	ns	18.00 ± 1.16	ns	1.35± 0.035	*	0.59 ± 0.009	ns	0.37 ± 0.009	*	0.09 ± 0.001	*
	100 Gy+25 mM NaCl	28.67 ± 0.88	ns	11.50 ± 0.76	ns	22.00 ± 0.56	ns	1.98 ± 0.079	ns	0.60 ± 0.013	*	0.53 ± 0.021	ns	0.09 ± 0.002	*
	200 Gy+25 mM NaCl	18.00 ± 1.16	*	10.97 ± 0.26	ns	19.6 7± 0.88	ns	1.03 ± 0.091	*	0.60 ± 0.003	*	0.28 ± 0.025	*	0.09 ± 0.000	*
	50 Gy+60 mM NaCl	17.50 ± 2.02	*	7.50 ± 1.26	*	15.00 ± 4.73	*	0.92 ± 0.092	*	0.36 ± 0.102	ns	0.25 ± 0.025	*	0.05 ± 0.013	ns
	100 Gy+60 mM NaCl	18.83 ± 0.73	*	6.50 ± 0.29	*	10.67 ± 0.88	*	0.95 ± 0.037	*	0.26 ± 0.009	ns	0.26 ± 0.010	*	0.04 ± 0.003	*
	200 Gy+60 mM NaCl	17.00 ± 1.16	*	6.83 ± 0.44	*	10.67 ± 0.88	*	0.91 ± 0.039	*	0.28 ± 0.009	ns	0.25 ± 0.011	*	0.05 ± 0.002	*
ILC 484	Control	19.67 ± 1.01	-	4.27 ± 0.56	-	21.00 ± 2.52	-	1.44 ± 0.23	-	0.26 ± 0.04	-	0.49 ± 0.08	-	0.067 ± 0.009	-
	25 mM NaCl salt	17.00 ± 0.58	ns	6.93 ± 0.12	*	33.00 ± 2.03	ns	1.79 ± 0.10	ns	0.42 ± 0.01	*	0.6 ± 0.03	ns	0.103 ± 0.003	*
	60 mM NaCl salt	14.00 ± 0.58	*	9.57 ± 0.54	*	8.33 ± 0.88	*	1.3 ± 0.12	ns	0.47 ± 0.05	*	0.35 ± 0.08	ns	0.028 ± 0.002	*
	50 Gy+25 mM NaCl	25.50 ± 1.32	*	6.47 ± 0.35	*	25.00 ± 1.73	ns	1.89 ± 0.16	ns	0.14 ± 0.01	*	0.64 ± 0.06	ns	0.022 ± 0.001	*
	100 Gy+25 mM NaCl	21.33 ± 1.20	ns	8.57 ± 0.35	*	24.33 ± 1.76	ns	1.53 ± 0.24	ns	0.26 ± 0.02	ns	0.45 ± 0.08	ns	0.034 ± 0.003	*
	200 Gy+25 mM NaCl	24.00 ± 1.15	*	8.50 ± 0.38	*	22.00 ± 1.15	ns	1.72 ± 0.05	ns	0.28 ± 0.02	ns	0.58 ± 0.02	ns	0.036 ± 0.003	*
	50 Gy+60 mM NaCl	15.33 ± 1.45	*	6.17 ± 0.67	*	13.00 ± 1.15	*	1.18 ± 0.04	ns	0.28 ± 0.02	ns	0.4 ± 0.02	ns	0.027 ± 0.002	*
	100 Gy+60 mM NaCl	14.33 ± 1.45	*	7.73 ± 0.24	*	10.67 ± 1.20	*	0.81 ± 0.36	ns	0.21 ± 0.01	ns	0.27 ± 0.12	ns	0.027 ± 0.001	*
	200 Gy+60 mM NaCl	16.00 ± 1.15	*	6.13 ± 0.49	*	12.67 ± 1.45	*	0.83 ± 0.37	ns	0.24 ± 0.02	ns	0.28 ± 0.13	ns	0.031 ± 0.003	*

Continued on the next page

ILC2555	Control	22.83 ± 0.73	-	10.17 ± 1.01	-	24.67 ± 0.88	-	1.26 ± 0.130	-	0.33 ± 0.110	-	0.24 ± 0.045	-	0.03 ± 0.006	-
	25 mM NaCl salt	23.83 ± 1.59	ns	8.67 ± 0.44	ns	20.33 ± 1.45	*	1.35 ± 0.231	ns	0.22 ± 0.092	ns	0.25 ± 0.066	ns	0.01 ± 0.003	*
	60 mM NaCl salt	21.17 ± 0.60	ns	7.33 ± 0.60	*	10.33 ± 0.88	*	0.97 ± 0.072	ns	0.25 ± 0.065	ns	0.19 ± 0.012	ns	0.03 ± 0.006	ns
	50 Gy+25 mM NaCl	23.17 ± 0.60	ns	10.73 ± 0.19	ns	25.67 ± 1.76	ns	1.04 ± 0.022	ns	0.37 ± 0.072	ns	0.24 ± 0.012	ns	0.04 ± 0.003	*
	100 Gy+25 mM NaCl	25.33± 2.19	ns	7.90 ± 0.70	*	24.67 ± 0.88	ns	1.48 ± 0.231	ns	0.49 ± 0.198	ns	0.34 ± 0.023	*	0.05 ± 0.006	*
	200 Gy+25 mM NaCl	18.50 ± 1.26	*	5.83 ± 0.52	*	11.67 ± 0.88	*	1.04 ± 0.134	ns	0.35 ± 0.045	ns	0.21 ± 0.021	ns	0.03 ± 0.003	ns
	50 Gy+60 mM NaCl	20.00 ± 2.08	ns	9.07 ± 0.23	ns	11.67 ± 0.88	*	1.24 ± 0.084	ns	0.26 ± 0.095	ns	0.26 ± 0.018	ns	0.03 ± 0.006	ns
	100 Gy+60 mM NaCl	20.00 ± 1.15	ns	6.60 ± 0.47	ns	10.67 ± 0.67	*	1.06 ± 0.054	ns	0.39 ± 0.042	ns	0.26 ± 0.009	ns	0.04 ± 0.003	ns
	200 Gy+60 mM NaCl	16.33 ± 1.20	*	3.97 ± 0.29	ns	10.00 ± 1.00	*	0.67 ± 0.021	*	0.28 ± 0.025	ns	0.18 ± 0.006	ns	0.03 ± 0.000	ns

Mean values ± SE are presented (n = 5). ns = non-significant difference, * denotes significant difference against the control in the same variety at $p < 0.05$ according to LSD test

under all treatments except combination of 50 Gy and 25 mM NaCl treatments. The number of leaves per plant increased by the treatments with the 25 mM NaCl and its contribution with all doses of γ-radiation in 'ILC 484', while the *γ*-radiation doses in combination with the 25 mM and 60 mM NaCl induced significant reduction in shoot length, number of leaves and shoot and root biomass comparing to the control and salt treatment in 'FLIP 97-263'. The combination of 100 Gy *γ*-radiation and 25 mM salt treatment induced significant increase in shoot fresh biomass comparing to the salt treatment only in 'FLIP 84-188' (Table 2).

4 DISCUSSION

All varieties used in the current study, except 'ILC 484', germinated in the control range when irradiated low doses of γ-radiation (50 Gy, 100, Gy 200 and 300 Gy) , which in agreement with Shah et al. (2008), who reported that germination was not affected in the desi variety Pb2000 at γ-radiation doses of 100 Gy, 200 Gy and 300 Gy. High doses of γ-radiation (400 Gy, 500 Gy and 600 Gy) on the other hand decreased the GP significantly compared with the low doses and the control. The inhibition of germination, seedling growth, and other biological responses were frequently observed (Abdelfattah Badr et al., 2014; Kim, Lee, Back, Kim, & Lee, 2000; Toker et al., 2005). The reduction of GP at high doses of γ-radiation, has been reported in many plants including chickpea (Joshi-Saha et al., 2015; Melki & Sallami, 2008; Shah et al., 2008). Low doses of irradiation, like low levels of other abiotic stresses, may increase the anti-oxidative capacity of the cells by producing ROS that mediate the acceleration of cell cycle entry to G0/G1 leading to a positive effect on the plant cell cycle machinery (Feher, Ötvös, Pasternak, & Pettkó-Szandtner, 2008; Sharma et al., 2012). On the contrary, high doses of γ-radiation may result in cell cycle arrest at G2/M phase during somatic cell division and/or damage in the genome (El-Azab et al., 2018; Preuss & Britt, 2003). However, the cytogenetics during germination under abiotic stress is not well understood and requires attention.

The retarded germination of seeds exposed to high doses of NaCl stress and the slow growth of seedlings under these treatments may be associated with slow cell division at the early emergence of seminal root and shot. It is widely accepted that the first action of abiotic stress on germination is moisture deficit resulting in poor plant stand at the early seedling phase and hampers early crop establishment (Kaydan & Yagmur, 2008; Shao, Chu, Jaleel, & Zhao, 2008). Mitotic index was approved as an efficient short-term genetic bioassay via the United States Environmental Agency through the Gene-Tox Program in 1981 (Waters & Auletta, 1981) and was used as an indicator to characterize the cell activity and proliferation (Scofield, Jones, & Murray, 2014). Low doses of γ-radiation induced an increase in the proportion of dividing cells, whereas higher doses resulted in reduction in mitotic activity. A dose-dependent increase in mitotic indices was observed in cowpea following exposure to γ-radiation ranging from 10 to 300 Gy (Girija, Gnanamurthy, & Dhanavel, 2013). Similar findings were also found in cowpea cultivars (Abdelfattah Badr et al., 2014) and in soybean cultivars (El-Azab et al., 2018). In plant root tips, arrest in cell cycle progression is caused by check points that mediate the entry of cells into S-phase and mitosis (De Veylder, Joubès, & Inzé, 2003). The cell often spontaneously continues cycle progression, but this is often followed by genome instability allowing cell survival at the cost of tolerating mutation including chromosomal abnormalities (Hartig & Beck, 2006).

As explained in the results section, the cytological effects of γ-radiation on cell division in the root tip mitosis was made on plants following exposure to the low doses (50, 100 and 200 Gy). Higher γ-radiation doses from (300 to 600 Gy) caused degradation of most nuclear membranes in the root meristematic cells of all varieties. This result is in agreement with Arian and Maqbool (2011) who reported that doses of 150 to 300 Gy induced oxidative damages and inhibition of cell division in chickpea root tip cells. The γ-radiation also affected the cell division phases forming different abnormality types. The total abnormalities percent showed a highly significant difference at all the studied varieties. The total number of abnormal cells increased with the increase of γ-radiation doses of all the studied varieties. Similar result was reported by (Wani, 2009) in chickpea following γ-radiation and ethyl methane sulphonate and their combination treatments.

Chromosomal abnormalities induced by γ-radiation include stickiness of chromosomes (Dhanavel, Gnanamurthy, & Girija, 2012). The highest value of sticky metaphase was recorded in 'ILC 464' at 200 Gy. Chromosome stickiness might be formed due to changes in specific non-histone proteins, histone proteins and DNA breaks induced during chromosome condensation (Piskadlo, Tavares, & Oliveira, 2017). The appearance of free and the lagging chromosomes was more frequent in all the treatments in the studied chick pea varieties except at 50 Gy in 'ILC 2555'. The lagging chromosomes at ana-telophase might be formed due to the failure of spindle fibers to push the respective chromosomes to the poles because of exposure to

γ-irradiations. The ataxia telangiectasia and Rad3-related (ATR) plays an essential role in suppressing replication stress from DNA damage. A mitosis-specific and R loop–driven ATR pathway supports faithful chromosome segregation, preventing formation of lagging chromosomes (Kabeche, Nguyen, Buisson, & Zou, 2018). Chromosomal bridges are commonly attributed to dicentric chromosomes originating from chromosome exchange after chromosome double strand breaks (Cornforth & Goodwin, 1991). Chromosome breakage is usually considered to involve the DNA molecule responsible for the linear stability of the chromosome. This aberration is the result of unfinished repair of DNA (Grant, 1978). Micronuclei usually arise from lagging chromosomes and fragments, which fail to reach the pole region in time and are included in the daughter cells as micronuclei (A Badr, 1986; Kumar, 1998). The micronuclei were more frequency observed in cells exposed to γ-radiation at low dose of 50 Gy, in all the varieties. The number of micronuclei could illustrate the individual sensitivity level to mutagens (Koteles, 1996; Köteles, Bojtor, Szirmai, Berces, & Otos, 1993).

All plants exposed to 60 mM NaCl treatment died before reaching maturity. This result is in agreement with Khan, Siddique, Munir, and Colmer (2015) who stated that salinity severely inhibited plant growth, and led to some tissue death resulting in plant deaths. Haleem (2012) reported that high concentrations of NaCl treatments at 50, 100 and 200 mM caused depression in plant growth, total soluble protein content, photosynthetic pigments content, nucleic acids contents and all yield characteristics, and concluded that seed irradiation with γ-rays moderates the adverse effect of salinity stress compared to non-irradiated seeds. Khan et al. (2015) stated that the 60 mM NaCl treatment also reduced stem and root dry mass of all chickpea genotypes when compared to their controls. Even at low (20 mM and 25 mM) salt concentration, chickpea growth was reduced significantly (Sadiki & Rabih, 2001). Salinity of 3 dS m^{-1} in field soils was reported to be the threshold for reduced shoot growth and yield in chickpea (Katerji, Van Hoorn, Hamdy, Mastrorilli, & Oweis, 2005; Rao, Giller, Yeo, & Flowers, 2002).

5 CONCLUSION

The γ-radiation doses above 300 Gy induced degradation of nuclear membranes, whereas lower doses did not affect or slightly enhanced mitotic activities but induced different types of chromosomal abnormalities. The total number of abnormal cells increased with the increase of γ-radiation doses in all the studied varieties. Gamma-rays induced various types of qualitative and quantitative chromosomal aberration including chromosome bridges, laggard chromosomes, stickiness, chromosome breakage and micronuclei. The salinity treatments at 25 mM NaCl and 60 mM NaCl reduced seedling's growth of all cultivars estimated as root and shoot length and biomass production. The application of γ-rays can moderate the adverse effect of low levels of salinity stress compared to non-irradiated seeds. The γ-radiation dose of 100 Gy alleviated the impact of NaCl salinity in chickpea plants at a concentration of 25 mM NaCl for all varieties, except 'FLIP 84-188'and 'FLIP 97-263'. On the other hand, the 60 mM NaCl treatment significantly reduced early growth of all cultivars and its effect was not alleviated by the γ-radiation application.

6 STATEMENTS AND DECLARATIONS

Conflict of interest: The authors declare no competing interests.

7 REFERENCES

Amri-Tiliouine, W., Laouar, M., Abdelguerfi, A., Jankowicz-Cieslak, J., Jankuloski, L., & Till, B. J. (2018). Genetic variability induced by gamma rays and preliminary results of low-cost TILLING on M2 generation of Chickpea (*Cicer arietinum* L.). *Frontiers in Plant Science, 9*, 1568. Retrieved from https://www.frontiersin.org/articles/10.3389/fpls.2018.01568/full

Arian, A., & Maqbool, A. F. (2011). Gross mutations and oxidative damages induced by high doses of gamma rays in chickpea (*Cicer arietinum* L.) root tip cells. *Institute of Plant Sciences, University of Sindh, Jamshoro*, 16-20

Badr, A. (1986). Effects of the s-triazine herbicide turbutryn on mitosis, chromosomes and nucleic acids in root tips of Vicia faba. *Cytologia, 51*(3), 571-577. Retrieved from https://www.jstage.jst.go.jp/article/cytologia1929/51/3/51_3_571/_article/-char/ja/

Badr, A., El-Shazly, H. H., & Halawa, M. (2014). Cytological effects of gamma radiation and its impact on growth and yield of M1 and M2 Plants of Cowpea Cultivars. *Cytologia, 79*(2), 195-206. Retrieved from https://www.jstage.jst.go.jp/article/cytologia/79/2/79_195/_article/-char/ja/

Bhat, T. A., & Wani, A. A. (2017). *Chromosome structure and aberrations*: Springer. https://doi.org/10.1007/978-81-322-3673-3

Brahmi, I., Mabrouk, Y., Charaabi, K., Delavault, P., Simier, P., & Belhadj, O. (2014). Induced mutagenesis through gamma radiation in chickpea (*Cicer arietinum* L.): developmental changes and improved resistance to the parasitic weed *Orobanche foetida* Poir. *International Journal, 2*(11),

670-684. Retrieved from https://www.researchgate.net/profile/Yassine-Mabrouk-2/publication/271705368_

Chopra, V. (2005). Mutagenesis: Investigating the process and processing the outcome for crop improvement. *Current Science*, 353-359. Retrieved from https://www.jstor.org/stable/24110583

Cornforth, M., & Goodwin, E. (1991). Transmission of radiation-induced acentric chromosomal fragments to micronuclei in normal human fibroblasts. *Radiation Research, 126*(2), 210-217. Retrieved from https://meridian.allenpress.com/radiation-research/article-abstract/126/2/210/39242/Transmission-of-Radiation-Induced-Acentric

De Veylder, L., Joubès, J., & Inzé, D. (2003). Plant cell cycle transitions. *Current Opinion in Plant Biology, 6*(6), 536-543. Retrieved from De Veylder, L., Joubès, J., & Inzé, D. (2003). Plant cell cycle transitions. Current opinion in plant biology, 6(6), 536-543. https://doi.org/10.1016/j.pbi.2003.09.001

Dhanavel, D., Gnanamurthy, S., & Girija, M. (2012). Effect of gamma rays on induced chromosomal variation in cowpea *Vigna unguiculata* (L.) Walp. *International Journal of Current Science, 2012*, 245-250.

El-Azab, E. M., Ahmed Soliman, M., Soliman, E., & Badr, A. (2018). Cytogenetic impact of gamma irradiation and its effects on growth and yield of three soybean cultivars. *Egyptian Journal of Botany, 58*(3), 411-422. Retrieved from https://journals.ekb.eg/article_7900.html

Feher, A., Ötvös, K., Pasternak, T. P., & Pettkó-Szandtner, A. (2008). The involvement of reactive oxygen species (ROS) in the cell cycle activation (G0-to-G1 transition) of plant cells. *Plant Signaling & Behavior, 3*(10), 823-826. https://doi.org/10.4161/psb.3.10.5908

Gaafar, R. M., Hamouda, M., & Badr, A. (2016). Seed coat color, weight and eye pattern inheritance in gamma-rays induced cowpea M2-mutant line. *Journal of Genetic Engineering and Biotechnology, 14*(1), 61-68. Retrieved from https://www.sciencedirect.com/science/article/pii/S1687157X15000669

Girija, M., Gnanamurthy, S., & Dhanavel, D. (2013). Cytogenetics effect of gamma rays on root meristem cells of *Vigna unguiculata* (L.). *European Journal of Experimental Biology, 3*(2), 38-41. Retrieved from https://www.researchgate.net/profile/D-Dhanavel/publication/333260847_Cytogenetics_effect_of_gamma_rays_on_root_meristem_cells_of_Vigna_unguiculata_L/links/5ce4dd0d458515712eba7214/Cytogenetics-effect-of-gamma-rays-on-root-meristem-cells-of-Vigna-unguiculata-L.pdf

Gnanamurthy, S., Mariyammal, S., Dhanavel, D., & Bharathi, T. (2012). Effect of gamma rays on yield and yield components characters R3 generation in cowpea (*Vigna unguiculata* (L.). Walp.). *International Journal of Plant Science, 2*(2), 39-42.

Grant, W. F. (1978). Chromosome aberrations in plants as a monitoring system. *Environmental Health Perspectives, 27*, 37-43. Retrieved from https://ehp.niehs.nih.gov/doi/abs/10.1289/ehp.782737

Haleem, M. A. (2012). Pre-exposure to gamma rays alleviates the harmful effect of salinity on cowpea plants. *Journal of Stress Physiology & Biochemistry, 8*(4). Retrieved from https://cyberleninka.ru/article/n/pre-exposure-to-gamma-rays-alleviates-the-harmful-effect-of-salinity-on-cowpea-plants

Hartig, K., & Beck, E. (2006). Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle. *Plant Biology, 8*(03), 389-396. Retrieved from https://www.thieme-connect.com/products/ejournals/abstract/10.1055/s-2006-923797

Jimenez-Lopez, J. C., Singh, K. B., Clemente, A., Nelson, M. N., Ochatt, S., & Smith, P. (2020). Legumes for global food security. *Frontiers in Plant Science, 11*, 926. Retrieved from https://www.frontiersin.org/articles/10.3389/fpls.2020.00926/full

Joshi-Saha, A., Reddy, K. S., Petwal, V., & Dwivedi, J. (2015). Identification of novel mutants through electron beam and gamma irradiation in chickpea (*Cicer arietinum* L.). *Journal of Food Legumes, 28*(2), 1-6.

Jukanti, A. K., Gaur, P. M., Gowda, C., & Chibbar, R. N. (2012). Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition, 108*(S1), S11-S26. Retrieved from https://www.cambridge.org/core/journals/british-journal-of-nutrition/article/nutritional-quality-and-health-benefits-of-chickpea-cicer-arietinum-la-review/BCD8920297E987AAABBC12BFF90EB0CF

Kabeche, L., Nguyen, H. D., Buisson, R., & Zou, L. (2018). A mitosis-specific and R loop–driven ATR pathway promotes faithful chromosome segregation. *Science, 359*(6371), 108-114. Retrieved from https://www.science.org/doi/abs/10.1126/science.aan6490

Kamble, G., & Patil, A. (2014). Comparative mutagenicity of EMS and gamma radiation in wild chickpea. *International Journal of Science and Technology, 3*, 166-180. Retrieved from https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.677.576&rep=rep1&type=pdf

Katerji, N., Van Hoorn, J., Hamdy, A., Mastrorilli, M., & Oweis, T. (2005). Salt tolerance analysis of chickpea, faba bean and durum wheat varieties: I. Chickpea and faba bean. *Agricultural Water Management, 72*(3), 177-194. Retrieved from https://www.sciencedirect.com/science/article/pii/S037837740400229X?casa_token=oeBq20KUQHMAAAAA:j0l_JkGs5I8-sr6ALn-R6e4YCB3y6jhNtNkK_eBgJFftC4JM8Jk4BtDwP2sSrS-DuPuAKdijgsg

Kaydan, D., & Yagmur, M. (2008). Germination, seedling growth and relative water content of shoot in different seed sizes of triticale under osmotic stress of water and NaCI. *African Journal of Biotechnology, 7*.

Khan, H. A., Siddique, K. H., Munir, R., & Colmer, T. D. (2015). Salt sensitivity in chickpea: growth, photosynthesis, seed yield components and tissue ion regulation in contrasting genotypes. *Journal of Plant Physiology, 182*, 1-12. https://doi.org/10.1016/j.jplph.2015.05.002

Kim, J.-S., Lee, E.-K., Back, M.-H., Kim, D.-H., & Lee, Y.-B. (2000). Influence of low dose ${\gamma} $ radiation on the physiology of germinative seed of vegetable crops.

Korean Journal of Environmental Agriculture, 19(1), 58-61. Retrieved from https://www.koreascience.or.kr/article/JAKO200019756103350.page

Koteles, G. (1996). The human lymphocyte micronucleus assay. A review on its applicabilities in occupational and environmental medicine. *Central European Journal of Occupational and Environmenta Medicine, 2*, 12-30.

Köteles, G., Bojtor, I., Szirmai, S., Berces, J., & Otos, M. (1993). Micronucleus frequency in cultured lymphocytes of an urban population. *Mutation Research/Genetic Toxicology, 319*(4), 267-271. Retrieved from https://www.sciencedirect.com/science/article/abs/pii/0165121893900145

Kumar, S. (1998). Effect of gamma rays, EMS, DES on meiosis in *Lathyrus sativus*. *Journal of. Cytology, 33*, 139-147. Retrieved from https://ci.nii.ac.jp/naid/10019329359/

Ladizinsky, G., & Adler, A. (1976). The origin of chickpea *Cicer arietinum* L. *Euphytica, 25*(1), 211-217. Retrieved from https://link.springer.com/article/10.1007/BF00041547

Marques, E., Krieg, C. P., Dacosta-Calheiros, E., Bueno, E., Sessa, E., Penmetsa, R. V., & von Wettberg, E. (2020). The impact of domestication on aboveground and belowground trait responses to nitrogen fertilization in wild and cultivated genotypes of Chickpea (*Cicer* sp.). *Frontiers in Genetics, 11*. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7738563/

Melki, M., Mhamdi, M., & Achouri, A. (2011). Chickpea response to low doses of gamma radiation. *Russian Agricultural Sciences, 37*(4), 318-321. Retrieved from https://link.springer.com/article/10.3103/S1068367411040136

Melki, M., & Sallami, D. (2008). Studies the effects of low dose of gamma rays on the behaviour of chickpea under various conditions. *Pakistan Journal of Biological Sciences: PJBS, 11*(19), 2326-2330. Retrieved from https://europepmc.org/article/med/19137865

Moreno, M.-T., & Cubero, J. (1978). Variation in *Cicer arietinum* L. *Euphytica, 27*(2), 465-485. Retrieved from https://link.springer.com/article/10.1007/BF00043173

Munns, R., Day, D. A., Fricke, W., Watt, M., Arsova, B., Barkla, B. J., . . . Foster, K. J. (2020). Energy costs of salt tolerance in crop plants. *New Phytologist, 225*(3), 1072-1090. Retrieved from https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.15864

Munns, R., & Gilliham, M. (2015). Salinity tolerance of crops–what is the cost? *New Phytologist, 208*(3), 668-673. Retrieved from https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.13519

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology, 59*, 651-681. Retrieved from https://www.annualreviews.org/doi/abs/10.1146/annurev.arplant.59.032607.092911

Nazarenko, M., & Izhboldin, O. (2017). Chromosomal rearrangements caused by gamma-irradiation in winter wheat cells. *Biosystems Diversity, 25*(1). Retrieved from https://cyberleninka.ru/article/n/chromosomal-rearrangements-caused-by-gamma-irradiation-in-winter-wheat-cells

Piskadlo, E., Tavares, A., & Oliveira, R. A. (2017). Metaphase chromosome structure is dynamically maintained by condensin I-directed DNA (de) catenation. *Elife, 6*, e26120. Retrieved from https://elifesciences.org/articles/26120

Preuss, S. B., & Britt, A. B. (2003). A DNA-damage-induced cell cycle checkpoint in *Arabidopsis*. *Genetics, 164*(1), 323-334. https://doi.org/10.1093/genetics/164.1.323

Rao, D., Giller, K., Yeo, A., & Flowers, T. (2002). The effects of salinity and sodicity upon nodulation and nitrogen fixation in chickpea (*Cicer arietinum*). *Annals of Botany, 89*(5), 563-570. Retrieved from https://academic.oup.com/aob/article/89/5/563/205764?login=true

Sadiki, M., & Rabih, K. (2001). Selection of chickpea (*Cicer arietinum*) for yield and symbiotic nitrogen fixation ability under salt stress. *Agronomie, 21*(6-7), 659-666. Retrieved from https://hal.archives-ouvertes.fr/hal-00886149/

Scofield, S., Jones, A., & Murray, J. A. (2014). The plant cell cycle in context. *Journal of Experimental Botany, 65*(10), 2557-2562. https://doi.org/10.1093/jxb/eru188

Shah, T. M., Mirza, J. I., Haq, M. A., & Atta, B. M. (2008). Radio sensitivity of various chickpea genotypes in M1 generation I-Laboratory studies. *Pakistan Journal of Botany, 40*(2), 649-665. Retrieved from https://d1wqtxts1xzle7.cloudfront.net/34204731/PJB402649-with-cover-page-v2.pdf?Expires=1643712109&Signature=f5dIrLXLvrmC1FqJRo6dTeC2-ZmygN6JXGkcojuRo~dpFt7zlbRhX7Z9SsozJPKgbdWosm2taOcu-qOE-mkspI8m43bgeg7d-Ly2lJa8iR10-WXnTWGl2Ybgrop6-hFvb69ApyTNjRU7s-DXISUaYDB680jDHwveI0Vkwpg3DQJYBrtFnl9mw-3ajxo1th0WX8L8b6RRWivH70gMbbpQeUVjX~hrwvAJ1iakk4QkO4Hdad02gfxNGNEXs6mEaRMD-tNnMg50ad-dU8xRJ-K8GJjXyFgbxdesLuOO22eLcaze2oQkl-yZyBfu-1nae4IPj96T1Zoiy041pH7sMxCdPqLz3Q__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA

Shao, H.-B., Chu, L.-Y., Jaleel, C. A., & Zhao, C.-X. (2008). Water-deficit stress-induced anatomical changes in higher plants. *Comptes Rendus Biologies, 331*(3), 215-225. https://doi.org/10.1016/j.crvi.2008.01.002

Sharma, P., Jha, A. á., Dubey, R. S., & PessarakliM, R. O. S. (2012). Oxidative damage and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany, 2012*, 1-26. Retrieved from https://downloads.hindawi.com/archive/2012/217037.pdf

Singh, B. D. (2005). Mutations in crop improvement. In: Singh, B. D. (ed). *Plant Breeding, Principles and Methods. Kalyani Publishers, Ludhiana,* , pp. 698–731.

Sohrabi, Y., Heidari, G., & Esmailpoor, B. (2008). Effect of salinity on growth and yield of Desi and Kabuli chickpea cultivars. *Pakistan Journal of Biological Sciences: PJBS, 11*(4), 664-667. Retrieved from https://europepmc.org/article/med/18817146

Soliman, M., Elkelish, A., Souad, T., Alhaithloul, H., & Farooq, M. (2020). Brassinosteroid seed priming with nitrogen supplementation improves salt tolerance in soybean. *Physiology and Molecular Biology of Plants, 26*(3), 501-511. Retrieved from https://link.springer.com/article/10.1007/s12298-020-00765-7

Sun, Z., Li, H., Zhang, Y., Li, Z., Ke, H., Wu, L., . . . Ma, Z. (2018). Identification of SNPs and candidate genes associated with salt tolerance at the seedling stage in cotton (*Gossypium hirsutum* L.). *Frontiers in Plant Science, 9*, 1011. Retrieved from https://www.frontiersin.org/articles/10.3389/fpls.2018.01011/full

Tabur, S., Avci, Z. D., & Özmen, S. (2021). Exogenous salicylic acid application against mitodepressive and clastogenic effects induced by salt stress in barley apical meristems. *Biologia, 76*(1), 341-350. Retrieved from https://link.springer.com/article/10.2478/s11756-020-00573-0

Toker, C., Uzun, B., Canci, H., & Ceylan, F. O. (2005). Effects of gamma irradiation on the shoot length of *Cicer* seeds. *Radiation Physics and Chemistry, 73*(6), 365-367. Retrieved from https://www.sciencedirect.com/science/article/pii/S0969806X05000721?casa_token=yH7l1OTAdUAAAAA:kR_9_MCIoPSbud0WjplZ2Wz6ovsPEjNNS7NSt9CYiBjUVAd8iujrB92hNmZ8zfzSnxzZshRzcA

Tshilenge-Lukanda, L., Kalonji-Mbuyi, A., Nkongolo, K., & Kizungu, R. (2013). Effect of gamma irradiation on morpho-agronomic characteristics of groundnut (*Arachis hypogaea* L.). *American Journal of Plant Sciences, 4*(11), 2186. Retrieved from https://www.scirp.org/html/39582.html

Upadhyaya, H. D., Dwivedi, S. L., Baum, M., Varshney, R. K., Udupa, S. M., Gowda, C. L., . . . Singh, S. (2008). Genetic structure, diversity, and allelic richness in composite collection and reference set in chickpea (*Cicer arietinum* L.). *BMC Plant Biology, 8*(1), 1-12. Retrieved from https://bmcplantbiol.biomedcentral.com/articles/10.1186/1471-2229-8-106

Van der Maesen, L. J. G. (1972). *Cicer L., A Monograph of the Genus, with Special References to Chickpea (Cicer arietinum L.). Its ecology and cultivation*. Mededlingen landbouw hogeschool (Communication Agricultural University), Wageningen.

Wani, A. A. (2009). Mutagenic effectiveness and efficiency of gamma rays, ethyl methane sulphonate and their combination treatments in chickpea (*Cicer arietinum* L.). *Asian Journal of Plant Sciences, 8*(4), 318-321. https://doi.org/10.3923/ajps.2009.318.321

Waters, M. D., & Auletta, A. (1981). The GENE-TOX program: genetic activity evaluation. *Jornal of Chemical Information and Computer Science, 21*(1), 35-38. https://doi.org/10.1021/ci00029a007

doi:10.14720/aas.2022.118.2.1867 Original research article / izvirni znanstveni članek

Symbiotic and physiological indicators of soybean inoculated of *Bradyrhizobium japonicum* single-strain in 7 days before sowing

Nadiya VOROBEY [1], Kateryna KUKOL [1], Petro PUKHTAIEVYCH [1, 2], Tetyana KOTS [1,3]

Received September 09, 2020; accepted June 13, 2022.
Delo je prispelo 9. septembra 2020, sprejeto 13. junija 2022

Symbiotic and physiological indicators of soybean inoculated of *Bradyrhizobium japonicum* single-strain in 7 days before sowing

Abstract: Results of investigation of soybean of the Almaz variety in inoculation with preparations based on nodule bacteria *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 B78, B157, D37, D87 are presented. Different periods of the soybean seeds inoculation were used - on the sowing day (control) and in 7 days before sowing (experimental variants). The differences between control and experimental plants in the formation and functioning of the symbiotic apparatus and its functional activity, depending on the period between from seed inoculation to sowing were analysed. It was determined that the number of root nodules in the control plants was higher. The mass of nodules at the stage of 3 true leaves exceeded the control by 1.5–2.0 times in plants inoculated in 7 days before sowing, and the intensity of nitrogen fixation by 1.7–6.6 times. At the budding-beginning of flowering stage, the mass and intensity of N_2 fixation by the nodules of control plants increased. As a result, the difference between the nitrogen fixing activity of control and experimental plants decreased significantly. Stimulating effect on aboveground mass of *Bradyrhizobium japonicum* strains with increased nitrogen fixing activity was noted. Optimal conditions for the formation and functioning of bean-rhizobial symbiosis were provided at the use of both of these terms of soybean inoculation. This reveals the possibility of effective application of early inoculation of soybean seeds with preparations based on nodule bacteria *Bradyrhizobium japonicum* active strains.

Key words: rhizobia; *Bradyrhizobium japonicum*; bacterial preparations; pre-sowing inoculation; nitrogen fixing activity; soybean

Simbiontski in fiziološki indikatorji soje, inokulirane sedem dni pred setvijo s sevom bakterije *Bradyrhizobium japonicum*

Izvleček: Predstavljeni so rezultati raziskave inokulacije soje, sorte Almaz, s simbiontsko bakterijo *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan; sevi 1982 B78, B157, D37, D87). Inokulacija semen soje je bila izvedena na dan setve (kontrola) in sedem dni pred setvijo (različna obravnavanja v poskusu). Ugotovljene so bile razlike med kontrolo in različnimi obravnavanji v tvorbi in delovanju simbiontskega aparata glede na čas inokulacije. Ugotovljeno je bilo, da je bilo število koreninskih nodulov pri kontrolnih rastlinah večje. Masa nodulov je na razvojni stopnji soje tretjega pravega lista presegala kontrolo pri rastlinah inokuliranih sedem dni pred setvijo za 1,5–2,0 krat , vezava zračnega dušika pa za 1,7–6,6 krat. Na razvojni stopnji začetka cvetenja sta se masa nodulov in jakost vezave N_2 pri kontroli povečali, s čemer se je značilno zmanjšala razlika med kontrolo in obravnavanji. Opazen je bil tudi stimulacijski učinek inokulacije s sevi *Bradyrhizobium japonicum* na nadzemno biomaso soje zaradi povečane vezave dušika. Optimalne razmere za tvorbo in delovanje te rizobijske simbioze s sojo so se pojavile pri obeh načinih inokulacije. To nakazuje možnost učinkovite uporabe zgodnje inokulacije semen soje s pripravki aktivnih sevov bakterije *Bradyrhizobium japonicum.*

Ključne besede: rizobiji; *Bradyrhizobium japonicum*; bakterijski pripravki; predsetvena inokulacija; aktivnost vezave zračnega dušika; soja

1 Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kyiv, Ukraine
2 Corresponding author, e-mail: azotfixation@gmail.com
3 Institute of the Ukrainian Language, National Academy of Sciences of Ukraine, Kyiv, Ukraine

1 INTRODUCTION

The nodule bacteria *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 are the basis of biological microbial preparations for inoculation of soybean, that are characterized by multifunctional effect on plants. Their application increases resistance of plant to abiotic and biotic factors, the number and mass of root nodules, the intensity of symbiotic nitrogen fixation, the chlorophyll content in leaves, improves crop productivity and grain quality. This reduces the use of expensive nitrogen fertilizers, and, as a consequence, reduces the negative impact on the environment *(*Patyka & Petrychenko, *2004;* Morgun & Kots*, 2008; Kots, 2011; Kots et al., 2016;* Kramarev & Artemenko, 2016*).*

Increasing the sown areas of soybean testifies its important role in the agricultural complex (Berbenets, 2019). Usually pre-sowing seeds inoculation is carried out on the sowing day or in the day before, which provides a period of 24 hours from preparation application on the seeds to getting inoculated seeds into the soil. It is difficult for growers with large sown areas to treat and sow seeds in the soil in one day (because it takes a long time, related consumables, equipment preparation, and human resources, etc.). Pesticides with which are treated seeds to kill fungal and bacterial infections can also have a negative effect on nodulating bacteria *Bradyrhizobium* (soybean inoculants). All this complicates the pre-sowing treatment of seeds with biologicals in industrial conditions. Therefore, in recent years, the soybean cultivation technology has begun to use seeds with pre-treatment by plant protection products and preparations of nodule bacteria *Bradyrhizobium japonicum*, which are compatible with fungicides and insecticides. Thus, today there is a problem of providing highly effective inoculants for early inoculation of pulses seeds, in particular soybean. They got the name pre-inoculants.

In the segment of soybean inoculants on the Ukrainian market there is a wide range of trade names of preparations of domestic and foreign production (Kokorina & Kozhemyakov, 2010; Kots & Mamenko, 2015). According to the main criteria (type of microorganisms, their titer), the domestic inoculants do not differ from the foreign ones. However, the presence of protectors (preservative, adhesive) in some foreign preparations, increases the treatment manufacturability, simplifies the process of soybean sowing (Slobodyanyuk, 2017).

Pre-inoculants are used as a special element of soybean growing technology, which is in demand today. Pre-inoculant has advantages over conventional inoculants, even with small seed volumes. It contains an additional component that is able to form a protective film and protect bacteria on the surface of seeds from harmful environmental factors. It promotes additional seeds nutrition, provides better germination, increases germination energy and allows pre-treatment of seeds with inoculant.

Currently known pre-inoculants, compatible with the original soybean seed treatment and with the possibility of early application of the preparation before sowing seed in soil in 24 hours – RhizoFlo 5 (Saatbau Linz, Austria), in 1–2 days – Rhizobophyte (Ukraine), in 7 days – Biobacter (Lallemand, Uruguay), in 5–15 days – Rizoform + Static (Schelkovo Agrohim, Russia), in 21 days – Bioboost Plus (Liquid, Canada), in 45 days – HiCoat Super Extender (Agrocenter BASF, Germany) and in 90 days – HiCoat Super (Becker Underwood, USA), Agribacter, Agribacter + Rise (Lallemand, Uruguay) and other (Agritema, 2017).

Improving the nodule bacteria biologicals for soybean in the direction of increase the time between seeds inoculation and their sowing will significantly increase their effectiveness and attractiveness as a tool for obtaining biological nitrogen (Laktionov et al., 2018), because biological nitrogen fixation is an unalterable way to provide plants with nitrogen and does not violate the natural environment ecology (*Kots et al., 2016)*.

Domestic preparations are also known on the Ukrainian market, including Rizoline + Rizosave inoculants with recommendation of early treatment of seeds (7–10 days before sowing). When using a tank mix with Rizoline 2 l t^{-1} + Rizosave 2 l t^{-1} and a fungicide Fever 0.4 l t^{-1}, there were obtained soybean yields higher than control on 2.8–3.3 c ha^{-1} (Slobodyanyuk, 2017).

Microbial preparations with natural film-forming compounds are known, whereby nodule bacteria on seeds are protected from negative external influences and remain viable for a long time. For example film-former for seed inlay, created on the basis of waste plant and animal origin, opens the possibility of seed inoculation in 25–30 days before sowing, maintaining the nodulating activity of rhizobia and increased yields depending on the variety by 12 % (Grishechkin & Golovina, 2014).

Many Ukrainian farmers recognized the advantages of modern inoculation processing technologies. The additional harvests gave them the opportunity to recoup costs, and to make significant profits. The most effective will be the preparation that provides the highest concentration of live bacteria on the seeds at the time of their entry into the soil. Bacterial titers of preparations for soybean inoculation, which are widely available on the domestic market, range from 1×10^9 to 5×10^9 at the time of production and 2×10^8 – $(2–4) \times 10^9$ at the

end of the shelf life of the preparation. High-quality two-component preparation for soybean HiCoat®Super of the American company Becker Underwood has the highest of the *Bradyrhizobium* genus bacteria concentration (1×10^{10} cells per 1 g of preparation), and the longest among all known inoculants bacterial life on seeds. This allows seeds inoculation in 90 days before sowing and initiates nodules formation already in the initial stages of plant development.

In the development modern preparations of nodule bacteria for legumes and pulses at the first stage it is important to study the period of viable bacteria on seeds to sowing, virulence and nitrogen fixing activity (NFA) due to pre-inoculation of seeds. Regarding the research of this problem, there are few publications, and the results obtained are debatable. Martyniuk S. et al. (2002) observed a sharp drop in the number of bacteria *Rhizobium lupini* (Schroeter, 1886) Eckhardt et al., 1931 363a and 367a on inoculated lupine seeds Oligarch variety (creator – Leningrad Scientific Research Institute of Agriculture 'Belogorka', Russia), within 24–48 hours after inoculation. However, after added to inoculant of polymeric protector polyvinylpyrrolidone (PVP) at a concentration of 5 % even after 168 hours, sufficient bacteria quantity remained to form an effective symbiosis, possibly due to the protective properties of this polymer and its action as an "adhesive". That is, the use of PVP is potentially able to increase the allowable time between seeds inoculation and sowing up to six days.

The scientists point to several factors that affect the effectiveness of early seeds treatment by inoculants. So, the most important of them are the ability of bacteria to survive on seeds, seeds storage conditions, as well as the influence of other products (compounds) used in inoculation (Anghinoni et al., 2017). There are data in the literature on the treatment of soybean seeds with bulk peat preparations based on active strains of *Bradyrhizobium japonicum* SEMIA 5079 and SEMIA 5080 in 5 days before sowing and on the sowing day. The authors found that both methods of bacterization at the absence of the fungicides use provided the formation of nodules number at the level of control plants (Zilli et al., 2010). This indicated the ability of rhizobia to survive on the seeds of *Glycine max* L. (Merrill) for five days. Regarding the fixation of molecular nitrogen, these researchers did not find significant differences between the variants depending on the duration from inoculation of seeds to sowing.

Bacterial preparations based on active strains of nodule bacteria can be effective for pre-sowing treatment of soybean seeds without the use of extenders. Thus, the aim of our investigation was to study the effectiveness formations and functioning of the symbiotic apparatus, growth and development of soybean plants depending on the duration of the period from seeds inoculation with nodule bacteria *Bradyrhizobium japonicum* to sowing without excipients in microbial preparations.

2 MATERIALS AND METHODS

The experiments were performed with the soybean (*Glycine max* L. (Merrill) seeds of Almaz variety (originator – Poltava State Agrarian Academy, Ukraine), included in the Register of plant varieties of Ukraine since 2007 and recommended for cultivation in the forest-steppe of Ukraine (early ripening, high plasticity to climatic conditions).

Inoculation of seeds was carried out with a liquid-phase preparation made on the basis different in symbiotic activity of nodule bacteria B78, B157, D37, D87 strains (obtained as result of intergeneric conjugation between *Escherichia coli* S17-1 (pSUP5011::Tn5*mob*) and strains 646, 634b from the collection of N_2-fixing microorganisms of the Institute of Plant Physiology and Genetics NAS of Ukraine. Restoration of physiological activity of *Bradyrhizobium japonicum* nodule bacteria after storage in the museum collection was carried out by standard microbiological methods (Netrusov et al., 2005).

The nodule bacteria were grown in biological tubes on a nutrient medium yeast mannitol agar (YMA) (Netrusov, 2005) for 7 days at +28 °C for preparing a liquid-phase preparation. Thereafter, the biomass of bacteria were washed off from agar, and transfered in glass flasks with liquid YM environment (10 ml of suspension per 350 ml of YM) and cultured during 7 days at a temperature of 28 °C and constant aeration. The bacterial titer of the preparation, which were used for inoculation of soybean seeds was $4.1–4.5 \cdot 10^9$ CFU (colony forming units) per g of the preparation.

Soybean seeds were externally sterilized for 15 min with 70 % ethanol, washed with running water, inoculated for 1 h by prepared liquid microbial preparations and sowed in the substrate. Variants with inoculation of soybean seeds in 7 days before the day of sowing (experimental variants) and with inoculation of seeds in 1 hour before sowing (control) were included in the experimental scheme.

Soybeans were grown on a sandy substrate (10 kg washed river sand, 8 plants in each pot on) with the introduction of Hellriegel nutrient mixture with 0.25 of nitrogen norm (1 norm was 708 mg Ca $(NO_3)_2 \cdot 4$ H_2O per kg of sand) (Grodzinsky & Grodzinsky, 1964). River sand is the sand extracted from riverbeds, which

is characterized by a high degree of purification and the absence of foreign inclusions. The pots with plants were placed on a specially equipped site of the Institute of Plant Physiology and Genetics NAS of Ukraine under conditions at natural light, temperature and artificial controlled irrigation (Figure 1). Sowing seeds – 18.05.2018, the first seedling – 23.05.2018. Repeatability of the experimental variants was 5 times.

Selection of plants for the analysis was carried out in the stages: 3 true leaves (on the 28th day after germination), budding-beginning of flowering (on the 35 days after germination), full flowering soybeans (on the 48 days after germination).

The nodulating ability of *Bradyrhizobium japonicum* was determined by counting the number and mass of root nodules in 10 plants of each variant of the experiment. Biometric indices – the mass of the aboveground part of plants and roots – in 15 plants of each variant of the experiment. N_2-fixation activity was determined by acetylene method in terms of acetylene regeneration activity by root nodules of soybean (Hardy et. al., 1968) and expressed in μmol of ethylene, produced by nodules of 1 plant for 1 hour. The roots with nodules were placed in hermetically sealed glass vials with a capacity of 75 cm^3, 10 % of acetylene of the total volume was injected through the rubber membrane. Incubation period with acetylene - 1 hour. A gas mixture containing ethylene, formed as a result of acetylene reduction by nitrogenase, was analyzed on Agilent Technologics 6850 Network GC System (USA) gas chromatograph with flame ionization detector. Separation of gases was performed on a column Supelco Porapak N at thermostat temperature +55 °C and detector +150 °C. The gas carrier was nitrogen (50 ml per 1 min). Sampling capacity for analysis was 1 cm^3. Pure ethylene was used as the standard. The amount of ethylene formed from acetylene for 1 h under the action of nitrogenase incubated sample (the nitrogen fixing activity) was represented in molar units of ethylene formed per 1 plant for 1 hour – μmol C_2H_4 (plant h)$^{-1}$. Experiment with determine of N_2-fixation activity was repeated five times.

The statistical processing of the obtained data was conducted using ANOVA and the Tukey HSD Test with the average values. The results were presented in the form of mean values and standard error (m ± SE). The difference between the data was considered significant, if $p \leq 0.05$.

3 RESULTS AND DISCUSSION

The ability to penetrate in the legume root through

Figure 1: The pots with plants on a specially equipped site of the Institute of Plant Physiology and Genetics NAS of Ukraine

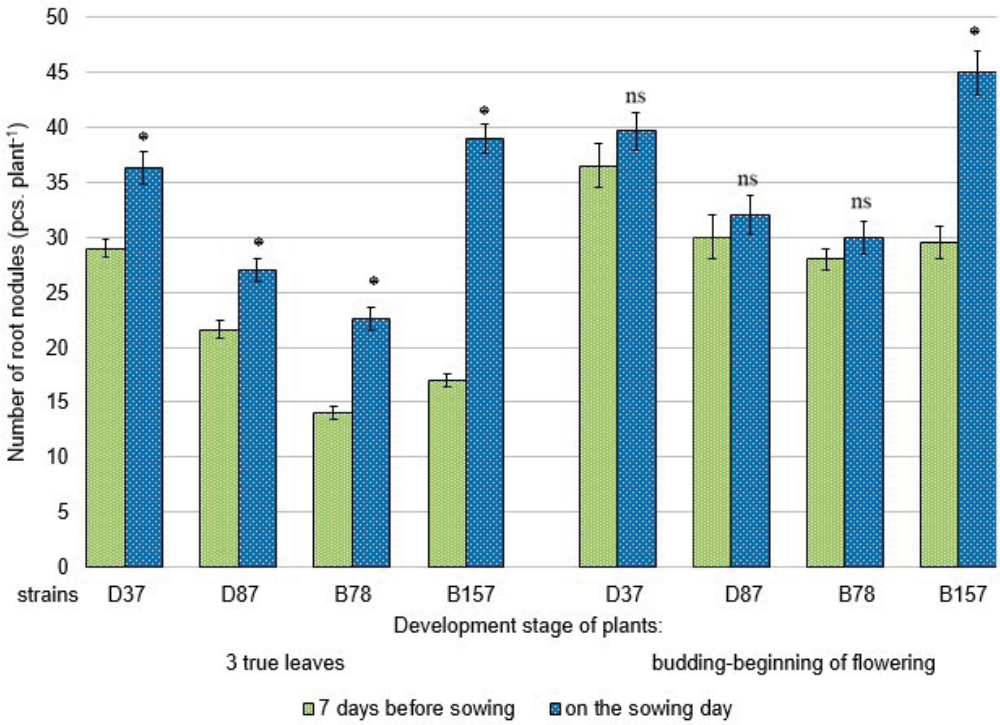

Figure 2: Root nodules number (pcs. plant^{-1}) in soybean Almaz variety depending on the period duration from seed treatment by strains of *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 D37, D87, B78, B157 to sowing (n = 10).
m ± SE. An asterisk (*) indicates statistically significant difference between treatments (paired columns) at $p \leq 0.05$; ns – no significant difference

root hairs, and in the process of complex morphophysiological changes to cause the formation of nodules indicates the virulence of nodule bacteria. Root nodules are the complex constructed organs of plants, the main structures of which are bacteria-infected tissue, where nitrogen is fixed, and the meristem.

We established that plants depending on the duration of the period from seeds inoculation to sowing and the inoculants on basis of *Bradyrhizobium japonicum* differed for number of root nodules formed. In the control plants (inoculated on the sowing day) formed more root nodules compared to the plants of the experimental variants (inoculation in 7 days before sowing). In particular, at stage of the 3 true leaves formation on soybean roots 22.6–39.0 nodules were counted. At the same time, in plants whose seeds were inoculation in 7 days before sowing, nodule number ranged from 14.0 to 29.0 per root (Figure 2). More intensive nodules formation was fixed in the budding-beginning of flowering period of soybean: 30.0–45.0 pieces per 1 root in variants with seeds inoculation in 1 hour before sowing and 28.0–36.5 pieces per 1 root in variants with seeds inoculation in 7 days before sowing.

Depending on the different time intervals between seed inoculation and sowing used in this study, no significant differences in the nodules location on the roots were observed. Symbiotic organs (nodules) formed mainly on the main root of soybean and branches of the first order at a depth of 1–17 cm and had a light pink color, indicating on the synthesis of leghemoglobin and nitrogen-fixing ability.

It should be noted the inoculating strains showed different virulence. When roots were infected by strain B78 the number of formed nodules was the lowest among the studied variants for both terms of preparations use. Probably due to the functional features of these rhizobia (reduced ability to survive on the seeds surface, less mobility and speed of penetration into the root meristem, etc.). Inoculation of soybeans with the preparation of nodule bacteria strain B157 was provided the largest number of root nodules in plants inoculated on the sowing day. In 7 days before sowing the largest nodules number was formed on the soybeans roots inoculated with nodule bacteria strain D37 (Figure 2).

It is known that after invasion of nodule bacteria

in the roots of plants is realized by the formation of bacteroids and the growth of meristem due to which the mass of the nodules increases (Spaink et al., 2002). At the stage of 3 true leaves, in plants which seeds were inoculated in 7 days before sowing mass of nodules was 1.2–2.0 times higher than that of the variants at the sowing day. At the budding-beginning of flowering stage, the difference in this index between the variants has been decreasing due to a more intensive increase in the mass of root nodules in variants with seed inoculation on the sowing day (Table 1).

Thus, in the variants with a prolonged period of 7 days from seeds inoculation to sowing, the nodule bacteria strains retained their functional activity, which

Table 1: Root nodules mass (mg plant^{-1}) in soybean Almaz variety depending on the period duration from seed treatment to sowing (n = 10)

	Development stage of plants:					
	3 true leaves			budding-beginning of flowering		
Inoculant strain	7 days before sowing	on the sowing day		7 days before sowing	on the sowing day	
D37	0.152 ± 0.006b	0.122 ± 0.010c	*	0.235 ± 0.012a	0.214 ± 0.013b	ns
D87	0.203 ± 0.005c	0.101 ± 0.006b	*	0.311 ± 0.016b	0.241 ± 0.020c	*
B78	0.097 ± 0.003a	0.063 ± 0.002a	*	0.220 ± 0.011a	0.143 ± 0.010a	*
B157	0.198 ± 0.016c	0.113 ± 0.008bc	*	0.305 ± 0.012b	0.292 ± 0.016d	ns

m ± SE, * – significant difference at $p \leq 0.05$; ns – no significant difference; interaction: a – not significant. For each strain and each variable, different letters a, b, c, d indicate significant differences

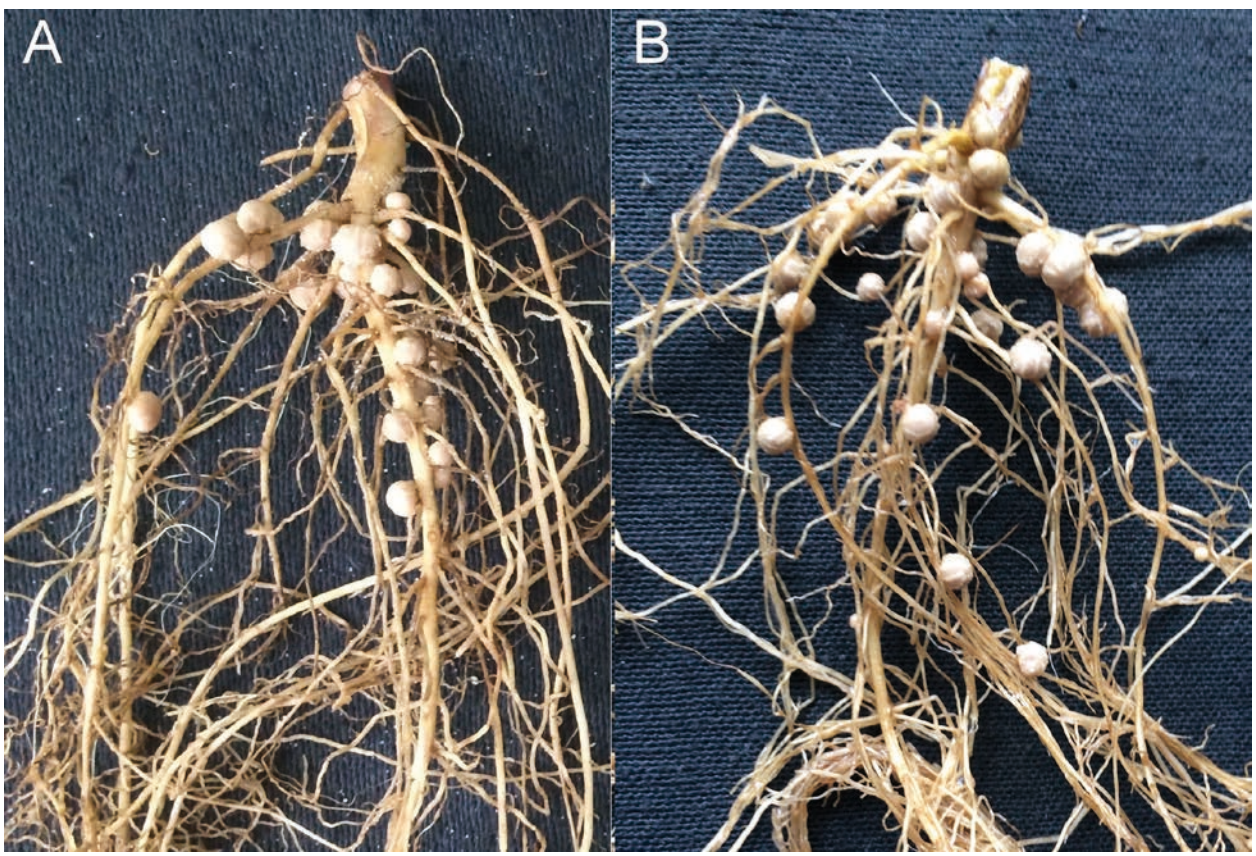

Figure 3: Nodules on soybean roots at seeds inoculation by the active strain *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 D87 at the budding-beginning of flowering stage, A – inoculation at the sowing day, B – inoculation in 7 days before sowing

was realized in the initial stages by active formation of root nodules (Figure 3).

Brazilian researchers, studying the effectiveness of early seeds inoculation of *Glycine max* L. (Merrill) in 5 and 10 days before sowing, using of chemical plant protection products, have established, that seeds treated with pesticides based on fludioxonil and thiamethoxane can be treated with bacterial preparations and stored for 10 days before sowing without negative impact on grain yield (Anghinoni et al., 2017). The same authors noted the influence of the duration from inoculation of soybean seeds to sowing in the soil on certain factors related to the nodulation. The issue of joint use of fungicides and inoculation requires the detailed study to ensure the effective formation and functioning of legume-rhizobial symbiosis and protection against phytopathogens of various etiologies. In model pot experiments in the Institute of Plant Physiology and Genetics NAS of Ukraine the effect of pesticides on the formation and functioning of the symbiotic apparatus of soybean plants was studied. A negative effect of a number of fungicides on the photosynthetic rate and nitrogen-fixing activity of soybean plants was studied. The strength of this effect depended on the preparation and the term of its use before sowing (Pavlyshche et al., 2017).

The time interval of 7 days between seeds inoculation and sowing is permissible in the case of preparations use based on mentioned active strains of nodule bacteria *Bradyrhizobium japonicum* with a high level of exopolysaccharides production. The latter can serve as natural substitutes for synthetic adhesive extenders, which are used in modern pre-inoculants. It is known that bacterial exopolysaccharides, forming a biofilm around rhizobial cells, provide their adsorption on the seed surface and protective function (Melnykova, 2019), thereby contributing to their preservation on seeds for a certain period of time and the restoration of physiological and symbiotic characteristics.

It is also actual studying the practical application of a wide spectrum water-soluble synthetic polymers as adhesive and film-formers as a part of biologicals for improvement of bacteria adhesion on a seed surface (by type of multicomponent formulations in the production of modern chemical treaters). Russian scientists have tested for this purpose low and high molecular mass sodium alginate (FMC polymer), hydroxypropyl methylcellulose (HPMC) (Colorcon, "Colorcon, Inc.", USA), polyethylene glycol (PEG), carbomer, polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP). Polymers are also capable prolonging the expiration date of microbial preparations, increase their compatibility with chemical plant protectors, resistance to ultraviolet radiation, temperature differences, drying, increase the survival of rhizobia on the seeds surface, which allows of pre-treatment. In the study of survival of 634b strain on the 'Belgorodska 7' (creator – Federal State Budgetary Educational Institution of Higher Education "V. Gorin Belgorod State Agricultural University", Russia) soybean variety seeds under the influence of polymers of different origin and composition it was shown that polyvinylpyrrolidone 10 % solution is the most effective of these compounds. Its use ensures the preservation of 10 times more number of viable rhizobia on the seeds in 10 days after inoculation compared to control (Laktionov et al., 2019).

The most important criterion for evaluating the effectiveness of symbiotic systems *Glycine max* – *Bradyrhizobium japonicum* is their molecular nitrogen fixation rate, which is based on the functioning of nodule bacteria enzyme nitrogenase and interrelated metabolic processes of symbiosis partners.

At the stage of 3 true leaves the nitrogen fixation of soybean root nodules was 1.7, 6.6, 4.5 and 1.8 times more intensive in plants which seeds were inoculated by D87, B78 and B157 strains in 7 days before sowing, compared with control plants (inoculation in 1 hour before sowing with similar preparations). The high level NFA of symbiotic systems when treating with rhizobia 7 days before sowing seems to be the result of a sufficient number of microbial cells for become infected after this period. Researchers have found that the viability of nodule bacteria on seeds without the use of pesticides depends on the plant species and the biological qualities of microorganisms (Gemell et al., 2005), as well as the duration and storage conditions of inoculated seeds (Deaker et al., 2012).

Pre-inoculation of soybean seeds (in 7 days before sowing) by preparation based on D87 strain provided the highest level of N_2 assimilation due to the formation of the largest nodules mass on the roots of plants (Figure 4).

At the budding-beginning of flowering stage the nitrogen fixation activity of soybean root nodules has increased in variants with seeds inoculation on the sowing day. As a result, the difference between the NFA indicators of control (inoculation at the sowing day) and experimental plants (inoculation in 7 days before sowing) has decreased and gradually to equalize. The identified features of the formation and functioning of the symbiotic apparatus of soybeans, formed due to the use of different terms between inoculation and sowing, indicate a significant role of adaptive properties of the microsymbiont during prolonged stay on the seeds before sowing and in the process of the formation of bean-rhizobial symbiosis. Therefore, the produce of

microbial preparations for pre-inoculation of seeds requires proper selection of strains of *Bradyrhizobium japonicum* taking into account their adaptive and physiological characteristics.

Thus, as a result of the application of mentioned time periods from seed inoculation to sowing, a symbiotic apparatus was formed on soybean roots, the nitrogen fixation rate of which changed during the growing season, that affected the plant supply by biological nitrogen.

Intense assimilation of N_2 by root nodules provokes in plants a growing demand for photoassimilates and causes their redistribution (Kirizii et al., 2007). The regulatory role of nitrogen fixation in plant metabolism can stimulate or slow the growth of aboveground mass and rhizogenesis. At the stage of 3 true leaves, the aboveground and the root mass of inoculated plants (on the sowing day) outweighed the corresponding indices of plants bacterized in 7 days before sowing. During the soybean growing season, the difference in root mass between plants of control and experimental variants decreased and was not significant at the budding-beginning of flowering stage (Table 2). The root system of plants in all variants was well developed, with a large number of lateral roots, which provided an increase in the soybean nutrition area surface.

Plant mass is one of the indices that characterizes the conditions of growth and development in different stages of the growing season. An actively functioning symbiotic apparatus is a more powerful sink of assimilates compared to vegetative growth. Therefore the photosynthetic apparatus is not always fully able to provide the needs of all growth meristems in assimilates when the balance between growth, photosynthesis and nitrogen fixation is disturbed.

In the early period of functioning of the bean-rhizobial symbiotic system of soybean (3 true leaves stage) in control and experimental plants there was a positive relationship between the intensity of nitrogen-fixing activity and aboveground mass growth (Figure 4; Table 3).

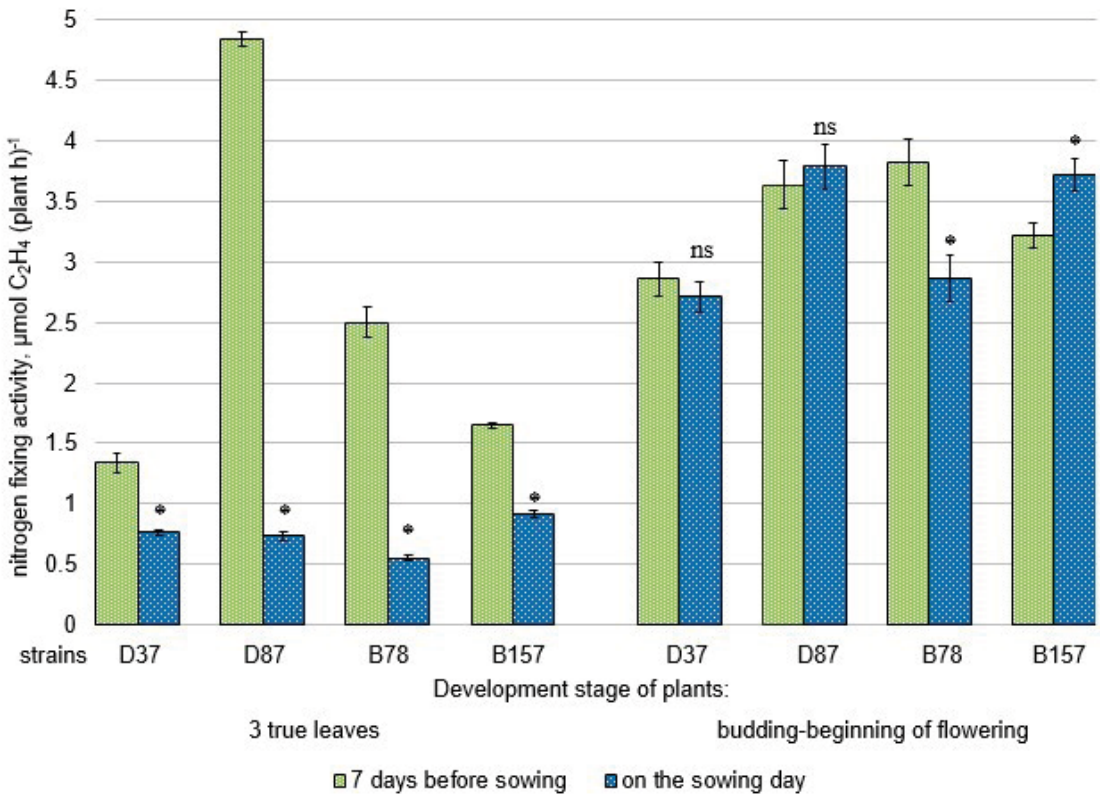

Figure 4: The nitrogen fixing activity, µmol C_2H_4 (plant h)$^{-1}$, of root nodules of the Almaz soybean variety plants depending on the period duration from seed treatment by *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 D37, D87, B78, B157 strains to sowing (n = 5).
m ± SE. An asterisk (*) indicates statistically significant difference between treatments (paired columns) at $p \leq 0.05$; ns – no significant difference

Table 2: The root mass (g plant^{-1}) of the Almaz soybean variety, under inoculation with nodule bacteria *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 (n = 15)

	Development stage of plants:					
	3 true leaves			budding-beginning of flowering		
Inoculant strain	7 days before sowing	on the sowing day		7 days before sowing	on the sowing day	
D37	2.09 ± 0.08a	2.30 ± 0.09a	*	3.34 ± 0.16a	3.08 ± 0.17a	ns
D87	2.04 ± 0.08a	2.28 ± 0.07a	*	3.33 ± 0.17a	3.45 ± 0.11a	ns
B78	2.35 ± 0.09b	2.50 ± 0.07ab	ns	3.19 ± 0.15a	2.95 ± 0.11a	ns
B157	2.08 ± 0.08a	2.31 ± 0.08a	*	3.14 ± 0.12a	2.86 ± 0.12a	ns

m ± SE, * – significant difference at $p \leq 0.05$; ns – no significant difference; interaction: a – not significant. For each strain and each variable, different letters a, b indicate significant differences

Table 3: The aboveground mass (g plant^{-1}) of the Almaz soybean variety, under inoculation with nodule bacteria *Bradyrhizobium japonicum* (Kirchner, 1896), Jordan, 1982 (n = 15))

	Development stage of plants:								
	3 true leaves			budding-beginning of flowering			full flowering		
Inoculant strain	7 days before sowing	on the sowing day		7 days before sowing	on the sowing day		7 days before sowing	on the sowing day	
D37	2.57a ± 0.12	2.50a ± 0.11	ns	3.86a ± 0.23	4.23a ± 0.13	ns	6.75a ± 0.28	6.82a ± 0.32	ns
D87	2.84a ± 0.18	2.73ab ± 0.11	ns	4.06a ± 0.28	4.60a ± 0.32	ns	7.62a ± 0.38	7.21a ± 0.30	ns
B78	2.87a ± 0.13	2.64ab ± 0.13	ns	3.77a ± 0.12	4.08a ± 0.30	ns	6.97a ± 0.32	7.18a ± 0.31	ns
B157	2.75a ± 0.13	2.93b ± 0.14	ns	3.70a ± 0.24	4.24a ± 0.32	ns	7.58a ± 0.23	7.23a ± 0.27	ns

m ± SE, * – significant difference at $p \leq 0.05$; ns – no significant difference; interaction: a – not significant. For each strain and each variable, different letters a, b indicate significant differences

Then, before the budding-beginning of flowering stage in control plants, the growth in aboveground mass accelerated and outpaced the growth of plants bacterized in 7 days before sowing with strains D37, D87, B78 and B157 by 9.6, 13.3, 8, 2 and 14.5 % respectively. There was an intensive increase in the vegetative mass of plants in all variants of the experiment from the budding stage to the full flowering stage. During the full flowering stage, which is related to the redistribution of assimilates and the formation of generative organs, the indicators of aboveground mass of control and experimental plants, taking into account the error of the experiment, also did not differ significantly. Thus, when applying seed bacterization on the sowing day, this indicator was in the range of 6.82–7.23 g plant^{-1} and with seed inoculation in 7 days before sowing – 6.75–7.58 g plant^{-1}. The dynamics of the aboveground mass formation of control and experimental plants was similar during the growing season. Optimal conditions for the formation and functioning of legume-rhizobial symbiosis for plants were provided using both of period of soybean inoculation.

In the vegetation experiment soybean were grown on a river sandy substrate with the introduction of Hellriegel nutrient mixture with 0.25 of nitrogen norm. It is probable that mineral nitrogen was used in the earlier stages of plant growth and development, and during the soybean flowering period the nutrition was mainly due to biologically N_2 (due to the functioning of the symbiotic apparatus of plants). Therefore, the stimulation of vegetative growth was more active in plants inoculated with preparations of nodule bacteria D87, B78 and B157 strains with increased nitrogen fixation intensity.

4 CONCLUSIONS

Thus, our studies have shown that inoculation of soybean seeds with microbial preparations based on

Bradyrhizobium japonicum on the sowing day caused the formation of more nodules on the plant roots. However, the mass of the formed nodules and the intensity of nitrogen fixation significantly dominated in plants inoculated in 7 days before sowing the seeds in the stage of the 3 true leaves only. The increase in the intensity of nitrogen fixation in control plants in the budding-beginning stage of flowering caused to the equalization of the difference of nitrogen fixation activity between the variants with different terms between inoculation and sowing. This allows the effective use of bacterial preparations based on active strains D37, B78, D87, B157 for pre-sowing treatment of soybean seeds (in 7 days before sowing) without the use of extenders. In the future, it is advisable to study the ability to nodulate and assimilate N_2 active strains of nodule bacteria (and preparations based on them without the use of extender) under conditions of longer delay of seeds sowing from their inoculation. The results obtained are important for elucidation of the possibility of introduction of nodule bacteria active strains obtained by biotechnological methods as a bacterial basis of preparations for pre-inoculation of soybean.

5 REFERENCES

Agritema. Catalog of products. (2017). Retrieved from https://agritema.com/wp-content/uploads/2017/09/AGRITEMA-Catalog_UA.pdf

Anghinoni, F. B. G., Braccini, A. L., Scapim, C. A., Anghinoni, G., Ferri, G. C., Suzukawa, A. K. & Tonin, T. A. (2017). Pre-inoculation with *Bradyrhizobium* spp. in industrially treated soybean seeds. *Agricultural Science, 8*(7), 582–590. https://doi.org/10.4236/as.2017.87044

Berbenets, O. V. (2019). World-wide production of soya as an inexhaustible source of vegetable proteins and Ukraine's place in the global trading market. *Agrosvit*, *10*, 41–45. https://www.doi.org/10.32702/2306-6792.2019.10.41

Deaker, R., Hartley, E., & Gemell, G. (2012). Conditions affecting shelf-life of inoculated legume seed. *Agriculture*, *2*(1), 38–51. https://doi.org/10.3390/agriculture2010038

Gemell, L. G., Hartley, E. J., & Herridge, D. F. (2005). Point-of-sale evaluation of preinoculated and custom-inoculated pasture legume seed. *Australian Journal of Experimental Agriculture*, *45*(3), 161–169. https://doi.org/10.1071/EA03151

Grishechkin, V. V. & Golovina, E. V. (2014). Use of new organic film-former (PPO) for conservation of viability of rhizobia at inoculation of seeds of soya and their influence on formation of nodules and productivity. *Legumes and Groat Crops*, *1*(9), 41–44.

Grodzinsky, A. M. & Grodzinsky, D. M. (1964). *Short reference book on plant physiology*, Kyiv: Nauk. Dumka.

Hardy, R. W. F., Holsten, R. D., Jackson, E. K. & Burns, R. C. (1968). The acetylene – ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology, 42*(8), 1185–1207. https://doi.org/10.1104/pp.43.8.1185

Kirizii, D. A., Vorobei, N. A. & Kots, S. Ya. (2007). Relationships between nitrogen fixation and photosynthesis as the main components of the productivity in alfalfa. *Russian Journal of Plant Physiology*. *54*(5), 666–671. https://doi.org/10.1134/S1021443707050032

Kokorina, A. L. & Kozhemyakov, A. P. (2010). *The Rhizobium-legume symbiosis and the use of microbiological preparations of complex action are an important reserve for increasing the productivity of arable land.* Saint Petersburg: All-Russia Research Institute for Agricultural Microbiology.

Kots, S & Mamenko, P. (2015). Soybean inoculation and incrustation: a review of application technology and market of prerarats. *Proposition. Special issue. Modern agrotechnologies for the use of biological products and growth regulators,* 24-28.

Kots, S. Ya. (2011). Current state of biological nitrogen fixation studies. *Physiology and Biochemistry of Cultivated Plants*, *43*(3), 212–225.

Kots, S. Ya., Vorobey, N. A., Kyrychenko, O. V., Melnykova, N. N., Mykhalkiv, L. M. & Pukhtaievych, P. P. (2016). *Microbiological Preparations for Agriculture*, Kyiv: Logos.

Kramarev, S. M. & Artemenko, S. F. (2016). The productivity of maize in crop short rotations with soybeans in the conditions of northern Steppe of Ukraine. *News of Dnipropetrovsk State Agrarian and Economic University*, *42*(4), 68–71.

Laktionov, Yu. V., Kosulnikov, Y. V., Dudnicova, D. V., Yahno, V. V. & Kojemyakov, A. P. (2019). Pre-sowing protection of inoculated soybean *Glycine max* (L.) Merr. seeds by water-soluble polymer compositions and their solid-phase modification. *Agricultural Biology*, *54*(5), 1052–1059 https://doi.org/10.15389/agrobiology.2019.5.1052eng

Laktionov, Yu. V., Kosulnikov, Yu. V. & Dudnikova, D. V. (2018). The effect of water-soluble polymers on the survival of nodule lupine bacteria (*Rhizobium lupini*). *Grain Economy of Russia*, *3*(57), 22–26. https://doi.org/10.31367/2079-8725-2018-57-3-22-26

Martyniuk, S., Oron, J., Martyniuk, M. & Wozniakowska, A. (2002). Effects of interactions between chemical seed dressings and *Bradyrhizobium japonicum* on soybean seeds. *Archives of Agronomy and Soil Science*, *48*(4), 305–310. https://doi.org/10.1080/03650340214202

Melnykova, N. M. (2019). Effect of rhizobial exopolysaccharides on soybean seed germination and nodule development in the soybean-rhizobia symbiosis. *Plant Physiology and Genetics*, *51*(5), 436–446. https://doi.org/10.15407/frg2019.05.436

Morgun, V. V. & Kots, S. Ya. (2008). Symbiotic nitrogen fixation and its significance in nitrogen plant nutrition: research status and prospects. *Physiology and Biochemistry of Cultivated Plants*, *40*(3), 187–205.

Netrusov, A. I., Yegorova, M. A., Zakharchuk, L. M., Kolotilova, N. N., Kotova, I. B., Semenova, E. V., … Judina, T. G. (2005). *Workshop on Microbiology*. Moscow: Akademiya Publ.

Patyka, V. P. & Petrychenko, V. F. (2004). Microbial nitrogen fixation in modern fodder production. *Feeds and Feed Production*, *53*, 3–6.

Pavlyshche, A. V., Kiriziy, D. A. & Kots, S. Ya. (2017). The re-

action of symbiotic soybean systems to the action of fungicides under various treatment. *Plant Physiology and Genetics*, *49*(3), 237–247. https://doi.org/10.15407/frg2017.03.237

Slobodyanyuk, O. (2017). The nodules for soybean. Rizoline is a new answer for an old question. *Agri Business Today*, *347*(5), 64–65.

Spaink, H., Kondorosi, A & Hooykaas, P. (2002). *The Rhizobiaceae Molecular Biology of Model Plant – Assosiated Bacteria*. Translated in rus. by Tikhonovich, I. A. & Provorov, N. A. St. Petersburg: Biont.

Zilli, J. E., Campo, R. J. & Hungria, M. (2010). Effectiveness of *Bradyrhizobium* inoculation at pre-sowing of soybean. *Pesquisa Agropecuaria Brasileira*, *45*(3), 335–337. https://doi.org/10.1590/S0100-204X2010000300015

doi:10.14720/aas.2022.118.2.2402 Review article / pregledni znanstveni članek

The usage of beneficial insects as a biological control measure in large-scale farming - a case study review on *Trichogramma* spp.

Aleksandar IVEZIĆ [1, 2], Branislav TRUDIĆ [3], Gordon DRAŠKIĆ [4]

Received November 02, 2021; accepted May 27, 2022.
Delo je prispelo 2. novembra 2021, sprejeto 27. maja 2022

The usage of beneficial insects as a biological control measure in large-scale farming - a case study review on *Trichogramma* spp.

Abstract: Large scale crops like maize, soybean, wheat and rice have changed the ecosystems worldwide, causing a major impact on global agricultural diversity. Intensive farming includes wide range of synthetic substances which are very often applied irrationally and excessively. Given the prevalence of large-scale farming in world agriculture, it is necessary to begin the transition from conventional crop protection to integrated pest management (IPM) in these agroecosystems. One of the most important components of IPM are biological control measures with augmentative release of commercially available species of the genus *Trichogramma* Westwood, 1833 (Hymentoptera: Trichogrammatidae) as potentially successful and environmentally friendly methods. Besides *Trichogramma,* many other beneficial organisms are constantly being tested as potential biocontrol agents such as *Chrysopa* spp. (Neuroptera: Chrysopidae) and *Orius* spp. (Hemiptera: Anthocoridae). Minimizing the use of chemicals and replacing them with biological plant protection is fully in line with the agriculture development strategy and confirmed to be achievable in practice. It is especially important to apply such tactical decisions in the production of large-scale crops, which, at the same time, represent the biggest polluters of the environment as well.

Key words: beneficial insects; biological control; Trichogramma spp.; large-scale crops; IPM

Uporaba koristnih žuželk kot merilo biotičnega varstva pri kmetovanju na velikih zemljiščih - pregledna raziskava na primeru parazitoidnih os iz rodu *Trichogramma*

Izvleček: Poljščine, kot so koruza, soja, pšenica in riž, ki se gojijo na velikih obdelovalnih zemljiščih, so globalno spremenile ekosisteme in imajo globalno največji vpliv na raznolikost v kmetijstvu. Intenzivno kmetijstvo uporablja širok spekter sintetičnih snovi, ki so pogosto uporabljene neracionalno in v prevelikem obsegu. Zaradi prevladovanja kmetovanja na velikih zemljiščih v svetovnem merilu je potrebno začeti s prehodom iz konvencionalnega varstva kmetijskih rastlin na integrirano zatiranje škodljivih organizmov (IPM) v agroekosistemih. Med najpomembnejšimi komponentami integriranega varstva rastlin so ukrepi biotičnega zatiranja škodljivcev s sproščanjem komercialno dostopnih vrst parazitoidnih os iz rodu *Trichogramma* Westwood, 1833 (Hymentoptera: Trichogrammatidae) kot potencialno učinkovitih in okolju prijaznih metod. Poleg vrst iz rodu *Trichogramma* se v biotičnem varstvu stalno preiskušajo mnogi drugi koristni organizmi, kot so tenčicarice (*Chrysopa* spp., Neuroptera: Chrysopidae) in plenilske stenice iz rodu *Orius* (Hemiptera: Anthocoridae). Zmanjševanje uporabe kemikalij in njihovo nadomeščanje z biotičnim varstvom rastlin je popolnoma v skladu z razvojno strategijo kmetijstva in je potrjeno lahko doseženo v praksi. Še posebej je pomembno uporabiti te metode v velikopovršinski pridelavi kmetijskih rastlin, ki hkrati predstavlja tudi enega izmed največjih onesnaževalcev okolja.

Ključne besede: koristne žuželke; biotični nadzor; *Trichogramma* spp.**;** veliko površinsko gojene kmetijske rastline; IPM

1 Department of Forecasting and Warning Service in Plant Protection, Agricultural extension service of Kikinda, Serbia
2 Corresponding author, e-mail: aleksandarivezic@yahoo.com
3 Forest Biodiversity and Restoration Team, Forestry Division, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, Rome, Italy
4 Persida Inc., A Specialized Software Solutions Provider, Brooklyn, NY, United States

1 INTRODUCTION

A combination of high commodity crop prices, rising global food demand and technological advances, has transformed the scale of global crop production (Hochman et al., 2014). Certain crops are becoming more prevalent taking into consideration monoculture mainstreaming in agricultural production globally. These large-scale crops which occupy most of the global agricultural area, have already transformed the land use dynamics and changed the ecosystems worldwide, and are still causing major impact on global agricultural diversity. Wheat (*Triticum* spp.), rice (*Oryza* spp.), corn (*Zea* spp.) and soybean (*Glycine* spp.) are prime examples. These four crops alone occupy approximately 50 % of the world's entire agricultural lands, while the remaining crops cover the rest (Ben Ari & Makowski, 2016).

Large scale farming (or intensive farming) is generally defined as highly mechanized and commercialized cropping activities with much greater use of external inputs (Tittonell et al., 2020). It also includes wide range of synthetic substances (fertilizers, fungicides, insecticides, and herbicides). These agrochemicals, used to prevent crop of diseases, manage the weeds and pests and boost plant growth, are very often applied irrationally and excessively. In such conditions side effects often occur. Inadequate use of pesticides can lead to pests' resistance, excessive pesticide residues in food, pollution of agroecosystems and suppression of beneficial organisms. Among all, these chemical agents are toxic for both wildlife and humans. Additionally, agrochemicals are often associated with reduction of populations of birds, amphibians and insects (bees, butterflies) by destroying their food source, contaminating soil and ground waters (Feshchenko, 2019).

As the volume and production of agriculture continue to grow to new global records, so does the environmental awareness of societies. There is an increasingly pronounced demand for production of high-quality food without pesticide residues and other toxic substances. There is also an ecological issue underlining the use of renewable energy sources and preservation of natural resources and environment. Modern trend of sustainable agricultural production imposes the need to change technological process of production with the application of techniques that pollute the environment less and contribute to the health security in general. Numerous studies and social initiatives are calling for conversion to more sustainable agricultural practices due to their favorable effect on ecosystems, biodiversity and human health (Siebrecht, 2020).

Given the prevalence of large-scale farming in world agriculture, it is necessary to begin the transition from conventional crop protection to integrated pest management (hereinafter: IPM). IPM is based on extremely controlled and justified use of chemical agents with the emphasis on the use of alternative ways for pest control, like biological control measures.

1.1 BIOLOGICAL CONTROL MEASURES

One of the most important components of IPM are biological control measures. Biological control or biocontrol is defined as a set of methods significant for pest control (insects, mites, weeds and plant diseases etc.) using their natural enemies. It relies on predation, parasitism, herbivory, or other natural mechanism, but also involves an active human influence (Flint & Dreistadt, 1998). Since the existing form of natural balance between pests and beneficial organisms is usually insufficient to achieve expected results in intensive farming, biological control requires the manipulation of beneficial insects by people to reduce the population of agricultural pests (Raspudić et al., 1999). In a strict ecological sense, applied biological control can be considered as a strategy to restore functional biodiversity in agroecosystems by adding missing entomophagous insects through classical and/or augmentative biocontrol techniques, but it can also be considered as a way of enhancing naturally occurring predators and parasitoids through conservation and habitat management (Altieri, 1994). Biological control is a self-sustaining strategy through which farmers rely on pest control through ecological services provided by restored functional biodiversity, thus avoiding dependence on costly pesticides (Polanczyk & Pratissoli, 2009).

The aim of this paper is to emphasize wide possibility of implementation of beneficial insects in large crop farming, to point out their great potential in agricultural practice and to discuss their high diversity worldwide.

1.2 TRADITIONAL KNOWLEDGE AND HISTORY OF BIOLOGICAL CONTROL

The use of natural enemies to reduce the impact of pests has a long history. There are antecedent historical events that trace the evolution of some of the fundamental concepts in the development of biological control, and several of these events show the remarkable and perceptive insight of man into the workings of nature (Den Bosch et al., 1982). The first description of use of biological control dates from around 300 AD, when

predatory ants were used for control of pests in citrus orchards in China, a method which is still used today in Asia. In the 1750s, the British and French transported mynah birds from India to Mauritius to control locusts. Early applied biological control programs began under the USDA's Department and later Bureau of Entomology established in 1881 (Polanczyk & Pratissoli, 2009).

The first introduction of an exotic braconid wasp parasite, *Cotesia glomerata* (Linnaeus, 1758) (Hymenoptera: Braconidae), against the imported cabbageworm, *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae), into the United States occurred in 1883 and the introduction of the famous predaceous vedalia beetle *Rodolia cardinalis* (Mulsant, 1850) (Coleoptera: Coccinellidae) to control the cottony cushion scale *Icerya purchasi* Maskell, 1878 (Hemiptera: Margarodidae), followed in 1888 (Clausen 1978). The Department's first large-scale biological control program did not begin until 1905 and involved explorations in Europe and Japan for natural enemies of the gypsy moth *Lymantria díspar* Linnaeus, 1758 (Lepidoptera: Lymantriidae), and browntail moth, *Euproctis chrysorrhoea* Linnaeus, 1758 (Lepidoptera: Lymantriidae), introduced into New England (Vail et al., 2001). It must be pointed out that from 1930 to 1940 there was a peak in biological control activity in the world with 57 different natural enemies established at various places, but World War II caused a sharp drop in biological control activity, and it did not regain popularity after war due to production of relatively inexpensive synthetic organic insecticides (Polanczyk & Pratissoli, 2009).

Today, there are hundreds of biological control products commercially available for pest control, but not all are sufficiently effective for large-scale farming. One of the potentially most successful and environmentally friendly methods for biological control of pests is the augmentative release of commercially available species of the genus *Trichogramma* Westwood, 1833 (Hymentoptera: Trichogrammatidae) (Stouthamer, 1993). These organisms have been identified and successfully used for inundative biological control of lepidopteran pests for more than 120 years (Smith, 1996; Van Lenteren, 2000). *Trichogramma* are egg parasitoids that attack more than 200 lepidopteran host species, including pest groups of borers, webworms, loopers, leafworms, fruitworms, cutworms, bollworms and armyworms (Knutson, 1998).

More than a thousand scientific papers have been published on *Trichogramma* and its usage as a biological control agent, making it one of the most researched natural enemies in the world. As a result, they have been widely used in inundative and inoculative biological control programs in more than 30 countries in agricultural crops (e.g. corn, cotton, sugarcane, rice, soybean, fruit trees, vegetables) and natural forests (Knutson, 1998).

1.3 Trichogramma spp. LIFE CYCLE

Trichogramma wasps primarily parasitize eggs of moths and butterflies (Lepidoptera). However, certain species of *Trichogramma* also parasitize eggs of beetles (Coleoptera), flies (Diptera), true bugs (Heteroptera), other wasps (Hymenoptera), lacewings and their relatives (Neuroptera) (Knutson, 1998). The adult female wasp uses chemical and visual clues to locate a host egg (Nordlund et al., 1981). Once a female finds a host egg, she drills a hole through the chorion (eggshell) and inserts two to three eggs into the host egg. Eggs of parasitoid hatch in about 24 hours and the parasite larvae develop very quickly. Larvae develop through three instars. During the third instar, dark melanin granules are deposited on the surface of the egg chorion, causing the host egg to turn black. Larvae than transform to the inactive pupal stage. After about 4-5 days, the adult wasps emerge from the pupae and escape the host egg by chewing a circular hole in the eggshell. The black layer inside the chorion and the exit hole are evidence of parasitism by *Trichogramma* (Ruberson et al., 1993).

2 Trichogramma IN MAIZE

Maize (*Zea* spp.) is one of the most abundantly produced cereal in the world. It is grown in every continent except Antarctica (Eckhoff et al., 2003). This large-scale crop has many pests and the European corn borer *Ostrinia nubilalis* Hubner, 1796 (Lepidoptera: Crambidae) is considered the major one worldwide (Mutuura & Monroe, 1970). The egg stages of many corn pests including *O. nubilalis*, are attacked by various species of *Trichogramma* (Ivezić & Trudić, 2021). More than 15 species and strains of native and exotic *Trichogramma* were evaluated in field and laboratory tests to determine those with a high preference for *O. nubilalis* egg clusters or other lepidopteran corn pests (Wang et al., 1999). Several species of *Trichogramma* have been identified as promising biological control agents of *O. nubilalis*, including *T. brassicae* Bezdenko, 1968 and *T. evanescens* Westwood, 1833 (Bigler, 1986; Hassan, 1993). Both species appear to be widespread across Europe and reared in commercial facilities for release as bioagents. In South Europe, these two *Trichogramma* species are considered to be the most abundant *Trichogramma* species in maize (Bohinc et al., 2015; Ivezić et al., 2021).

In order to ensure production of effective parasites, mass rearing facilities developed rearing techniques with stringent quality control procedures. This requires controlled environments, artificial diets and ovipositional substrates, mechanized equipment and operations performed by work units (Knutson, 1998). This process selects the strain of *Trichogramma* with the most efficient ability to fly, locate and parasitize the eggs of a targeted host. After selecting the best strain/colony, *Trichogramma* pupae is used to colonize the crop. Pupae can be programmed to enter a condition of arrested development called diapause. Once in diapause, wasp pupae can be stored for up to 9 months so the large demand for *Trichogramma* during the summer can be met (Bigler, 1994). Cardboard capsules containing host eggs with developing *Trichogramma* are applied to the corn field and can be distributed from the ground or air. Capsules either fall to the ground or are caught in the corn plant. The capsules protect the *Trichogramm*a from predators and weather extremes until the adults emerge from the host egg and escape through tiny holes in the capsules. Released *Trichogramma* are at different developmental stages so that adults emerge from the capsules over several days. This increases the time interval between applications (Knutson, 1998).

Releases of *Trichogramma* are performed manually or mechanically (ground and air) (Li, 1994). Since manual applications have shown as time-consuming process, aerial applications are more acceptable option. One such experiment was performed in Poland, where the use of ultralight aircraft proved to be an effective option (Bzowska-Bakalarz et al., 2020). The results indicate that the low-height aerial application allows precise dosing and satisfactory distribution of bioagents. The efficacy of 60–85 % (depending on the year) of the gyroplane-based spraying operations was comparable with those monitored for ground application. These results show a promising alternative for the application of *Trichogramma*, especially in large-scale crops, because the ground application in intensive agriculture requires too much time and energy (Bzowska-Bakalarz et al., 2020).

Other caterpillar pests of corn, such as the southwestern corn borer *Diatraea grandiosella* Diar, 1911 and American cotton bollworm *Helicoverpa zea* Boddie, 1850, are attacked by native or introduced species of *Trichogramma* (*T. pretiosum* Riley, 1879, *T. deion* Pinto and Oatman, 1986, *T. thalense* Pinto and Oatman, 1985) throughout the world (Manandhar & Wright, 2015). Some companies sell *Trichogramma* for control of these pests, but research to support this usage is lacking. At this point, European corn borer is still the most controlled pest of maize with wasps of the genus *Trichogramma*.

3 *Trichogramma* IN RICE

Rice (*Oryza* spp.) is one of the most important crops in the world, being produced in many locations and under a variety of climatic conditions. Since sizable portions of certain crops are used for purposes other than human consumption, rice is the most important food crop, directly feeding more people than any other crop (Rao et al., 2017). Traditionally, countries in Asia have the largest share in world rice production, but this crop is becoming increasingly important in Africa and Latin America. Meanwhile, the expansion of rice crops poses a challenge for agricultural workers as they constantly face many obstacles, such as pests and diseases (Afifah et al., 2019). Key pests of rice are striped rice stemborer *Chilo suppressalis* Walker 1863 (Lepidoptera: Crambidae), yellow stem borer *Scirpophaga incertulas* Walker 1863 (Lepidoptera: Crambidae), pink stem borer *Sesamia inferens* Walker 1856 (Lepidoptera: Noctuidae), rice leafroller *Cnaphalocrocis medinalis* Guenee, 1854 (Lepidoptera: Crambidae), rice planthopper *Nilaparvata lugens* Stal, 1854 (Hemiptera: Delphacidae) and rice green semilooper *Naranga aenescens* Moore, 1881 (Lepidoptera: Noctuidae) (Tang et al., 2017). Among these, yellow stem borer is considered to be the most important pest of rain-fed lowland and flood-prone rice ecosystems (Barthakur, 2010; Ko et al., 2014). Populations of these pests substantially increased within one decade, therefore, the appropriate, effective, and inexpensive control measures are needed for the continuity of high rice production (Gao et al., 2012). Optimized methods with less environmental impact and high sustainability are in demand, such as releasing biological control agents. As one of the most important natural enemies worldwide, the use of *Trichogramma* wasps in rice fields is the subject of constant research (Afifah et al., 2019). Although *Trichogramma* has been studied for management of key lepidopteran pests in rice, these wasps aren't still commercially used in intensive rice production. Recent findings from China indicate that *Trichogramma* releases may be considered practical for control of striped rice stemborer and rice leafroller. However, it is less clear whether yellow stem borer can also be controlled by *Trichogramma* wasps as less studies have been done on this species so far (Tang et al., 2017). From field surveys conducted in Indian rice fields, there are indicators that yellow stem borer eggs may not be effectively parasitized under natural conditions (Hikjm, 1988; Chakraborty, 2012). On the other hand, more positive results have been reported from a

field survey in China showing rather high parasitism rates of yellow stem borer eggs in the range of 46.7 % to 79.1 % (Guo et al., 2002; Samara et al., 2008). In general, there have been a very few attempts to control yellow stem borers by inundative releases of *Trichogramma* (Tang et al., 2017). Positive results were obtained from Indonesia where *T. japonicum* Ashmead, 1904 showed the potential to become candidate for control of white rice stem borer (Yunus, 2018), while promising results were also obtained in Egypt in the control of rice stem borer *Chilo agamemnon* Bleszynski, 1962 (Lepidoptera: Crambidae) due to inundative release of *T. evanescens* (Sherif et al., 2008).

There are rich communities of beneficial insects, spiders, and diseases that attack insect pests of rice, but just a few of them are commercially applied. Certain results indicate that the application of *Trichogramma* wasps shows promising results in the control of rice pests, which has consequently aroused great interest among consumers and rice producers. However, for wider commercial application of these organisms in rice production positive results are lacking, and the use of these organisms is mainly done for the purpose of research.

4 *Trichogramma* IN SOYBEAN

The largest producers of soybean (*Glycine* spp.) in the World are Brazil, the USA, Canada, China and Argentina (https://www.fas.usda.gov/commodities/soybeans). There is a significant variety of insects that may be found in soybean fields at any given time of the season, and among them is a large number of different pests, many of which can cause significant yield losses. As in the above-mentioned crops, different species of the genus *Trichogramma* are present in soybean fields, both as native populations and as introduced biocontrol agents (Bueno et al., 2008). Some of these species are important natural enemies of key pests of soybean production such as sunflower looper *Rachiplusia nu* Guenee, 1852 (Lepidoptera: Noctuidae), velvetbean caterpillar *Anticarsia gemmatalis* Hubner, 1818 (Lepidoptera: Erebidae), soybean budborer *Crocidosema aporema* Walsingham, 1914 (Lepidoptera: Tortricidae) and cotton earworm *Helicoverpa armigera* Hubner, 1808 (Lepidoptera: Noctuidae) (Bortolotto et al., 2015).

In Southern Brazil three species of *Trichogramma* spp. are well known and used for controlling the number of velvetbean caterpillar: *T. pretiosum, T. acacioi* Brun, Moraes and Soares, 1984 and *T. rojasi* Nagaraja and Nagarkatti 1973 (Foerster et al., 2015). In Uruguay, six species of *Trichogramma* have been identified from collections of different crops (Basso et al., 2020). Among the reported species, *T. pretiosum* is the most widely distributed in Uruguay and parasitizing a great number of lepidopteran pests (Basso et al., 1999a; Basso et al., 1999b; Basso & Pintureau, 2004). Given the prevalence, in last decades this species was introduced as biological agent in soybean crops (Basso et al., 2020). The selection of *T. pretiosum* was based on the fact that, in the laboratory, it presented the highest fertility parasitizing eggs of *A. gemmatalis* deposited on soybean plants, when compared to *T. exiguum* Pinto and Platner, 1978 and *T. galloi* Zucchi, 1988, species also present in Uruguay (Basso et al., 2020). In this country a multi-year study was conducted to compare conventional practice with different doses of the egg parasitoid. Although the best results were obtained with the application of chemical insecticides, two releases of *T. pretiosum* by terrestrial methods, 20 days apart, or 4 weekly applications by means of a drone, reached the best results below the thresholds of sanitary intervention, both options with 200,000 parasitoids per hectare (Basso et al., 2020). The application of *T. pretiosum* under the inundative biological control method appears as a real alternative to chemical insecticides for the control of the main lepidopteran pests in soybean crops in Uruguay (Basso et al., 2020). These results showed that biological tool such as egg parasitoids of the genus *Trichogramma* can differentiate and value production of soybean. Beside Latin America, *T. pretiosum* is present in almost all biogeographic regions in the world. With 240 host records in the Americas, it is one of the most commonly collected species, especially in agricultural and other disturbed habitats (Pinto, 1998).

The efforts of large soybean producers to implement alternative ways and eco-friendly methods for pest control management indicate that environmental awareness is constantly growing, but also that future production of large- scale crops should be much more based on the use of beneficial insects and biological control measures in general.

5 *Trichogramma* AS A BIOAGENT IN OTHER CROP SPECIES

Besides in large scale crops, augmentation of *Trichogramma* has been promoted for pest control in cotton, apple, spruce, avocado, tomato and potato production (Olkowski & Zhang, 1990). In Europe, *T. evanescens* is widely used for control of codling moth *Cydia pomonella* Linnaeus, 1758 (Lepidoptera: Tortricidae) in apples (Knutson 1998), but also as an effective tool to decrease population of potato tuber moth *Phthori-*

maea opercullea Zeller, 1873 (Lepidoptera: Gelechiidae) (Saour, 2004). Three *Trichogramma* species, *T. cacoeciae* Marchal, 1927, *T. evanescens*, and *T. principium* Sugonjaev and Sorokina, 1976, are proved as effective candidates in parasitizing potato tumber moth eggs (Saour, 2004). In the USA, parasitism of tomato fruit pests (*H. armigera* or tomato leafminer *Tuta absoluta* Meyrick, 1917 (Lepidoptera: Gelechiidae) by native *T. pretiosum* in tomatoes is considered in the treatment thresholds for these pests with insecticides (Hoffman et al., 1990). Augmentation of *T. pretiosum* is an effective control tactic in Mexico and is a part of the integrated pest program for fresh market tomatoes (Trumble & Alvarado-Rodriguez, 1991). In some countries like China, the Philippines, India, and Taiwan, *T. chilonis* Ishii, 1941 is already being used as a biological control agent in sugarcane plantations. In Indonesia (Grieshop et al., 2014), *T. chilonis* was first developed to address the problem of stem borer in several sugarcane plantations in Java which was later introduced to Lampung after similar problems arose (*Afifah et al., 2019*). The results showed that the release of 150,000 eggs *Trichogramma* spp. per hectare could reduce the population of sugarcane shoot borer *Chilo infuscatellus* Snellen, 1890 (Lepidoptera: Crambidae) while 250,000 eggs are required per hectare to control sugarcane stem borer *C. terrenellus* Pagenstecher, 1900 (Lepidoptera: Crambidae) (Cascone et al., 2015). Besides being able to parasitize *Chilo* spp. *T. chilonis* is also capable of parasitizing *Agrotis* spp. (Lepidoptera: Noctuidae), sugarcane gray borer *Tetramorea schistaceana* Snellen, 1891 (Lepidoptera: Tortricidae), rice leafroller *Cnaphalocrosis medinalis* Guenee, 1854 (Lepidoptera: Crambidae), *H. armigera*, soyabean pod borer *Leguminivora glycinivorella* Obraztsov, 1960 (Lepidoptera: Tortricidae) and beet armyworm *Spodoptera exigua* Hubner, 1808 (Lepidoptera: Noctuidae) (Li-Ying, 1994).

In California, two avocado pests, the omnivorous looper *Sabulodes aegrotata* Guenee, 1857 (Lepidoptera: Geometridae) and the avocado leafroller *Amorbia cuneana* Walsingham, 1879 (Lepidoptera: Tortricidae), can be managed by releasing *T. platneri* Nagarkatti, 1975 in every fourth avocado tree (Olkowski & Zhang, 1990). Large field studies in Canada have shown that two releases, each with 30 million *T. minutum* individuals per acre, resulted in 60 to 80 % egg parasitism of spruce budworm *Choristoneura fumiferana* Clemens, 1865 (Lepidoptera: Tortricidae) in white spruce stands (Olkowski & Zhang, 1990).

The actual rates of release vary considerably, even for the same pest, crop, and country. This range is probably related to the range in dimensional volume of the crop. For example, the total rates of release for *T. brassicae* alone, which is reared from small host eggs against European corn borer in Europe, range from 150,000 to 2.8 million wasps/ha (El-Wakeil et al., 2020). Rates

Table 1: Host and country of origin of the *Trichogramma* species and strains (adapted from Tabone et al., 2010)

Species*	Strain	Host and taxonomy*	Country of Origin
Trichogrammatoidea bactrae Nagaraja, 1979	Bac-1	*Plutella xylostella* Linnaeus, 1758 (Lepidoptera: Plutellidae)	Thailand
Trichogramma bourarachae Pintureau and Babaul, 1988	Bou-1	*Vanessa cardui* Linnaeus, 1785 (Lepidoptera: Nymphalidae)	Morocco
Trichogramma bourarachae	Bou-2	*Helicoverpa armigera* (Lepidoptera: Noctuidae)	Portugal
Trichogramma buesi Voegele, 1982	Bue-1	*Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae)	Canada
Trichogramma chilonis	Chi-1	Plutella xylostella	Japan
Trichogramma chilonis	Chi-3	Ephestia kuehniella	Taiwan
Trichogramma dendrolimi Matsumara, 1926	Den-1	*Lobesia botrana* Denis and Schiffermu ller, 1775 (Lepidoptera: Tortricidae)	Italy
Trichogramma evanescens	Eva-1	*Pectinophora gossypiella* Saunders, 1884 (Lepidoptera: Gelechiidae)	Egypt
Trichogramma oleae Voegele and Pointe, 1979	Ole-1	*Prays oleae* Bernard, 1794 (Lepidoptera: Plutellidae)	France
Trichogramma ostriniae	Ost-2	Ephestia kuehniella	Moldova
Trichogramma principium Sugonjaev and Sorokina, 1976	Pri-1	*Earias insulana* Boisduval, 1833 (Lepidoptera: Nolidae)	Syria

*Detailed explanation of the species (author and taxonomy) is provided only for those species which are not mentioned in previous text

in several millions of wasps/ha are generally cited in arboreal situations such as forestry, and in fruit or nut orchards, whereas those in agricultural crops such as corn, cotton, and tomato, range from 500 to more than 1 million wasps/ha, with averages of 200,000-600,000 wasps/ha (El-Wakeil et al., 2020). China often reports lower rates than other countries, possibly because of the frequent use of large host eggs (Wang, 2013).

When determining the effectiveness of certain *Trichogramma* species in biological control, it was found that the rate of parasitism does not only depend on the selected *Trichogramma* species, but also on the choice of the appropriate strain. Certain strains within the same species may have different laboratory and field performances, or different preferences towards the same or different hosts. Tabone et al. (2010) analyzed the rate of parasitism of different *Trichogramma* species and strains and pointed out significant differences within the diversity of *Trichogramma* species in certain regions and on different hosts (Table 1.). These results very effectively represent the preference of *Trichogramma* parasitoids for Lepidoptera, both among different species and among different strains within the same *Trichogramma* species.

6 GENERALIST PREDATORS

The scientific community uses all available resources in order to explore the natural potential of entomofauna and introduce less-toxic solutions in pest management programs. Many other beneficial organisms are constantly being tested as potential biocontrol agents such as species of the genus *Chrysopa* spp. (Neuroptera: Chrysopidae) and *Orius* spp. (Hemiptera: Anthocoridae).

Chrysopids commonly known as lacewings, occur in numerous agricultural and horticultural zones of the northern hemisphere. Adults are free-living and usually non-predatory in nature, surviving on nectar and pollen, while three larval stages are highly predatory (Bellows & Fisher, 1999). They are active predators of a wide variety of pests including aphids, chinch bugs, mealybugs, scales, whiteflies, leafhoppers, lepidopterous eggs and larvae, and mites (Principi & Canard, 1984). The efficacy in biological control of aphids as well as other arthropod pests has been recognized for more than 250 years (Dhandapani et al., 2016).

Inundative releases of the common green lacewing *Chrysoperla carnea* Stephens, 1836 (Neuroptera: Chrysopidae) on cotton provided effective results in control of American cotton bollworm *Helicoverpa zea* Boddie, 1850 (Lepidoptera: Noctuidae) and tobacco budworm *Heliotihis virescens* Fabricius, 1777 (Lepidoptera: Noctuidae) (Ridgway & Jones, 1969). Releases of *C. carnea* eggs in field cages at rates of 50,000 and 100,000 per acre can significantly reduce the population of tobacco budworm and increase the yield (Ridgway & Jones, 1969). Green lacewing has also been effective on potato aphid *Macrosiphum euphorbie* Thomas, 1878 (Hemiptera: Aphididae) and buckthorn aphid *Aphis nastrurtii* Kaltenbach, 1843 (Hemiptera: Aphididae) (Capinera, 2001). A species that is very common in corn fields is *Chrysoperla oculata* Ruzicka, 1997. The most suitable prey for *C. oculata* in corn fields is corn leaf aphid *Rhopalosiphum maidis* Fitch, 1856 (Hemiptera: Aphididae) an important pest of corn (Bellows & Fisher, 1999). Many species of the genus *Chrysopa*, such as red-lipped green lacewing *C. rufilabris* Burmeister, 1839, *C. externa* Hagen, 1861 and *C. perla* Linnaeus, 1758 are important predators and are used as biological control agents worldwide. Among the above-mentioned species, most attempts have evaluated the efficacy of *C. carnea* in augmentation releases in the field or in the greenhouses (Ridgway & Mcmuphy, 1984; Nordlund et al., 2001).

In the context of biological control, the genus *Orius* includes several species that have found their place in commercial pest control in agriculture. This genus is represented by very tiny true bugs commonly known as minute pirate bugs and flower bugs (Riudavets & Castane, 1994). They play a key role in the management of various agricultural pests in greenhouse and field environments. They can be found in numerous crops, pastureland and surrounding areas (cotton, soybean, bean, potato, wheat, alfalfa, maize, orchards, other vegetables and ornamental crops), as well as in trees, shrubs, weeds and many wild plants. They prey on thrips, aphids, mites, whiteflies, moths and other tiny arthropods and insect eggs. *Orius* are very effective predators and can thus provide biological pest control in a variety of cropping systems (Brust & Yurchak, 2021).

The species insidious flower bug *Orius insidiosus* Say, 1832 (Hemiptera: Anthocoridea) is one of the most important predators in corn field. The ability of *O. insidiosus* to search, find and destroy European corn borer and corn earworm eggs was investigated in numerous studies. *O. insidiosus* is an important natural enemy of corn earworm in corn, cotton and sorghum. A study conducted in the USA, revealed European corn borer larvae sustain high mortality in field corn and that *O. insidiosus* was the most important predator of these larvae in western Maryland (Brust & Yurchak, 2021). Their population peak coincides with corn pollen-shedding and sulking, during which they feed on second-generation of European corn borer larvae and corn pollen. Therefore, successful biological control of European

corn borer larvae by *O. insidiosus* is linked to arthropod prey and corn pollen (Brust & Yurchak, 2021).

Orius insidiosus adults and nymphs are common in soybean fields. Its population dynamics in soybean fields have been linked to thrips population levels and soybean flowering. Nymphs and adults eat soybean aphids in the field. Experimental findings suggest that under certain conditions, *O. insidiosus* can effectively suppress aphid population growth and that they may be key factors influencing aphid population dynamics in soybeans in some areas within the USA. In addition to soybean aphids, soybean thrips are believed to be one of the most important thrips prey of *O. insidiosus* in soybean. It is believed that soybean thrips serve as an important prey resource for *O. insidiosus* in soybeans and may be important in sustaining *O. insidiosus* populations before the arrival of soybean aphids. *O. insidiosus* is known to feed on eggs and first instar of green cloverworm *Hypena scabra* Fabricius, 1789 (Lepidoptera: Erebidae) as well (Brust &Yurchak, 2021).

Rice predators include spiders, ants, some insect families such as Carabidae, plant bugs, amphibians, dragonflies and other beetles and water bugs. However, the most abundant ones are the spiders (Wopereis et al., 2008). Their ability to hunt in a variety of habitats in combination with high abundance, positions spiders as potentially effective biocontrol agents (Symondson et al., 2002). Dispersal by running and ballooning allows spiders to colonize agricultural fields soon after disturbance due to agricultural practices such as ploughing and seed sowing (Radermacher et al., 2020). This applies in particular to agricultural systems with multiple cropping cycles per year and asynchronous planting practice (Marc et al., 1999). In fact, spiders are among the most abundant arthropod predators in rice ecosystems and assumed to contribute to the control of pest species such as plant and leafhoppers (Sigsgaard, 2007). With the ability to capture prey of different feeding guilds, including herbivores and detritivores, spiders may play an important role soon after planting rice fields when herbivore populations still are low (Radermacher et al., 2020). Generalist predators in agricultural systems such as spiders may link aboveground herbivore and belowground detrital systems using prey of both systems (Scheu, 2001; Snyder & Wise, 2001; Wise et al., 2006).

7 CONCLUSIONS

Although the use of beneficial insects may seem simple at first, effective pest biocontrol is determined by many factors. Those factors are adequate selection of parasites and predators, the quality and fitness of used bioproduct, the numbers released and the timing of the release, the release method, complex interactions between the parasite/predator, the target pest, the crop and environmental conditions (Knutson, 1998). However, a fundamental step in the development of any biological control program utilizing beneficial insects is the identification and choice of species and/or strains to use; not all species (or populations) perform equally well, in terms of mass rearing or field dispersal and performance (Ivezić et al., 2018). Therefore, the first step for the implementation of beneficial insects in the program of biological control of pests is the accurate identification and genetic characterization of native species, since autochthonous species are likely to be best adapted to environmental conditions in a specific ecosystem (Whitman & Nordlund, 1994).

In addition to native populations of beneficial insects, biological control also involves the introduction of natural enemies that are not typical of certain areas. The introduction of natural enemies is used when a pest of exotic origin is the target of the biocontrol program (White, 2019). Pests are constantly being imported into countries where they are not native, either accidentally, or in some cases, intentionally. Many of these introductions do not result in establishment or if they do, the organism may not become pest. However, it is not uncommon for some of these introduced organisms to become pests due to a lack of natural enemies to suppress their population number. In these cases, introduction of natural enemies can be highly effective. Once the country of origin of the pest is determined, exploration in the native region can be conducted to search for promising natural enemies (White, 2019). If such enemies are identified, they may be evaluated for potential impact on the pest organism in the native country or alternatively, introduced into the new country for further study. They first need to be placed in a quarantine for one or more generations to be sure that no undesirable species are accidentally imported (diseases, hyperparasitoids etc.). Additional permits are required for interstate shipment and field release (White, 2019).

Besides inundative and inonculative activities for commercial usage of beneficial insects, it is also necessary to preserve the autochthonous residential species populations. Conservation of habitats and preservation of biodiversity as one of the main prerequisites for successful implementation of biological control strategies can be defined as an identification and modification of human influence that allows natural enemies to express their potential to suppress pests (Rechcigl & Rechcigl, 2020). Numerous studies and experience have shown that conserving natural enemies is of tremendous im-

portance in the safe and economical management of insect pests and doing so has to be a major component of a producer's management activities (Rechcigl & Rechcigl, 2020). Likewise, the most important component of biological control is establishing the list of indigenous species of organisms for biological control, which includes only those beneficial species that are indigenous or ubiquitous in specific region. Such an approach has been done in Slovenia and represents an additional safeguard that practically prevents the use of beneficent species that could in any way endanger the common domestic flora and fauna (Trdan et al., 2020).

Over the last decades large scale farming led to increasing production, but also caused substantial environmental degradation as such increases were mostly based on its expansion onto natural areas and greater use of external inputs and other forms of intensified use (IPBES, 2019). Achieving sustainability of agricultural production is one of the key challenges for humanity. Minimizing the use of chemicals and replacing them with biological plant protection is firstly fully in line with the agriculture's development strategy and secondly, confirmed to be achievable in practice. It is especially important to apply such tactical decisions in the production of large-scale crops, which, at the same time, represent the biggest polluters of the environment in general. The use of beneficial insects in biological control is common worldwide but its potential has not been explored for many pests and in many geographic regions. Even in Europe certain countries with very intensive agriculture do not currently use the full potential of these organisms in their agroecological systems. In addition, a recent survey found that very few governmental Extension Services currently provide recommendations for controlling pests with *Trichogramma* spp. or any other beneficial insects (Ivezić & Trudić, 2021). The successful use of augmentative releases of biocontrol agents in pest management programs will depend on a sound and thorough research program, favorable economics, commercial investment, and the development of an extension program to transfer this technology to crop consultants and large scale growers. Mainstreaming agroecology and its biological control alternatives among large scale farmers is urgently needed, but it requires addressing specific questions in research, technology and policy development to support sustainable transitions.

8 ACKNOWLEDGEMENT

This review paper is done under the scope of RIS fellowship programme (September 2021-December 2021, Warsaw, Poland) of The European Institute of Innovation and Technology (EIT), University of Warsaw, Warsaw, Poland.

9 REFERENCES

Afifah, L., Muhammad Bayfurqon, F. and, Latifatus Siriyah S. (2019). Control of Rice Stem Borer *Scirpophaga* sp. using *Trichogramma* sp. *Indonesian Journal of Community Engagement*. *5*(1), 99-107. http://doi.org/10.22146/jpkm.34180

Afifah, L., Fawzy, B., and Siti, S. (2019). Control of Rice Stem Borer *Scirpophaga* sp. Using Trichogramma sp. *Journal Pengabdian Kepada Masyarakat (Indonesian Journal of Community Engagement)*, *5*(99), https://doi.org/10.22146/jpkm.34180

Altieri, M.A. (1994). *Biodiversity and pest management in agroecosystems*. New York, Haworth Press. 213p.

Basso, C., Grille, G., and Pintureau, B. (1999a). Eficacia de *Trichogramma exiguum* Pinto & Platner y de *T. pretiosum* Riley en el control de *Argyrotaenia sphaleropa* (Meyrick) y de *Bonagota cranaodes* (Meyrick) en la vid en el Uruguay. *Agrociencia Uruguay*, *3*, 20-6.

Basso, C., Pintureau, B., and Grille, G. (1999b). Taxonomic study of two *Trichogramma* species from Uruguay (Hym: Trichogrammatidae). *Boletin de Sanidad Vegetal Plagas*, *25,* 373-82.

Basso, C. and Pintureau, B. (2004). Las especies de *Trichogramma* del Uruguay (Hymenoptera: Trichogrammatidae). *Revista de la Sociedad Entomológica Argentina*, *63*(1-2), 71-80.

Basso, C., Chiaravalle, W., and Maignet, P. (2020). Effectiveness of *Trichogramma pretiosum* in controlling lepidopterous pests of soybean crops. *Agrociencia Uruguay*. https://doi.org/10.31285/AGRO.24.419

Bellows, T.S. and Fisher, T.W. (1999). *Handbook of Biological Control. Principles and Application of Biological Control*, Academic Press San Diego. California, 1047p.

Ben Ari, T. and Makowski, D. (2016). Analysis of the trade-off between high crop yield and low yield instability at the global scale. *Environmental Research Letters*, https://doi.org/10.1088/1748-9326/11/10/104005

Bigler, F. (1986). Mass production of *Trichogramma maidis* Pint. et Voeg. and its field application against *Ostrinia nubilalis* Hbn. in Switzerland. *Journal of Applied Entomology*, *101*, 23–29.

Bigler, F. (1994). Quality control in *Trichogramma* production. In *Biological Control with Egg Parasitoids*, eds. E. Wajnberg and S. A. Hassan, pp. 93-111. Oxon, U.K.: CAB International. https://doi.org/10.1111/j.1439-0418.1986.tb00829.x

Bohinc, T., Schmidt, S., Monje, J.C., Trdan, S. (2015). Prva najdba parazitoidne ose *Trichogramma brassicae* Bezdenko, 1968 (Hymenoptera, Trichogrammatidae) v Sloveniji. *Acta agriculturae Slovenica*, *105*(2), 323-327. https://doi.org/10.14720/aas.2015.105.2.15

Bortolotto, O.C., Pomari-Fernandes, A., Bueno, RCOF., Bueno, AF., Cruz, YKS., Sanzovo, A., Ferreira, R.B. (2015). The

use of soybean integrated pest management in Brazil: A review. *Agronomy Science and Biotechnology*, *1*(1), 25-32. https://doi.org/10.33158/ASB.2015v1i1p25

Brust, G. and Yurchak, V. (2021). *Minute Pirate Bug: A Beneficial Generalist Insect Predator***.** University of Meriland Extension. https://extension.umd.edu/resource/minute-pirate-bug-beneficial-generalist-insect-predator

Bueno, A.F., Bueno, RCOF., Parra, JRP., and Vieira S.S. (2008). Effects of pesticides used in soybean crops to the egg parasitoid *Trichogramma pretiosum*. *Ciência Rural, 38*, 1495–1503. https://doi.org/10.1590/S0103-84782008000600001

Bzowska-Bakalarz, M., Bulak, P., Beres, P.K., Czarnigowska, A., Czarnigowski, J., Karamano, B., Pniak, M., and Bieganowski, A. (2020). Using gyroplane for application of *Trichogramma* spp. against the European corn borer in maize. *Pest Management Science*. https://doi.org/10.1002/ps.5762

Capinera, J. (2001). *Handbook of Vegetable Pests*. Gulf Professional Publishing. 729 p.

Cascone, P. et al. (2015). Improving the efficiency of *Trichogramma achaeae* to Control Tuta absoluta. *BioControl*, *60*(6), 761-771. https://doi.org/10.1007/s10526-015-9684-1

Chakraborty, K. (2012). Relative composition of egg parasitoid species of yellow stem borer *Scirpophaga incertulas* Wlk. in paddy field at Uttar Dinajpur, West Bengal, India. *Current Biology, 6*, 42–52.

Clausen, C.P. (1978). Introduced parasites and predators of arthropod pests and weeds. USDA, *Agriculture Handbook*, 454p.

Deka, S. and Barthakur, S. (2010). Overview on current status of biotechnological interventions on yellow stem borer *Scirpophaga incertulas* (Lepidoptera: Crambidae) resistance in rice. *Biotechnology Advances, 28*, 70–81. https://doi.org/10.1016/j.biotechadv.2009.09.003

Dhandapani, N., Sarkar, P., and Mishra, G. (2016). Chrysopids. In: *Ecofriendly Pest Management for Food Security*. Academic Press, Elsevier, Inc. pp. 311-327. https://doi.org/10.1016/B978-0-12-803265-7.00010-5

Eckhoff, S. R., M.R. Paulsen, and S. C. Yang. (2003). 'MAIZE'. In *Encyclopedia of Food Sciences and Nutrition* (Second Edition), edited by Benjamin Caballero, 3647–53. Oxford: Academic Press. https://doi.org/10.1016/B0-12-227055-X/00725-2

El-Wakeil, N., Mahmoud, S., and Mohamed, A.H. (2020). Cottage Industry of Biocontrol Agents and Their Applications Practical Aspects to Deal Biologically with Pests and Stresses Facing Strategic Crops: Practical Aspects to Deal Biologically with Pests and Stresses Facing Strategic Crops. https://doi.org/10.1007/978-3-030-33161-0

Feshchenko, A. (2019). Large Scale Agriculture. Impact of Intensive Farming on Biodiversity. https://www.largescaleagriculture.com/home/news-details/impact-of-intensive-farming-on-biodiversity/

Flint, M.L. and Dreistadt, S.H. (1998). The Illustrated Guide to Biological Pest Control. In *Natural Enemies Handbook*. Clark, JK (ed). University of California Press. ISBN 978-0-520-21801-7.

Foelix, R.F. (2011). *Biology of spiders*, 3rd ed. Oxford University Press, New York.

Foerster, M.R., Marchioro, C.A., and Foerster, L.A. (2015). How *Trichogramma* survives during soybean offseason in Southern Brazil and the implications for its success as a biocontrol agent. *BioControl, 60*, 1–11. https://doi.org/10.1007/s10526-014-9616-5

Gao, X.H., Tian, J.H., and Chen, P. (2012). Population dynamics of *Tryporyza incertulas* (Walker) in Menghai, Yunnan from 2001–2011. *Journal of Yunnan University, 34*, 367–372.

Giraud, M. (2015). *Trichogramma achaeae* as an IPM tool in tomato greenhouses. *Pure final congress*, Poznan, Poland.

Grieshop, M., Flinn, P., Nechols, J. and Schöller, M. (2014). Host-foraging success of three species of *Trichogramma* (Hymenoptera: Trichogrammatidae) in a simulated retail environment'. *Journal of Economic Entomology, 100*, 591–98. https://doi.org/10.1603/0022-0493(2007)100[591:HSOTSO]2.0.CO;2

Guo, H.F., Fang, J.C., Xie, Y.F., Du, Z.W. (2002). Egg parasitism of rice stem borers in regions with different rice stem borer occurring patterns. *Chinese Journal of Biological Control*, *18*, 13–16.

Hassan, SA. (1981). Mass-production and utilization of *Trichogramma*: 2. Four years successful biological con- trol of the European corn borer. *Mededelingen van de Faculteit Landbouwwetenschappen. Rijksuniversiteit Gent*, *46*(2), 417–427.

Hassan, S.A. (1993). The mass rearing and utilization of *Trichogramma* to control lepidopterous pests: achievements and outlook. *Pesticide Science*, *37*(4), 387–391. https://doi.org/10.1002/ps.2780370412

Hikjm, I.S. (1988). Seasonal parasitism by egg parasites of the yellow rice borer, *Scirpophaga incertulas* (Lepidoptera Pyralidae). *Entomophaga*, *33*, 115–124. https://doi.org/10.1007/BF02372319

Hochman, G., Deepak, R., Govinda, T., and Zilberman, D. (2014). Quantifying the causes of the global food commodity price crisis. *Biomass and Bioenergy, 68*, 106–14. https://doi.org/10.1016/j.biombioe.2014.06.012

Hoffman, M.P., Wilson, L.T., Zalom, F.G., and Hilton, R.J. (1990). Parasitism of *Heliothis zea* (Lepidoptera: Noctuidae) eggs: effect on pest management decision rules for processing toma- toes in the Sacramento Valley of California. *Environmental Entomology*, *19*, 753-763. https://doi.org/10.1093/ee/19.3.753

IPBES 2019. Global Assessment Report on Biodiversity and Ecosystem Services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services.

Ivezić, A., Rugman-Jones, P., Stouthamer, R., and Ignjatović-Ćupina, A. (2018). Molecular identification of *Trichogramma* egg parasitoids of *Ostrinia nubilalis* in the north eastern Serbia. *Archives of Biological Sciences*, *70*(3), 425-432. https://doi.org/10.2298/ABS171103002I

Ivezić, A. and Trudić, B. (2021). Parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae), natural enemies of European corn borer *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae). *Journal of Central European Agriculture* (in press). https://doi.org/10.5513/JCEA01/22.4.3247

Knutson, A. (1998). *The Trichogramma Manual*. Texas (TX):

The Texas A&M University Systems.

Ko, K. et al. (2014). Evaluation for potential *Trichogramma* (Hymenoptera: Trichogrammatidae) strains for control of the striped stem borer (Lepidoptera: Crambidae) in the greater Mekong subregion. *Journal of economic entomology*, *107*(3), 955-963. https://doi.org/10.1603/EC13481

Kot, J. (1964). Experiments in the biology and ecology of species of the genus *Trichogramma* Westw. and their use in plant protection. *Ekologia Polska – Seria A*, *12*(15), 1-61.

Li-Ying, Li. (1994). Worldwide Use of Trichogramma for Biological Control on Different Crops: A Survey. In Hassan. S. A. & Wajnberg. E. (Ed.), *Biological Control with Egg Parasitoids* (pp. 37-51). Wallingford: CAB International.

Manandhar, R. and Mark, G. Wright. (2015). Enhancing biological control of corn earworm, *Helicoverpa* and *Thrips* through habitat management and inundative release of *Trichogramma pretiosum* in corn cropping systems. *Biological Control, 89*, 84–90. https://doi.org/10.1016/j.biocontrol.2015.05.020

Marc, P., Canard, A., and Ysnel, F. (1999). Spiders (Araneae) useful for pest limitation and bioindication. *Agriculture, Ecosystems & Environmen*t, *74*, 229–273. https://doi.org/10.1016/S0167-8809(99)00038-9

Murali-Baskaran, R.K., Sharma, K.C., Sridhar, J., Jain, L., Kumar, J. (2021). Multiple releases of *Trichogramma japonicum* Ashmead for biocontrol of rice yellow stem borer, *Scirpophaga incertulas* Walker). *Crop Protection.* https://doi.org/10.1016/j.cropro.2020.105471

Mutuura, A. and Monroe, E. (1970). Taxonomy and distribution of the European corn borer and allied species: genus *Ostrinia* (Lepidoptera: Pyralidae), pp. *1-112 in Memoirs of the Entomological Society of Canada*, edited by D. P. Pielou. Entomological Society of Canada, Ottawa. https://doi.org/10.4039/entm10271fv

Nordlund, D.A., Cohen, A.C., and Smith, R.A. (2001). Mass-rearing. Release Techniques and Augmentation. In *Lacewings in the Crop Environment*. Mcewen PK, New TR, Whittington AE (eds). Cambridge University Press, Cambridge, 303-319. https://doi.org/10.1017/CBO9780511666117.015

Nordlund, D.A., W. J. Lewis, H.R. Gross, Jr., and M, Beevers. (1981). Kairomones and their use for management of entomophagus insects. XII. The stimulatory effects of host eggs and the importance of host-egg density to the effective use of kairomones for *Trichogramma pretiosum*. Riley. *Journal of Chemical Ecology,* 7, 909-17. https://doi.org/10.1007/BF00987616

Nurindah, D.A. and Sunarto, S. (2020). Overview of the use of biocontrol agents in the control of Indonesian sugarcane borers. *IOP Conference Series: Earth and Environmental Science, 418*, 012061. https://doi.org/10.1088/1755-1315/418/1/012061

Obrycki, J.J., Hamid, M.N., Sajap, A.S., and Lewis, L.C. (1989). Suitability of corn insect pests for development on survival of *Chrysoperla carnea* and *Chrysoperla oculata* (Nuoroptera: Chrysapidae). *Environmental Entomology*, *18*, 1126-1130. https://doi.org/10.1093/ee/18.6.1126

Olkowski, W. and A. Zhang. (1990). *Trichogramm*a- a modern day frontier in biological control. *The IPM Practitioner*, *12*, 1-15.

Pappas, M. and Broufas Koveos, D.S. (2011). Chrysopid predators and their role in biological control. *Journal of Entomology*. https://doi.org/10.3923/je.2011.301.326

Pinto, J.D. (1998). *Systematics of the North American species of Trichogramma Westwood (Hymenoptera: Trichogrammatidae*). Washington (DC): Entomological Society of Washington, 289p. (Memoirs of the Entomological Society of Washington; 22).

Polanczyk, R.A. and Pratissoli, D. (2009). Biological control of agricultural pests: Principles and field applications. *Revista Ceres, 56*(4), 410-419.

Principi, M.M. and Canard, M. (1984). Feeding Habits. In *Biology of Chrysopidae*. Canard M, Semeria Y, New TR (eds). Dr W. Junk Publishers, The Hague, The Nederlands 76-92.

Radermacher, N., Hartke, T.R., Villareal, S., et al. (2020). Spiders in rice-paddy ecosystems shift from aquatic to terrestrial prey and use carbon pools of different origin. *Oecologia, 192*, 801–812. https://doi.org/10.1007/s00442-020-04601-3.

Radermacher, N., Hartke, T.R., Villareal, S., and Scheu, S. (2020). Spiders in ricepaddy ecosystems shift from aquatic to terrestrial prey and use carbon pools of diferent origin. *Oecologia*, *192*, 801–812. https://doi.org/10.1007/s00442-020-04601-3.

Rao, A. N., S. P. Wani, M. S. Ramesha, and J. K. Ladha. (2017). Rice Production Systems. In *Rice Production Worldwide*, edited by Bhagirath S. Chauhan, Khawar Jabran, and Gulshan Mahajan, 185–205. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-47516-5_8.

Raspudić, E., Ivezić, M., and Mlinarević, M. (1999). Utjecaj transgenih Bt hibrida kukuruza na kukuruznog moljca (*Ostrinia nubilalis* Hübner). Sažeci priopćenja 43. seminara iz Zaštite bilja Opatija, *Glasnik zaštite bilja*, *1*, 6-7.

Rechcigl, J.E. and Rechcigl, N.A. (2000). *Biological and biotechnological control of insects pest*. Boca Raton: CRC Press; 2000. 375 p. https://doi.org/10.1201/9781439822692

Ridgway, R. and Jones, S.L. (1969). Inundative releases of *Chrysopa carnea* for control of *Heliothis* on cotton. *Journal of Economic Entomology.* https://doi.org/10.1093/JEE/62.1.177

Ridgway, R.L. and Mcmuphy, W.L. (1984). Biological Control in the Field. In: *Biology of Chrysopidae*. Canard M, Semeria Y, New TR (eds). Dr W. Junk Publishers, The Hague, The Nederlands 220-228.

Riechert, S.E. and Lockley, T. (1984). Spiders as biological control agents. *Annual Review of Entomology*, *29*, 299–320. https://doi.org/10.1146/annur ev.en.29.010184.001503

Riudavetes, J. and Castane, C. (1994). Abundance and host plant preferences for oviposition of *Orius* spp. (Heteroptera: Anthocoridae) along the Mediterranean coast of Spain. *IOBC/wprs Bulletin*, *17*(5), 230-236.

Ru, N., Whitcomb, W.H., and Murphey, M. (1976). Culturing of *Chrysopa rufilabris* (Neuroptera: Chrysopidae). *The Florida Entomologist*, *59*(1), 21-26. https://doi.org/10.2307/3493163

Ruberson, J.R. and Kring, T.J. (1993). Parasitism of developing eggs by *Trichogramma pretiosum* (Hymenoptera:

Trichogrammatidae): Host age preference and suitability. *Biological Control*, *3*, 39-46. https://doi.org/10.1006/bcon.1993.1007

Samara, R.Y., Monje, J.C., and Zebitz, C.P.W. (2008). Comparison of different European strains of *Trichogramma aurosum* (Hymenoptera: Trichogrammatidae) using fertility life tables. *Biocontrol Science and Technology, 18*, 75–86. https://doi.org/10.1080/09583150701749789

Saour, G. (2004). Efficacy assessment of some *Trichogramma* species (Hymenoptera: Trichogrammatidae) in controlling the potato tuber moth *Phthorimaea operculella* Zell. (Lepidoptera: Gelechiidae). *Journal of Pest Science*, *77*, 229–234. https://doi.org/10.1007/s10340-004-0058-1

Scheu, S. (2001). Plants and generalist predators as links between the below-ground and above-ground system. *Basic and Applied Ecology, 13*, 3–13. https://doi.org/10.1078/1439-1791-00031

Scheu, S. (2002). The soil food web: structure and perspectives. *The European Journal of Soil Biology, 38*, 11–20. https://doi.org/10.1016/S1164-5563(01)01117-7

Sherif, M.R., Hendawy, A., and El-Habashy, M.M. (2008). Utilization of *Trichogramma evanescens* (Ashmead) for controlling rice stem borer, *Chilo agamemnon* Bles. in rice fields in Egypt. *Egyptian Journal of Biological Pest Control, 18*, 11–16.

Siebrecht, N. (2020). Sustainable agriculture and its implementation gap - overcoming obstacles to implementation. *Sustainability*. http://dx.doi.org/10.3390/su12093853

Sigsgaard, L. (2007). Early season natural control of the brown planthopper, *Nilaparvata lugens*: the contribution and interaction of two spider species and a predatory bug. *Bulletin of Entomological Research*, *97*, 533– 544. https://doi.org/10.1017/S0007485307005196.

Smith, S.M. (1996). Biological control with *Trichogramma*: advances, successes, and potential of their use. *Annual Review of Entomology*, *41*, 375–406. https://doi.org/10.1146/annurev.en.41.010196.002111

Snyder, W.E. and Wise, D.H. (2001). Contrasting trophic cascades generated by a community of generalist predators. *Ecology*, *82*, 1571–1583. https://doi.org/10.1890/0012-9658(2001)082[1571:CTCGBA]2.0.CO;2

Stouthamer, R., Breeuwert, J.A., Luck, R., and Werren, J. (1993). Molecular identification of microorganisms associated with parthenogenesis. *Nature, 361*(6407), 66–68. https://doi.org/10.1038/361066a0

Symondson, WOC., Sunderland, K.D., and Greenstone, M.H. (2002). Can generalist predators be effective biocontrol agents? *Annual Review of Entomology*, *47*, 561–594. https://doi.org/10.1146/annurev.ento.47.091201.145240

Tabone, E., Bardon, C., Desneux, N., and Wajnberg, E. (2010). Parasitism of different *Trichogramma* species and strains on *Plutella xylostella* L. on greenhouse cauliflower. *Journal of Pest Science*, *83*, 251–256. https://doi.org/10.1007/s10340-010-0292-7

Tang, R., Babendreier, D., Zhang, F., Kang, M., Song, K., and Hou, M.L. (2017). Assessment of *Trichogramma japonicum* and *T. chilonis* as potential biological control agents of yellow stem borer in rice. *Insects, 8*(1), 19. https://doi.org/10.3390/insects8010019

Tittonell, P., Piñeiro, G., Garibaldi, L.A., Dogliotti, S., Olff, H., and Jobbagy, E.G. (2020). Agroecology in large scale farming - A research agenda. *Frontiers in Sustainable Food Systems*, *214*. https://doi.org/10.3389/fsufs.2020.584605

Trdan., S, Laznik, Ž., Bohinc, T. (2020). Thirty years of research and professional work in the field of biological control (predators, parasitoids, entomopathogenic and parasitic nematodes) in Slovenia: A review. *Applied Sciences*, 10(21), 1-12. https://doi.org/10.3390/app10217468

Trumble, J.T. and Alvarado-Rodriguez, B. (1991). Development and economic evaluation of an IPM program for fresh market tomato production in Mexico. *Agriculture, Ecosystems and Environment, 43*, 267-284. https://doi.org/10.1016/0167-8809(93)90091-3

Vail, P.V., Coulson, J.R., Kaufmann, W.C. and Dix, M.E. (2001). History of biological control programs in the United States Department of Agriculture. *American Entomologist*, *47*, 24-49. https://doi.org/10.1093/ae/47.1.24

Van den Bosch, R., Messenger, P.S., and Gutierrez, A.P. (1982). The History and Development of Biological Control. In: *An Introduction to Biological Control*. Springer, Boston, MA. https://doi.org/10.1007/978-1-4757-9162-4_3

Van Driesche, R.G. and Bellows, T.S. (1996). *Biological control*. New York (NY): Chapman and Hall. https://doi.org/10.1007/978-1-4613-1157-7

Van Lenteren, J.C., Babendreier, D., Bigler, F., Burgio, G., Hokkanen, H.M.T., Kuske, S., Loomans, A.J.M., Menzler-Hokkanen, I., van Rijn, PCJ., and Thomas M.B., et al. (2003). Environmental risk assessment of exotic natural enemies used in inundative biological control. *BioControl*, *48*(1), 3–38. https://doi.org/10.1023/A:1021262931608

Van Schelt, J. and Ravensberg, W.J. (1991). Some aspects on the storage and application of *Trichogramma maidis* in corn. In: Wajnberg E, Vinson SB, editors. *The Third International Symposium on Trichogramma and other Egg Parasitoids*; San Antonio, USA; p. 239–242.

Van Lenteren, JC. (2000). Success in biological control of arthropods by augmentation of natural enemies. In: Gurr GM, Wratten SD, editors. *Biological control: measures of success*. Dordrecht, Netherlands: Kluwer Academic Publishers; p. 77–103. https://doi.org/10.1007/978-94-011-4014-0_3

Wang, B., Ferro, D.N., and Hosmer, D.W. (1999). Effectiveness of *Trichogramma ostriniae* and *T. nubilale* for controlling the European corn borer *Ostrinia nubilalis* in sweet corn. *Entomologia Experimentalis et Applicata*, *91*, 297–303. https://doi.org/10.1046/j.1570-7458.1999.00496.x

Zhenying, W., He, K., Zhang, F., Lu, X., and Babendreier, D. (2014). Mass rearing and release of *Trichogramma* for biological control of insect pests of corn in China. *Biological Control*, *68*, 136–44. https://doi.org/10.1016/j.biocontrol.2013.06.015

White, B. (2019). *Biological Control of Insects Pests*. ED-Tech Press, United Kingdom 298.

Whitman, D. and Nordlund, D.A. (1994). Plant chemicals and the location of herbivorous arthropods by their natural enemies. In: Ananthakrishnan TN (editor). *Functional Dynamics of Phytophagous Insects*. New Delhi, India: Oxford and IBH Publishing, pp. 207–248.

Wise, D.H., Moldenhauer, D.M., and Halaj, J. (2006). Using stable isotopes to reveal shifts in prey consumption by generalist predators. *Ecological Applications*, *16*, 865–876. https://doi.org/10.1890/1051-0761(2006)016[0865:USITRS]2.0.CO;2

Wopereis, M.C.S., Defoer, T., Idinoba, P., Diack, S., and Dugué, M.J. (2008). Participatory Learning and Action Research (PLAR) for Integrated Rice Management (IRM) in *Inland Valleys of Sub-Saharan Africa: Technical Manual. AfricaRice Training Series*. Cotonou, Benin: Africa Rice Center. 128 pp.

Yuan, X., Song, L., Zhang, J., Zang, L., Zhu, L., Ruan, C., and Sun, G. (2012). Performance of four Chinese *Trichogramma* species as biocontrol agents of the rice striped stem borer, *Chilo suppressalis*, under various temperature and humidity regimes. *Journal of Pest Science*, *85*, 497–504. https://doi.org/10.1007/s10340-012-0456-8

Yunus, M. (2018). *Effectiveness of Trichogramma japonicum Utilization for Biological Control Agents on Scirpophaga Incertulas in Indonesia*'. https://doi.org/10.3923/ajcs.2018.31.39