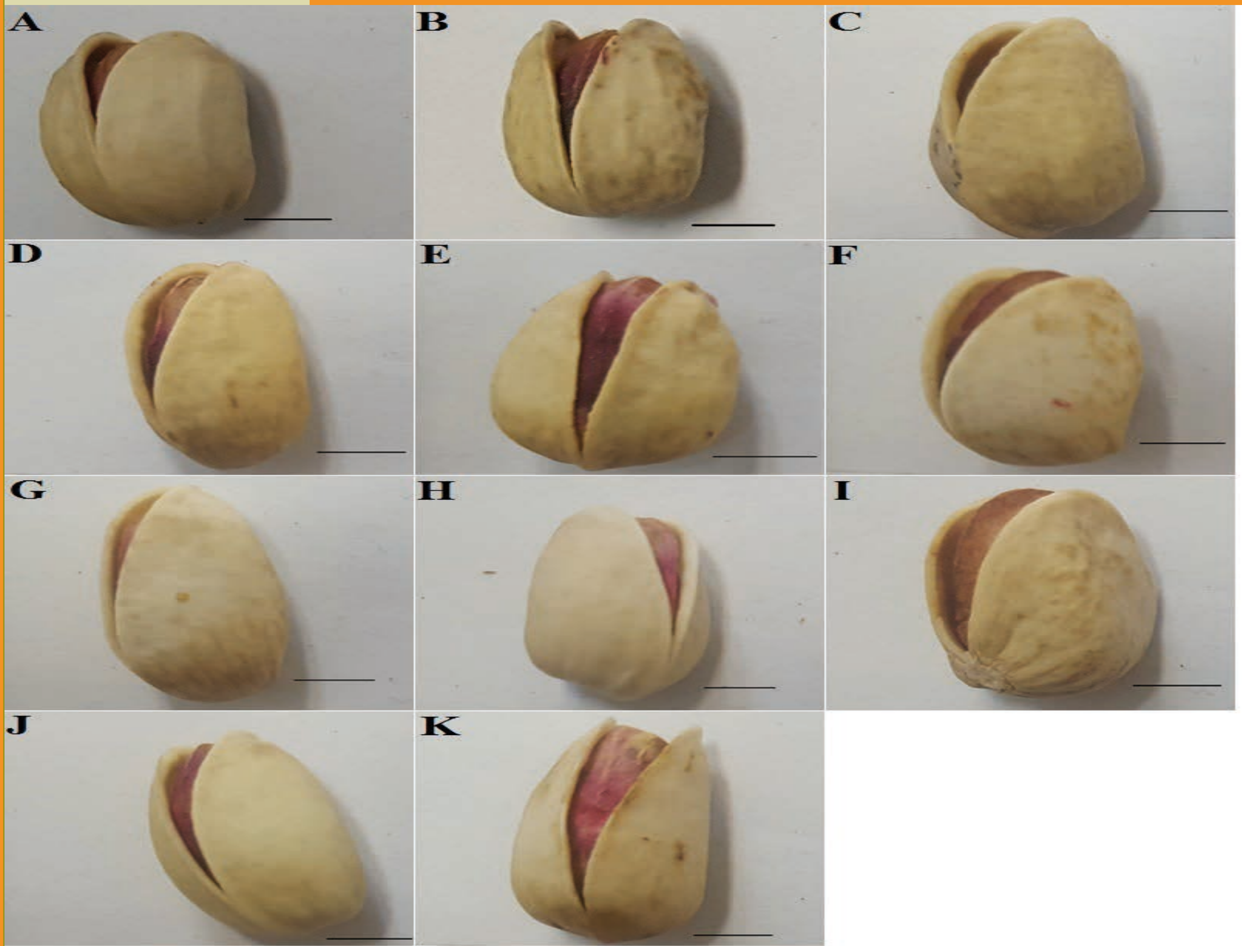

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Ovitek: Oblika plodov preučevanih sort pistacije (črke označujejo sorto glede na preglednico 1; merilo 5 mm); (foto: Fariba Sharifnia, 1–10)
Cover: Fruit shape of the investigated pistachio cultivars (the letters indicate cultivars name according to Table 1, scale bar 5 mm); (photo: Fariba Sharifnia, 1–10)

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Phenolic contents, antioxidant activity and colour density of Slovak Pinot Noir wines

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Phenolic contents, antioxidant activity and colour density of Slovak Pinot Noir wines

Abstract: Recent studies show that wine contains more than thousand different compounds that could come from grapes, or could be formed in the process of winemaking and maturing. The most abundant compounds in wines are polyphenols, which affect sensory properties such as colour, taste and aroma, but also has antioxidant properties. The aim of this study was to determine total polyphenol and total anthocyanin contents, and to evaluate antioxidant effects and wine colour density of red wines 'Pinot Noir' produced in Slovakia. Thirteen analysed, bottled, quality dry 'Pinot Noir' wines with origin in various Slovak wine regions were purchased in retail network, to provide that analysed samples of wine would have the same properties as wines that are consumed by common consumers. The content of total polyphenols in analysed 'Pinot Noir' wines ranged from 1458 to 3324 mg GAE l^{-1}, while contents of total anthocyanins ranged from 43.6 to 279.6 mg l^{-1}. Antioxidant activities ranged from 80.2 % to 85.3 % inhibition of DPPH and wine colour density ranged from 0.679 to 1.495. The highest total polyphenol content, total anthocyanin content, and wine colour density was determined in wines from the south Slovakia winegrowing region, while the highest antioxidant activity in wines from Nitra winegrowing region. Results did not show significant differences among studied parameters in wines from different winegrowing regions. Results showed that Slovakian 'Pinot Noir' wines have characteristics comparable with 'Pinot Noir' wines from other countries.

Key words: wine; polyphenols; antioxidant activity; anthocyanins; 'Pinot Noir'

Vsebnost fenolov, antioksidacijska aktivnost in obarvanost slovaških vin iz sorte Pinot Noir

Izvleček: Sedanje raziskave kažejo, da vsebujejo vina več kot tisoč različnih spojin, ki izvirajo iz grozdja ali pa se lahko tvorijo v procesu pridelave in zorenja vina. Najpogostejše spojine v vinu so polifenoli, ki vplivajo na senzorične lastnosti vina kot so barva, okus in aroma, imajo tudi antioksidativne lastnosti. Namen raziskave je bil določiti celokupno vsebnost polifenolov in antocianinov in ovrednostiti obarvanost rdečih vin, ki se pridelujejo na Slovaškem iz sorte Pinot Noir. Analizirano je bilo trinajst ustekleničenih kakovostnih suhih vin črnega pinoja, ki so izvirala iz različnih vinorodnih območij Slovaške, pridobljenih iz prodaje na drobno, da bi se zagotovili vzorci vina z enakimi lastnostmi kot jih ima vino v splošni porabi. Vsebnost celokupnih polifenolov v analiziranih vzorcih črnega pinoja je bila v območju od 1458 do 3324 mg GAE l^{-1}, medtem, ko je bila vsebnost celokupnih antocianinov v območju od 43,6 do 279,6 mg l^{-1}. Antioksidacijska aktivnost je bila v območju od 80,2 % do 85,3 % inhibicije DPPH, obarvanost vina pa je bila v območju od 0,679 do 1,495. Največje vsebnosti celokupnih polifenolov in antocianinov in največja obarvanost so bile določene v vzorcih vina iz južne Slovaške, največja antioksidacijska aktivnost pa v vinih iz vinorodnih območij Nitre. Izsledki niso pokazali značilnih razlik v preučevanih parametrih vin iz različnih vinorodnih območij, pokazali pa so, da so lastnosti slovaškega črnega pinoja primerljivi s črnimi pinoji iz drugih dežel.

Ključne besede: vino; polifenoli; antioksidacijska aktivnost; antocianini; 'Pinot Noir'

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1 INTRODUCTION

The wine contains a number of polyphenolic substances that can affect its important sensory properties, such as colour, taste, bitterness and astringency (Ivanova-Petropulos et al., 2015). Phenolic substances are involved mainly in the colour changes of grapes, and play a key role in determining the quality of the wine. Antioxidant properties of phenolic compounds have positive impact on the wine stability. Their concentration in wine is affected by temperature and time of maceration, presence of SO_2, pH, and process of micro-oxygenation (Mulero et al., 2015). Main phenolic compounds in red wines are tannins, anthocyanidins, flavonols, flavan-3-ols, and stilbenes (Moreno and Peinado, 2012).

In viniculture, polyphenolic compounds play a very important role, because they affect the character, quality, taste, and colour of red wines (Li et al., 2009). The main source of polyphenols in wines are grape berries. They are in skin, pulp, seeds, and grape juice (Jackson, 2008). The final composition of polyphenolic compound in wine depends mainly on their content in grapes, which depends on many factors, such as climatic conditions, extraction, as well as winemaking technologies, and chemical reactions during the aging of wine (Atanacković et al., 2012).

Colour is one of the most important properties of red wines. Main cause of the red colour of wine are anthocyanins and their derivatives, which are formed during the fermentation process. Colour of red wine is influenced by many factors, including type and content of anthocyanins, pH, free SO_2 content, and extent of polymerization and co-pigmentation (Versari et al., 2008). During the first two years of wine maturation, monomeric anthocyanins go through a wide series of reactions, in which new pigments, important for colour stability, are formed. Although anthocyanins are odourless and almost tasteless, they can interact with other aromatic substances, and thus affect the taste of wine. Anthocyanins are water soluble flavonoid pigments, which contribute to the red, violet, or blue colour of the grapes, depending on the pH (He et al., 2012). Monomeric forms of anthocyanins are responsible for the red colour of young wines, and contribute to the development of red polymer pigments during the wine maturation (Versari et al., 2008). Main monomeric anthocyanins of red wines are 3-O-monoglucosides, which include delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, and malvidin-3-O-glucoside (Jackson, 2008).

The antioxidant activity of anthocyanins is considered to be one of their most important physiological functions (Yang et al., 2009). Intake of anthocyanins has been linked to a number of human health benefits. They have strong antioxidant properties, and act as protective agents against many chronic diseases (Welch et al., 2008).

'Pinot Noir' is intended mainly for the production of quality varietal wines in the category of the late harvest to grape selection. It has a genetically lower content of anthocyanins. The usual alcohol content in these wines is about 13 vol. %. The wines are lighter brick colour and their aroma is distinctly fruity reminiscent of cherries, plums, and forest fruits. Wines made from this variety are usually extractive with a pleasant taste of tannins and are suitable for archiving (Pavloušek, 2007). In ordinary vintages, it provides soft, velvety, alcoholic, full-bodied wines with a delicious almond bouquet. They reach their peak at the bottle maturity that, according to the year and quality, sometimes appears only after several years (Malík et al., 2005). According to the Vineyard Register of the Slovak Republic (2020), the total area of bearing vineyards as of 31.7.2020 was 11090 ha. Red grapevine varieties represent 3226 ha, ‚Pinot Noir' represents 223 ha. In the last decade, there has been a decrease of the total vineyard area by 23.2 %. According to OIV (2020), wine production in Slovakia in 2020 was cca 300000 hl, with Pinot Noir representing less than 1 % of it.

The aim of this study was to determine and evaluate properties and their mutual correlations in Slovak wines Pinot Noir, and to compare them with other Slovak red wines.

2 MATERIALS AND METHODS

2.1 SAMPLES

Analysed, bottled, quality dry Pinot Noir (PN) wines and their characteristics are mentioned in Table 1. Wine samples with origin in various Slovak wine-growing regions (WR) were purchased in retail network, to provide that analysed samples of wine would have the same properties as wines that are consumed by common consumers.

2.2 CHEMICALS AND INSTRUMENTS

The chemicals used for all analysis were: Folin-Ciocalteau reagent, monohydrate of gallic acid p. a., anhydrous sodium carbonate p. a., citric acid p. a., disodium hydrogenphosphate dodecahydrate, 35 % hydrochloric acid p. a., ethanol p. a., methanol p. a., 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical p. a., Trolox (pure).

Table 1: Characteristics of analysed wines

Sample	Producer	Winegrowing region	Quality	Vintage
PN-LC1	Karpatská Perla, Šenkvice	Little Carpathian	Grape selection	2011
PN-LC2	Mrva a Stanko, s. r. o., Trnava	Little Carpathian	Grape selection	2012
PN-LC3	VPS, s. r. o., Pezinok	Little Carpathian	Grape selection	2013
PN-LC4	Lacko & Majtán, Malacky	Little Carpathian	Quality	2012
PN-SS1	Villa Víno Rača, a. s., Bratislava	South Slovak	Late harvest	2013
PN-SS2	Víno Matyšák, s. r. o., Pezinok	South Slovak	Grape selection	2012
PN-SS3	Vinárske závody Topoľčianky, s. r. o.	South Slovak	Quality	2013
PN-SS4	Vinárske závody Topoľčianky, s. r. o.	South Slovak	Late harvest	2013
PN-SS5	Víno Nitra / Ch. Modra, Trnava	South Slovak	Grape selection	2012
PN-N1	Muráni-Víno Čajkov, s. r. o., Čajkov	Nitra	Cabinet	2010
PN-N2	Agropest, s. r. o., Veľký Cetín	Nitra	Grape selection	2012
PN-N3	Pivnica Radošina, s.r.o. Trnava	Nitra	Grape selection	2012
PN-N4	PD Mojmírovce	Nitra	Grape selection	2012

PN – Pinot Noir, LC – Little Carpathian winegrowing region, SS – South Slovakia winegrowing region, N – Nitra winegrowing region

All analysed parameters – total polyphenol content, total anthocyanin content, antioxidant activity and wine colour density in wines were analysed by UV/VIS spectrophotometry (spectrophotometer Shimadzu UV/VIS – 1240, Shimadzu, Japan).

2.3 WINE ANALYSIS

2.3.1 Determination of total polyphenol content

Total polyphenol content (TPC) was assessed by the modified method of Singleton & Rossi (1965) using of 20 % solution of NA_2CO_3, Folin-Ciocalteau reagent and distilled water. 1 ml of wine sample was pipetted into 50 ml volumetric flask and diluted with 25 ml of distilled water. Then, 2.5 ml Folin-Ciocalteau reagent was added to dilute the mixture, and after 3 minutes, 1.5 ml of 20 % solution of Na_2CO_3 was added. Then, the sample was filled with distilled water to volume 50 ml, and after mixing, left at the laboratory temperature for 2 hours. The blank and calibration solutions of gallic acid were prepared by the same procedure. The absorbance of samples solutions was measured against blank solution at 765 nm. The content of total polyphenols in wines was calculated as the amount of gallic acid equivalent (GAE) in mg per 1 litre of wine (mg GAE.l^{-1}).

2.3.2 Determination of antioxidant activity

Antioxidant activity (AA) was assessed by the method of Brand-Williams et al. (1995) using of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical. Exactly, 3.9 ml of DPPH solution was pipetted into cuvette. The absorbance of DPPH solution was measured at 515.6 nm. Then, 0.1 ml of wine sample was added, stirred, and left for 10 minutes. After 10 minutes, absorbance at 515.6 nm was measured, and antioxidant activity was expressed as % inhibition of DPPH, and also as Trolox equivalent calculated from the calibration curve (TE l^{-1})

2.3.3 Determination of total anthocyanin content

Total anthocyanin content (TAC) was assessed by the modified pH differential method of Lapornik et al. (2005), based on the transformation of all anthocyanins to red coloured flavylium cation by reduction of the pH of wine samples with HCl solution to values 0.5-0.8. The total anthocyanin content was calculated from the difference of absorbance values of both solutions (diluted original and a sample of wine acidified with HCl) and expressed as the amount of malvidin-3-monoglucoside in mg l^{-1} of wine.

2.3.4 Determination of wine colour density

Wine colour density (WCD) was measured by the method of Sudrand (1958) as the sum of the absorbance at 420 nm and 520 nm. The absorbance of the wine samples was measured in 0.2 cm path length glass cells. WCD was also expressed in the absorbance unit (AU) considering dilution factor (R = 5), for obtaining better comparison with other authors.

All chemical analyses were performed as four parallels. Results were expressed by average ± standard deviation.

2.4 STATISTICAL ANALYSIS

MS Excel 2016 and XLSTAT were used to perform statistical analysis. To obtain statistically significant information about the differences between the tested samples, nonparametric Kruskal-Wallis test was conducted (Addinsoft, 2014).

3 RESULTS AND DISCUSSION

All studied parameters – total polyphenols content (TPC), total anthocyanins content (TAC), antioxidant activity (AA) and wine colour density (WCD) of the Slovak Pinot Noir wines are described in Table 2.

Total polyphenols content (TPC) in analysed wines ranged from 1458 to 3324 mg GAE l^{-1}, reaching an average TPC 2334 mg GAE.l^{-1}. TPC in Slovak Pinot Noir wine was about the same as TPC in Argentinian Pinot Noir wines (2319 mg GAE l^{-1}), higher than TPC in Croatian (1825 mg GAE l^{-1}), Italian (2029 mg GAE l^{-1}), French (2062 mg GAE l^{-1}) and Chilean Pinot Noir wines (1759 mg GAE l^{-1}), but lower than TPC in Czech (8714 mg GAE l^{-1}) and French Pinot Noir wines (3545 mg GAE.l^{-1}) (Landrault et al., 2001; Mlček et al., 2019; Šeruga et al., 2011; Van Leeuw et al., 2014). Previous studies of Slovak red wines showed about the same TPC in Blaufränkisch wines – 2003 mg GAE.l^{-1}, Saint Laurent wines – 2297 mg GAE $l^{-1)}$, Cabernet Sauvignon wines – 2424 mg GAE l^{-1}, and higher TPC in Slovak Alibernet wines – 3057 mg GAE l^{-1} (Bajčan et al., 2012; Bajčan et al., 2015; Bajčan et al., 2016).

According to the average TPC, wines from SSWR reached the highest content (2543 mg GAE l^{-1}), followed by wines from LCWR (2418 mg GAE l^{-1}) and wines from NWR (1990 mg GAE l^{-1}). However, the results did not show significant differences in TPC among Pinot Noir wines from different vineyard areas in Slovakia, as shown in Figure 1.

Total anthocyanins content (TAC) in analysed wines ranged from 43.6 to 279.2 mg l^{-1}, reaching an average TAC 153.3 mg l^{-1}. TAC in Slovak Pinot Noir wines was higher than TAC in Uruguayan Pinot Noir wines

Table 2: Total polyphenols content, total anthocyanins content, antioxidant activity and wine colour density in analysed Pinot Noir wines from Slovakia

Sample	TPC (mg GAE l^{-1})	TAC (mg l^{-1})	AA (%)	AA (mmol TE l^{-1})	$WCD_{0.2}$	$WCD_{1.0}$ (AU)
PN-LC1	3138 ± 52	128.5 ± 1.4	81.9 ± 0.3	1.015 ± 0.004	0.956 ± 0.011	4.78 ± 0.055
PN-LC2	3039 ± 26	167.4 ± 1.4	80.5 ± 0.5	0.992 ± 0.006	1.049 ± 0.008	5.245 ± 0.04
PN-LC3	2035 ± 26	229.6 ± 1.8	83.9 ± 0.5	1.050 ± 0.006	0.771 ± 0.015	3.855 ± 0.075
PN-LC4	1458 ± 25	82.5 ± 1.2	84.5 ± 0.4	1.061 ± 0.006	0.918 ± 0.014	4.59 ± 0.07
Average LCWR	2418 ± 816[a]	152 ± 71.4[a]	82.7 ± 1.9[a]	1.030 ± 0.034[a]	0.924 ± 0.135[a]	4.618 ± 0.675[a]
PN-SS1	2604 ± 13	271.3 ± 9.8	80.7 ± 0.5	0.995 ± 0.006	1.495 ± 0.021	7.475 ± 0.105
PN-SS2	2690 ± 24	69.8 ± 1.4	85.3 ± 0.4	1.074 ± 0.005	0.679 ± 0.006	3.395 ± 0.03
PN-SS3	1777 ± 24	279.2 ± 1.4	83.8 ± 0.3	1.048 ± 0.004	0.959 ± 0.008	4.795 ± 0.04
PN-SS4	3324 ± 26	159.4 ± 2.1	81.0 ± 0.4	1.000 ± 0.005	1.113 ± 0.016	5.565 ± 0.08
PN-SS5	2318 ± 25	101.5 ± 1.6	82.7 ± 0.4	1.029 ± 0.006	1.045 ± 0.009	5.225 ± 0.045
Average SSWR	2543 ± 751[a]	176.2 ± 101.7[a]	82.7 ± 2.2[a]	1.029 ± 0.036[a]	1.058 ± 0.396[a]	5.291 ± 1.98[a]
PN-N1	1995 ± 25	43.6 ± 2.1	84.3 ± 0.6	1.057 ± 0.007	0.832 ± 0.007	4.16 ± 0.035
PN-N2	1943 ± 12	87.6 ± 2.8	80.2 ± 0.5	0.987 ± 0.006	1.156 ± 0.016	5.78 ± 0.08
PN-N3	1895 ± 24	199.5 ± 1.4	84.1 ± 0.4	1.053 ± 0.005	0.779 ± 0.01	3.895 ± 0.05
PN-N4	2125 ± 25	173.6 ± 2.1	83.6 ± 0.6	1.042 ± 0.007	0.923 ± 0.015	4.615 ± 0.075
Average NWR	1990 ± 112[a]	126.1 ± 75.Z[a]	83.1 ± 2.0[a]	1.035 ± 0.034[a]	0.923 ± 0.183[a]	4.613 ± 0.915[a]
Total average	2334 ± 577	153.3 ± 76.4	82.8 ± 1.7	1.031 ± 0.030	0.975 ± 0.210	4.875 ± 1.05

Different letters indicate significant differences ($p < 0.05$) among different winegrowing regions.

(78.1 mg l^{-1}), and lower than TAC in Australian Pinot Noir wines (190 mg l^{-1}, 232 mg l^{-1}) (Carew et al., 2013; Piccardo et al., 2019; Song et al., 2014).

Previous studies of Slovak red wines showed higher TAC in Blaufränkisch wines – 266.1 mg l^{-1}, Saint Laurent wines – 264.0 mg l^{-1}, Cabernet Sauvignon wines – 220.6 mg l^{-1}, and Alibernet wines – 403.0 mg l^{-1}. (Bajčan et al., 2012; Bajčan et al., 2015; Bajčan et al., 2016).

According to the average TAC, wines from SSWR reached the highest content (176.2 mg l^{-1}), followed by wines from LCWR (152 mg l^{-1}) and wines from NWR (126 mg l^{-1}). However, the results did not show significant differences in TAC among Pinot Noir wines from different vineyard areas in Slovakia, as shown in Figure 1.

Antioxidant activity (AA) in analysed wines ranged from 80.2 % (0.987 mmol TE l^{-1}) to 85.3 % (1.074 mmol TE l^{-1}), reaching an average AA 82.8 % (1.031 mmol TE l^{-1}.) AA in Slovak Pinot Noir wines was higher than AA in South American Pinot Noir wines (47.93 - 66.70 %.), but lower than AA in Croatian Pinot Noir wines (4.3 mmol TE l^{-1}) (Granato et al., 2011; Šeruga et al., 2011). Previous studies of Slovak red wines showed about the same AA in Blaufränkisch wines – 83.8 %, Saint Laurent wines – 81.2 %, Cabernet Sauvignon wines – 78.8 %, and lower average AA in Slovak Alibernet wines – 74.5 % (Bajčan et al., 2012; Bajčan et al., 2015; Bajčan et al., 2016).

According to the average AA, wines from NWR reached the highest content (83.1 %), followed by wines from LCWR (82.7 %) and wines from SSWR (82.7 %). However, the results did not show significant differences in TAC among Pinot Noir wines from different vineyard areas in Slovakia, as shown in Figure 1.

Wine colour density (WCD) in analysed wines ranged from 0.679 (3.395 AU) to 1.459 (7.475 AU), reaching an average WCD 0.975 (4.875 AU). Song et al., (2014) reported about the same average WCD in Australian Pinot Noir wines (3.61 - 8.47 AU). WCD in Slovak Pinot Noir wines was higher than WCD in Australian Pinot Noir wines (2.4 - 3.7 AU) (Carew et al., 2013). Previous studies of Slovak red wines showed higher WCD in Cabernet Sauvignon wines – 1.399 and Alibernet wines – 2.317 (Bajčan et al., 2015; Bajčan et al., 2016).

According to the average WCD, wines from SSWR reached the highest content (1.058), followed by wines from LCWR (0.924) and wines from NWR (0.923). However, the results did not show significant differences in WCD among Pinot Noir wines from different vineyard areas in Slovakia, as shown in Figure 1.

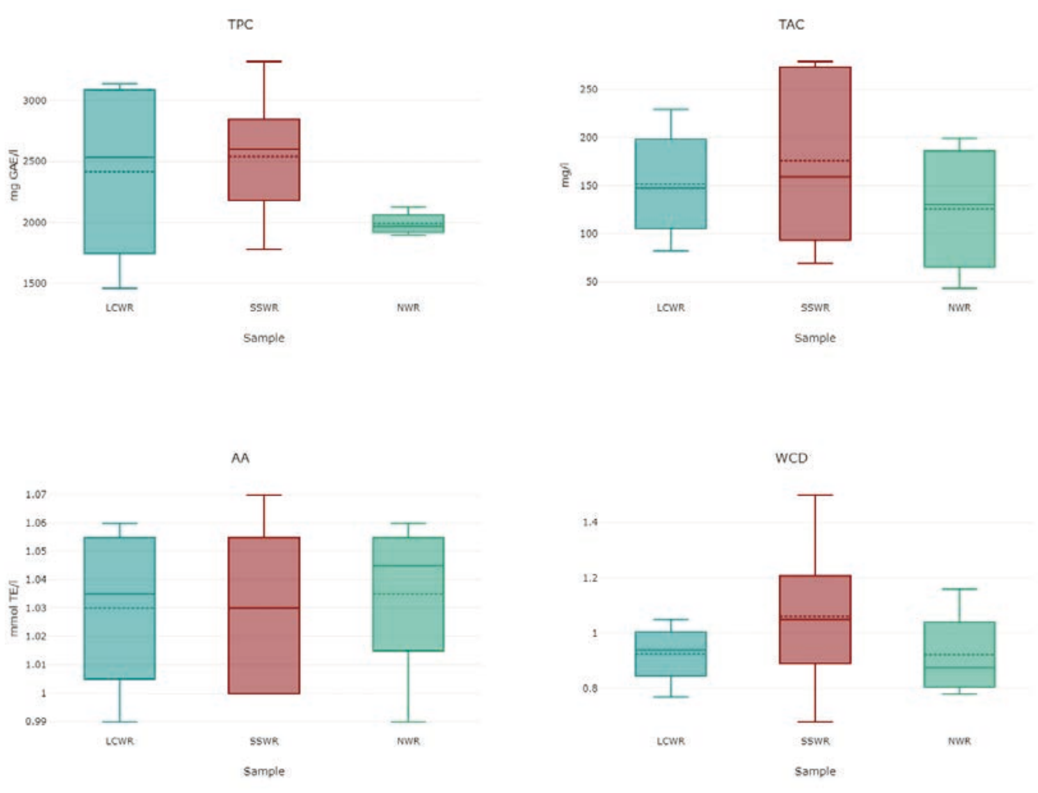

Figure 1: Differences among individual properties of wines from different winegrowing regions

In order to examine the mutual relations among analysed parameters, the linear regressions were made. Results are shown in Figure 2. The statistical evaluation of the obtained results confirmed strong negative linear correlation between AA and WCD (r = -0.825), which is in accordance with previous reports by Bajčan et al. (2016) for Slovak Cabernet Sauvignon wines and for Slovak Alibernet wines (Bajčan et al., 2015). Furthermore, there were not confirmed correlations between TPC and TAC (r = 0.01), between TPC and AA (r = -0.052), between TPC and WCD (r = 0.277), between TAC and AA (r = -0.171), and between TAC and WCD (r = 0.038). Bajčan et al. (2015) and Bajčan et al. (2016) reported moderate positive correlations between TPC and TAC (r = 0.542), TAC and WCD (r = 0.600), and TPC and WCD (r = 0.697) in Slovak Cabernet Sauvignon wines and moderate positive correlation between TPC and TAC (r = 0.447), TAC and WCD (r = 0.660), moderate negative correlation between TAC and AA (r = -0.532), and strong positive correlation between WCD and TPC (r = 0.887), and strong negative correlation between TPC and AA (r = -0.917) in Slovak Alibernet wines. Based on our results, it can be stated that there are no strong correlations between the individual monitored properties of wines, except for AA and WCD. These correlations are unusual and in disagreement with other authors. Granato et al. (2011) reported moderate positive correlation between TPC and AA (r = 0.59) in Australian Pinot Noir wines. Šeruga et al. (2011) reported strong positive correlation between TPC and AA in Croatian Pinot Noir wines (r = 0.9885).

4 CONCLUSIONS

Total phenolic contents, total anthocyanin contents, antioxidant activities and wine colour densities of Pinot Noir wines from three vineyard regions of Slovakia was determined in this study. Studied Pinot Noir wines showed high antioxidant activity, content of polyphenols and anthocyanins, the substances that contribute to the various health benefits.

The highest total polyphenol content, total anthocyanin content, and wine colour density was determined in wines from South Slovakia winegrowing region, while the highest antioxidant activity in wines from Nitra winegrowing region. At the end, Slovak Pinot Noir wines showed lower wine colour density in comparison to other Slovak wines. Results did not show significant differences among studied parameters in wines from different winegrowing regions. Based on statistical evaluation, strong negative correlation between antioxidant activity and wine colour density was determined.

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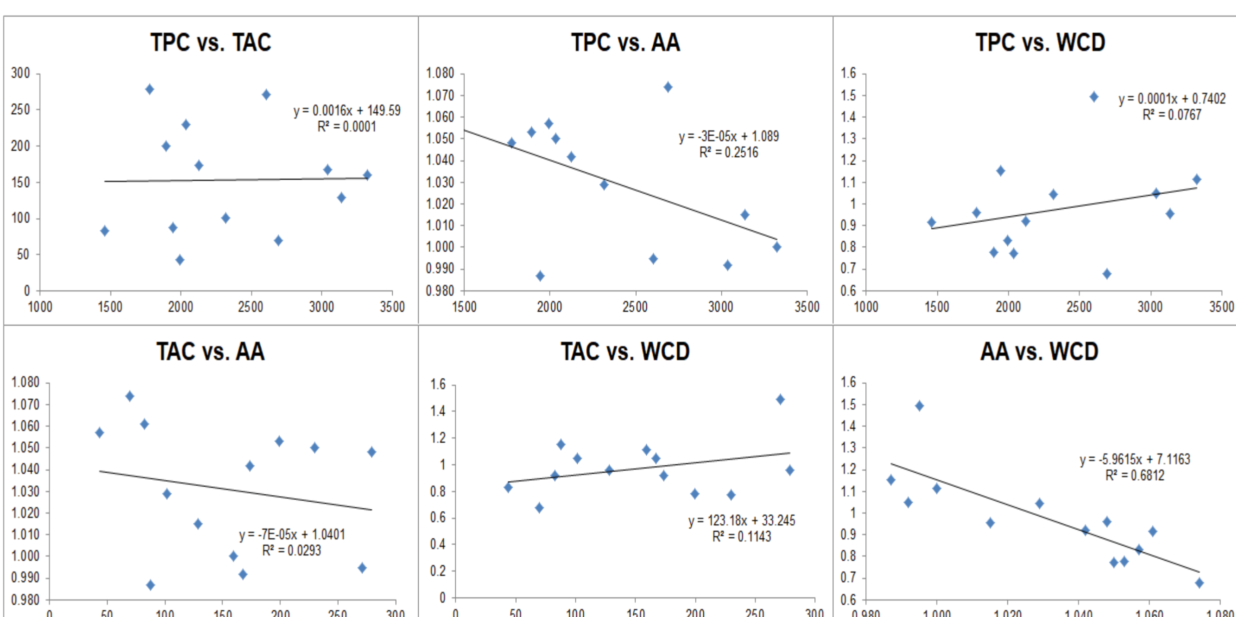

Figure 2: Correlations among analysed parameters

6 REFERENCES

Addinsoft. (2014). *XLSTAT, Analyse de données et statistique avec MS Excel*. Addinsoft, NY, USA.

Atanacković, M., Petrović, A., Jović, S., Gojković-Bukarica, L., Bursać, M., Cvejić, J. (2012). Influence of winemaking techniques on the resveratrol content, total phenolic content and antioxidant potential of red wines. *Food Chemistry*, *131*(2), 513-518. https://doi.org/10.1016/j.foodchem.2011.09.015

Bajčan, D., Čéryová, S., Tomáš, J. (2012). Antioxidant properties of the bestselling Slovak red wines. *Journal of Microbiology*, *Biotechnology and Food Sciences*, *1*(4), 455-465. https://doi.org/10.15414/jmbfs.2015.4.special3.5-8

Bajčan, D., Šimanský, V., Tóth, T., Árvay, J. (2015). Colour, Phenolic content and antioxidant activity of the Slovak Alibernet red wine samples. *Journal of Microbiology, Biotechnology and Food Sciences*, *4*(3), 5-8. https://doi.org/10.15414/jmbfs.2015.4.special3.5-8

Bajčan, D., Vollmannová, A., Šimanský, V., Bystrická, J., Trebichalský, P., Árvay, J. Á. J., Czako, P. (2016). Antioxidant activity, phenolic content and colour of the Slovak cabernet sauvignon wines. *Potravinarstvo Slovak Journal of Food Sciences*, *10*(1), 89-94. https://doi.org/10.5219/534

Brand-Williams, W., Cuvelier, M. E., Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, *28*(1), 25-30. https://doi.org/10.1016/s0023-6438(95)80008-5

Carew, A. L., Smith, P., Close, D. C., Curtin, C., Dambergs, R. G. (2013). Yeast Effects on Pinot noir Wine Phenolics, Color, and Tannin Composition. *Journal of Agricultural and Food Chemistry*, *61*(41), 9892–9898. doi:10.1021/jf4018806

Granato, D., Katayama, F. C. U., de Castro, I. A. (2011). Phenolic composition of South American red wines classified according to their antioxidant activity, retail price and sensory quality. *Food Chemistry*, *129*(2), 366–373. doi:10.1016/j.foodchem.2011.04.085

He, F., Liang, N. N., Mu, L., Pan, Q. H., Wang, J., Reeves, M. J., Duan, C. Q. (2012). Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expression. *Molecules*, *17*(2), 1571-1601. https://doi.org/10.3390/molecules17021571

Ivanova-Petropulos, V., Hermosín-Gutiérrez, I., Boros, B., Stefova, M., Stafilov, T., Vojnoski, B., Kilár, F. (2015). Phenolic compounds and antioxidant activity of Macedonian red wines. *Journal of Food Composition and Analysis*, *41*, 1-14. https://doi.org/10.1016/j.jfca.2015.01.002

Jackson, R. S. (2008). *Wine science: principles and applications*. Academic press. https://doi.org/10.1006/abio.1995.1141

Landrault, N., Poucheret, P., Ravel, P., Gasc, F., Cros, G., Teissedre, P.-L. (2001). Antioxidant Capacities and Phenolics Levels of French Wines from Different Varieties and Vintages. *Journal of Agricultural and Food Chemistry, 49*(7*), 3341–3348.* doi:10.1021/jf010128f

Lapornik, B., Prošek, M., Wondra, A. G. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering*, *71*(2), 214-222. https://doi.org/10.1016/j.jfoodeng.2004.10.036

Li, H., Wang, X., Li, Y., Li, P., Wang, H. (2009). Polyphenolic compounds and antioxidant properties of selected China wines. *Food Chemistry*, *112*(2), 454-460. https://doi.org/10.1016/j.foodchem.2008.05.111

Malík, F. 2005: *Víno Malých Karpát*. Bratislava: Albert Marenčin vydavateľstvo.

Mlček, J., Adámková, A., Škrovánková, S., Adámek, M., Ontrášová, M. (2019). Comparison of antioxidant activity, content of polyphenols and flavonoids in liturgical and common wines. *Potravinarstvo Slovak Journal of Food Sciences*, *13*(1), 218–223. https://doi.org/10.5219/1030

Moreno, J., & Peinado, R. (2012). *Enological Chemistry*. Academic Press. https://doi.org/10.1016/c2011-0-69661-9

Mulero, J., Martínez, G., Oliva, J., Cermeño, S., Cayuela, J. M., Zafrilla, P., Barba, A. (2015). Phenolic compounds and antioxidant activity of red wine made from grapes treated with different fungicides. *Food Chemistry*, *180*, 25-31. https://doi.org/10.1016/j.foodchem.2015.01.141

OIV. (2020). *World wine production- first estimates.* Retrieved from: https://www.oiv.int/public/medias/7541/en-oiv-2020-world-wine-production-first-estimates.pdf

Pavloušek, P. (2008). *Encyklopedie révy vinné*. Computer Press.

Piccardo, D., Favre, G., Pascual, O. (2019) Influence of the use of unripe grapes to reduce ethanol content and pH on the color, polyphenol and polysaccharide composition of conventional and hot macerated Pinot Noir and Tannat wines. *European Food Research and Technology, 245*, 1321–1335. https://doi.org/10.1007/s00217-019-03258-4

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(3), 144-158.

Song, J., Smart, R. E., Dambergs, R. G., Sparrow, A. M., Wells, R. B., Wang, H., Qian, M. C. (2014). Pinot Noir wine composition from different vine vigour zones classified by remote imaging technology. *Food Chemistry*, *153*, 52-59. https://doi.org/10.1016/j.foodchem.2013.12.037

Sudrand, P. (1958). Interpretation des dabsorption des vins rouges. An. *Technology and Agriculture*, *7*, 203-208.

Šeruga, M., Novak, I., Jakobek, L. (2011). Determination of polyphenols content and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods. *Food Chemistry*, *124*(3), 1208-1216. https://doi.org/10.1016/j.foodchem.2010.07.047

Van Leeuw, R., Kevers, C., Pincemail, J., Defraigne, J. O., Dommes, J. (2014). Antioxidant capacity and phenolic composition of red wines from various grape varieties: Specificity of Pinot Noir. *Journal of Food Composition and Analysis*, *36*(1-2), 40–50. doi:10.1016/j.jfca.2014.07.001

Versari, A., Boulton, R. B., Parpinello, G. P. (2008). A comparison of analytical methods for measuring the color components of red wines. *Food Chemistry*, *106*(1), 397-402. https://doi.org/10.1016/j.foodchem.2007.05.073

Vinohradnícky register SR/ ÚKSUP. (2020). *Vinohradnícky register, štatistický prehľad, vinársky rok 2019/2020* Retrieved from: file:///C:/Users/admin/Downloads/Vinohradnicky_register_statistika_vin_rok_2019_2020_web%20(2).pdf

Welch, C. R., Wu, Q., Simon, J. E. (2008). Recent advances in anthocyanin analysis and characterization. *Current Analytical Chemistry*, *4*(2), 75-101. https://doi.org/10.2174/157341108784587795

Yang, J., Martinson, T. E., Liu, R. H. (2009). Phytochemical profiles and antioxidant activities of wine grapes. *Food Chemistry*, *116*(1), 332-339. https://doi.org/10.1016/j.foodchem.2009.02.021

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Evaluation of nuts morphology and composition of fatty acids in certain Iranian *Pistacia vera* L. (Anacardiaceae) cultivars

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Evaluation of nuts morphology and composition of fatty acids in certain Iranian *Pistacia vera* L. (Anacardiaceae) cultivars

Abstract: Fruits of various Pistachio (*Pistacia vera* L.) cultivars are widely used in food industries for its inimitable color, taste and nutrient value. We elevated fruit morphology and kernel fatty acids composition of eleven Iranian cultivars of pistachio. Oils of kernels were extracted using cold press method, and composition of the oil fatty acids in the methyl ester form was detected using gas chromatography (GC). For morphological study, nine qualitative and quantitative traits were evaluated. The quantitative ones widely differed among the studied cultivars, and ANOVA test revealed the significant variations ($p = 0.00$) for all of them. Moreover, the qualitative traits varied among the cultivars. We characterized 11 fatty acid components representing about 99.56 to 100 % of the total oil composition. The principal fatty acids for all the cultivars were: oleic, linoleic and palmitic acids, while their amounts differed among the cultivars. In this regard, unsaturated fatty acids comprised the major oil part, 87.46 to 88.89 %. Oleic acid (53.11-70.99 %) and palmitic acid (9.09 to 10.55 %) were detected as the unsaturated and saturated fatty acids in all the evaluated cultivars. The quality index of oils were determined according to oleic/ linoleic acids ratio, which highly varied among the cultivars. According to UPGMA tree and PCO plot, we divided the investigated cultivars into four chemotypes, and each of them was characterized by the certain oil composition.

Key words: saturated fatty acid; unsaturated fatty acid; pistachio; gas chromatography; Iran

Ovrednotenje morfologije oreščkov in sestave maščobnih kislin v nekaterih iranskih sortah pistacije, *Pistacia vera* L. (Anacardiaceae)

Izvleček: Plodovi/semena različnih sort pistacije (*Pistacia vera* L.) se naširoko uporabljajo v prehrambeni industriji zaradi njihove neposnemljive barve, okusa in hranilne vrednosti. V raziskavi smo dali poudarek na morfologijo plodov in sestavo maščobnih kislin v jedrcih enajstih iranskih sort pistacije. Olja iz jedrc so bila hladno stisnjena, sestava maščobnih kislin je bila v obliki metil estrov določena s plinsko kromatografijo (GC). V morfološki raziskavi je bilo ovrednoteno devet količinskih in kakovostnih lastnosti. Količinske lastnosti so se med sortami zelo razlikovale in ANOVA test je odkril med vsemi značilne razlike ($p = 0.00$). Tudi kakovostne lastnosti so se med sortami razlikovale. Določili smo 11 maščobnih kislin, ki so predstavljale okrog 99,56 do 100 % celokupne sestave olja. Najpomembnejše maščobne kisline v vseh sortah so bile oleinska, linolenska in palmitinska kislina, pri čemer se je njihova količina v posameznih sortah razlikovala. V tem pogledu so nenasičene maščobne kisline sestavljale večji del olja, od 87,46 do 88,89 %. Oleinska kislina (53,11-70,99 %) in palmitinska kislina (9,09-10,55 %) sta bili ugotovljeni kot nenasičena in nasičena maščobna kislina v vseh ovrednotenih sortah. Kakovostni indeks olja, določen kot razmerje med oleinsko in linolensko kislino, se je med sortami zelo razlikoval. Glede na razvrstitve v UPGMA drevesu in PCO polju smo preučene sorte razdelili v štiri kemotipe, od katerih je imel vsak posebno sestavo olja.

Ključne besede: nasičena maščobna kislina; nenasičena maščobna kislina; pistacija; plinska kromatografija; Iran

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1 INTRODUCTION

The genus *Pistacia* L. belongs to Anacardiaceae, order Sapindales according to APG III (2009). Phylogenetic analyses according to phenotypical characteristics revealed that the genus definite as a monophyletic group and comprises of two sections: *Pistacia* and *Lentiscus* (AL-Saghir, 2009).

Taxa of the genus are deciduous or evergreen and dioecious trees, with stems up to 9 m high. The leaves are pinnately-compound containing round-ovate to elliptical leaflets. Female as well as male flowers are apetalous, wind-pollinated, subtended by small bracts and bracteoles, arranged in panicles or racemes inflorescences. In male flowers, 4-5 anthers are arranged on a disc. Female flowers have a short, 3-fided style and produce a drupe fruit (AL-Saghir, 2006; Khatamsaz, 1989).

According to several studies (Parfitt and Badenes, 1997; Kafkas and Perl-Treves, 2001; Kafkas et al., 2002), the genus had been originated in Central Asia more than 75 million years ago, and has two genetic diversity centers (1) Mediterranean region of Europe, Northern Africa, as well as the Middle East, and (2) West (Eastern slopes of Zagros mountains in Iran) and Central Asia (Crimea to the Caspian Sea).

Pistacia vera L. (cultivated pistachio) belongs to section *Pistacia* and based on RAPD molecular data, *P. khinjuk* Stocks and *P. vera* are closely related taxa (Al-Saghir, 2009).

Zohary (1952) believed that pistachio is the ancestral species and other *Pistachia* taxa are probably its derivatives. It is the only *Pistachia* commercially cultivated species, and the others are mostly employed as rootstocks (Bozorgi et al., 2013)

Pistachio is ecologically adapted to a wide range of soil conditions and is probably more tolerant to saline and alkaline soil than most other crops. Besides, these trees grow in hot and dry desert-like habitats (Tous and Ferguson, 1996).

Based on the FAO (2010) reports, Iran, USA, Turkey and Syria are considered as the major producers of pistachio in the world.

Pistachio has several bioactive compounds, which the body of human can assimilate and use them (Noguera-Artiaga et al., 2019). For example, its fruit is considered as the food material with the largest antioxidant capacity and also a rich source of phenolic metabolites (Noguera-Artiaga et al., 2019; Dreher, 2012). The nuts of this tree contain several flavonoids such as cyanindin-3-O-glucoside, quercetin, kaempferol and epicatechin.

Moreover, Mandalari et al. (2013) suggested that polyphenol compounds of this nut is biologically accessible during simulated human digestion, consequently nearly 91 % of its total amount release in the gastric organ.

Several studies (Kasliwal et al., 2015; Kocyigit et al., 2006; Dreher, 2012) revealed that pistachio nuts have a larger amount of monounsaturated fatty acids and a lower ratio of polyunsaturated to saturated fatty acids, in comparison with other nuts. It reveals that pistachio has cholesterol-reducing potential, and its low glycemic index reduces the diabetes risk.

The physical properties (morphology) of fruit such as length, width, diameter and color are considered as the important features which influence consumer preference in pistachio fruit (Zarei et al., 2014).

Although, there have been some studies on the fruit morphological characteristics and composition of fatty acids of pistachio cultivars from Iran (Roozban et al., 2006; Mazinani et al., 2012; Abdoshahi et al., 2011; Esteki et al., 2019; Yahyavia et al., 2020) and other countries (Dogan ,et al. 2010; Satil et al. 2003; Arena et al. 2007), these studies did not include all pistachio cultivars. So in the current evaluation, we studied the morphological characteristics and composition of the fatty acids in eleven Iranian pistachio cultivars. The aims of the study were: (1) to determine morphological variability in qualitative and quantitative fruits characteristics, (2) to study fatty acids composition of kernels, and (3) to detect quality index of kernels oil. As far as we could search, two cultivars have been studied for the first time in the world, including: 'Fakhri' and 'Menghar-Kalaghi'.

2 MATERIAL AND METHODS

2.1 PLANT SAMPLES

Plant materials of the current study were the fruits of eleven pistachio cultivars which were harvested from Semnan province (Table 1).

We harvested pistachio fruits and after morphological examinations, removed their shells and dried in an oven at 55 °C for 72 h.

2.2 MORPHOLOGICAL STUDIES

In order to compare the fruits of cultivars morphologically, nine qualitative and quantitative characteristics were studied: fruit length, width, length/width ratio, and diameter, epicarp color, kernel coat color, kernel color and endocarp apical shape. The quantitative traits were measured based on the method described

Table 1: Codes, names and localities of cultivars.

Code	Name of cultivars	Localities
A	Kalleh Ghochi-white	Semnan province,Damghan,Saleh Abad village.
B	Shahpasand white	Semnan province, Damghan, Saleh Abad village.
C	Akbari red	Semnan province, Damghan, Saleh Abad village.
D	Khanjari	Semnan province, Damghan, Saleh Abad village.
E	Kalleh-Ghochi red	Semnan province, Damghan, Saleh Abad village.
F	Shahpasand red	Semnan province, Damghan, Saleh Abad village.
G	Fakhri	Semnan province, Damghan, Saleh Abad village.
H	Akbari white	Semnan province, Damghan, Saleh Abad village.
I	Abasali	Semnan province, Damghan, Saleh Abad village.
K	Ahmad Aghaei	Semnan province, Damghan, Saleh Abad village.
L	Menghar Kalaghi	Semnan province, Damghan, Saleh Abad village.

by Gavit (1990). The seed dimension measurements including width and length were performed by a standard ruler. The fruit length was measured parallel to hilum, while the fruit width was measured at the fruit broadest part. We investigated the qualitative characteristics according to descriptive terminology of Stearn (1985).

2.3 OILS EXTRACTION AND PREPARATION OF THEIR METHYL ESTERS

The oil extraction was performed by pressing of 100 g pistachio kernels of each cultivar using Oilmaster machine by cold press method. The process was done two times and the very tinny and fine kernels parts in the extracted oil were separated by filtration. Then, the filtered oil was centrifuged (Saber-Tehrani et al., 2013). We prepared fatty acids methyl esters dissolving of 0.4 g pistachio fruit oil in 4 ml of isooctane and methylated in 0.2 ml of 2 M methanolic KOH. The prepared oils were kept at −18 °C for further analyses.

2.4 FATTY ACIDS IDENTIFICATION

Analysis of fatty acid methyl ester was done on a Shimadzu (Nexis 2030) gas-chromatography (GC) equipped with Dikmacap 2330 FID (Flame Ionization Detector) detector, *fused silica* capillary column (60 m × 0.25 mm i. d., 0.25 µm film thickness). The carrier gas was helium at a flow rate of 2 ml min^{-1} in a split ratio of 1 : 60. Injector and detector temperatures were kept at 250 and 260 °C, respectively. The column temperature was initially kept at 60 °C for 2 min and then amplified to 200 °C at a rate of 10 °Cm min^{-1} and hold at the final temperature 240 °C for 7 min. We detected the fatty acid methyl esters by retention time comparison and equivalent chain length with respect to standard FAMEs. For this, 1.0 µl of FAMEs dissolved in petroleum ether was injected directly into gas chromatograph for analysis using a split ratio of 30 : 1. Besides, we computed the relative percentages of detected fatty acids from the GC peak area. We detected the quality index of kernels fatty acids using the ratio of oleic to linoleic acids (O/L). The index is commonly used as a measure to predict the shelf life and stability of the oil (Esteki et al., 2019).

2.5 STATISTICAL ANALYSES

We expressed the morphological data as mean ± standard deviation. In addition, one-way analysis of variance (ANOVA) test was carried out to evaluate the morphological quantitative variables significant variations (p = 0.00) among the studied cultivars.

For clustering analyses of the evaluated cultivars, we standardized the quantitative data (mean = 0, variance = 1) and used for Principal Coordinate Ordination (PCO), Unweighted Paired Group using Average method (UPGMA) and Principal Correspondence Analysis (PCA) by MVSP according to Talebi et al. (2020).

3. RESULTS

3.1 MORPHOLOGICAL STUDY

The investigated morphological traits have been summarized in Table 2. Fruit qualitative morphological traits varied among the evaluated cultivars (Fig. 1). The epicarp color varied as yellowish pink (Kalleh-Ghochi white, Khanjari, Akbari white and Ahmad-Aghaei cultivars), purple (Shahpasand white, Akbari red, Kalleh-Ghochi red and Fakhri cultivars), pink (Shahpasand red and Abasali cultivars) and yellowish orange (Menghar-Kalaghi cultivar).

In addition, we registered kernel coat color as purple (Kalleh-Ghochi white and Ahmad- Aghaei cultivars), purple brown (Shahpasand white and Khanjari cultivars), pink (Kalleh-Ghochi red, Fakhri, Akbari white, Abasali and Menghar-Kalaghi cultivars) and purple pink (Akbari red and Shahpasand red cultivars).

The color of kernels observed as yellowish (Kalleh-Ghochi white, Khanjari, Fakhri, Abasali and Ahmad-Aghaei cultivars), pea green (Shahpasand white, Akbari red, Kalleh-Ghochi red and Shahpasand red cultivars) and green (Akbari white and Menghar-Kalaghi cultivars).

Besides, quantitative variables changed among the investigated cultivars. In this regard, the largest (3 cm) and smallest (1.8 cm) fruit lengths were observed in Kalleh-Ghochi red, Ahmad-Aghaei and Menghar-Kalaghi cultivars, respectively.

The broadest (1.7 cm) fruit belonged to Menghar-Kalaghi cultivar, while the narrowest (1.1 cm) was recorded in Shahpasand red cultivar. Moreover, the longest (1.8 cm) and shortest (1 cm) fruit diameters belonged to Shahpasand white and Ahmad-Aghaei cultivars, respectively. Moreover, the ANOVA test revealed significant difference (p = 0.00) for all the quantitative morphological characteristics (Table 2).

Fig. 1: Fruit shape of the investigated pistachio cultivars (the letters indicate cultivars name according to table 1, scale bar 5 mm)

Table 2: Qualitative and quantitative fruit morphological characteristics of the studied pistachio cultivars

Characteristics	Kalleh-Gghochi white	Shahpasand white	Akbari red	Khanjari	Kalleh-Ghochi red	Shahpasand red	Fakhri	Akbari white	Abasali	Ahmad Aghaei	Menghar Kalaghi	ANOVA
Quantitative characteristics												
Fruit length	2 ± 0.005	2.5±0.028	2.4±0.10	2.4±0.057	1.8±0.057	2.0±0.00	2.3±0.05	2.3±0.057	2.1±0.10	1.8±0.057	3.0±0.057	F=115.631, P=0.000
Fruit width	1.5±0.02	1.6±0.05	1.4±0.05	1.3±0.057	1.5±0.10	1.1±0.10	1.4±0.00	1.5±0.00	1.5±0.057	1.2±0.05	1.7±0.057	F=25.762, P=0.000
Fruit length/width ratio	1.3±0.05	1.63±0.05	1.7±0.05	1.8±0.17	1.2±0.057	1.8±0.15	1.6±0.00	1.53±0.00	1.4±0.05	1.5±0.10	1.76±0.05	F=16.960, P=0.000
Fruit diameter	1.3±0.02	1.8±0.04	1.2±0.04	1.3±0.05	1.4±0.05	1.3±0.17	1.4±0.05	1.3±0.05	1.2±0.05	1±0.057	1.5±0.05	F=26.01, P=0.000
Qualitative characteristics												
Epicarp color	Yellowish Pink	Purple	Purple	Yellowish Pink	Purple	Pink	Purple	Yellowish Pink	Pink	Yellowish Pink	Yellowish Orange	----
Kernel coat color	Purple	Purple Brown	Purple Pink	Purple Brown	Pink	Purple Pink	Pink	Pink	Pink	Purple	Pink	----
Kernel color	Yellowish	Pea Green	Pea Green	Yellowish	Pea Green	Pea Green	Yellowish	Green	Yellowish	Yellowish	Green	----
Endocarp apical shape	Obtuse	Mucronatus	Mucronatus	Mucronatus	Obtuse	Mucronatus	Mucronatus	Mucronatus	Mucronatus	Mucronatus	Mucronatus	----
Endocarp apical symmetry	Obtuse	Symmetrical	Symmetrical	Symmetrical	Obtuse	Asymmetrical	Symmetrical	Symmetrical	Asymmetrical	Asymmetrical	Asymmetrical	----

3.2 FATTY ACIDS COMPOSITION

The oil composition, unsaturated and saturated fatty acids percentages, of the evaluated pistachio cultivars kernels are listed in Table 3. The amounts of mono and polyunsaturated and saturated fatty acids differed from 87.46 to 89.68 %, and 10.48 to 12.01%, respectively.

The oleic, linoleic and palmitic acids were detected the principal fatty acids for all the cultivars. However, the amounts of other fatty acids did not exceed more than 1.6 %.

Oleic (omega-9) and linoleic (omega-6) acids were the most abundant unsaturated fatty acids. The oleic acid, first main polyunsaturated fatty acid, ranged from 53.11 (Menghar-Kalaghi cultivar) to 70.99 % (Kalleh-Ghochi white cultivar), with the general mean of 60.78 %.

The second main fatty acid was linoleic, which its percentages ranged from 15.01 (Kalleh-Ghochi white cultivar) to 33.11 %(Menghar-Kalaghi cultivar), with the average amount of 25.75 %.

The palmitic acid was the first main saturated fatty acid which ranged from 9.09 % (Kalleh-Ghochi red cultivar) to 10.55 % (Shahpasand red cultivar), and the average amount for all the cultivars was 9.9 %.

We estimated the quality index of the studied cultivars oils based on oleic/linoleic acids ratio and reported that Kalleh-Ghochi white cultivar contained the largest amount (4.72) and Menghar-Kalaghi cultivar had the lowest value (1.60). According to UPGMA tree (Fig 2), the studied cultivars were divided into 4 chemotypes; I) Kalleh-Ghochi white cultivar, II) Shahpasand white, Akbari red and Shahpasand red cultivars, III) Khanjari, Kalleh-Ghochi red, Abasali and Akbari white cultivars, and IV) Fakhri, Ahmad-Aghaei and Menghar-Kalaghi cultivars. In addition, the PCA and PCO plots produced similar results (Fig. 3, 4). According to both plots, axis 1 act as a cut factor and divided the studied cultivars into two clades. Then, each clade was subdivided into two groups: Kalleh-Ghochi white cultivar was grouped separately in both plots. However, other cultivars were clustered in three groups.

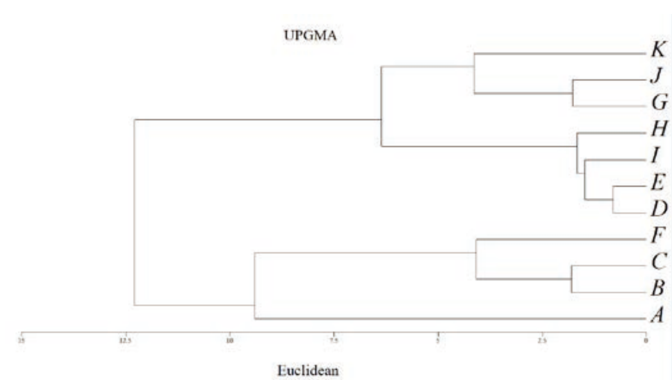

Fig. 2: UPGMA tree of the investigated pistachio cultivars based on the fatty acids compositions (letters indicated the cultivars name according to Table 1)

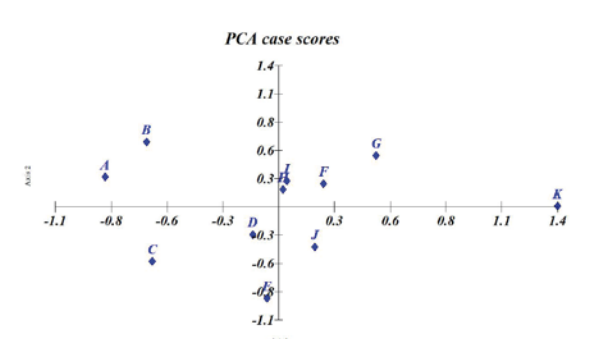

Fig.3: PCA plot of the studied cultivars of pistachio according to fatty acids compositions (letters indicated the cultivars name according to Table 1)

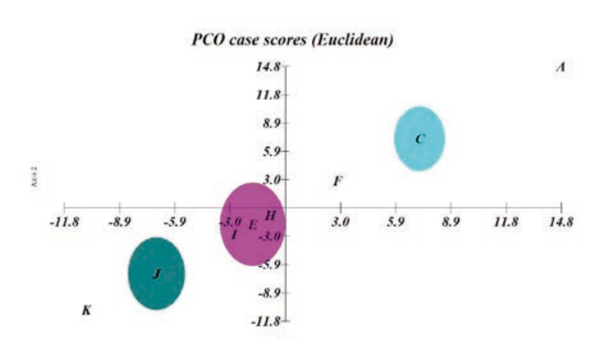

Fig. 4: PCO plot of the evaluated pistachio cultivates according to fatty acids composition (letters indicated the cultivars name according to Table 1)

Table 3: Fatty acids composition of the evaluated pistachio cultivars

Compositions	Kalleh-Ghochi white	Shahpasand white	Akbari red	Khanjari	Kalleh-Ghochi red	Shahpasand red	Fakhri	Akbari white	Abasali	Ahmad Aghaei	Menghar Kalaghi
Unsaturated fatty acids (%)											
Palmitoleic acid (C16:1)	0.8	0.75	0.61	0.67	0.65	0.82	0.85	0.73	0.8	0.75	1.14
Linoleic acid (C18:2c)	15.01	20.93	21.03	27.21	27.54	23.52	30.11	26.03	27.49	31.31	33.11
Oleic acid (C18:1c)	70.99	64.85	66.3	59.71	60.16	62.57	55.97	59.96	58.67	56.63	53.11
Oleic/ Linoleic acids ratio	4.72	3.09	3.15	2.19	2.18	2.78	1.85	2.30	2.13	1.80	1.60
Linolenic acid (C18:3n3)	0.4	0.39	0.35	0.35	0.3	0.35	0.36	0.41	0.49	0.41	0.45
Cis-10 Heptadecenoic acid (C17:1)	0.09	0.08	0.08	0.09	0.09	0.08	0.08	0.09	0.07	0.08	0.1
Cis-11-Eicosedienic acid (C20:1)	0.46	0.45	0.52	0.43	0.49	0.4	0.4	0.42	0.56	0.5	0.39
Total Unsaturated fatty acids	87.75	87.46	88.89	88.46	89.23	87.76	87.81	87.64	88.07	89.23	88.3
Saturated fatty acids											
Myristic acid (C14:0)	0.1	0.18	0.08	0.11	0.09	0.1	0.09	0.09	0.1	0.1	0.08
Palmitic acid (C16:0)	9.9	10.3	9.27	9.61	9.09	10.55	10.51	9.96	10.02	9.4	10.39
Margaric acid (C17:0)	0.05	0.05	0.05	0.05	0.04	0.04	0.06	0.11	0.04	0.05	0.05
Stearic acid (C18:0)	1.59	1.55	1.3	1.35	1.14	1.12	1.2	1.2	1.32	1.11	0.84
Arachidic acid (C20:0)	0.17	0.15	0.14	0.14	0.12	0.12	0.15	0.17	0.13	0.14	0.12
Total saturated fatty acids	11.81	12.23	10.84	11.26	10.48	11.93	12.01	11.53	11.61	10.8	11.48
Oil total	99.56	99.68	99.73	99.72	99.71	99.67	99.78	99.17	99.65	100	99.78

4 DISCUSSION

We elevated the fruit morphological characteristics and kernels fatty acids compositions in eleven cultivars of pistachio from Iran, the first pistachio producer of the world. Because, these findings are extremely important for both pistachio producers and consumers.

We selected all the cultivars from the same region in Iran, to eliminate the effects of environmental factors. According to different investigations the morphological and phytochemical features of pistachio nuts depend on habitat characteristics (Zur et al., 2008; Arena et al., 2007).

Morphological characteristics of fruit and kernels highly varied among the populations. Knowledge of morphological properties are very essential in equipment designing for sorting, transportation and storing of pistachio fruits (Kashaninejada et al., 2006).

Among the studied pistachio samples, Menghar-Kalaghi cultivar possess the largest dimensions (including length, width and diameter) fruits, while the smallest pistachio fruits belonged to Ahmad-Aghaei cultivar. The fruits size of other cultivars were between the fruits size of Menghar-Kalaghi and Ahmad-Aghaei cultivars.

Zarei et al. (2014) studied fruit morphological characteristics of certain (Akbari, Kalleh-Ghuchi, Ohadi and Sephid) cultivars of pistachio and reported Akbari and Kalle-Ghouchi cultivars produce bigger fruit rather than the others. However, in the current research Menghar-Kalaghi cultivar possess the bigger fruit rather than Akbari and Kalle-Ghouchi cultivars. It seems that the cultivar may be useful in genetic breeding program of pistachio.

In addition, the color of fruit epicarp, kernel coat and kernel differed among the cultivars. It seems that different types of anthocyanins and some flavonoids such as lutein derivatives exist on the fruit are responsible for pistachio fruit color (Dreher, 2012).

Unsaturated fatty acids represent 87-89 % of total fatty acids composition in the investigated pistachio cultivars. Among these fatty acids, oleic and linoleic acid play a significant role with amount of 53-70 % and 15.01-33.11 %, respectively. Givianrad et al. (2011) suggested that the kernel oil of pistachio has been definite as an oleiclinoleic oil and could be used in culinary and food industries. Because, the oleic acid is most abundant fatty acid, and it was followed by linoleic acid.

However, the percentages of oleic and linoleic acids differed among the studied cultivars nearly 1.33 and 2.2 –folds, respectively. This profoundly affects the quality of pistachio oil. According to Roozban et al. (2006), the quality of pistachio fruit is depended on composition of its fatty acids composition, chiefly with oleic and linoleic acids amounts.

Oleic acid has several usages in food industries. For example, it acts as food preservative and foods that prepared with the acid remains longer, even out of the refrigerator. Moreover, the acid possess the fungistatic property against a wide spectrum of saprophytic yeasts and moulds. This mono-unsaturated fatty acid possess several usages in hygiene products such as lotions, creams, lipsticks, detergents and soaps as softening agent and emollient(Saber-Tehrani et al., 2013).

The highest and the lowest amounts of oleic/ linoleic acids ratio were reported from Kalleh-Ghochi white and Menghar-Kalaghi cultivars, respectively. This ratio is called the quality index, and usually applied as a measure to predict the stability and shelf life of the fruit oil. Recently, Esteki et al. (2019) have suggested that the oxidative rancidity of pistachio oils develops with an increase in polyunsaturated fatty acids level. So, the higher amount of unsaturation fatty acids leads to the lower oil quality. A higher ratio reveals longer shelf life and chemical stability.

The quality index value varied nearly 3-times among the cultivars and fruits of Kalleh-Ghochi white cultivar have the longest shelf life and chemical stability, while the reverse pattern was found for Fakhri, Ahmad-Aghaei and especially Menghar-Kalaghi cultivars. Similar results were reported by Esteki et al. (2019), which suggested that the large variation exists in fatty acids composition among the evaluated cultivars and also in quality index according to the oleic/linoleic acid ratio. Because oleic acid is considered as a monounsaturated acid and its higher amounts leads to a higher oxidative stability and consequently a large shelf life.

The main fatty acids of the oil were the same among the investigated cultivars. The findings agreed with previous investigations of Iranian and Turkish cultivars. For example, in several researches (Esteki et al., 2019; Yahyavia et al., 2020; Roozban et al., 2006; Mazinani et al., 2012; Abdoshahi et al., 2011) various Iranian pistachio cultivars including Qazvini, Ahmad-Aghaei, Akbari, Chrok, Kalle-Ghouchi, Ohadi, Damgani, Momtaz and Fandoghi were evaluated and the same fatty acids (oleic, linoleic and palmitic acids) were reported as the major fruit oil fatty acids. In addition, similar results were obtained from fatty acids composition of Turkish pistachio cultivars (Dogan et al., 2010; Arena et al., 2007; Satil et al., 2003).

These findings revealed that the kind of main chemical composition of pistachio kernel oil was comparatively homogeneous and have limited diagnosis value for cultivar identification. However, the observed quantitative variations in fatty acid may be related to small genetic divergence of the cultivars.

Farzad-Amirebrahimi et al. (2017) analyzed genetic diversity of 28 Iranian cultivars of pistachio using ISSR molecular marker and reported that 8 % of total genetic variations belonged to among populations and the rest (92 %) related to within population's one. In this regard, they suggested that the low among population's differences could be due to low genetic divergence in the primary parental populations.

All of our harvested cultivars were selected from Damghan in North-east of Iran, and it seems that all of them have the same parental taxon. According to previous investigations (Aalami et al., 1996; Mirzaei et al., 2005; Ahmadi-Afzadi et al., 2007) *Pistacia vera* 'Sarakhs' is distributed as self-grown forests in North-east of the country and has very small genetic divergence with pistachio cultivars. Therefore, it seems that the Iranian pistachio cultivars have been originated from the same taxon.

Results of clustering analyses revealed that the studied cultivars were classified into four chemotypes. Each chemotype was characterized by a special chemical profile. For example, chemotype I (containing Kalleh-Ghochi white cultivar) possess the highest amount of oleic acid and lowest percentage of linoleic acid. In chemotype III (including Khanjari, Kalleh-ghochi red, Abasali and Akbari white cultivars), the percentages of the oil principal fatty acids were nearly equal. However, these cultivar grouping were not in agreement with results of previous Inter Simple Sequence Repeat (Noroozi et al., 2009) and Amplified Fragment Length Polymorphism (Ahmadi-Afzadi et al., 2007) molecular studies on the certain studied cultivars.

5 CONCLUSION

We elevated fruit morphology and kernel fatty acids composition of eleven Iranian cultivars of pistachio. Quantitative morphological characteristics varied among the cultivars and ANOVA test revealed significant difference for all of quantitative ones. The largest and the smallest fruit sizes belonged to Menghar-Kalaghi and Ahmad-Aghaei cultivars, respectively. Unsaturated fatty acids constituent the great part of fatty acid composition. Although the major fatty acids (oleic, linoleic and palmitic acids) of oil were the same among the cultivars, their value differed among them. The quality index of oil (oleic/ linoleic acids ratio) varied among the cultivars and its highest and lowest amounts were reported from Kalleh-Ghochi white and Menghar-Kalaghi, respectively. The index usually applied as a measure to predict the stability and shelf life of the fruit oils.

6 REFERENCES

Aalami, A., Nayeb, M. (1996). *Using isozyme for genetic diversity analysis of Iranian pistachio*. M.Sc. Thesis, Faculty of Agriculture, Tarbiat Modares University, Iran.

Abdoshahi, A., Mortazavi, S. A., Shabani, A. A., Elhamirad, A. H., Taheri, M. (2011). Evaluation of protein, fat and fatty acids content of the pistachio (*Pistacia vera* L.) cultivars of Damghan, Iran. *International Journal of Nuts and Related Sciences*, *2*(4), 15-24.

Ahmadi-Afzadi, M., Sayed Tabatabaei, B. E., Mohammadi, S. A., Tajabadipur, A. (2007). Comparison of genetic diversity in species and cultivars of pistachio (*Pistacia* sp. L.) based on amplified fragment length polymorphism (AFLP) markers. *Iranian Journal of Biotechnology*, *5*(3), 147-152.

AL-Saghir, M. G. (2009). Evolutionary history of the genus *Pistacia* (Anacardiaceae), *International Journal of Botany*, *5*(3), 255-257. https://doi.org/10.3923/ijb.2009.255.257

APG III. (2009). An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society*, *161*, 105–121. https://doi.org/10.1111/j.1095-8339.2009.00996.x

Arena, E., Campisi, S., Fallico, B., Maccarone, E. (2007). Distribution of fatty acids and phytosterols as a criterion to discriminate geographic origin of pistachio seeds. *Food Chemistry*, *104*, 403–408. https://doi.org/10.1016/j.foodchem.2006.09.029

Bozorgi, M., Memariani, Z., Mobli, M. Salehi Surmaghi, M. H., Shams-Ardekani, M. R., Rahimi, R. (2013). Five *Pistacia* species (*P. vera, P. atlantica, P. terebinthus, P. khinjuk,* and *P. lentiscus*): A review of their traditional uses, *The Scientific World Journal*, 33 pp. https://doi.org/10.1155/2013/219815

Dreher, M. L. (2012). Pistachio nuts: Composition and potential health benefits. *Nutrition Reviews*, *70*(4), 234-240. https://doi.org/10.1111/j.1753-4887.2011.00467.x

Dogan, A., Çelik, F., Balta, F., Javidipour, I., Yavic A. (2010). Analysis of fatty acid profiles of pistachios (*Pistacia vera* L.) and native walnuts (*Juglans regia* L.) from Turkey. *Asian Journal of Chemistry*. *22*(1), 517-521.

Esteki, M., Ahmadi, P., Heyden, Y. V., Simal-Gandara, J. (2019). Fatty acids-based quality index to differentiate worldwide commercial pistachio cultivars. *Molecules*, *24*, 58. https://doi.org/10.3390/molecules24010058

FAO, Food and Agriculture Commodities, (2010). http://www.fao.org/es/ess/top/commodity.html

Farzad-Amirebrahimi, F., Mahmoodnia-Meimand, M., Karimi, H. R., Malekzadeh, K. & Tajabadipour A. (2017). Genetic diversity assessment of male and female pistachio genotypes based on ISSR markers. *Journal of Plant Molecular Breeding*, *5*(1), 31–39. Doi: 10.22058/JPMB.2017.63965.1132.

Gavit, N. C. (1990) *A contribution to the study of systematic seed* *morphology of South Gujarat plants*. Ph. D. Thesis, South Gujarat University, Surat.

Givianrad M. H., Saffarpour S. &Beheshti, P. (2011). Fatty acid and triacylglycerol compositions of *Capparis spinosa* seed oil. *Chemistry of Natural Compounds*, *47*(5), 798–799. https://doi.org/10.1007/s10600-011-0063-6

Kafkas, S., Perl-Treves, R. (2001). Morphological and Molecular Phylogeny of *Pistacia* species in Turkey, *Theoretical and Applied Genetics*, *102*, 908-915. https://doi.org/10.1007/s001220000526

Kashaninejada, M., Mortazavi, A., Safekordi, A., Tabil, L.G. (2006). Some physical properties of pistachio (*Pistacia vera* L.) nut and its kernel. *Journal of Food Engineering*, *72,* 30-38. https://doi.org/10.1016/j.jfoodeng.2004.11.016

Kasliwal, R. R., Bansal, M., Mehrotra, R., Yeptho, K. P., Trehan, N. (2015). Effect of pistachio nut consumption on endothelial function and arterial stiffness. *Nutrition*, *31*, 678–685. https://doi.org/10.1016/j.nut.2014.10.019

Khatamsaz, M. (1988). *Flora of Iran, no.3, Anacardiaceae*. Ministry of Agriculture and Natural Resources, research Institute of Forests and Rangelands, Tehran.

Kocyigit, A., Koylu, A. A., Keles, H. (2006). Effects of pistachio nuts consumption on plasma lipid profile and oxidative status in healthy volunteers. *Nutrition, Metabolism Cardiovascular Diseases*, *1693*, 202–209. https://doi.org/10.1016/j.numecd.2005.08.004

Mandalari, G., Bisignano, C., Filocamo, A., Chessa, S., Sarò, M., Torre, G., Faulks, R. M. Dugo, P. (2013). Bioaccessibility of pistachio polyphenols, xanthophylls, and tocopherols during simulated human digestion. *Nutrition*, *29*(1), 338–344. https://doi.org/10.1016/j.nut.2012.08.004

Mazinani, S. Elhami Rad, A. H., Khaneghah, A. M. (2012). Determination and comparison of the amount of tocopherolic and phenolic compounds and fatty acids profile in edible nuts (Pistachio, Almond and Walnut) oil. *Advances in Environmental Biology*, *6*, 1610–1619

Mirzaei, S., Bahar, M., Sharifnabi, B. (2005). A phylogenetic study of Iranian wild pistachio species and some cultivars using RAPD markers. *Acta Horticulture*, *726*, 39-43. https://doi.org/10.17660/ActaHortic.2006.726.3

Noguera-Artiaga, L., García-Romo, J. S., Rosas-Burgos, E. C., Cinco-Moroyoqui, F. J., Vidal-Quintanar, R. L., Carbonell-Barrachina, A. A. & Burgos-Hernández, A. (2019). Antioxidant, antimutagenic and cytoprotective properties of Hydrosos pistachio nuts. *Molecules*, *24*(23), 4362; https://doi.org/10.3390/molecules24234362

Noroozi, S., Baghizadeh, A., Jalali Javaran, M. (2009). The genetic diversity of Iranian pistachio (*Pistacia vera* L.) cultivars revealed by ISSR markers. *Biological Diversity and Conservation*, *2*, 50-56.

Parfitt, D. E. & Badenes, M. L. (1997). Phylogeny of the gecnus *Pistacia* as determined from analysis of the chloroplast genome. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(15), 7987-7992. https://doi.org/10.1073/pnas.94.15.7987

Saber-Tehrani M., Givianrad M. H., Aberoomand-Azar P., Waqif-Husain S., and Jafari Mohammadi S. A. (2013). Chemical composition of Iran's *Pistacia atlantica* cold-pressed oil. *Journal of Chemistry*, https://doi.org/10.1155/2013/126106

Roozban, M. R., Mohamadi, N. & Vahdati, K. (2006). Fat content and fatty acid composition of four Iranian pistachio (*Pistacia vera* L.) varieties grown in Iran. *IV International Symposium on Pistachios and Almonds: Tehran, Iran*; *726,* 573–577. https://doi.org/10.17660/ActaHortic.2006.726.96

Stearn, W. T. (1985). *Botanical Latin: history, grammar, syntax, terminology and vocabulary*, 3rd ed. David & Charles, Newton Abbot, UK

Satil, F., Azcan, N. & Baser, K. H. C. (2003). Fatty acid composition of pistachio nuts in Turkey. *Chemistry of Natural Compounds*, *39*(4), 322–324. https://doi.org/10.1023/B:CONC.0000003408.63300.b5

Talebi, S. M., Amini, F., Askary, M., Farahani, S. & Matsyura, A. (2020). Seed morphology and fatty acids composition among flax populations. *Brazilian Journal of Botany*, https://doi.org/10.100 7/s40415-020-00601-y

Tous, J. & Ferguson L. (1996). Mediterranean Fruits. In: J. Janick, Ed., *Progress in New Crops*, ASHS Press, Ar- lington, , pp. 416-430.

Yahyavia, F., Alizadeh-Khaledabada, M., Azadmard-Damirchi, S. (2020). Oil quality of pistachios (*Pistacia vera* L.) grown in East Azarbaijan, Iran. *NFS Journal, 18*, 12–18. https://doi.org/10.1016/j.nfs.2019.11.001

Zarei, M., Davarynejad, Gh., Abedi, B., Kafi, M., Biabani, A. (2014). Changes in physical properties, chemical composition and antioxidant activity of four pistachio cultivars at ten maturity stages. *Advances in Environmental Biology*, *8*(10), 106-115.

Zohary, M. (1952). A monographical study of the genus *Pistacia*. *Palestine Journal of Botany (Jerusalem Series)*, *5*(4), 187–228.

Zur, K., Heier, A., Blaas, K.W., Fauhl-Hassek, C. (2008). Authenticity control of pistachios based on 1H- and 13C-NMR spectroscopy and multivariate statistics. *European Food Research and Technology*, *227*, 969–977. https://doi.org/10.1007/s00217-007-0804-8.

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Investigation about wetting ability (surface tension) of water used for preparation of pesticide solutions

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Investigation about wetting ability (surface tension) of water used for preparation of pesticide solutions

Abstract: The investigation about surface tension of water used for preparation of pesticide solutions reveals it is quite diverse and changeable without any logical correlation towards location, time, and type of water source. Moreover, spraying with solutions with lower surface tension give bigger flow rates due to the lower resistance of fluid to the nozzles. The conducted trials show that plant surfaces with more rough texture require to be sprayed with pesticide solutions with lower surface tension. The wax content of the surfaces has no significant impact on surface tension requirement.

Key words: surface tension; pesticides; plant protection products; sprayers; wetting ability

Preučevanje omočitvene sposobnosti (površinske napetosti) vode za pripravo raztopin pesticidov

Izvleček: Raziskave glede površinske napetosti vode uporabljene za pripravo raztopin pesticidov so odkrile, da je ta zelo raznolika in, da se spreminja brez logične povezave glede na lokacijo, čas in vir vode. Škropljenje z raztopino z manjšo površinsko napetostjo daje večje pretoke zaradi manjšega upora tekočine v šobah. Izvedeni poskusi kažejo, da površine rastlin z bolj grobo teksturo zahtevajo škropljenje z raztopino pesticidov z manjšo površinsko napetostjo. Vsebnost voska na površini nima značilnega vpliva glede zahtev o površinski napetosti raztopine pesticidov.

Ključne besede: površinska napetost; pesticidi; pripravki za zaščito rastlin; razpršilci; omočitvena sposobnost

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1 INTRODUCTION

Using of pesticide solution with low surface tension (good wetting ability) is crucial for achievement of satisfactory level of pesticide effectiveness (Crease & Thacker, 1991; Ellis et al., 2001,). If the surface tension is too high (wetting ability – too low), pesticide solutions will be at the form of drops on the sprayed surfaces (they will not cover all surface and can be easily dropped down) on one hand and they will not penetrate fully in the rough surfaces from the other. (Ellis et al., 2001). In both cases, the effectiveness of pesticides can be dramatically lowered, especially when pesticide solutions are sprayed in relatively low temperatures (which increase surface tension of the water) and on plant parts with rough surface textures (which require solutions with lower surface tension for full cover in order pesticide solution fully to penetrate into of the surfaces plant tissues - cuticle). The spraying of plants with pesticide solutions over the point of run-off can cause significant risk for contamination of soil and waters (Bergström, 1990).

Traditionally it is expected that common tap water or water from rivers or lakes used for preparing of pesticide solutions have a constant surface tension only slightly depending from temperature (Gittens, 1969; Grisso et al., 1988). Different surface tension means different flow rates and respectively different sprayed dose rates (Matthews, 2008).

The main aim of present investigation was to reveal that the surface tension of the water is highly diverse and changeable and it depends on numerous factors and cannot be predicted in any way unlike common acceptance (Claussen, 1967,; Pallas & Pethica, 1983; Kalová & Mareš, 2015;). Also, the common acceptance that plant surfaces with high content of wax like cabbage leaves require to be sprayed with pesticide solution with lower surface tension (better wetting ability) also was wrong – the solutions with lower surface tension is required for surfaces with more rough textures, not with higher wax content (Bartell & Zuidema, 1936; Hess and Foy, 2000; Wagner et al., 2003). The spraying of solutions with different surface tension can significantly change the flow rates of the sprayers and must be taken into account during treatments with plant protection products (Semiao et.al., 1996; Miller and Ellis, 2000; De Schampheleire et al., 2009)

2 MATERIALS AND METHODS

Water samples from different sources in Bulgaria were taken during 2018 year. The sources were:

- tap water from different towns and villages (from different districts of given town / village);

- river and dam lake water from sources situated nearby different towns and villages in Bulgaria;

- well water which although rarely, also is used sometimes for making pesticide solutions (especially during summer drought) from sources situated in different towns and villages;

The 3 samples from one source (location) were taken four times in different time different seasons – spring (in May), winter (in February) , summer (in August) and autumn ((in October). The surface tension of water samples was established by tensiometer K6 Du Noüy Ring (Macy, 1935), produced by Krüss Scientific, at 24 °C checked with digital thermometer and average results was presented (used) for each taken sample.

Samples (leaves, barks, fruits) from different plants (mainly agricultural, but also decorative and forest plants) in different BBCH growth stages were taken and were sprayed with solutions made from distilled water and organosillicone surfactant Silwet L 77, produced by Momentive Performance Materials with different surface tension at 24 °C (Stevens et al., 1988). The ability of solutions to form even film, to spread easily and uniformly over the treated surfaces (wetting ability) was examined. A liquid with high wetting ability forms a thin, continuous film when it spreads over the surface which allow compete cover of the treated surface with pesticide solutions for maximal effectiveness (maximum cover, exposure and retention) from one side and minimal pollution of the environment (minimal amounts of pesticides fall on soils and waters from treated plants) – from the other (Prado et al., 2016; Zhu et al.,2019). Lower surface tension means better wetting ability (Bonn et al., 2009). Effective wetting ability requires the surface tension of the adhesive ($Y_{adhesive}$) to

$$\gamma_{LV} \cos\theta = \gamma_{SV} - \gamma_{SL}$$

Complete wetting when $\theta = 0$, or when $\cos\theta = 1$

Thus $\gamma_{LV} = \gamma_{SV} - \gamma_{SL}$

Or $\gamma_{LV} \leq \gamma_{SV}$

Or $\gamma_{ADHESIVE} \leq \gamma_{SUBSTRATE}$

Figure 1: Wetting surface requirement

be less than or equal to that of the substrate ($Y_{substrate}$) (Yuan & Lee, 2013) (Figure. 1)

The flow rate with solution with different surface tension was examined by hand compression Knapsack Sprayer Matabi super green 12 , produced by Goizper. The size of droplets was visually determined by water sensitive papers, produced by Syngenta by method described by Turner and Huntington (1970); Salyani and Fox (1999) and Cerruto et al.(2016) and by digital micrometer (KINEX ABSOLUTE ZERO, 0-25 MM, 0,001MM, DIN 863, IP 65) produced by KINEX Measuring. Nozzles were set to produce spray plum consisting of relatively uniform droplets with diameter of 25 µm or 300 µm.

3 RESULTS AND DISCUSSION

The results from conducted trials with measurements of the surface tension from different water sources are presented bellow as tables indicated location of the taken water samples, geographical coordinates, height above the sea level (m), date of taking samples and the average value surface tension of each sample.

3.1 RESULTS OF TAP WATER ANALYSIS

The presented Table 1 shows that surface tension of water is absolutely changeable and of upredictable value and can vary in remote places or on a very short distance. For example – town of Plovdiv, Bulgaria, has approximately 102 square kilometers area. The tap water from different residential districts has different surface tension. The same situation is in the towns of Karlovo and Sopot in Central Bulgaria, where the distance between towns is only 4 km or town of Stamboliiski and village of Joakim Gruevo, where the distance between them is 2 km. The surface tension value of tested tap water was completely different and unpredictable.

Table 1: Surface tension of tap water samples taken in Bulgaria during 2018 in different seasons (winter, spring, summer and autumn)

Location	Coordinates	Height above the sea level (m)	Date of taking sample	Measured surface tension (mN m^{-1})
Town of Karlovo	42°63′66″N 24°79′98″E	452	10.02.2018	64
Town of Karlovo	42°63′66″N 24°79′98″E	452	10.05.2018	58
Town of Karlovo	42°63′66″N 24°79′98″E	452	10.08.2018	71
Town of Karlovo	42°63′66″N 24°79′98″E	452	10.10.2018	57
Village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′95″E	444	10.02.2018	78
Village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′95″E	444	10.05.2018	55
Village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′95″E	444	10.08.2018	58
Village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′95″E	444	10.10.2018	64
Town of Stamboliiski	42°13′25″N 24°52′20″E	200	03.02.2018	63
Town of Stamboliiski	42°13′25″N 24°52′20″E	200	03.05.2018	72
Town of Stamboliiski	42°13′25″N 24°52′20″E	200	03.08.2018	71
Town of Stamboliiski	42°13′25″N 24°52′20″E	200	03.10.2018	65

Village of Joakim Gruevo, town of Stambiliiski district	42°11′93″N 24°55′83″E	289	03.02.2018	69
Village of Joakim Gruevo, town of Stambiliiski district	42°11′93″N 24°55′83″E	289	03.05.2018	72
Village of Joakim Gruevo, town of Stambiliiski district	42°11′93″N 24°55′83″E	289	03.08.2018	68
Village of Joakim Gruevo, town of Stambiliiski district	42°11′93″N 24°55′83″E	289	03.10.2018	64
Town of Jambol	42°47′78″N 26°49′22″E	114	20.02.2018	70
Town of Jambol	42°47′78″N 26°49′22″E	114	20.05.2018	70
Town of Jambol	42°47′78″N 26°49′22″E	114	20.08.2018	74
Town of Jambol	42°47′78″N 26°49′22″E	114	20.10.2018	68
Town of Varna	43°22′20″N 27°88′18″E	80	25.02.2018	72
Town of Varna	43°22′20″N 27°88′18″E	80	25.05.2018	58
Town of Varna	43°22′20″N 27°88′18″E	80	25.08.2018	71
Town of Varna	43°22′20″N 27°88′18″E	80	25.10.2018	70
Agricultural University, town of Plovdiv	42°13′35″N 24°76′68″E	164	01.02.2018	71
Agricultural University, town of Plovdiv	42°13′35″N 24°76′68″E	164	01.05.2018	70
Agricultural University, town of Plovdiv	42°13′35″N 24°76′68″E	164	01.08.2018	72
Agricultural University, town of Plovdiv	42°13′35″N 24°76′68″E	164	01.10.2018	65
town of Plovdiv, residential district Trakia	42°13′68″N 24°79′46″E	164	01.02.2018	63
town of Plovdiv, residential district Trakia	42°13′68″N 24°79′46″E	164	01.05.2018	70
town of Plovdiv, residential district Trakia	42°13′68″N 24°79′46″E	164	01.08.2018	63
town of Plovdiv, residential district Trakia	42°13′68″N 24°79′46″E	164	01.10.2018	70
town of Plovdiv, residential district Komatevo	42°10′38″N 24°70′54″E	164	05.02.2018	72
town of Plovdiv, residential district Komatevo	42°10′38″N 24°70′54″E	164	05.05.2018	63
town of Plovdiv, residential district Komatevo	42°10′38″N 24°70′54″E	164	05.08.2018	68
town of Plovdiv, residential district Komatevo	42°10′38″N 24°70′54″E	164	05.10.2018	71
town of Plovdiv, residential district Karshiaka	42°16′16″N 24°74′23″E	164	01.02.2018	65

town of Plovdiv, residential district Karshiaka	42°16′16″N 24°74′23″E	164	01.05.2018	72
town of Plovdiv, residential district Karshiaka	42°16′16″N 24°74′23″E	164	01.08.2018	70
town of Plovdiv, residential district Karshiaka	42°16′16″N 24°74′23″E	164	01.10.2018	65
Town of Sopot	42°65′04″N 24°76′51″E	417	10.02.2018	58
Town of Sopot	42°65′04″N 24°76′51″E	417	10.05.2018	70
Town of Sopot	42°65′04″N 24°76′51″E	417	10.08.2018	67
Town of Sopot	42°65′04″N 24°76′51″E	417	10.10.2018	70
Town of Kalofer	42°61′11″N 24°97′78″E	666	10.02.2018	67
Town of Kalofer	42°61′11″N 24°97′78″E	666	10.05.2018	66
Town of Kalofer	42°61′11″N 24°97′78″E	666	10.08.2018	69
Town of Kalofer	42°61′11″N 24°97′78″E	666	10.10.2018	70

3.2 RESULTS OF RIVER WATER ANALYSIS

From Table 2 we can see that just like in the case of tap water, the samples from river water also have different surface tension in different seasons. The water from river Nevolia in the village of Vasil Levski, in February had surface tension of 71 mN m^{-1}, the sample taken from same river, at the same time in the town of Bania, which is situated lie about 7 km south, had surface tension of 59 mN m^{-1}. The tap water however taken from house 5 m away from the river Nevolia during February has 78 mN m^{-1}surface tension. The results confirm the unpredictable nature of the surface tension value towards river waters just like tap waters. Some early investigations reveal direct connection between natural waters contaminations and changes in the surface tension (Pockels, 1893). Certain contaminants can alter water surface tension significantly. The value of surface tension is almost independent of pH of the water (Sridhar & Reddy, 1984; Beattie et al., 2014).

3.3 RESULTS OF LAKE WATER ANALYSIS

Just like river and tap water samples, the results show absolutely changeable and unpredictable surface tension values for lake. For example, the tap water of the village of Vasil Levski in February had 78 mN m^{-1} surface tension, the value of this index of river water sample taken from river 5 meters away was 71 mN m^{-1}, the surface tension of dam lake water taken from dam lake Vasil Levski situated approximately 1,8 km away from the river was 65 mN m^{-1}.

3.4 RESULTS OF WELL WATER ANALYSIS

The tap water of the village of Vasil Levski in February had 78 mN m^{-1} surface tension, the value of this index of river water sample taken from river 5 meters away was 71 mN m^{-1}, the surface tension of dam lake water taken from dam lake Vasil Levski situated approximately 5 km away from the river was 65 mN m^{-1}, however the water sample taken from well situated 20 m away from place where river sample and tap water sample were taken, had 57 mN m^{-1} surface tension. The previously conducted research also confirmed that surface tension of water of soils (underground water) is less than that of pure water (Tschapek et al., 1978). From the results for well waters in present study, is obvious that this value sometimes is less, sometimes is bigger. For sure is completely unpredictable and changeable. However, in present study only freshwater sources were examined. Other study shows that according to the seawater sample analysis there is similar uncertainty in prediction on surface tension as sweet water (Nayar et al., 2014).

Table 2: Surface tension of river water samples taken in Bulgaria during 2018 in different seasons (winter, spring, summer and autumn)

Location	Coordinates	Height above the sea level (m)	Date of taking sample	Measured surface tension (mN m-1)
River Nevolia, village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′99″E	444	10.02.2018	71
River Nevolia, village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′99″E	444	10.05.2018	56
River Nevolia, village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′99″E	444	10.08.2018	74
River Nevolia, village of Vasil Levski, town of Karlovo district	42°60′80″N 24°88′99″E	444	10.10.2018	62
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.02.2018	59
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.05.2018	63
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.08.2018	68
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.10.2018	72
River Strjama, town of Banija	42°54′20″N 24°82′08″E	295	10.02.2018	60
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.05.2018	74
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.08.2018	75
River Nevolia, town of Banija	42°56′33″N 24°83′02″E	295	10.10.2018	57
River Jantra, town of Gabrovo	42°89′09″N 25°32′42″E	392	27.02.2018	71
River Jantra, town of Gabrovo	42°89′09″N 25°32′42″E	392	27.05.2018	68
River Jantra, town of Gabrovo	42°89′09″N 25°32′42″E	392	27.08.2018	65
River Jantra, town of Gabrovo	42°89′09″N 25°32′42″E	392	27.10.2018	65
River Osam, town of Trojan	42°91′52″N 24°71′01″E	380	27.02.2018	64
River Osam, town of Trojan	42°91′52″N 24°71′01″E	380	27.05.2018	65
River Osam, town of Trojan	42°91′52″N 24°71′01″E	380	27.08.2018	55
River Osam, town of Trojan	42°91′52″N 24°71′01″E	380	27.10.2018	65

Table 3: Surface tension of dam lake water samples taken in Bulgaria during 2018 in different seasons (winter, spring, summer and autumn)

Location	Coordinates	Height above the sea level (m)	Date of taking sampels	Measured surface tension (mN m^{-1})
Dam lake Vasil Levski, village of Vasil Levski, Karlovo district	42°61′48″N 24°91′16″E	444	10.02.2018	65
Dam lake Vasil Levski, village of Vasil Levski, Karlovo district	42°61′48″N 24°91′16″E	444	10.05.2018	60
Dam lake Vasil Levski, village of Vasil Levski, Karlovo district	42°61′48″N 24°91′16″E	444	10.08.2018	67
Dam lake Vasil Levski, village of Vasil Levski, Karlovo district	42°61′48″N 24°91′16″E	444	10.10.2018	70
Dam lake Murla, town of Sopot	42°64′74″N 24°77′21″E	417	10.02.2018	55
Dam lake Murla, town of Sopot	42°64′74″N 24°77′21″E	417	10.05.2018	55
Dam lake Murla, town of Sopot	42°64′74″N 24°77′21″E	417	10.08.2018	65
Dam lake Murla, town of Sopot	42°64′74″N 24°77′21″E	417	10.10.2018	68
Dam lake Kovatchevo, village of Kovatchevo, town of Stara Zagora district	42°21′83″N 24°20′90″E	133	20.02.2018	59
Dam lake Kovatchevo, village of Kovatchevo, town of Stara Zagora district	42°21′83″N 24°20′90″E	133	20.05.2018	70
Dam lake Kovatchevo, village of Kovatchevo, town of Stara Zagora district	42°21′83″N 24°20′90″E	133	20.08.2018	67
Dam lake Kovatchevo, village of Kovatchevo, town of Stara Zagora district	42°21′83″N 24°20′90″E	133	20.10.2018	65
Dam lake Zrebchevo, town of Nikolaevo	42°63′19″N 25°84′95″E	274	20.02.2018	58
Dam lake Zrebchevo, town of Nikolaevo	42°63′19″N 25°84′95″E	274	20.05.2018	68
Dam lake Zrebchevo, town of Nikolaevo	42°63′19″N 25°84′95″E	274	20.08.2018	57
Dam lake Zrebchevo, town of Nikolaevo	42°63′19″N 25°84′95″E	274	20.10.2018	73
Dam Lake Radetski, village of Radetski, town of Nova Zagora District	42°28′51″N 26°09′65″E	156	20.02.2018	66
Dam Lake Radetski, village of Radetski, town of Nova Zagora District	42°28′51″N 26°09′65″E	156	20.05.2018	70
Dam Lake Radetski, village of Radetski, town of Nova Zagora District	42°28′51″N 26°09′65″E	156	20.08.2018	70
Dam Lake Radetski, village of Radetski, town of Nova Zagora District	42°28′51″N 26°09′65″E	156	20.10.2018	65
Dam Lake Matza, village Matza, town of Polski Gradetz district	42°22′14″N 26°15′20″E	190	20.02.2018	71
Dam Lake Matza, village Matza, town of Polski Gradetz district	42°22′14″N 26°15′20″E	190	20.05.2018	74
Dam Lake Matza, village Matza, town of Polski Gradetz district	42°22′14″N 26°15′20″E	190	20.08.2018	71
Dam Lake Matza, village Matza, town of Polski Gradetz district	42°22′14″N 26°15′20″E	190	20.10.2018	70

Table 4: Surface tension of well water samples taken in Bulgaria during 2018 in different seasons (winter, spring, summer and autumn)

Location	Coordinates	Height above the sea level (m)	Date of taking sampels	Measured surface tension (mN m^{-1})
Village of Radetski, town of Nova Zagora district	42°28′39″N 26°11′45″E	156	20.02.2018	71
Village of Radetski, town of Nova Zagora district	42°28′39″N 26°11′45″E	156	20.05.2018	58
Village of Radetski, town of Nova Zagora district	42°28′39″N 26°11′45″E	156	20.08.2018	72
Village of Radetski, town of Nova Zagora district	42°28′39″N 26°11′45″E	156	20.10.2018	70
Village of Vasil Levski, town of Karlovo district	42°60′84″N 24°88′83″E	444	10.02.2018	57
Village of Vasil Levski, town of Karlovo district	42°60′84″N 24°88′83″E	444	10.05.2018	68
Village of Vasil Levski, town of Karlovo district	42°60′84″N 24°88′83″E	444	10.08.2018	55
Village of Vasil Levski, town of Karlovo district	42°60′84″N 24°88′83″E	444	10.10.2018	71

3.5 RESULTS ON ANALYS OF REQUIREMENTS FOR DIFFERENT PLANTS ABOUT WETTING ABILITY OF SPRAYS IN PESTICIDES APPLYCATION

Figures 2, 3 and 4 shows the wetting requirements of the leaves of different cultures in four different growing stages.

From presented results above can be clearly seen that wetting ability of the sprayed liquid does not depend on plant surface structures wax content, but on the roughness of plant surface texture. The cabbage leaves although have a high wax content but are relative smooth (Lee et al., 1988), require to be sprayed with solutions with surface tension 25 mN m^{-1} for full wetting (Figure 4). However, the bean leaves with less wax content but more rough textures for full wetting require solutions with surface tension 21 mN m^{-1} (Figure 4). The tomato leaves in earlier growing stages when are more rough require solutions with 22 mN m^{-1} surface tension, while in late growing stages when they develop more smooth texture need to be sprayed with solutions with 30 mN m^{-1} surface tension. The leaves of apples (*Malus domestica* Borkh.), potato (*Solanum tuberosum* L.), maize (*Zea mays* L.) and cauliflower (*Brassica oleracea* L. ssp. *oleracea* convar. *Botrytis* (L.) Alef) which are relativelly smooth in all phenophases require to be sprayed with solutions with surface tension close to 22 mN m^{-1} (Figure 3).

There is obvious difference between leaves of cabbage and cauliflower, leaves of the second have more rough texture and for full wetting required solutions with lower surface tension. The test with bark also reveals that for good wetting of more rough textured plant surfaces – liquids with lower surface tension is required.

3.6 RESULTS OF ANALYSIS OF IMPACT OF WATER SURFACE TENSION ON NOZZLE FLOW RATE

Figure 5 shows flow rates obtained with Matabi sprayer with solutions with different surface tensions.

The nozzle of the sprayer was adjusted to spray with droplets with the size of 25 and 300 microns (diameter). The size of the droplets was visually determined by water sensitive papers and digital micrometer. The difference is obvious - the same sprayer with the same nozzle adjusted to a constant position and sprayed droplets with the same size but with solutions with different surface tension (wetting ability) achieved different flow rates. When sprayer works with distilled water (66 mN m^{-1} surface tension) 500 ml m^{-1} flow rate was achieved. The same sprayer, with the same nozzle adjustment working with the same water but with addition of organosilicone surfactant Silwet L 77 at 0.01 % concentration (22 mN m^{-1} surface tension), achieved

900 ml m^{-1} flow rate – 80 % more solution pass through nozzle. With increase of the droplet size from 25 to 300 microns, and addition of organosilicone surfactant Silwet L 77 to the distilled water again at 0.01 % (22 mN m^{-1} surface tension), only 37 % more solution pass from the nozzle.

In both cases (25 and 300 microns size of droplets) the addition of the same amount of organosilicone surfactant (0.01 % concentration) to the distilled water decreased the surface tension to the same level (22 mN m^{-1}) and dramatically increased the flow rate of the sprayer nozzle.

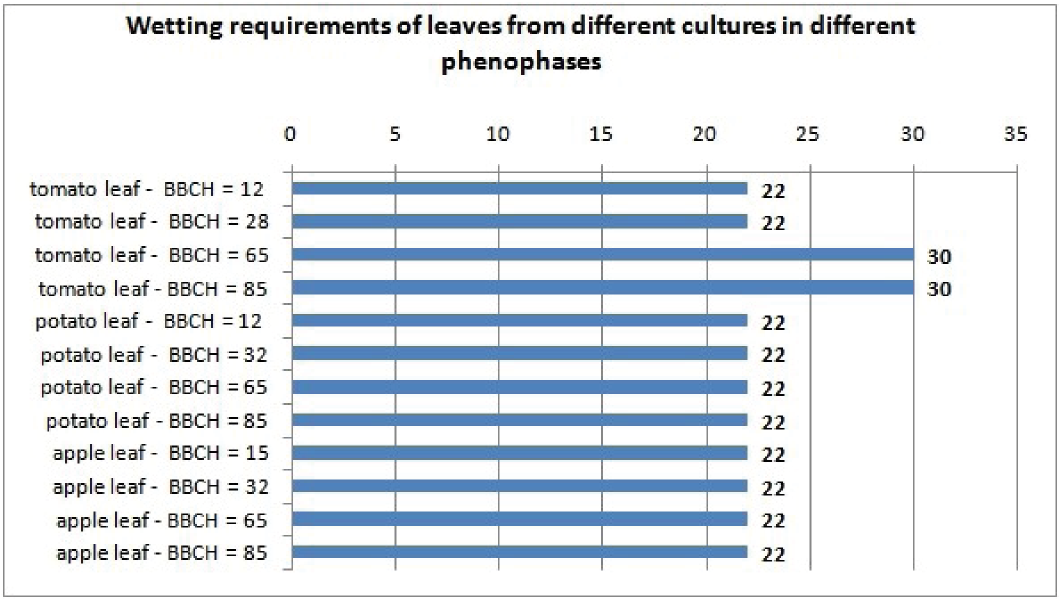

Figure 2: Wetting ability requirements of the crop leaves – on y axis are presented different plant leaves taken in different growth stages (BBCH stages); on x axis is measured surface tension (mN m^{-1}) of pesticides spray needed for full coverage and penetration in the texture of the leaves.

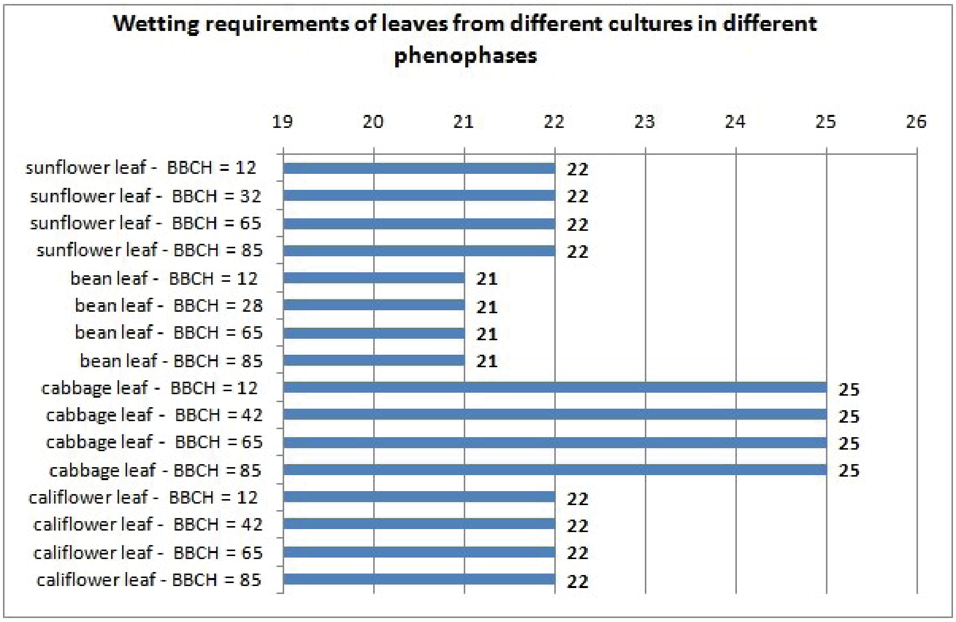

Figure 3: Wetting ability requirements of the crop leaves – on y axis are presented different plant leaves taken in different growth stages (BBCH stages); on x axis is measured surface tension (mN m^{-1}) of pesticides spray needed for full coverage and penetration in the texture of the leaves

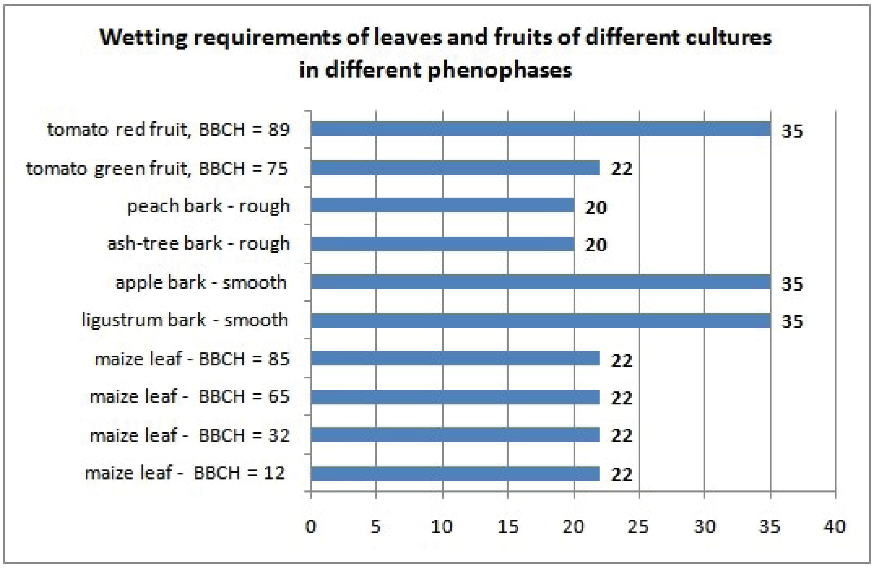

Figure 4: Wetting ability requirements of the crop leaves – on y axis are presented different plant leaves taken in different growth stages (BBCH stages); on x axis is measured surface tension (mN m^{-1})of pesticides spray needed for full coverage and penetration in the texture of the leaves

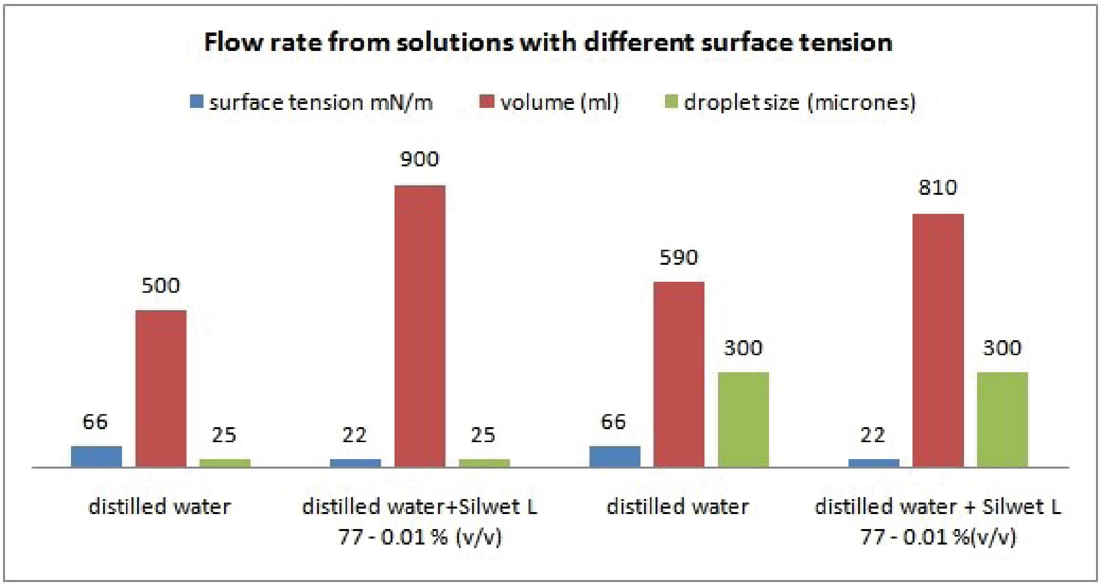

Figure 5: Flow rates (ml min^{-1}) of solutions with different surface tensions - left two columns small droplets (25 µm) and right two big droplets (300 µm)

4 CONCLUSION

The conducted tests proved that surface tension of the water used for preparation of the pesticide spray was not of constant value and depended not only from temperature, but also on many other factors and could be completely different from different sources and time periods (season, weather) without any logical correlation with location, time and type of water source. Sprayed surfaces with more rough texture require to be treated with pesticide solutions with lower surface tension. The solutions with lower surface tension give a bigger nozzle flow rates than solutions with higher surface tension, especially when spray consists of smaller size droplets. The addition of wetting agent to the pesticide solutions can dramatically increase the flow rate from the sprayer.

5 REFERENCES

Bartell, F. E., & Zuidema, H. H. (1936). Wetting characteristics of solids of low surface tension such as talc, waxes and resins. *Journal of the American Chemical Society*, *58*(8), 1449-1454. https://doi.org/10.1021/ja01299a041

Beattie, J. K., Djerdjev, A. M., Gray-Weale, A., Kallay, N., Lützenkirchen, J., Preočanin, T., & Selmani, A. (2014). pH and the surface tension of water. *Journal of Colloid and Interface Science*, *422*, 54-57. https://doi.org/10.1016/j.jcis.2014.02.003

Bergström, L. (1990). Use of lysimeters to estimate leaching of pesticides in agricultural soils. *Environmental Pollution*, *67*(4), 325-347. https://doi.org/10.1016/0269-7491(90)90070-S

Grisso, R. D., Hewett, E. J., Dickey, E. C., Schnieder, R. D., & Nelson, E. W. (1988). Calibration accuracy of pesticide application equipment. *Applied Engineering in Agriculture*, *4*(4), 310-315. https://doi.org/10.13031/2013.26624

Bonn, D., Eggers, J., Indekeu, J., Meunier, J., & Rolley, E. (2009). *Wetting and Spreading. Reviews of Modern Physics*, *81*(2), 739. https://doi.org/10.1103/RevModPhys.81.739

Cerruto, E., Failla, S., Longo, D., & Manetto, G. (2016). Simulation of water sensitive papers for spray analysis. *Agricultural Engineering International: CIGR Journal*, *18*(4), 22-29.

Claussen, W. F. (1967). Surface tension and surface structure of water. *Science*, *156*(3779), 1226-1227. https://doi.org/10.1126/science.156.3779.1226

Crease, G. J., Hall, F. R., & Thacker, J. R. M. (1991). Reflection of agricultural sprays from leaf surfaces. *Journal of Environmental Science & Health Part B*, *26*(4), 383-407. https://doi.org/10.1080/03601239109372744

De Schampheleire, M., Nuyttens, D., Baetens, K., Cornelis, W., Gabriels, D., & Spanoghe, P. (2009). Effects on pesticide spray drift of the physicochemical properties of the spray liquid. *Precision Agriculture*, *10*(5), 409-420. https://doi.org/10.1007/s11119-008-9089-6

Ellis, M. B., Tuck, C. R., & Miller, P. C. H. (2001). How surface tension of surfactant solutions influences the characteristics of sprays produced by hydraulic nozzles used for pesticide application. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *180*(3), 267-276. https://doi.org/10.1016/S0927-7757(00)00776-7

Gittens, G. J. (1969). Variation of surface tension of water with temperature. *Journal of Colloid and Interface Science*, *30*(3), 406-412. https://doi.org/10.1016/0021-9797(69)90409-3

Hess, F. D., & Foy, C. L. (2000). Interaction of Surfactants with Plant Cuticles1. *Weed Technology*, *14*(4), 807-813. https://doi.org/10.1614/0890-037X(2000)014[0807:IOSWPC]2.0.CO;2

Kalová, J., & Mareš, R. (2015). Reference values of surface tension of water. *International Journal of Thermophysics*, *36*(7), 1396-1404. https://doi.org/10.1007/s10765-015-1907-2

Lee, C. H., Hwang, I. J., & Kim, J. K. (1988). Macro-and microstructure of Chinese cabbage leaves and their texture measurements. *Korean Journal of Food Science and Technology*, *20*(6), 742-748. https://doi.org/10.9721/KJFST.2011.43.6.742

Macy, R. (1935). Surface tension by the ring method. Applicability of the du Nouy apparatus. *Journal of Chemical Education*, *12*(12), 573 https://doi.org/10.1021/ed012p573.

Matthews, G. (2008). *Pesticide application methods*. John Wiley & Sons.

Miller, P. C. H., & Ellis, M. B. (2000). Effects of formulation on spray nozzle performance for applications from ground-based boom sprayers. *Crop Protection*, *19*(8-10), 609-615. https://doi.org/10.1016/S0261-2194(00)00080-6

Nayar, K. G., Panchanathan, D., McKinley, G. H., & Lienhard, J. H. (2014). Surface tension of seawater. *Journal of Physical and Chemical Reference Data*, *43*(4), 043103. https://doi.org/10.1063/1.4899037

Pallas, N. R., & Pethica, B. A. (1983). The surface tension of water. *Colloids and Surfaces*, *6*(3), 221-227. https://doi.org/10.1016/0166-6622(83)80014-6

Pockels, A. (1893). Relations between the surface-tension and relative contamination of water surfaces. *Nature, 48*, 152–154. https://doi.org/10.1038/048152a0

Prado, E. P., Raetano, C. G., do Amaral Dal, M. H. F., Chechetto, R. G., Ferreira Filho, P. J., Magalhaes, A. C., & Miasaki, C. T. (2016). Effects of agricultural spray adjuvants in surface tension reduction and spray retention on Eucalyptus leaves. *African Journal of Agricultural Research*, *11*(40), 3959-3965. https://doi.org/10.5897/AJAR2016.11349

Salyani, M., & Fox, R. D. (1999). Evaluation of spray quality by oiland water-sensitive papers. *Transactions of the ASAE*, *42*(1), 37. https://doi.org/10.13031/2013.13206

Semiao, V., Andrade, P., & da GraCa Carvalho, M. (1996). Spray characterization: numerical prediction of Sauter mean diameter and droplet size distribution. *Fuel, 75*(15), 1707-1714. https://doi.org/10.1016/S0016-2361(96)00163-9

Sridhar, M. K. C., & Reddy, C. R. (1984). Surface tension of polluted waters and treated wastewater. *Environmental Pollution Series B, Chemical and Physical*, *7*(1), 49-69. https://doi.org/10.1016/0143-148X(84)90037-5

Stevens, P. J. G., Gaskin, R. E., & Zabkiewicz, J. A. (1988). Silwet L-77: a new development in spray adjuvants. In *Proceedings of the New Zealand Weed and Pest Control Conference (Vol. 41, pp. 141-145*). https://doi.org/10.30843/nzpp.1988.41.9880

Tschapek, M., Scoppa, C. O., & Wasowski, C. (1978). The surface tension of soil water. *Journal of Soil Science, 29*(1), 17-21. https://doi.org/10.1111/j.1365-2389.1978.tb02026.x

Turner, C. R., & Huntington, K. A. (1970). The use of a water sensitive dye for the detection and assessment of small spray droplets. *Journal of Agricultural Engineering Research, 15*(4), 385-387. https://doi.org/10.1016/0021-8634(70)90099-5

Wagner, P., Fürstner, R., Barthlott, W., & Neinhuis, C. (2003). Quantitative assessment to the structural basis of water repellency in natural and technical surfaces. *Journal of Experimental Botany*, *54*(385), 1295-1303. https://doi.org/10.1093/jxb/erg127

Yuan, Y., & Lee, T. R. (2013). Contact angle and wetting properties. In *Surface science techniques* (pp. 3-34). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-34243-1_1

Zhu, F., Cao, C., Cao, L., Li, F., Du, F., & Huang, Q. (2019).
Wetting behavior and maximum retention of aqueous

surfactant solutions on tea leaves. *Molecules*, *24*(11), 2094. https://doi.org/10.3390/molecules24112094

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First report of an invasive pest, *Phyllonorycter populifoliella* (Lepidoptera: Gracillariidae) from Ladakh

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First report of an invasive pest, *Phyllonorycter populifoliella* (Lepidoptera: Gracillariidae) from Ladakh

Abstract: *Phyllonorycter populifoliella* (Treitschke 1883), is an invasive pest and is first reported on poplar trees, from the eastern region of Ladakh, India. The details of the taxonomic identification based on genital morphology are presented. Besides, host range, feeding habits and level of infestation in different hamlets of Ladakh are also presented. This study is important for further understanding the pest biology, its diversity and management by adopting control strategies. It is also important to restrict its dispersal to other states of the Indian union and to devise pest management strategies for this pest.

Key words: Ladakh; *Phyllonorycter populifoliella*; poplar; invasive pest

Prvo poročilo o invazivnem škodljivcu na topolu, listnem zavrtaču *Phyllonorycter populifoliella* (Lepidoptera: Gracillariidae), na območju Ladakha

Izvleček: Listni zavrtač *Phyllonorycter populifoliella* (Treitschke 1883) je invazivna vrsta, o kateri prvič poročamo o pojavljanju na topolih v Ladakhu, Indija. Predstavljene so podrobnosti taksonomske določitve škodljivca na podlagi zunanje zgradbe genitalij. Poleg tega so predstavljeni še spekter njegovih gostiteljskih rastlin, habitati, v katerih se prehranjuje in stopnja napadenosti rastlin s tem škodljivcem v različnih zaselkih Ladakha. Raziskava je zelo pomembna za boljše razumevanje bionomije škodljivca, njegove diverzitete in odločanju o strategijah njegovega zatiranja. Pomembna je tudi za omejevanje njegovega širjenja na druga območja Indije in pri snovanju strategij za zatiranje tega škodljivca.

Ključne besede: Ladakh; *Phyllonorycter populifoliella;* topol; invazivni škodljivec

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1 INTRODUCTION

Ladakh is a cold desert region that spans over 70,000 km^2. This is India's high altitude, cold arid zone, which has harsh climatic conditions. This area, however, is endowed with unique flora and fauna, with poplars and willows serving as the main timber trees of the silviculture-agroforestry system. The poplar species in Ladakh include *Populus euphractica* Oliv., *P. alba* L., *P. nigra* L., *P. ciliata* Wall. ex Royle and *P. balsamifera* L. belonging to order Malpighiales and family Salicaceae. Poplar trees are used for wood as raw material for manufacturing furniture, doors, windows and other decorative objects. Additionally, these trees help to prevent soil erosion (Naithani & Nautiyal, 2012). Poplar trees meet the increased demand for fuelwood during harsh winters when temperatures drop below -30 °C (Kumar & Singh, 2012).

Several insect pests attack poplars around the world. Among them are defoliating beetle, *Chrysomela populi* (Lin., 1767) (Coleoptera: Chrysomelidae), tent caterpillar, *Malacosoma indica* Walker, 1855 (Lepidoptera: Lasiocampidae), Indian gypsy moth, *Lymantria abfuscata* Walker, 1865 (Lepidoptera: Erebidae) and poplar petiole gall aphid, *Pemphigus* spp. Passerini (Hemiptera: Aphididae) are some of the most common insect pests of poplar trees in Ladakh (Kumar et al., 2007). The poplar leaf blotch miner *Phyllonorycter populifoliella* (Treitschke, 1883) (Lepidoptera: Gracillariidae) has recently emerged as a threat to poplar plantations in this region (REF). Graccillariidae is a large family of leaf-mining insects that includes 107 recognised genera and 1993 species that feed on 7868 host plants (Anonymous, 2019). The majority of species can be found in temperate climates; 257 species have been described from the Palaearctic region and 81 from Neoarctic (De Prins & De Prins, 2009). The larvae breed in the leaf mesophyll (Davis & Robinson, 1998) and were first discovered in 1989 near the Kharkiv region of Ukraine (Sulkhanov, 1990).

In Graccillariidae family, larval development occurs up to four stages (Trägardh, 1913), known as hypermetamorphosis or heteromorphic development (Wagner et al., 2000). The initial larval stages feed on tree sap, while the later stages feed on tissues. The later larval stages contain well-developed chewing mandibles and hypognathous mouthparts and are called tissue feeding forms (Trägardh, 1913).

1.1 BIOLOGY OF *PHYLLONORYCTER* spp.

They are small insects and disperse rapidly from one locality to another in Ladakh, since there are no strict quarantine regulations around borders with China and Pakistan. The genus *Phyllonorycter* comprises over 380 species from all the zoogeographical regions (De Prins & De Prins, 2005). They have been found on 112 different plant genera in 31 different families (Lopez-Vaamonde et al., 2003; De Prins & De Prins, 2009). The larvae of *Phyllonorycter* spp. feed internally on living tissues of the plant and devour the parenchyma cells. The pupa and all pre-imaginal stages of the *Phyllonorycter* genus grow within a tentiform mine (Davis & Robinson, 1998). The larvae live inside the galleries, which provide them with shelter during adverse climatic conditions and protects them from natural enemies (Connar & Taverner, 1997).

During favourable conditions, moths are multivoltine and insect outbreaks occur on native plants (Bengtsson & Johansson, 2011; De Prins & Kawahara, 2012). The successive generations cause significant damage to woody plants (Kirichenko et al., 2019). The adult's small size allows them to pass through small crevices and holes in windowpanes or can be seen glued on the door entrances. The early larval instars invade all types of poplars and mine the leaves of the hosts. They make tentiform mines on the lower surface of the leaves. Generally, larvae grow on the underside of leaf and occasionally feed and make mines on the upper side of the leaf (Mutanen et al., 2007).

Our study reports the presence of this pest and infestation for the first time from Ladakh, India. The genital morphology of *P. populifoliella* was used to determine its taxonomic status. There has been no previous report on the possible presence of this pest in Ladakh, India.

2 MATERIALS AND METHODS

The study was conducted in Ladakh, India. Poplar is one of the important sources of timber in this region. There are about 10 species of poplars grown at different altitudes, including *Populus euphratica* , *P. alba, P ciliata* etc. The main surveyed areas in this study included Khaltsi, Phyang, Basgo, Stakna, Saspol, Minji, Silichee, Karbuthang and Hardas (Figure 1). Surveys were carried out in June and August 2018. Every three weeks, the poplar trees were selected randomly from the selected locations to observe the leaf miner damage on newly flushed leaves. Twenty shoots were observed from the lower canopy (1.0 m), at random from 50 trees per location and the number of leaves per shoots per tree was counted to calculate per cent infestation acord-

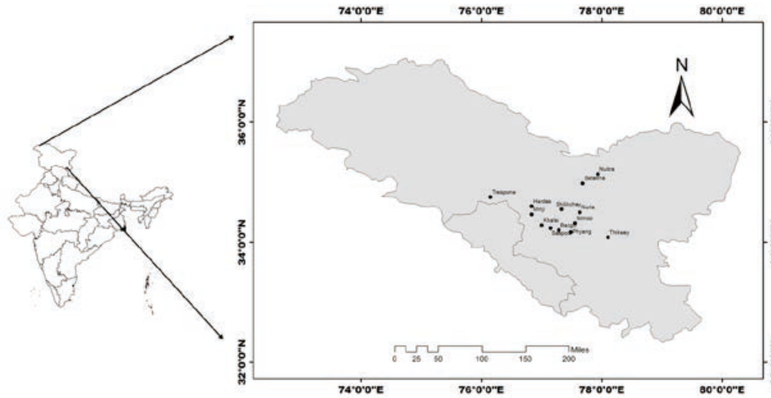

Figure 1: Survey locations for the survey of *Phyllonorycter populifoliella* on poplar trees in Ladakh, India

ing to Peña et al. (2000). The larvae were collected from the infested leaves and reared in plastic containers. The adults emerging were recorded on daily basis.

The data on infestation was subjected to analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) to understand the significant difference across different locations (SPSS (v.15.1; SPSS 168 Inc., Chicago, IL).

The infested leaves were excised and brought to High Altitude Entomology Laboratory at Satakna, Leh, Ladakh, and then kept in polystyrene jars glued with nylon lids on the top. The infested leaves were maintained at ambient temperature and periodically inspected for adult emergence for taxonomic identification. To study the morphology of genitalia, the methodology of Robinson (1976) was followed with slight modifications. The abdomens were cut and macerated in 10 % KOH solution for 18 hours to dissolve the extra body tissues. The samples were processed in 80 % ethanol. The genitalia were observed under a stereoscope binocular microscope (Olympus 598472, Japan).

3 RESULTS AND DISCUSSION

3.1 TAXONOMY

Poplar leaf blotch miner, p populifoliella (Treitschke, 1883), has been regarded as one of the most important pests globally (Selikhovkin, 2010). The species of the genus *Phyllonorycter* are difficult to identify because of the small size of adults and their wing pattern is rather similar across the species. In the middle Volga area of Russia, 13 species of the genus *Phyllonorycter* were identified based on female genitalia (Mishchenko, 2014). The descriptions of female genitalia were in accordance with Kuznetsov (1981). Noreika (1997) published the diagnostic keys of some species of this genus based on the structure of male genitalia. Rumyantsev (1934) confirmed the specimens of leaf miner in St. Petersburg as *P. populifoliella* based on genital morphology. The adults of *P. populifoliella* are relatively small (wingspan 6–9 mm). The forewings are white in colour with brown and ochre chess designs. The hind wings are white and translucent. Both front and hind wings are heavily fringed. The head is covered with a large tuft of white-colored hairs (Figure 2). The larvae pass through five instars and pupation takes place in the mines of the infested leaves. The entire development (from egg to adult) takes about 10–14 days. The larvae feed on poplar leaves, mining the bottom surface of leaves to form a tentiform mine without a fold (Figure 3). Pupation takes place in a rounded cocoon within a mine in the infested leaves. The second generation occurs in the second half of July. The pupa of the second generation undergoes diapause. High rates of infestations were observed in the study areas. The lowest infestation of 30 % was recorded in Nurla village, while the maximum infestation of 55.50 % recorded in the Nubra valley of Ladakh (Table 1).

Taxonomic keys to some species of the genus Phyllonorycter:

1 Presence of asymmetrical basal process of sacculus with a straight spine------2

- Absence of asymmetrical basal process of sacculus-------------------------------3

2 Cucullus provided with short dense hairs, corpus bursae with not well developed signum -------------------*Phyllonorycter mespilella*.

- Cucullus not provided with dense hairs. Valve long and narrow, sharp apical valval spines ---------------------*Phyllonorycter trifasciella*.

3 Spines not present on apical parts of valve. Valve wide, slightly asymmetrical with small projection on right cucullus, corpus bursae with well-developed signum---------------------*Phyllonorycter populifoliella*.

Table 1: Infestation (% per branch of lower area) by *Phyllonorycter populifoliella* on poplar trees at different locations in Ladakh, Indiations in Ladakh, India

Location	Infestation (%)
Mathoo	30.50 ± 2.9[fg]
Satakna	45.25 ± 4.2[d]
Tikhsey	42.35 ± 2.5[d]
Chachoot	50.00 ± 3.9[c]
Nubra	55.50 ± 5.5[b]
Goma	44.35 ± 5.0[d]
Bazgo	34.54 ± 3.5[f]
Nurla	30.00 ± 2.8[f]
Saspool	50.50 ± 6.0[a]
Nimoo	35.00 ± 2.8[f]
Khalsi	42.50 ± 6.2[d]
Phyang	40.20 ± 3.9[de]
Minji	32.00 ± 2.6[f]
Shilichee	28.80 ± 3.0[g]
Hardas	35.50 ± 4.1[f]
Trespone	47.25 ± 5.0[d]
Leh city	60.50 ± 5.8[a]

Values (Mean ± SE) with similar letters within a row do not differ significantly at $p \leq 0.05$ (DMRT)

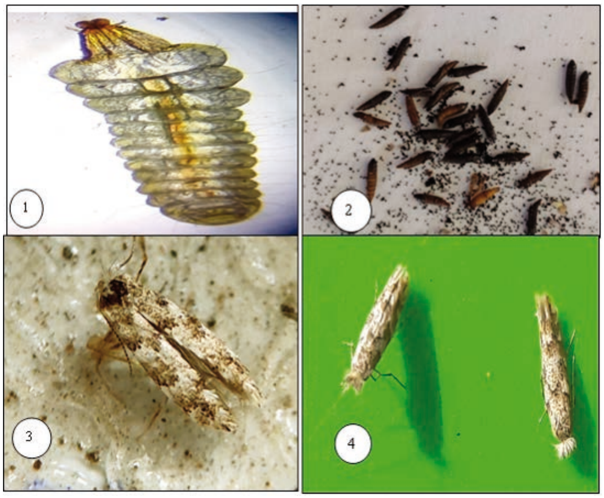

Figure 2: Life stages of *Phyllonorycter populifoliella*: 1. Larva, 2. Pupae, 3 and 4. Adults (Female-left, Male-right)

3.2 MALE AND FEMALE GENITALIA OF *PHYLLONORYCTER POPULIFOLIELLA*

Male genitalia with tegument is heavily sclerotized, half ring-shaped; juxta not clearly visible; valvae are wide and slightly asymmetrical with a small projection on left valva; sacculus is narrow and cucullus is wide with short and thin hairs on inner side; aedeagus is long, narrow, cylindrical-shaped with its basal end round and swollen (Figure 4).

Female genitalia has spherical corpus bursae, signum is spherical having two teeth; ostium bursae are without sclerotisation; anterior apophyses are longer than the posterior apophyses; papilla analis is trapezoidal, setose; ductus bursae are delicate, more or less zig-zag in shape with sac-like structure hanging on one side. The anterior and posterior margins of abdominal segment VII are not sclerotised (Figure 4).

3.3 LOCALITIES OF SPECIMENS EXAMINED

India: Ladakh, District Kargil and Leh; Minji, 34°28'N and 76° 50'E; Shilichee 34° 33' N and 76° 80' E; Hardas 34° 36' N and 76° 50' E. Trespone 34°45' N and 76°09' E; Bazgo 34° 12' N and 76° 80' E; Phyang 34°10' N and 77° 29' E; Satakna 33° 59' N and 77° 41' E; Khalsi 34° 17' N and 77°0 ' E; Nubra 34°68' N and 77°56' E; Nurla 34°30' N and 76°98' E; Thiksey 34°05' N and 77°66' E; Nimoo 34°19' N and 77°33' E; Saspool 34°14' N and 77°9' E.

3.4 INFESTATION

The P. populifoliella was recorded across the tested regions. The lower surfaces of the leaves and leaves of the lower branches were highly infested (Figure 3).

3.5 HOST RANGE

The main hosts of *Phyllonorycter populifoliella,* include black poplars such as *Populus nigra*, balsam poplars, such as *P. balsamifera, P. suaveolens* Fisch. ex Poit. & A.Vilm. and *P. laurifolia* Ledeb. (Ellis 2020; Ermolaev et al. 2020). The species of poplars in this study could not be identified due to the presence of several exotic hybrids in Ladakh.

Figure 3: Damage symptoms of *Phyllonorycter populifoliella* infestation on poplar leaves

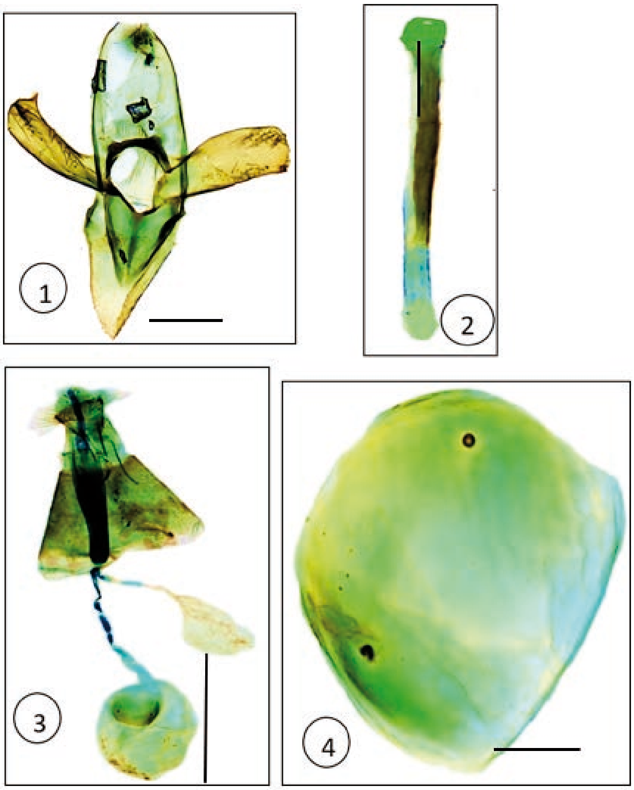

Figure 4: *Genital morphology of Phyllonorycter populifoliella: 1. Male genitalia; 2. Aedeagus: 3. Female genitalia; 4. Signum. Scale bars: 1-2 (200 µm), 3 (500 µm), 4 (300 µm)*

3.6 DISTRIBUTION

Austria, Belgium, Denmark, France, Germany, Italy, Russia, Spain, Turkey, Ukraine, United Kingdom.

3.7 DAMAGE

Though the leaf miner infestation does not cause immediate defoliation, reduced photosynthetic leads to reduced tree growth. Further, defoliation levels depend on the season and age and type of infested host (Raimondo et al., 2003), and summer defoliation has a strong impact on tree growth (Raimondo et al., 2003). Repeated defoliation over consecutive years can kill the trees. In addition, defoliation induced by leaf miners diminishes the aesthetic value of poplars.

4 CONCLUSION

This is for the first time that the poplar leaf miner *P. populifoliella* has been reported in the region of Ladakh, India. Though the pest could have been in the region much earlier, research was not conducted on this pest. In Ladakh, there are four native poplar species and many exotic poplars (Naithani & Nautiyal 2012). The presence of *P. populifoliella* in Ladakh can be attributed to the diversity of native and exotic species of poplars in this region, which are the main hosts of this insect. Till now, this pest has remained undiscovered due to the less density of poplars. Since poplars are the major source of firewood in the region, the discovery of new insect pests puts a major challenge to this industry. Further, in-depth studies on biology, pest behaviour and management strategies need to be worked out to contain this pest.

5 REFERENCES

Anonymous. (2019). *Global taxonomic database of Gracilleridae*. www.Gracilleridae.net (Assessed on July 04, 2020)

Bengtsson B.A., Johansson, R. (2011). *Fjärilar: Bronsmalar – rullvingemalar*. Lepidoptera: Roeslerstammiidae –Lyonetiidae. ArtDatabanken, Sveriges lantbruksuniversitet, Uppsala, pp. 494.

Connor E.F., Taverner M.P. (1997). The evolution and adaptive significance of the leaf-mining habit. Oikos, 79, 6-25. https://doi.org/10.2307/3546085

Davis D.R., Robinson G.S. (1998). The Tineoidea and Gracillarioidea. In: Kristensen, N.P. editor. *Handbook of Zoology IV/35, Lepidoptera, Moths and Butterflies. Vol. 1. Evolution, Systematics, and Biogeography.* Walter de Gruyter, Berlin, New York, pp. 91–117. https://doi.org/10.1515/9783110804744.91

De Prins J., Kawahara A. (2012). Systematics, revisionary taxonomy, and biodiversity of Afrotropical Lithocolletinae (Lepidoptera: Gracillariidae). *Zootaxa, 3594, 1–283.* https://doi.org/10.11646/zootaxa.3594.1.1

De Prins J., De Prins W. (2009). *Global Taxonomic Database of Gracillariidae (Lepidoptera)*. Royal Museum for Central Africa, Belgian Biodiversity Platform, Tervuren, Brussels, Belgium. Available from http://gc.bebif.be. (Assessed on July 10, 2020)

De Prins W., De Prins J. (2005). *Gracillariidae*, In World Catalog of Insects Apollo Books, Stenstrup, vol. 6.

Kirichenko N, Augustin S., Kenis, M. (2019). Invasive leaf miners on woody plants: a global review of pathways, impact, and management. *Journal of Pest Science*, 92, 93–106. https://doi.org/10.1007/s10340-018-1009-6

Kumar P.A., Namgyal D., Mir M.S., Bilal, A.S. (2007). Major insect pest associated with forest plantations in cold arid region, Ladakh of Jammu and Kashmir. *Journal of Entomological Research, 31*(2), 155–162.

Kumar D., Singh, N.B. (2012). Status of poplar introduction in India. *ENVIS Forestry Bulletin*, 12, 9–14.

Kuznetsov V.I. (1981). Family Gracillariidae Leaf Blotch Miners," In: Medvedev, G.S. editor. *A Key to the Insects of the European Part of the USSR*, (Nauka, Leningrad,), vol. IV, part 2,149–311.

Lopez-Vaamonde C., Godfray H.C.J., Cook J.M. (2003). Evolutionary dynamics of hostplant use in a genus of leaf mining moths. *Evolution*, *57*, 1804–1821. https://doi.org/10.1111/j.0014-3820.2003.tb00588.x

Mishchenko A.V. (2014). A Review of the Leaf Blotch Miners of the Genus Phyllonorycter Hübn. (Lepidoptera, Gracillariidae) in the Middle Volga Area, with a Key to the Species Using morphological Characters of the Female Genitalia. *Entomological Review, 94*(9), 1342–1347. https://doi.org/10.1134/S0013873814090176

Mutanen M., Itämies J., Kaila, L. (2007). *Heliozela resplendella* (Stainton, 1851) and *H. hammoniella Sorhagen*, 1885: two valid species distinguishable in the genitalia of both sexes and life histories (Heliozelidae). *Nota Lepidopterologica*, 30(1), 79–92.

Naithani H.B., Nautiyal S. (2012). Indian Poplars with special reference to indigenous species. *Forestry Bulletin*, 12(1), 1-8

Noreika R.V. (1997). Family Gracillariidae—Leaf Blotch Miners, In: Lehr, P.A. editor. *A Key to the Insects of the Russian Far East, (Dal'nauka, Vladivostok,), 5*(1), 373–429.

Peña J.E., Hunsberger A., Schaffer, B. (2000). Citrus leafminer (Lepidoptera: Gracillariidae) density: Effect on yield of 'Tahiti' lime. *Journal of Economic Entomology, 93*, 374–379. https://doi.org/10.1603/0022-0493-93.2.374

Raimondo F., Ghirardelli L.A., Nardini A., Salleo, S. (2003). Impact of the leaf miner Cameraria ohridella on photosynthesis, water relations and hydraulics of Aesculus hippocastanum leaves. *Trees - Structure and Function, 17,* 376–382. https://doi.org/10.1007/s00468-003-0248-0

Robinson G.S. (1976). The preparation of slides of Lepidoptera genitalia with special reference to the Microlepidoptera. *Entomologist´s Gazette, 27*, 127–132.
Rumyantsev P.D. (1934). Biology of the Poplar Leaf Blotch Miner (*Lithocolletis populifoliella* Tr.) in Moscow, *Zoologicheskii Zhurnal, 12*(2), 257–279.
Selikhovkin A.V. (2010). Specific features of popu-lation dynamics of the poplar leaf blotch miner *Phyllonorycter populifoliella* Tr. (Gracillariidae)," *Izvestiya Sankt-Peterburgskoi sotekhnicheskoi Akademii, 192*, 220–235.
Sulkhanov A.V. (1990). Species composition and spatial distribution of parasites of the poplar moth *Lithocolletis populifoliella* Tr.. *Biologicheskie Nauki* 7, 33–40.
Trägardh I. (1913). Contributions towards the comparative morphology of the trophi of the lepidopterous leaf-miners. *Arkiv för Zoologi*, 8, 148.

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Response of onion crop to bulb set size and planting date under mulching in dry Mediterranean environment

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Response of onion crop to bulb set size and planting date under mulching in dry Mediterranean environment

Abstract: The present pot experiment under open field conditions was conducted to evaluate the response of onion crop to bulb set size and planting date using mulching. Two different sizes of onion sets at planting (large (6-10 g) and small (2-6 g)) and three different planting dates (February, March, and April) with two soil coverings (with and without straw mulching) were tested. Treatments were replicated three times. Onion was not exposed to any drought stress during the course of the experiment.

Results indicated that the larger bulb sets which were planted earlier under mulching, maximised the total bulb yield (Yield, 44.0 t ha^{-1}), water use efficiency (WUE, 8.37 kg m^{-3}), and irrigation water use efficiency (IWUE, 9.57 kg m^{-3}). Moreover, findings revealed that onion crop appreciably respond to smaller bulb sets when they were planted earlier under mulching. Onion bulb responses were predicted to be linearly increased with the earliness in planting date, with an obvious better preference under mulching and heavier bulb sets. Hence, adopting early planting date with mulching is suggested for sustainable crop production and for enhancing water use efficiency in dry Mediterranean area.

Key words: total bulb yield; bulb shape index; water use efficiency; irrigation water use efficiency; trend analysis

Odziv pridelka čebule na velikost čebulčkov, datuma sadnje in mulčenja v suhem sredozemskem okolju

Izvleček: Lončni poskus na prostem je bil izveden za vrednotenje odziva pridelka čebule v odvisnosti od velikosti čebulčkov in datuma sadnje z uporabo mulčenja. Za sadnjo sta bili uporabljeni dve velikosti čebulčkov, velika (6-10 g) in mala (2-6 g), tri datumi sadnje (februar, marec, in april) in dva načina prekrivanja tal (z in brez zastrtja s slamo). Obravnavanja so bila s tremi ponovitvami. V času poskusa čebula ni bila izpostavljena sušnemu stresu.

Rezultati so pokazali, da je zgodnja sadnja večjih čebulčkov dala ob prekrivanju tal večji celokupni pridelek čebul (44,0 t ha^{-1}), imela je boljšo učinkovitost izabe vode (WUE; 8,37 kg m^{-3}), in večjo učinkovitost namakanja (IWUE; 9,57 kg m^{-3}). Izkazalo se je tudi, da je sadnja drobnejših čebulčkov dala dober pridelek, če so bili posajeni zgodaj in, če so bila tla prekrita. Napovedan odziv pridelka čebule se je linearno povečeval z zgodnostjo sadnje, predvsem pa z velikostjo čebulčkov in prekrivanjem tal. Zaradi naštetega priporočamo za trajnostno pridelavo čebule v suhem sredozemskem območju zgodnjo sadnjo in prekrivanje tal z mulčenjem.

Ključne besede: celokupen pridelek čebule; velikostni indeks čebul; učinkovitost izrabe vode; učinkovitost namakanja; analiza trendov

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1 INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important crops worldwide. The ecological conditions such as temperature and photoperiod largely affect its growth and production. Also, the cultural practices such as planting date, size of onion bulb sets at planting, and irrigation water availability have an impact on crop production (Brewster, 2008; Khokhar, 2014; Mubarak and Hamdan, 2018a). Onion crop thrives best when temperatures are cool during early development period and then warmer and sunny during maturity. Hence, planting date has a profound impact on onion crop growth and development. Early planting date tends to have a longer onion growing season before bulb initiation ensuring larger plants. However, large plants are more likely to become sensitive to the cold stimulus resulting in bolting (formation of seed stalk followed by flowering), which represents a highly unfavourable feature for onion bulb production. Large plants are also related with split bulbs. However, late-date-planted onions start forming bulbs before reaching satisfactory plant growth to support the final size of bulbs. This would produce very small bulbs, and therefore, decreasing the bulb yield (Brewster, 2008; Rohini and Paramaguru, 2016).

Onion crop has a shallow rooting system, and therefore, it is considered as a sensitive crop to water stress than other crops with deeper roots. Water use efficiency (WUE) is usually used to recognize the cultural practices by which the yield per unit water can be optimized. In the dry regions where water resources are limited as in the dry Mediterranean region, improving water use efficiency and crop yield represents a main challenge for agricultural water management. In this context, soil mulching could be considered as a key water-saving technique that would help in meeting both water scarcity and sustainable crop production. Mulching can decrease the loss of soil water through evaporation and maintains soil water content, and thereby reducing irrigation water requirements, promoting rooting system development, and increasing crop growth, development and yield (Vavrina and Roka, 2000; Gimenez et al., 2002; Hamma, 2013; Mutetwa and Mtaita, 2014; Mubarak and Hamdan, 2018b; Mubarak, 2020).

There have been several studies conducted on the effects of onion bulb sets at planting on both seed production (Singh and Sachan, 1999; Abedin et al., 1999; Khokhar et al., 2001; Ashrafuzzaman et al., 2009), and the total onion bulb yield at harvest (Addai et al., 2014; Addai and Anning, 2015). They reported that large onion sets produced the greatest vegetative development and total bulb yield. In other word, the larger the onion bulb sets, the higher the total bulb yield.

In the dry Mediterranean area, bulb sets as directly planted in the soil is a common practice, probably the simplest method employed to establish onion in the field as compared with planting using the seeds. Farmers plant onion bulb sets in the spring and harvest in the summer. The production period between April and August is characterized by no rainfall (Ragab and Prudhomme, 2002; Turner, 2004). Moreover, the Mediterranean climate is extremely variable with hot and dry summer, and cold and wet to dry winters. The Middle East and North Africa are dry areas, with only 1 % of the renewable water resources (Joffre and Rambal, 2001; Turner, 2004; Ceccarelli et al., 2007). The increasing climatic change have intensified the vulnerability to drought (Giorgi and Lionello, 2008; Somot et al., 2008; FAO, 2011; Polade et al., 2014). An increase by 1.25-2.5 °C in temperature is predicted in winter, and the precipitation between October and March will decrease by 10-15 % in the southern Mediterranean countries (Ragab and Prudhomme, 2002).

As the crop production is already limited by the water availability and local climate, moving towards feasible tools (such as using mulching) and agronomic practices (such as changing planting date) adapted to climate change is urgently needed for improving irrigation and cultivation period of crop (FAO, 2011; Khokhar, 2014; Zinkernagel et al., 2015). In this context, and responding to the Sustainable Development Goals (SDGs) putted forward by United Nations to adapt to climate change and to sustain agricultural production, the present work aimed to evaluate the combined effects of different planting date, bulb set size, and mulching on onion crop production. The obtained outcomes may encourage the introduction of alternative and more effective practices, to stimulate onion farmers in the region to adopt using straw mulching in their fields and to select bulb sets of uniform and large sizes for planting. This would sustain onion crop production with efficient water use in the dry Mediterranean area.

2 MATERIALS AND METHODS

A pot experiment was conducted under open field conditions at the Agricultural Experimental Station, near Damascus, Syria (33°20′ N, 36°26′ E, altitude 600 m), for different planting seasons in 2017. The site is located within a dry Mediterranean area, in which the long-period average of the total annual rainfall is about 120 mm. Some climatic data for the studied site collected during the growing seasons was fairly close to those averaged over the last 16 years (from 2000 to 2016) as can be shown in Table 1. For this reason, testing different planting seasons during one year (2017) seemed somewhat adequate.

The soil is classified as a clay loam, containing on average 29.5 % clay, 42.7 % silt, and 27.8 % sand. Both volumetric soil water contents at permanent wilting point (*PWP*) and field capacity (*FC*) are 0.18 and 0.36 $m^3 m^{-3}$, respectively. The chemical and physical soil properties are: pH 8.0; ECe 0.34 ds m^{-1}; organic matter 1.20 %; available P 5.7 ppm; NO_3^- 28.3 ppm; NH_4^+ 12.6 ppm.

Pots with dimensions of 25 × 30 cm and containing 8 kg of soil were used in the experiment. Three bulb sets of onion (*Allium cepa* 'Selmouni Red') were planted in each pot. The pots were set outdoors under natural climatic conditions. Plants were thinned after germination to two bulbs per pot, getting a plant density of about 400000 plants ha^{-1}.

Three different planting dates separated with 28 days were tested: PD1 (on February 8th), PD2 (on March 8th), and PD3 (on April 5th). At each planting season, the experiment was laid out following a 2 × 3 factorial experiment arranged in a randomized complete block design with two sizes of onion sets and two types of soil covering, with three replicates. The sizes of onion sets composed of large sets (OS1: 6-10 g), and small sets (OS2: 2-6 g). The soil covering comprised two distinct types. The first one (M1) was with straw mulching using 40 g pot^{-1} (about 8 t ha^{-1}); and the second one (M2) was without mulching. In all pots, plants received 100 % of the crop evapotranspiration; and the root zone was replenished to the field capacity. Irrigation water was applied three times/week. Each experiment was started on the planting day with a wet soil at field capacity as measured by pot's mass. The pots were weighed before and after each irrigation event. The water amount depleted (mm) between two successive irrigation events (ETc) was regulated by weight and estimated using (Eq.

$$ETc = \frac{Mt_1 - Mt_1}{\rho_w \times A} \qquad (1)$$

1) as:

where Mt_1 : the mass of the pot (kg) after irrigation (the soil water content in the pot was at the field capacity); Mt_2 : the mass of the pot (kg) just before the next irrigation event; ρ_w : the water density (g cm^{-3}); and A : the pot soil surface area (m^2). The daily crop evapotranspiration (mm day^{-1}) was estimated by dividing the ETc calculated using Eq. (1) by the number of days between two successive irrigations. The seasonal crop evapotranspiration was the summation of the daily ETc, which represented the total crop water requirements during a growing season.

For each planting season, phosphorous and potassium were applied as basal dose at planting day; but nitrogen fertilizer was divided into two equally split applications added during early vegetative stage. Irrigation was stopped when more than 50 % of leaf-head was hung and turned yellow. The onions were lifted to field cure about two weeks after. After the leaves were completely dried, they were cut leaving about 2.0 cm tops above the bulb. The length (BL), diameter (BD), and mass of both matured onion bulbs from each pot were measured. The shape index (Sh I) was calculated as the ratio of bulb length to diameter (BL/BD). The total bulb yield (Yield, t ha^{-1}) was estimated. Water use efficiency (WUE, kg m^{-3}) was estimated by dividing yield by the seasonal crop evapotranspiration. Irrigation wa-

Table 1: Some climatic data for the experimental station as averaged over the last 16 years (from 2000 to 2016), and those measured during the year 2017

Year	Variable	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.
2000-2016 average	T_{min} (⁰C)	4.0	6.8	10.1	14.1	17.6	19.3	20.4
	T_{max} (⁰C)	15.7	20.6	25.3	30.4	35.0	37.4	37.4
	$T_{average}$ (⁰C)	10.6	15.0	18.1	23.6	27.7	29.4	28.7
	RH (%)	75.0	64.1	60.9	56.5	56.3	60.7	60.2
	Rainfall (mm)	31.0	31.6	5.9	4.2	0.0	0.0	0.0
2017	T_{min} (⁰C)	4.0	6.2	9.7	14.4	17.2	20.6	20.0
	T_{max} (⁰C)	14.7	18.7	26.2	31.6	35.7	40.6	38.5
	$T_{average}$ (⁰C)	9.1	14.0	19.2	24.9	28.4	31.1	28.9
	RH (%)	69.3	74.4	63.1	57.9	56.3	56.0	59.0
	Rainfall (mm)	11.6	42.6	0.0	0.0	0.0	0.0	0.0

T_{min}: minimum temperature, T_{max}: maximum temperature, $T_{average}$: average temperature, RH: relative air humidity ter use efficiency (IWUE, kg m^{-3}) was also calculated by dividing yield by the irrigation water amount.

The two-way analysis of variance (ANOVA) was conducted using the DSAASTAT add-in (Onofri, 2007), for each planting season. A combined analysis of data over seasons was carried out to examine the interaction between planting season and the studied treatments (Gomez and Gomez, 1984). Mean comparison was made only for data after achieving the combined analysis using the LSD test at the 1 % level. Trend analysis (regression analysis) was also performed.

3 RESULTS AND DISCUSSION

Table 2 summarizes the effects of tested factors (planting date, onion set size, and soil cover system) on the measured traits of onion crop (BD, Sh I, Yield, WUE, and IWUE).

3.1 BULB SHAPE INDICATORS

Two indicators were used in this study to represent the shape of onion bulbs: bulb diameter (BD) and the shape index (Sh I). Both indicators were found to be highly affected by the main effects of all studied factors according to the ANOVA (Table 2). Since no significant interaction was observed, the data under each factor were pooled over the other factors for mean comparison purposes (Table 3).

Results indicated onion sets planted early in February (PD1) produced bulbs with larger diameter of 3.82 cm. Then, BD significantly decreased by 26 and 39 % compared with those planted later in March (PD2) and April (PD3), respectively. With regard to onion set size, the heavier set (OS1) produced bulbs with diameter 30 % larger than OS2. Moreover, onion sets grown under mulching produced bulbs with mean diameter significantly larger than those grown without mulching (Table 3).

Results showed that the minimum and maximum mean values of shape index (Sh I) were obtained from onion sets which were planted in PD1 and PD3 (or PD2), respectively. Also, using heavier onion sets (OS1) resulted in a decrease in Sh I by about 10 % relative to lighter onion sets (OS2). When mulching was used, Sh I significantly decreased by 20 % compared with no-mulching conditions (Table 3). As the tested variety is an oval- to elongated-shape onion, the better the bulb shape for marketing purposes, the lower the shape index. Hence, the suggested agricultural practice to obtain better shape index is to plant onion sets early in February using mulching and larger onion sets. Similar results about the role of mulching in enhancing the bulb shape indicator were reported by Mubarak and Hamdan (2018b).

Table 2: Analysis of variance of the combined data of crop responses as affected by planting date, onion set size, and soil cover system (significance of *F-test*)

Source of variance	*df*	BD	Sh I	Yield	WUE	IWUE
Planting date (PD)	2	***	***	***	***	***
Rep. within PD	6					
Onion set size (OS)	1	***	***	***	***	***
Soil cover system (M)	1	***	***	***	***	***
PD × OS	2	ns	ns	***	***	***
PD × M	2	ns	ns	ns	***	***
M × OS	1	ns	ns	***	***	***
PD × M × OS	2	ns	ns	ns	ns	ns
Pooled error	18					
Total	35					
CV (%)		5.17	9.21	5.51	5.85	5.94

*** = significant at 1‰ level, ns = non-significant at 1 % level,
df = degree of freedom, BD = Bulb diameter, Sh I = shape index, Yield = total bulb yield, WUE = water use efficiency, and IWUE = irrigation water use efficiency

Table 3: Mean comparisons of crop responses as influenced by planting date, soil cover system, and onion set size

	BD (cm)	Sh I (cm cm^{-1})	Yield (t ha^{-1})	WUE (kg m^{-3})	IWUE(kg m^{-3})
Planting date					
PD1 (February)	3.82 a	1.26 b	30.77 a	5.08 a	5.73 a
PD2 (March)	2.82 b	1.64 a	25.99 b	4.00 b	4.08 b
PD3 (April)	2.33 c	1.64 a	20.02 c	2.78 c	2.78 c
$LSD_{0.01}$	0.18	0.16	1.66	0.27	0.29
Onion set size					
OS1 (6-10 g)	3.36 a	1.43 b	31.73 a	4.89 a	5.18 a
OS2 (2-6 g)	2.62 b	1.60 a	19.45 b	3.02 b	3.21 b
$LSD_{0.01}$	0.15	0.13	1.35	0.22	0.24
Soil cover system					
M1 (with mulching)	3.37 a	1.36 b	31.16 a	5.46 a	5.83 a
M2 (without mulching)	2.61 b	1.67 a	20.03 b	2.45 b	2.56 b
$LSD_{0.01}$	0.15	0.13	1.35	0.22	0.24
Interactions					
PD1× OS1	— y	— y	37.89 a	6.23 a	7.02 a
PD1× OS2	—	—	23.64 c	3.93 c	4.44 c
PD2× OS1	—	—	32.44 b	4.98 b	5.08 b
PD2× OS2	—	—	19.54 d	3.02 e	3.08 d
PD3× OS1	—	—	24.87 c	3.45 d	3.45 d
PD3× OS2	—	—	15.17 e	2.12 f	2.12 e
$LSD_{0.01}$			2.34	0.38	0.41
PD1× M1	— y	— y	— y	6.94 a	7.93 a
PD1× M2	—	—	—	3.23 d	3.53 c
PD2× M1	—	—	—	5.52 b	5.65 b
PD2× M2	—	—	—	2.47 e	2.51 d
PD3× M1	—	—	—	3.92 c	3.92 c
PD3× M2	—	—	—	1.65 f	1.65 e
$LSD_{0.01}$				0.38	0.41
M1× OS1	— y	— y	38.07 a	6.67 a	7.12 a
M1× OS2	—	—	24.24 b	4.25 b	4.55 b
M2× OS1	—	—	25.40 b	3.11 c	3.25 c
M2× OS2	—	—	14.65 c	1.79 d	1.87 d
$LSD_{0.01}$			1.91	0.31	0.34

y"—" = Interaction is not significant.
In each column and studied factor, means followed by different letters are significantly different according to LSD at 1 % level. BD = Bulb diameter, Sh I= shape index, Yield = total bulb yield, WUE = water use efficiency, and IWUE = irrigation water use efficiency

3.2 TOTAL YIELD OF ONION BULBS (YIELD)

The ANOVA revealed that Yield was significantly influenced by the main effects of all studied factors at the 1 % level.

Results demonstrated that early planting date significantly enhanced the total yield of bulbs. An increase of 54 % in yield was obtained when onion sets were planted in February compared with the current practice followed by farmers (in April) (Table 3). This could be due to the fact that onion sets which were planted in February were subjected to cool temperatures during the vegetative stage, favouring photosynthesis, and consequently enhancing crop production. In fact, onion is a vegetative overwintering stage in its life cycle, i.e., it thrives best when temperatures are cool during early growth stages (Brewster, 2008). Cool temperatures during the vegetative stages allow having vigorous growth that ensures increased photosynthetic capacity by onion leaves and ultimately attain the maximum bulb development. Similar results were also documented by Mohanty (2002), Hamma (2013), Rohini and Paramaguru (2016), and Mubarak (2020).

In addition, using larger onion sets resulted in a considerable increase in yield (about 63 %) compared with smaller onion sets. Several studies reported similar results that the large sets produce heavier bulbs than the small sets (Ansari et al., 2009; Addai et al., 2014; Addai and Anning, 2015).

A further significant increase of 56 % in yield could be obtained under mulching relative to non-mulching conditions. The results are in accordance with similar findings previously reported (Vavrina and Roka, 2000; Igbadun et al., 2012; Hamma, 2013; Mutetwa and Mtaita, 2014; Mubarak and Hamdan, 2018b). In fact, mulch decreases evaporation from soil surface, providing more available water for plants (Igbadun et al., 2012). This could moderate the severity of wetting-drying cycle between irrigations, and therefore, yield could be improved (Vavrina and Roka, 2000; Gimenez et al., 2002; Mubarak and Hamdan, 2018b). Moreover, mulching could improve soil fertility and soil physical properties, and consequently, yield could be augmented under mulching (Khaledian et al., 2010; Khaledian et al., 2011).

On the other hand, the effect of planting date × onion set size interaction on yield was also highly significant (Table 2). Trend analysis showed that yield was predicted to be increased linearly with the earliness of planting date, for each tested size of onion sets (Fig. 1A). The slope of representative line under OS1 is about 1.5 times greater than that under OS2. That is to say that yield increased with the earliness of planting date in a higher rate using larger onion sets than using smaller sets. This could explain the existence of the interaction between planting date and onion set size.

For presentation and discussion purposes, the responses of yield to different planting dates under both mulching and non-mulching conditions were demonstrated in Figure 1B. Trend analysis showed also the linear relationship between yield and planting date ($p \leq 0.01$). Parallel representative lines confirmed the lack of interaction between planting date and soil cover system (PD × M) as found by ANOVA (Table 2).

The interaction effect of soil cover system and onion set size (M × OS) on yield was significant ($p \leq 0.001$). The maximum yield was obtained using larger onion sets planted under mulching (38.07 t ha^{-1}); while the minimum one was obtained using smaller onion sets without mulching (14.65 t ha^{-1}). Also, results indicated that using mulching enhanced the response of yield to smaller onion sets, with a significant increase of about 65 % (Table 3).

Consequently, both experimental data and developed linear equations could be used for accurate yield prediction in order to sustain onion crop production under similar climatic conditions. For example, the recommended agricultural management to maximize yield is to plant larger onion sets early in February under mulching conditions. In such management, Yield could be attained about 44 t ha^{-1}.

3.3 WATER USE PARAMETERS

The irrigation water applied to PD1, PD2, and PD3 were, 710, 800, and 910 under non-mulching conditions, and 460, 565, and 640 mm under mulching, respectively. Seasonal crop evapotranspiration (ETc), as calculated by Eq. (1), under non-mulching conditions were 775, 811, and 910 mm, while under mulching were 525, 578, and 640 mm, for PD1, PD2, and PD3, respectively. Both crop water use and irrigation water amount were considerably reduced when mulching was used. An important water portion of about 30 % was saved under mulching relative to non-mulching conditions. Similar findings were also documented by Mubarak and Hamdan (2018b). Furthermore, early planting date resulted in significant decreases in both crop water consumption and irrigation water amount. These findings emphasize the importance of both early planting date and mulching in water resource management for irrigation purposes. The irrigation water saving due to both early planting and mulching could be utilized to irrigate additional cropped area.

The analysis of variance demonstrated that both WUE and IWUE were highly significantly affected by the main effects of the three tested factors. The two-factor interactions were also highly significant (Table 2).

Onion sets planted early in PD1 recorded maximum WUE and IWUE. After that, a gradual reduction in both efficiencies was recorded. Also, both WUE and IWUE were optimised when larger onion sets were used with an important increase of 62 % relative to the use of smaller sets. In addition, when mulching was used, both WUE and IWUE were higher than those without mulching (Table 3).

Trend analysis showed that both WUE and IWUE

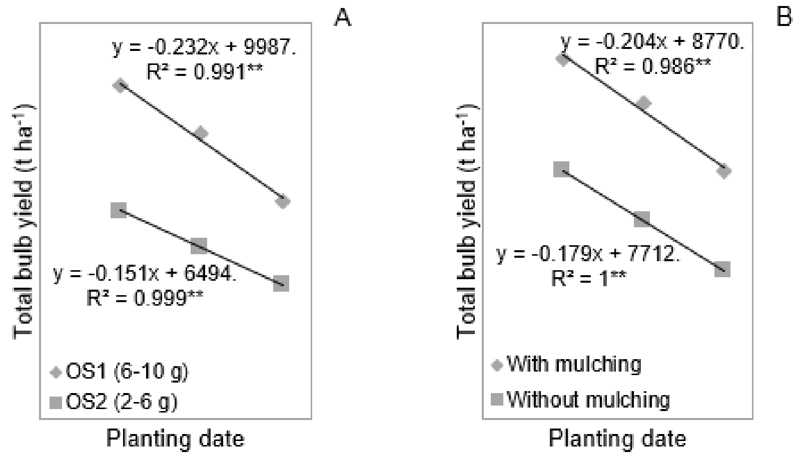

Fig. 1: Response of total onion bulb yield to plant. date under (A) the two sizes of onion sets at planting, and (B) the two types of soil covering. Regression equations were fitted and coefficients of determination (R^2) were given. ** = significant at 1 % level

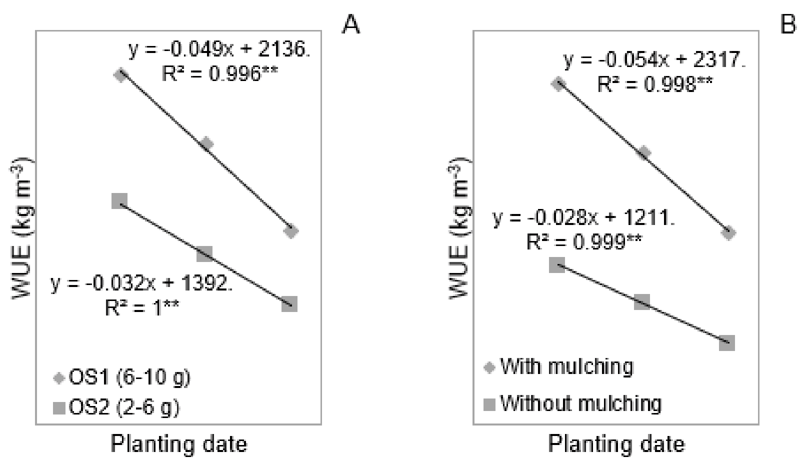

Fig. 2: Response of water use efficiency (WUE) to plant. date under (A) the two sizes of onion sets at plant., and (B) the two types of soil covering. Regression eq. were fitted and coefficients of determination (R^2) were given. ** = significant at 1 % level

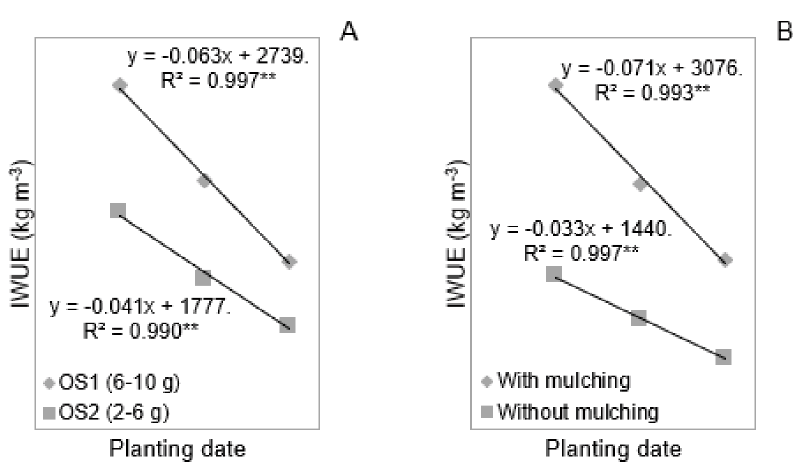

Fig. 3: Response of irrigation water use efficiency (IWUE) to planting date under (A) the two sizes of onion sets at planting, and (B) the two types of soil covering. Regression equations were fitted and coefficients of determination (R^2) were given. ** = significant at 1 % level

are gradually increased with the early planting date, with significant values of coefficient of determination (R^2) at the 1 ‰ level, under both sizes of onion sets (Fig. 2A for WUE, and Fig. 3A for IWUE). For each efficiency, both representative lines were not parallel, but did not intersect over the tested period. The slope of fitting line under OS1 was about 1.5 times higher than that under OS2. That is to say that the magnitude of improvements in both WUE and IWUE provoked by the earliness in planting date, could be multiple if larger onion sets are planted. This could illustrate the existence of the planting date × onion set size interaction (PD × OS).

The same trend was detected in the responses of WUE and IWUE to planting date under both types of soil covering (Figs. 2B and 3B, for WUE and IWUE respectively). That is, the degree of enhancements in both WUE and IWUE encouraged by the earliness in planting date could be doubled if mulching is used. The difference in response rate could explain the interaction between planting date and soil cover system (PD×M).

On the other hand, the soil cover system×onion set size interaction effect (M × OS) confirmed the important role of mulching in increasing both WUE and IWUE. The highest values of WUE (6.67 kg m^{-3}) and IWUE (7.12 kg m^{-3}) were obtained when larger onion sets grown under mulching. Significant decreases were observed when smaller sets or/and non-mulching was used. For instance, WUE was reduced by 75 % when smaller sets without mulching was used (Table 3).

Such experimental data and developed linear functions could be invested for predicting the targeted values of WUE and IWUE under the dry Mediterranean climate. For example, the best agricultural management suggested to have an efficient use of water (maximum values of both efficiencies) is to plant large onion sets under mulching in February with no-water stress. Under such conditions, WUE and IWUE could be reached 8.37 and 9.57 kg m^{-3}, respectively. Furthermore, another important result is that the yield of smaller onion sets could be significantly enhanced when they were planted earlier and if mulching is used. This finding is in agreement with similar results previously obtained on the importance of mulching. For example, Mubarak and Hamdan (2018b) demonstrated that an increase of about 134 % in water use efficiency could be achieved when mulching was used compared with non-mulching conditions, whatever the selected level of irrigation

4 CONCLUSIONS

The following conclusions and recommendations can be attained in the studied agro-pedo-climatic context:

Onion crop was found to be responsive to early planting date, larger onion sets at planting, and using straw mulching, so that both bulb shape indicators and the total bulb yield were significantly enhanced, compared with those obtained under the traditional agricultural practices (planting in April without mulching). Both water use efficiency and irrigation water use efficiency were also increased considerably; and the seasonal crop water use and irrigation water amount were found to be obviously decreased.

Results indicated that early planting date and using mulching improved the response of onion crop to smaller onion sets. This could be an appropriate agronomic alternative to meet the ever increasing demand for onions and to save irrigation water in the dry Mediterranean area.

Onion bulb responses were predicted to be increased linearly with the early planting date, but with obvious preferences using mulching and larger onion sets. Both experimental data and developed equations could be invested for predicting onion crop responses under similar environment without conducting any additional trials. Moreover, they could be used as a tool for rational management of onion crop production and limited water resources.

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6 REFERENCES

Abedin, M. J., Rahim, M. A., Islam, K. S., Haider, M. A. (1999). Effect of planting date and bulb size on the yield and quality of onion seed. *Bangladesh Journal of Seed Science and Technology, 3,* 25-28.

Addai, I. K. (2014). Effects of cultivar and culb size on growth and bulb yield of onion (*Allium cepa* L.) in the northern region of Ghana. *British Journal of Applied Science and Technology, 4*(14), 2090-2099. https://doi.org/10.9734/BJAST/2014/8458

Addai, I. K., Anning, D. K. (2015). Response of onion (*Allium cepa* L.) to bulb size at planting and NPK 15:15:15 fertilizer application rate in the Guinea Savannah. *Journal of Agronomy, 14*(4), 304-309. https://doi.org/10.3923/ja.2015.304.309

Ansari, N., Teixeira da Silva, J., Yazdani, N. (2009). Effect of onion set size and cultivar on production of green bunch onion (*Allium cepa*). *Middle Eastern and Russian Journal of Plant Science and Biotechnology. 3,* 5-9.

Ashrafuzzaman, M., Nasrul Millat, M., Razi Ismail, M., Uddin, M. K., Shahidullah, S. M., Meon, S. (2009). Paclobutrazol and bulb size effect on onion seed production. *International Journal of Agriculture and Biology, 11,* 245–250.

Brewster, J. L. (2008). *Onions and other vegetable alliums*. 2nd Ed., CAB International, Wallingford, United Kingdom. https://doi.org/10.1079/9781845933999.0000

Ceccarelli, S., Grando, S., Baum, M. (2007). Participatory plant breeding in water-limited environments. *Experimental Agriculture, 43,* 411-435. https://doi.org/10.1017/S0014479707005327

FAO (2011). *Climate change, water and food security*. Rome: FAO.

Gimenez, C., Otto, R. F., Castilla, N. (2002). Productivity of leaf and root vegetable crops under direct cover. *Scientia Horticulturae, 94,* 1-11. https://doi.org/10.1016/S0304-4238(01)00356-9

Giorgi, F., Lionello, P. (2008). Climate change projections for the Mediterranean region. *Global and Planetary Change, 63(2-3),* 90-104. https://doi.org/10.1016/j.gloplacha.2007.09.005

Gomez, K. A., Gomez, A. A. (1984). *Statistical Procedures for Agricultural Research* (2nd ed.). New York, NY: John Wiley & Sons.

Hamma, I. L. (2013). Growth and yield of onion as influenced by planting dates and mulching types in Samaru, Zaria. *International Journal of Advance Agricultural Research, 1,* 22-26.

Igbadun, H. E., Ramalan, A. A., Oiganji, E. (2012). Effects of regulated irrigation deficit and mulch on yield, water use and crop water productivity of onion in Samaru, Nigeria. *Agricultural Water Management, 109*, 162-169. https://doi.org/10.1016/j.agwat.2012.03.006

Joffre, R., Rambal, S. (2001). Mediterranean Ecosystems. In: eLS. John Wiley & Sons Ltd, Chichester. https://doi.org/10.1038/npg.els.0003196

Khaledian, M. R., Mailhol, J. C., Ruelle, P., Mubarak, I., Perret, S. (2010). The impacts of direct seeding into mulch on the energy balance of crop production system in the SE of France. *Soil and Tillage Research, 106*(2), 218-226. https://doi.org/10.1016/j.still.2009.10.002

Khaledian, M. R., Mailhol, J. C., Ruelle, P., Mubarak, I., Maraux, F. (2011). Nitrogen balance and irrigation water productivity for corn, sorghum and durum wheat under direct seeding compared with conventional tillage in the Southeastern France. *Irrigation Science, 29*(2), 413-422. https://doi.org/10.1007/s00271-010-0250-4

Khokhar, K. M., Hussain, S. I., Mahmood, T., Hidayatullah, M. H., Bhatti, M. H. (2001). Effect of set size on bulb yield, maturity and bolting in local and exotic cultivars of onion during autumn season. *Sarhad Journal of Agriculture, 17,* 355-358.

Khokhar, K. M. (2014). Flowering and seed development in onion-A Review. *Open Access Library Journal, 1:* e1049. http://dx.doi.org/10.4236/oalib.1101049

Mohanty, B. K. (2002). Variability, heritability, interrelationship and path analysis in onion. *Haryana Journal of Horticultural Sciences 31(1-2),* 84-87.

Mubarak, I. (2020). Improving water productivity and yield of onion crop by combining early planting and straw mulch under different irrigation levels in dry Mediterranean region. *Advances in Horticultural Science, 34(1),* 49-60.

Mubarak, I., Hamdan, T. (2018a). Onion crop response to different irrigation and N-fertilizer levels in dry Mediterranean region. *Advances in Horticultural Science*, *32(4)*, 495-501.

Mubarak, I., Hamdan, T. (2018b). Onion crop response to regulated deficit irrigation under mulching in dry Mediterranean region. *Journal Horticultural Research*, *26(1)*, 87-94. https://doi.org/10.2478/johr-2018-0010

Mutetwa, M., Mtaita, T. (2014). Effects of mulching and fertilizer sources on growth and yield of onion. *Journal of Global Innovation in Agricultural and Social Sciences, 3,* 102-106. https://doi.org/10.17957/JGIASS/2.3.561

Onofri, A. (2007). Routine statistical analyses of field experiments by using an Excel extension. National Conference Italian Biometric Society. Proceeding 6th (Pisa). In: "*La statistica nelle scienze della vita e dell'ambiente*"; *20-22,* 93-96.

Polade, S. D., Pierce, D. W., Cayan, D. R., Gershunov, A., Dettinger, M. D. (2014). The key role of dry days in changing regional climate and precipitation regimes. *Scientific Reports, 4,* 43-64. https://doi.org/10.1038/srep04364

Ragab, R., Prudhomme, C. (2002). Climate change and water resources management in arid and semi arid regions: prospective and challenges for the 21st century. *Biosystems Engineering, 81*, 3-34. https://doi.org/10.1006/bioe.2001.0013

Rohini, N., Paramaguru, P. (2016). Seasons' influence on bulb, seed yield and quality of aggregatum onion. *International Journal of Farm Sciences, 6(1),* 174-183.

Singh, S. R., Sachan, B. P. (1999). Interaction of bulb size and spacing on seed yield and yield attributing trait of onion (*Allim cepa* L.) cv. Kalyanpur Round Red. *Scientia Horticulturae, 6,* 125–128.

Somot, S., Sevault, F., Deque, M., Crepon, M. (2008). 21st century climate change scenario for the Mediterranean using a coupled atmosphere-ocean regional climate model. *Global Planet Change, 63(2-3),* 112-126. https://doi.org/10.1016/j.gloplacha.2007.10.003

Turner, N.C. (2004). Sustainable production of crops and pastures under drought in a Mediterranean environment. *Annals of Applied Biology*, *144*, 139-147. https://doi.org/10.1111/j.1744-7348.2004.tb00327.x

Vavrina, C. S., Roka, F. M. (2000). Comparison of plastic mulch and bareground production and economics for short-day onions in a semitropical environment. *HortTechnology, 10,* 326-330. https://doi.org/10.21273/HORTTECH.10.2.326

Zinkernagela, J., Schmidta, N., Kahlen, K. (2015). Changing thermal growing season and climatic water balance affect irrigation and cultivation period of vegetables. *Procedia Environmental Sciences, 29,* 51-52. https://doi.org/10.1016/j.proenv.2015.07.153

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Marker-trait association study for root-related traits in chickpea (*Cicer arietinum* L.)

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Abstract: Root structure modification can improve important agronomic traits including yield, drought tolerance and nutrient deficiency resistance. The aim of the present study was to investigate the diversity of root traits and to find simple sequence repeat (SSR) markers linked to root traits in chickpea (*Cicer arietinum* L.). This research was performed using 39 diverse accessions of chickpea. The results showed that there is significant variation in root traits among chickpea genotypes. A total of 26 alleles were detected 26 polymorphic bands were produced by 10 SSR markers in the eight linkage groups (LG). The results indicated that there is substantial variability present in chickpea germplasm for root traits. By analyzing the population structure, four subpopulations were identified. PsAS2, AF016458, 16549 and 19075 SSR markers on LG1, LG3, LG2 and LG1 linkage group respectively were associated with root traits**.** The research findings provide valuable information for improving root traits for chickpea breeders.

Key words: linkage groups; drought tolerance; population structure; SSR markers; subpopulations; variation

Raziskava povezave genskih označevalcev in lastnosti korenin pri čičerki (*Cicer arietinum* L.)

Izvleček: Sprememba zgradbe korenin lahko izboljša pomembne agronomske lastnosti vključno s pridelkom, strpnost za sušo in odpornost na pomanjkanje hranil. Namen raziskave je bil preučiti raznolikost lastnosti korenin in najti enostavne označevalce ponavljajočih se zaporedij (SSR) povezanih z lastnostmi korenin pri čičerki (*Cicer arietinum* L.). Raziskava je bila opravljena na 39 različnih akcesijah čičerke. Rezultati so pokazali, da obstaja značilna spremenljivost v lastnostih korenin med genotipi čičerke. Celokupno je bilo ugotovljeno 26 alelov. 10 SSR označevalcev je dalo 26 polimorfnih prog v osmih povezanih skupinah (LG). Izsledki so pokazali, da obstaja v dednem materialu čičerke znatna variabilnost v lastnostih korenin. Z analizo zgradbe populacije so bile ugotovljene štiri podpopulacije. PsAS2, AF016458, 16549 in 19075 SSR označevalci v LG1, LG3, LG2 in LG1 povezanih skupinah so bili povezani z lastnostmi korenin. Ugotovitve raziskave prispevajo žlahtniteljem čičerke pomembne informacije za izboljšanje lastnosti korenin.

Ključne besede: povezane skupine**;** toleranca na sušo; zgradba populacije; SSR označevalci; podpopulacije; variabilnost

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1 INTRODUCTION

Chickpea (*Cicer arietinum* L., 2n = 16) as a third major legume in the world widely used for food and fodder. Numerous biotic and abiotic stresses affect the production and yield of chickpea of which drought is one of the most important abiotic constraints. Drought causes heavy production losses, about 45–50 % in chickpea (Ahmad et al., 2005).

For drought management, genetic improvement over crop options for better adaptation to drought can be a sustainable and low-cost solution. But, it is very difficult to understand the maintenance of potential yield under drought stress, due to the different mechanisms used by plants to maintain growth under limited water resource, (Tuberosa & Salvi, 2006**).** The major challenges in identifying drought tolerance genotypes is drought interaction with the environment and its quantitative inheritance (Varshney et al., 2014).

Root structure modification can improve important agronomic traits including yield, drought tolerance and nutrient deficiency resistance (Tuberosa et al., 2002; Beebe et al., 2006; Ghanem et al., 2011). Despite, approximately small populations and inaccurate phenotyping cause it difficult to make large scale use of root genetic information in plant breeding (de Dorlodot et al., 2007). From now, correct phenotyping and characterization of root traits is necessary for translating novel physiological and genetic progresses into a conception of the role of root systems in increasing yield and productivity (especially in dry environments). The effect of diverse root features on drought tolerance were found to be high under final drought stress condition, mainly in environment where plant only confide in the stored soil water (Ludlow & Muchow, 1990; Kashiwagi et al., 2006; Passioura, 2006; Wasson et al., 2014). For example, Kirkegaard et al. (2007) indicated using root traits and soil moisture assessments in the field, that a 30 cm enhance in root depth increased the uptake of 10 mm more underground soil moisture and thus increased the yield by 0.5 t ha^{-1} grain yield. it also was demonstrated that Large root system effect on shoot biomass production and harvest index (HI) under terminal drought stress (Kashiwagi et al., 2006; Zaman-Allah et al., 2011). Although plant breeders are aware of the worth of the root system offering, but due to the low heritability of root traits, high variation in expression in different soils and soil moisture environments, and the difficulty of measuring these traits in the field has been less pay attention to these traits selection (Tuberosa et al., 2002; Malamy, 2005; Gaur et al., 2008).

Genetic diversity has been investigated using diverse types of *DNA markers,* including SSR in chickpea (Sefera et al., 2011; Keneni et al., 2012; Ghaffari et al., 2014; Hajibarat et al., 2015). *DNA* markers have been found for many agronomic traits (Thudi et al., 2014a).

Majority of the breeding attempts made in chickpea have been, and are being, focused on improving yield, resistance to diseases like *Ascochyta* blight and *Fusarium* wilt (Varshney et al., 2014a) and on tolerance to various abiotic stresses (such as drought (Varshney et. al., 2014; Jaganathan et al., 2015), cold (Mugabe et. al., 2019) and heat tolerance (Jha et al., 2018)). However even with the value of root traits and their critical roles in drought and heat adaptation in chickpea (Maphosa et. al., 2020), their genetic control has been less studied. Consequential associations between markers and quantitative traits led to the identification of locus significantly associated with drought tolerance. The root phenotyping problems has reduced the identity of root trait genomic locus in chickpea thus the aim of this research was to identify of the SSR markers associated with root-related traits in a various chickpea germplasm.

2 MATERIAL AND METHODS

Plant material contains 39 chickpea genotypes, including accessions from ICARDA (International Center for Agricultural Research in the Dry Areas) chickpea germplasm (Table 1). These entries were selected based on the results of previous drought tolerance trials in Kabuli type chickpea genotypes.

2.1 GENOTYPING

2.1.1 DNA extraction and SSR primers, PCR and agarose gel electrophoresis

Genomic DNA was extracted from young leaflets of chickpea genotypes plant leaves (4 plants of each genotypes) using a CTAB method according Doyle and Doyle (1987) with a slight modification. On the basis of their locations on the eight linkage groups (LGs) of the integrated genetic linkage map of chickpea (*Cicer arietinum* L.), altogether 10 SSR markers were select (Sefera et al., 2011) (Table 2). PCR was carried out in a 14 µl reaction mixture that contain 100 ng of DNA, 100 pmol of each primer (forward and reverse), 7µl of CinnaGen PCR master mix, 2 X (0.08 units μl^{-1} Taq DNA polymerase in reaction buffer, 3 mmol $MgCl_2$, and 1.6 mmol dNTPs). The amplifications were performed with a Thermal Cycler (Applied Bio Rad, Foster City, CA, USA), with an initial denaturation at 94 ^{0}C for 240 sec that was followed by 10 cycles of: at 94 ^{0}C for 30 s, 45 s at annealing temperature (Ta) (Table 2), 120 s at 72 ^{0}C, and then was followed by 25 cycles of: 30 s at 94 ^{0}C, 45 s at Ta, 120 s at 72 ^{0}C and a final extension step at 72 ^{0}C for 420 s In 2.5 % agarose gel by 1X TBE running buffer, amplified fragments were resolved and quantity one software (Bio-Rad, CA 94547, USA) analyzed images.

Table 1: The list of genotypes used in the present study

NO.	Genotype Name	Pedigree
1	FLIP97-706C	X04TH62/X03TH-130XFLIP97-116
2	FLIP03-77C	X04TH65/X03TH-133XFLIP96-154
3	FLIP03-130C	X04TH65/X03TH-133XFLIP96-154
4	FLIP06-158C	X04TH65/X03TH-133XFLIP96-154
5	FLIP07-19C	X04TH66/X03TH-134XFLIP97-116
6	FLIP07-20C	X04TH66/X03TH-134XFLIP97-116
7	FLIP07-22C	X04TH66/X03TH-134XFLIP97-116
8	FLIP07-28C	X04TH67/X03TH-135XFLIP99-34
9	FLIP07-31C	X04TH67/X03TH-135XFLIP99-34
10	FLIP07-44C	X04TH76/X03TH-144XFLIP97-116
11	FLIP07-239C	X04TH77/X03TH-145XFLIP99-34
12	FLIP07-261C	X04TH79/X03TH-147XFLIP96-154
13	FLIP07-280C	X04TH110/X03TH-178XFLIP97-116
14	FLIP08-46C	X04TH110/X03TH-178XFLIP97-116
15	FLIP08-200C	X04TH114/X03TH-182XFLIP97-116
16	FLIP09-70C	X04TH115/X03TH-183XFLIP99-34
17	FLIP09-81C	X04TH117/X03TH-185XFLIP96-154
18	FLIP09-85C	X04TH123/FLIP97-205XFLIP97-116
19	FLIP09-90C	X04TH124/FLIP97-229XFLIP99-34
20	FLIP09-98C	X04TH126/FLIP98-229XFLIP96-154
21	FLIP09-148C	X04TH129/FLIP98-233XFLIP99-48
22	FLIP09-149C	X05TH7/X04TH-126XFLIP01-18
23	FLIP09-189C	X05TH106/FLIP97-131XFLIP00-14
24	FLIP09-191C	X05TH106/FLIP97-131XFLIP00-14
25	FLIP09-192C	X05TH106/FLIP97-131XFLIP00-14
26	FLIP09-194C	X05TH106/FLIP97-131XFLIP00-14
27	FLIP09-214C	X05TH131/FLIP97-118XFLIP00-17
28	FLIP09-216C	X05TH152/FLIP98-107XUC27
29	FLIP09-218C	X04TH31/X03TH-31XFLIP97-116
30	FLIP09-219C	X06TH100/FLIP02-47XFLIP98-230
31	ILC482	ILC482
32	FLIP 82-150C	X79TH101/ILC 523 X ILC 183
33	FLIP88-85C	X85 TH143/ILC 629 x FLIP 82-144C
34	FLIP93-93C	X89TH258/ (FLIP 85-122CXFLIP 82-150C)/FLIP 86-77C
35	FLIP07-180C	X04TH12/X03TH-12XFLIP99-48
36	FLIP09-88C	X04TH40/X03TH-40XFLIP99-34
37	FLIP09-115C	X04TH50/X03TH-50XFLIP99-34
38	FLIP09-337C	X04TH53/X03TH-53XFLIP97-116
39	FLIP09-386C	X04TH59/X03TH-59XFLIP99-48

Table 2: The list of genotypes used in the present study

NO.	Marker name	Primer sequences(5'-3')	Linkage Group	Annealing temperature (^{0}C)
1	19075	F:CACGAGTACAACATGGAGTGAAG R: CAAGCTCAACCTCCTCATACC	LG1	57.75
2	18363	F:CATGCATGGAGTTGGAAGAG R: GTCCCAAAATGCAGCCAATA	LG3	55.6
3	16549	F:CAATGAGATGCTGGCGATAA R: GTTCGGTGTTGTGGGTTTTT	LG2	55.7
4	C24	F:GCTACTGGAGGAGGCTTTCA R: GCCTTCTACACAACGGCTTC	LG4	58.2
5	PsAS2	F: CTAATCACACGTTTAGGACCGG R: CGAAATCCAAACCGAACCTAATCC	LG1	58.9
6	PSAB60	F:AATTAATGCCAATCCTAAGGTATT R: GGTTGCACTATTTTCGTTCTC	LG6	53.95
7	PD23	F:ATGGTTGTCCCAGGATAGATAAR: GAAAACATTGGAGAGTGGAGTA	LG 5	54.9
8	PSAD147	F: AGCCCAAGTTTCTTCTGAATCC R: GAAAACATTGGAGAGTGGAGTA	LG7	57.55
9	17605	F:CGCCCTTCATCATCATCTTC R: AAATTCGCAGAGCGTTTGTTAC	LG 8	57.35
10	AF016458	F:CGCCCTTCATCATCATCTTC R: CGAATCTTGGCCATGAGAGTTGC	LG 3	57

2.2 PHENOTYPING

2.2.1 Root sample extraction and processing

The experiment was conducted in Glasshouse at Ilam university. The average daily temperature was 25/16 ^{0}C (day/night), and the humidity was 70 %. Experiment was carried out in completely randomized design (CRD) with four replications. The seeds of each genotype were sown in split drain pipes (SDP) with 60 cm height and 10 cm diameter. The soil used in SDP was a mixture of sand and Jons Innes No-2 (1:1 ratio). Each SDP was put together with a single plant. The plants were harvested 30 days after germination. Plants were harvested on 35 day after germination based on taproot length increments for the growth period (Chen et al., 2017).

2.2.1 Root-related traits

Chickpea root samples were taken to record root traits. Using a water shower, the soil was separated from the roots and then the fresh mass of the roots was measured. Then, by floating the root samples in water in a tray, organic debris and weed roots were removed manually from chickpea roots. The fresh soil and roots were thereupon dried in an oven at 65 °C for 72 hours and the percentage of soil and root moisture was obtained. The root characteristics are showed in Table 3.

2.3 STATISTICAL ANALYSIS

Analysis of variance was performed with the SAS 9.2 software to evaluate the factor 'GENOTYPE". The genotype means were compared by a Duncan's multiple range post hoc test and used for the association analyses.

2.4 ASSOCIATION ANALYSES

The polymorphism information content (PIC) value was calculated using $PIC = 1\text{-}\Sigma\ (P_{ij})^2$ (Where Pij is the frequency of jth allele in ith primer and summation extends over 'n' patterns) (Nei , 1973) for each primer. PIC describe content of 'gene diversity'.

NTSYSpc 2.02e was used to compute Jaccard similarity coefficients to report genetic relationships among the chickpea genotypes. Also using this software and based on genetic distances, cluster analysis was carried out using the unweighted pair-group (UPGMA) method and the dendrograms were drawn (Rohlf, 2000).

The marker–trait association between the SSR markers and each of root related traits tested using TASSEL 4.0. (Bradbury et al., 2007). General linear model (GLM) and mixed linear model (MLM) approaches used for association analysis. Covariates in GLM and MLM analyses were the corresponding *Q* values. Manhattan plots present association between a SSR marker and phenotypic trait that was significant at $p \leq 0.05$. STRUCTURE version 2.3.4 used for determine the population structure of the 39 accessions using the Bayesian clustering method (Pritchard et al., 2000). The STRUCTURE analysis separated the population based on ΔK method (Evanno et al., 2005).

Table 3: The root related trait measured in the present study

No.	Trait	Formula	Unit of measurement	References
1	Root length (RL)	Total RL of each sample was measured using a ruler.	cm	-
2	Root fresh mass (RFM)	The fresh weight of the roots was measured with a digital scale to the nearest thousandth	g	-
3	Root dry mass (RDM)	The roots were kept for oven drying at 70 ◦C for 72 h (to constant mass) then was estimated.	g	Ramamoorthy et al., 2017
4	Dry mass of plant shoots (SDM)	The shoots were kept for oven drying at 70 ◦C for 72 h (to constant mass) then SDW was estimated	g	Ramamoorthy et al., 2017
5	Root volume (RV)	$RV = B - C$	cm^3	-
6	Root area (RA)	$RA = 2 \times SQRT \langle RV \times 3.14 \times RL \rangle$	cm^2	Akhavan et al., 2012
7	Root fineness (RF)	$RF = \frac{RL}{RV}$	cm root /root fresh mass	Hajabbasi, 2001
8	Root diameter (Rd)	$Rd = SQRT \frac{\langle 4 \times RFW \rangle}{\langle RL \times 3.14 \rangle}$	cm	Schenk & Barber, 1979
9	root length (SRL) Specific	$SRL = \frac{RL}{RDW}$	cm root length cm^{-3} soil volume	Mahanta et al., 2014
10	Root water content (RWC)	$RWC = \frac{RFW - RDW}{RDW}$	g	Lovelli et al., 2012
11	Root length density (RLD)	$RLD = \frac{RL}{SV}$	cm RL cm^{-3} soil volume	Mahanta et al., 2014
12	Specific root volume (SRV)	$SRV = \frac{RDW}{SV}$	g RDW cm^{-3} soil volume)	Hasanabadi et al., 2010
13	Root tissue density (RTD)	$RTD = RDW \times RV$	g RDW× cm^3 soil volume	Paula & Pausas, 2011
14	Root volume density (RVD)	$RVD = \frac{RFW}{SV}$	cm m^{-3}	Hajabbasi, 2001
15	Root area density (RAD)	$RAD = RL \times RD \times 3.14$	$cm^2\, cm^{-3}$	Akhavan et al., 2012
16	Root density (RD)	$RD = \frac{RDW}{RV}$	g cm^{-3}	Akhavan et al., 2012

B =water and root volume, C = water volume, SQRT = root square

3 RESULTS

3.1 ANALYSIS OF ROOT TRAITS DATA

Root morphological traits differed significantly among genotypes. All of 16 measured root related traits differed significantly among genotypes ($p \leq 0.001$) (Table 4). The average root length was 50.69 cm and ranged from 27 to 72 cm (Table 5). The variation (Coef. Var.) in RL among genotypes was 20.7 % (Table 5). Root volume (RV) and root fresh mass (RFM) varied significantly among genotypes (Table 5). RV ranged from 3.75 cm^3 (FLIP07-28C) to 22 cm^3 (FLIP07-31C), with an average root volume of 11.5 cm^3. The root fresh mass (RFM) averaged 10.93 g across all genotypes. RFM varied among genotypes and ranged from 2.69 g (FLIP07-28C) to 22.52 g (FLIP09-192C). Root dry mass (RDM) was 0.15 g (ILC482) to 3.93g (FLIP09-192C) (average 1.33 g). The average leaf dry mass (LDM) was 0.91g, ranging from 0.17 g (FLIP07-31C) to 2.28 g (FLIP09-192C), and root fineness (RF) ranged from 2.07 FLIP97-706C to 13 (FLIP88-85C) (mean 4.95 cm root / root fresh mass). The average specific root length (SRL) was 50.37 cm and ranged from 15 cm (FLIP97-706C) to 238.71 cm (FLIP 82-150C). Root water content (RWC) averaged 8.30 g across all genotypes. RWC ranged from 2.58 (FLIP07-31C) to 30.88 (FLIP07-20C). The average root tissue density (RTD) ranging from 0.61 (ILC482) to 86.53 (FLIP07-31C) (mean 16.47 g RDW × cm^3 soil volume). Root diameter (Rd) ranged from 0.038 cm (FLIP88-85C) to 0.27 cm (FLIP09-192C), with an average root volume of 0.12 cm. The average root area (RA) was 83.69 cm^2 and ranged from 36.83 cm^2 (FLIP07-28C) to 127.68 cm^2 (FLIP07-31C). The average root density(RD) was 0.52 g cm^{-3} and ranged from 0.29 g cm^{-3} (ILC482) to 0.71 g cm^{-3} (FLIP07-31C). Root length density (RLD) ranging from 0.05 (FLIP07-28C) to 0.13 (FLIP07-20C) (mean 0.094 cm RL cm^{-3} soil volume). The average specific root volume (SRV) was 0.0025 g RDM cm^{-3} soil volume and ranged from 0.0028 (ILC482) to 0.0073 g RDM cm^{-3} soil volume (FLIP09-192C). Root volume density (RVD) ranged from 0.0050 cm m^{-3} (FLIP07-28C) to 0.042 cm m^{-3} (FLIP09-192C), with an average RVD of 0.020 cm m^{-3}. Root area density (RAD) averaged across all genotypes 82.14 cm^2 cm^{-3}. RAD ranged from 30.20 cm^2 cm^{-3} (FLIP07-28C) to 129.19 cm^2 cm^{-3} (FLIP09-192C).

3.2 SSR MARKER SCREENING AND GENETIC DIVERSITY ASSESSMENT

Using the SSR marker system the genetic diversity of 39 chickpea genotypes analyzed. Detected alleles were 26. 2-3 bands with an average number of 2.6 alleles per locus observed. AF016458، 17605، PSAD147، 19075، 16549 and PD23 had 3 alleles.

All of the amplification products (100 %) showed polymorphism, denoted high variation among chickpea accessions at the DNA level. Size of fragments produced varied from 110 to 150 bp (Table 6). The highest PIC was for primer 16549 and PSAD147 (0.54) and the lowest PIC was for the primer C24 (0.38). Hence, primer 16549 and PSAD147 were effective and useful markers for determining the genetic differences among the chickpea genotypes (Table 6).

The cluster analysis showed that the 39 accessions were divided into five clusters (Fig. 1). The first cluster included FLIP97-706C and FLIP03-77C. The second cluster included only FLIP09-148C. The third cluster included FLIP09-85C, FLIP09-90C, FLIP09-98C, FLIP09-115C, FLIP09-337C and FLIP09-386C. The forth cluster included FLIP03-130C, FLIP09-214C, FLIP09-216C, FLIP09-218C, FLIP09-219C, ILC482, FLIP 82-150C, FLIP88-85C, FLIP93-93C, FLIP07-180C and FLIP09-88C. The fifth cluster included FLIP06-158C, FLIP07-20C, FLIP07-239C, FLIP07-280C, FLIP08-200C, FLIP09-149C, FLIP09-189C, FLIP09-191C and FLIP09-192C.

3.3 POPULATION STRUCTURE

The marker segregation data was used for the population clustering, the STRUCTURE analysis separated the population into four cluster (Fig. 2). The 39 chickpea genotypes were grouped in to four subpopulations, as viewed in STRUCTURE analysis (Fig. 2).

Genotypes 39, 38, 20, 19, 18 and 37, respectively, were named as FLIP09-386C, FLIP09-337C, FLIP09-98C, FLIP09-90C, FLIP09-85C and FLIP09-115C, respectively. Genotypes 31, 32, 33, 28, 30, 27, 29, 26 and 34 respectively with the letters ILC482, FLIP 82-150C, FLIP88-85C, FLIP09-216C, FLIP09-219C, FLIP09-214C, FLIP09-218C, FLIP09 -194C and FLIP93-93C belonged to the second subpopulation. Genotypes 13, 11, 12, 14, 16, 6, 17, 15, 8, 5, 7, 9 and 10 respectively with the names FLIP07-280C, FLIP07-239C, FLIP07-261C, FLIP08-46C, FLIP09-70C, FLIP07-20C, FLIP09-81C, FLIP08-200C, FLIP07-28C, FLIP07-19C, FLIP07-22C, FLIP07-31C and FLIP07-44C were in the third subpopulation and genotypes 23, 3, 24, 25, 22, 2 , 1, 4, 35, 36 and 21 respectively with the letters FLIP09-189C, FLIP03-130C, FLIP09-191C, FLIP09-192C, FLIP09-149C, FLIP03-77C, FLIP97-706C, FLIP06-158C, FLIP07-180C, FLIP09 -88C and FLIP09-148C were also included in the fourth subpopulation (Figure 2).

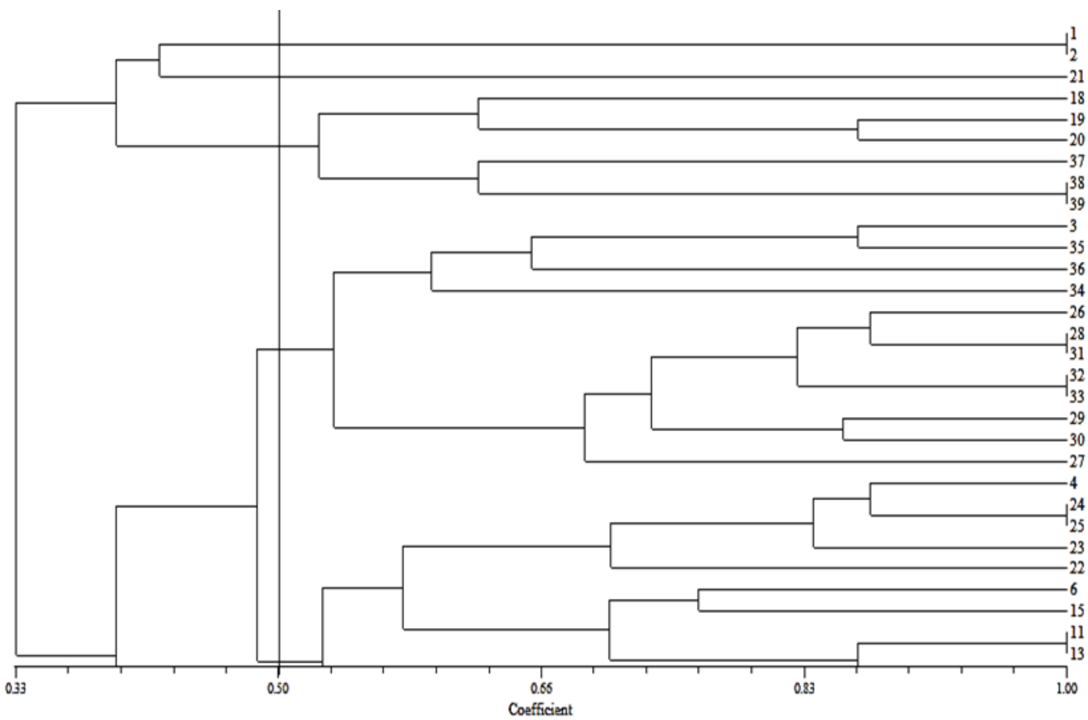

Figure 1: A dendrogram based on SSR markers of the 39 chickpea genotypes by UPGMA method

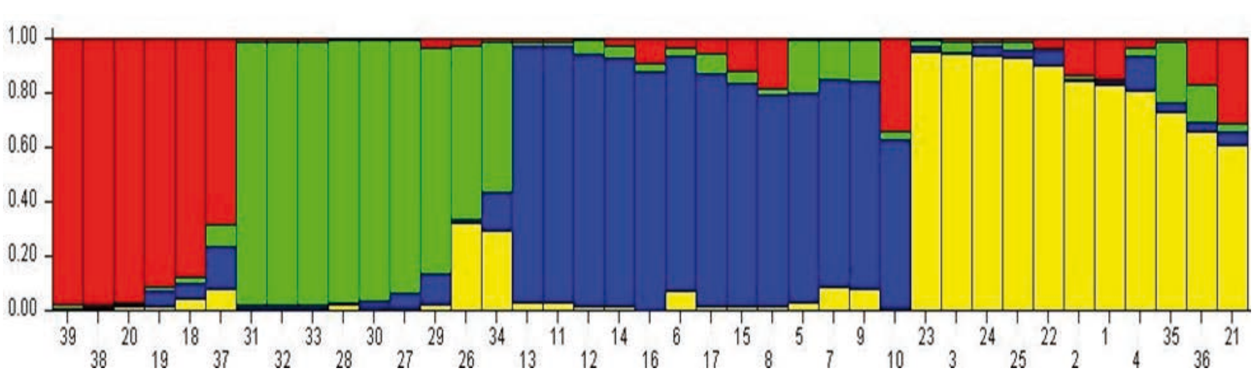

Figure 2: Genetic relatedness of 39 genotypes of chickpea with 10 SSR primer combinations as analyzed by the STRUCTURE program

3.4 ASSOCIATION ANALYSIS

The markers with minor allele frequency less than 5 %, remove so 21 marker loci retained for association analysis (Table 7). As in table 7 seen, AF016458 significantly associated with root fresh masst, root diameter, root volume density, root area, root length density, root area density, root length and root flavor. The 16549 marker was significantly associated to root fresh mass, root volume density, root area, root volume, root fineness and root area density. Significant associations were observed to the marker 19075 with root flavor. PsAS2 was significantly associated with root flavor, root volume density, root area, root volume, root fresh mass and root area density.

4 DISCUSSION

Several putative root traits contributing to drought

Table 4: Analysis of variance of root morphological traits in 39 chickpea genotypes

Source of variation	Degree of freedom	Mean square															
		RTD	RWC	SRL	RF	LDW	RDM	RFM	RV	RL	RAD	RVD	SRV	RLD	RD	RA	Rd
genotype	38	387.26**	30.67**	4018.71**	10.72**	0.69**	1.26**	48.61**	40.71**	399.72**	1308.67**	0.0001**	0.000 004**	0.001 **	0.027**	1193.69**	0.005**
Experimental error	113	16.65	2.97	73.50	0.84	0.03	0.04	2.97	2.90	7.95	54.59	0.00001**	0.0000 001	0.000 02	0.001	55.13	0.0007

RL: Root length, RFM: Root fresh mass, RDM: Root dry mass, DMS: Dry mass of plant shoots, RV: Root volume, RA: Root area, RF: Root fineness, Rd: Root diameter, SRL: specific root length RWC: Root water content, RLD: Root length density, SRV: Specific root volume, RTD: Root tissue density, RVD: Root volume density, RAD: Root area density, RD: Root density. **: significant at 0.01

Table 5:. Descriptive statistics of 16 measured root traits in 39 chickpea genotypes grown in a greenhouse condition

Variable	Mean	SE Mean	Coef. Var. (%)	Minimum	Maximum
RL (cm)	50.69	0.85	20.7	27	72
RV (cm^3)	11.30	0.29	32.1	3.75	22
RFM (g)	10.93	0.31	35.14	2.69	22.52
RDM (g)	1.33	0.051	47.85	0.15	3.93
LDW (g)	0.91	0.036	49.98	0.17	2.28
RF (cm root /root fresh mass	4.95	0.15	38.41	2.07	13
SRL (cm)	50.37	3.02	74.17	15	238.71
RWC (g)	8.30	0.31	46.22	2.58	30.88
RTD (g RDM× cm^3 soil volume)	16.47	0.95	71.70	0.61	86.53
Rd (cm)	0.12	0.0036	37.91	0.038	0.27
RA (cm^2)	83.69	1.52	22.41	36.83	127.68
RD (g cm^{-3})	0.52	0.0074	17.56	0.29	0.71
RLD (cm RL cm^{-3} soil volume)	0.094	0.0016	20.75	0.05	0.13
SRV (g RDM cm^{-3} soil volume)	0.0025	0.000095	47.85	0.0028	0.0073
RVD (cm m^{-3})	0.020	0.00058	35.14	0.0050	0.042
RAD ($cm^2 cm^{-3}$)	82.14	1.57	23.70	30.20	129.19

RL: Root length, RFM: Root fresh mass, RDM: Root dry mass, DMS: Dry massof plant shoots, RV: Root volume, RA: Root area, RF: Root fineness, Rd: Root diameter, SRL: Specific root length C: Root water content, RLD: Root length density, SRV: Specific root volume, RTD: Root tissue density, RVD: Root volume density, RAD: Root area density, RD: Root density

Table 6: The number and size range of bands produced by the SSR primers among the 39 chickpea genotypes

Marker name	Number of observed alleles	Polymorphism information content (PIC)
19075	3	0.57
18363	2	0.47
16549	3	0.66
C24	2	0.38
PsAS2	2	0.48
PSAB60	2	0.48
PD23	3	0.59
PSAD147	3	0.66
17605	3	0.65
AF016458	3	0.54
Mean	2.6	0.55

resistance in chickpea has been found (Benjamin and Nielsen, 2006; Fukai et al., 1995; Ali et al., 2005; Kashiwagi et al., 2008). Phenotypic selection for root traits is difficult because of the laborious, time-consuming and destructive methods involved in root studies (Gaur et al., 2008). An effort has been made in this study to identify the markers showed association with root traits in chickpea using a diverse set of genotypes. All of the measured root related traits differed significantly among genotypes ($p \leq 0.001$) (Table 4). The variation (Coef. Var) in all root-related traits (17.56-74.17 % (Table 5)) observed in the genotypes in the present study justifies its use for association analysis. Breseghello & Sorrells (2006) suggested use of diverse genotypes for the purpose of association mapping.

FLIP09-192C had the highest root fresh mass, root dry mass, the average leaf dry mass, root diameter, the average specific root volume, root volume density and root area density and FLIP07-31C had the highest root

Table 7: Marker-trait associations with MLM and GLM models

Traits	Marker name	No. of Associations	P. value		R^2(%)
			MLM	GLM	
Root fresh mass	PsAS2	2	0.047	0.039	44.9
Root fresh mass	AF016458	3	-	0.042	44.1
Root fresh mass	16549	3	0.021	0.038	45.2
Root diameter	AF016458	3	0.042	0.045	34.8
Root volume density	16549	3	0.025	0.037	45
Root volume density	AF016458	3	-	0.048	42.2
Root volume density	PsAS2	2	0.047	0.039	44.8
Root flavor	AF016458	3	0.039	0.025	37.2
Root flavor	16549	3	0.039	0.042	32.5
Root flavor	19075	2	0.034	0.046	31.7
Root flavor	PsAS2	2	-	0.044	32
Root area	AF016458	3	-	0.038	40.1
Root area	PsAS2	2	0.046	0.016	49
Root area	16549	3	0.027	0.014	49.9
Root length density	AF016458	3	0.038	0.029	31.7
Root volume	16549	3	0.050	0.017	51.6
Root volume	PsAS2	2	0.029	0.019	50.9
Root area density	16549	3	0.036	0.025	46.2
Root area density	AF016458	3	0.041	0.019	48.9
Root area density	PsAS2	2	-	0.026	45.9
Root length	AF016458	3	0.049	0.030	31.6

volume, the average leaf dry mass, root water content, the average root area. Generally, tolerant genotypes have high root growth vigor and deeper soil root proliferation under drought stress, allowing them to extract water from all soil depths and maintain yield and HI (Maphosa et al., 2020). The marker segregation data grouped FLIP07-31C and FLIP09-192C in the third and fourth subpopulation respectively.

In this research, a total of 26 alleles with a mean of 2.6 alleles per locus were found. Also, the mean PIC value was 0.55 (Table 6). So that according to indicated genetic diversity among cultivated chickpea genotypes was lesser than the wild chickpea genotypes (Ghaffari et al., 2014 and Hajibarat et al., 2015) and the wild chickpea species showed greater PIC value and number of allele count per locus (Upadhyaya et al. (2008) and Ghaffari et al. (2014)).

The 39 genotypes used for association analysis were split in to four distinct subpopulations at K = 4 (Fig. 2). Genotypes in a subpopulation often have similar pedigrees (Table 1). The presence of subpopulations within a population can be due to reasons such as the different geographical origin of the genotypes, natural or human selection, or genetic drift (Flint-Garcia et al., 2003; Buckler & Thornsberry, 2002).

In the present study, a total of 10 SSR markers have been used for genotyping the 39 chickpea The microsatellite markers showing association with root traits were detected using TASSEL software. A total of 21 marker-trait association have been found in this study at $p < 0.05$. The markers, PsAS2, AF016458, 16549 and 19075 on LG1, LG3, LG2, LG1 linkage group respectively was linked with root fresh mass root diameter, root volume density, root area, root length density, root area density, root length and root flavor.

Several QTLs controlling root traits have been reported (Kale et. al., 2015; Gaur et al., 2008; Varshney et al., 2014). Chandra et al. (2004) reported that a SSR marker, TAA 170, was associated with root mass and root length under drought stress in chickpea. Li et al. (2018) found that several SNPs from auxin-related genes were associated with yield and yield-related traits under drought condition. H6C-07 (on LG3) and H5G01 (on LG4) markers found that associated with QTLs for many drought-related traits (Hamwieh et al., 2013). Thudi et al. (2014b) discovered over 200 SSR, DArT, and SNP markers associated with drought-related traits. The most of highly expressed ESTs encoded proteins involved in cellular organization, protein metabolism, signal transduction, and transcription in the chickpea under drought stress (Jain & Chattopadhyay, 2010). The role of hypothetical abscissic acid and stress ripening (ASR) protein NP_001351739.1 in mediating drought responses as a transcription factor were recognized in chickpea (Sachdeva et al., 2020). A "QTL-hotspot" containing quantitative trait loci (QTL) for several root and drought tolerance traits was transferred through marker assisted backcrossing into JG 11, a leading variety of chickpea (*Cicer arietinum* L.) in India from the donor parent ICC 4958. some introgression lines were identified that may be released as improved variety with enhanced drought tolerance (Varshney et al., 2013).

5 CONCLUSIONS

In conclusion, this study demonstrated the existence of genetic diversity exists in the current chickpea germplasm for root traits. The present study has helped in identification of significant marker-trait associations on LG1, LG2 and LG3. This shows that these chromosomes are potential candidate ones for emphasizing future studies. The research findings provide valuable information for marker-assisted selection improving root traits after validation for chickpea breeders.

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Authors' Contributions: Zahra Shekari: Collection of experimental data. Zahra Tahmasebi: supervision of the study and writing of manuscript. Homayon Kanoni: review of the manuscript. Ali Asherf Mehrabi: molecular and statistical analysis.

7 REFERENCES

Ahmad, F., Gaur, P., & Croser, J. (2005). Chickpea (*Cicer arietinum* L.). In '*Genetic resources, chromosome engineering and crop improvement–grain legumes*'.(Eds R Singh, P Jauhar) pp. 185–214. https://doi.org/10.1201/9780203489284.ch7

Akhavan, S., Shabanpour, M., & Esfahani, M. (2012). Soil compaction and texture effects on the growth of roots and shoots of wheat. *Journal of Water and Soil, 26*(3), 727–735. doi: 10.22067/JSW.V0I0.14941.

Beebe, S. E., Rojas-Pierce, M., Yan, X., Blair, M. W., Pedraza, F., Munoz, F., .. & Lynch, J. P. (2006). Quantitative trait loci for root architecture traits correlated with phosphorus acquisition in common bean. *Crop Science*, *46*(1), 413-423. https://doi.org/10.2135/cropsci2005.0226

Benjamin, J. G., & Nielsen, D. C. (2006). Water deficit effects on root distribution of soybean, field pea and chickpea. *Field Crops Research*, *97*(2-3), 248-253. https://doi.org/10.1016/j.fcr.2005.10.005

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*, *23*(19), 2633 2635. https://doi.org/10.1093/bioinformatics/btm308

Breseghello, F., & Sorrells, M. E. (2006). Association mapping of kernel size and milling quality in wheat (Triticum aestivum L.) cultivars. *Genetics*, *172*(2), 1165-1177. https://doi.org/10.1534/genetics.105.044586

Buckler IV, E. S., & Thornsberry, J. M. (2002). Plant molecular diversity and applications to genomics. *Current Opinion in Plant Biology*, *5*(2), 107-111. https://doi.org/10.1016/S1369-5266(02)00238-8

Chandra, S., Buhariwalla, H.K., Kashiwagi, J., Harikrishna, S., Rupa Sridevi, K., Chandra, S., Buhariwalla, H. K., Kashiwagi, J., & Harikrishna, S. (2004). Identifying QTL-linked markers in marker-deficient crops. In 4th International Crop Science Congress. *Markers*, *2*(38.1), 235.

Chen, Y., Ghanem, M. E., & Siddique, K. H. (2017). Characterising root trait variability in chickpea (*Cicer arietinum* L.) germplasm. *Journal of Experimental Botany*, *68*(8), 1987-1999. https://doi.org/10.1093/jxb/erw368

de Dorlodot, S., Forster, B., Pagès, L., Price, A., Tuberosa, R., & Draye, X. (2007). Root system architecture: opportunities and constraints for genetic improvement of crops. *Trends in Plant Science*, *12*(10), 474-481. https://doi.org/10.1016/j.tplants.2007.08.012

Doyle, J. J., & Doyle, J. L. (1987). *A rapid DNA isolation procedure for small quantities of fresh leaf tissue* (No. RESEARCH).

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, *14*(8), 2611-2620. Gaur, P.M., Krishnamurthy, L. and Kashiwagi, J. 2008. Improving drought-avoidance root traits in chickpea (*Cicer arietinum* L.)-current status of research at ICRISAT. *Plant Production Science, 11*(1), 3-11. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Fukai, S., & Hammer, G. L. (1995). Growth and yield response of barley and chickpea to water stress under three environments in southeast Queensland. II. Root growth and soil water extraction pattern. *Australian Journal of Agricultural Research*, *46*(1), 35-48. https://doi.org/10.1071/AR9950035.

Gaur PM, Krishnamurthy L, Kashiwagi J. (2008). Improving drought-avoidance root traits in chickpea (*Cicer arietinum* L.)—current status of research at ICRISAT. *Plant Production Science*, *11*(1), 3-11. https://doi.org/10.1626/pps.11.3

Ghaffari, P., Talebi, R., & Keshavarzi, F. (2014). Genetic diversity and geographical differentiation of Iranian landrace, cultivars, and exotic chickpea lines as revealed by morphological and microsatellite markers. *Physiology and Molecular Biology of Plants*, *20*(2), 225-233. https://doi.org/10.1007/s12298-014-0223-9

Ghanem, M. E., Hichri, I., Smigocki, A. C., Albacete, A., Fauconnier, M. L., Diatloff, E., .. & Pérez-Alfocea, F. (2011). Root-targeted biotechnology to mediate hormonal signalling and improve crop stress tolerance. *Plant Cell Reports*, *30*(5), 807-823. https://doi.org/10.1007/s00299-011-1005-2

Hajabbasi, M. A. (2001). Tillage effects on soil compactness and wheat root morphology. *Journal of Agricultural Science and Technology*, *3*(1), 67-77.

Hajibarat, Z., Saidi, A., Hajibarat, Z., & Talebi, R. (2015). Characterization of genetic diversity in chickpea using SSR markers, start codon targeted polymorphism (SCoT) and conserved DNA-derived polymorphism (CDDP). *Physiology and Molecular Biology of Plants*, *21*(3), 365-373. https://doi.org/10.1007/s12298-015-0306-2

Hamwieh, A., Imtiaz, M., & Malhotra, R. S. (2013). Multi-environment QTL analyses for drought-related traits in a recombinant inbred population of chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics*, *126*(4), 1025-1038. https://doi.org/10.1007/s00122-012-2034-0

Hasanabadi, T., Ardakani, M. R., Rejali, F., Paknejad, F., Eftekhari, S. A., & Zargari, K. (2010). Response of barley root characters to co-inoculation with *Azospirillum lipoferum* and *Pseudomonas flouresence* under different levels of nitrogen. *American-Eurasian Journal of Agricultural and Environmental Science*, *9*(2), 156-162. ISSN : 1818-6769

Jaganathan, D., Thudi, M., Kale, S., Azam, S., Roorkiwal, M. & Gaur, P.M., et al. (2015). Genotyping-by-sequencing based intra-specific genetic map refines a "QTL hotspot" region for drought tolerance in chickpea. *Molecular Genetics and Genomics*, *290*(2), 559-71. https://doi.org/10.1007/s00438-014-0932-3

Jain, D., & Chattopadhyay, D. (2010). Analysis of gene expression in response to water deficit of chickpea (*Cicer arietinum* L.) varieties differing in drought tolerance. *BMC Plant Biology*, *10*(1), 1-14. https://doi.org/10.1186/1471-2229-10-24

Jha, U. C., Jha, R., Bohra, A., Parida, S. K., Kole, P. C., Thakro, V., .. & Singh, N. P. (2018). Population structure and association analysis of heat stress relevant traits in chickpea (*Cicer arietinum* L.). *3 Biotech*, *8*(1), 43. https://doi.org/10.1007/s13205-017-1057-2

Kale, S.M., Jaganathan, D., Ruperao, P., Chen, C., Punna, R., Kudapa, H., (2015). Prioritization of candidate genes in "QTL-hotspot" region for drought tolerance in chickpea (*Cicer arietinum* L.). *Scientific Reports*, *5*(1),15296. https://doi.org/10.1038/srep15296

Kashiwagi, J., Krishnamurthy, L., Crouch, J. H., & Serraj, R. (2006). Variability of root length density and its contributions to seed yield in chickpea (*Cicer arietinum* L.) under terminal drought stress. *Field Crops Research*, *95*(2-3), 171-181. https://doi.org/10.1016/j.fcr.2005.02.012

Kashiwagi, J., Krishnamurthy, L., Gaur, P. M., Chandra, S., & Upadhyaya, H. D. (2008). Estimation of gene effects of the drought avoidance root characteristics in chickpea (*C. arietinum* L.). *Field Crops Research*, *105*(1-2), 64-69. https://doi.org/10.1016/j.fcr.2007.07.007

Keneni, G., Bekele, E., Imtiaz, M., Dagne, K., Getu, E., & Assefa, F. (2012). Genetic diversity and population structure of Ethiopian chickpea (*Cicer arietinum* L.) germplasm accessions from different geographical origins as revealed by microsatellite markers. *Plant Molecular Biology Reporter*, *30*(3), 654-665. https://doi.org/10.1007/s11105-011-0374-6

Kirkegaard, J. A., Lilley, J. M., Howe, G. N., & Graham, J. M. (2007). Impact of subsoil water use on wheat yield. *Australian Journal of Agricultural Research*, *58*(4), 303-315. https://doi.org/10.1071/AR06285

Li, Y., Ruperao, P., Batley, J., Edwards, D., Khan, T., Colmer, T. D., .. & Sutton, T. (2018). Investigating drought tolerance in chickpea using genome-wide association mapping and genomic selection based on whole-genome resequencing data. *Frontiers in Plant Science*, *9*, 190. https://doi.org/10.3389/fpls.2018.00190

Lovelli, S., Perniola, M., Di Tommaso, T., Bochicchio, R., & Amato, M. (2012). Specific root length and diameter of hydroponically-grown tomato plants under salinity. *Journal of Agronomy*, *11*(4), 11. https://doi.org/10.3923/ja.2012.101.106

Ludlow, M. M., & Muchow, R. C. (1990). A critical evaluation of traits for improving crop yields in water-limited environments. *Advances in Agronomy*, *43*, 107-153. https://doi.org/10.1016/S0065-2113(08)60477-0

Mahanta, D., Rai, R. K., Mishra, S. D., Raja, A., Purakayastha, T. J., & Varghese, E. (2014). Influence of phosphorus and biofertilizers on soybean and wheat root growth and properties. *Field Crops Research*, *166*, 1-9. https://doi.org/10.1016/j.fcr.2014.06.016

Malamy, J.E., 2005. Intrinsic and environmental response pathways that regulate root system architecture. *Plant, Cell & Environment*, *28*(1), 67-77. https://doi.org/10.1111/j.1365-3040.2005.01306.x

Maphosa, L., Richards, M. F., Norton, S. L., & Nguyen, G. N. (2020). Breeding for abiotic stress adaptation in chickpea (*Cicer arietinum* L.): A comprehensive review. *Crop Breeding, Genetics and Genomics*, *4*(3). https://doi.org/10.20900/cbgg20200015

Mugabe, D., Coyne, C. J., Piaskowski, J., Zheng, P., Ma, Y., Landry, E., ... & Abbo, S. (2019). Quantitative trait loci for cold tolerance in chickpea. *Crop Science*, *59*(2), 573-582. https://doi.org/10.2135/cropsci2018.08.0504

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences*, *70*(12), 3321-3323. https://doi.org/10.1073/pnas.70.12.3321

Passioura, J. (2006). Increasing crop productivity when water is scarce—from breeding to field management. *Agricultural Water Management*, *80*(1-3), 176-196. https://doi.org/10.1016/j.agwat.2005.07.012

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959. https://doi.org/10.1093/genetics/155.2.945

Ramamoorthy, P., Lakshmanan, K., Upadhyaya, H. D., Vadez, V., & Varshney, R. K. (2017). Root traits confer grain yield advantages under terminal drought in chickpea (*Cicer arietinum* L.). *Field crops research*, *201*, 146-161. https://doi.org/10.1016/j.fcr.2016.11.004

Rohlf, F.J. (2000). *NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1*. New York: Exeter Software. https://doi.org/10.1016/j.fcr.2016.11.004

Sachdeva, S., Bharadwaj, C., Singh, R. K., Jain, P. K., Patil, B. S., Roorkiwal, M., & Varshney, R. (2020). Characterization of ASR gene and its role in drought tolerance in chickpea (*Cicer arietinum* L.). *PloS One*, *15*(7), e0234550. https://doi.org/10.1371/journal.pone.0234550

Schenk, M. K., & Barber, S. A. (1979). Root characteristics of corn genotypes as related to p uptake 1. *Agronomy Journal*, *71*(6), 921-924. https://doi.org/10.2134/agronj1979.00021962007100060006x

Sefera, T., Abebie, B., Gaur, P. M., Assefa, K., & Varshney, R. K. (2011). Characterisation and genetic diversity analysis of selected chickpea cultivars of nine countries using simple sequence repeat (SSR) markers. *Crop and Pasture Science*, *62*(2), 177-187. https://doi.org/10.1071/CP10165

Thudi, M., Gaur, P. M., Krishnamurthy, L., Mir, R. R., Kudapa, H., Fikre, A., .. & Varshney, R. K. (2014a). Genomics-assisted breeding for drought tolerance in chickpea. *Functional Plant Biology*, *41*(11), 1178-1190. https://doi.org/10.1071/FP13318

Thudi, M., Upadhyaya, H. D., Rathore, A., Gaur, P. M., Krishnamurthy, L., Roorkiwal, M., .. & Varshney, R. K. (2014b). Genetic dissection of drought and heat tolerance in chickpea through genome-wide and candidate gene-based association mapping approaches. *Plos One*, *9*(5), e96758. https://doi.org/10.1371/journal.pone.0096758

Tuberosa, R., & Salvi, S. (2006). Genomics-based approaches to improve drought tolerance of crops. *Trends in Plant Science*, *11*(8), 405-412. https://doi.org/10.1016/j.tplants.2006.06.003

Tuberosa, R., Salvi, S., Sanguineti, M. C., Landi, P., Maccaferri, M., & Conti, S. (2002). Mapping QTLs regulating morpho-physiological traits and yield: Case studies, shortcomings and perspectives in drought-stressed maize. *Annals of Botany*, *89*(7), 941-963. https://doi.org/10.1093/aob/mcf134

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. https://doi.org/10.1186/1471-2229-8-106

Varshney, R. K., Gaur, P. M., Chamarthi, S. K., Krishnamurthy, L., Tripathi, S., Kashiwagi, J., .. & Jaganathan, D. (2013). Fast-track introgression of "QTL-hotspot" for root traits and other drought tolerance traits in JG 11, an elite and leading variety of chickpea. *The Plant Genome*, 6(3). https://doi.org/10.3835/plantgenome2013.07.0022

Varshney, R. K., Thudi, M., Nayak, S. N., Gaur, P. M., Kashiwagi, J., Krishnamurthy, L., .. & Viswanatha, K. P. (2014). Genetic dissection of drought tolerance in chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics*, *127*(2), 445-462. https://doi.org/10.1007/s00122-013-2230-6

Wasson, A. P., Rebetzke, G. J., Kirkegaard, J. A., Christopher, J., Richards, R. A., & Watt, M. (2014). Soil coring at multiple field environments can directly quantify variation in deep root traits to select wheat genotypes for breeding. *Journal of Experimental Botany*, *65*(21), 6231-6249. https://doi.org/10.1093/jxb/eru250

Yang, T., Fang, L., Zhang, X., Hu, J., Bao, S., Hao, J., .. & Zong, X. (2015). High-throughput development of SSR markers from pea (*Pisum sativum* L.) based on next generation sequencing of a purified Chinese commercial variety. *PLoS One*, *10*(10), e0139775. https://doi.org/10.1371/journal.pone.0139775

Yusuf Ali, M., Johansen, C., Krishnamurthy, L., & Hamid, A. (2005). Genotypic variation in root systems of chickpea (*Cicer arietinum* L.) across environments. *Journal of Agronomy and Crop Science*, *191*(6), 464-472. https://doi.org/10.1111/j.1439-037X.2005.00177.x

Zaman-Allah, M., Jenkinson, D. M., & Vadez, V. (2011). A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. *Journal of Experimental Botany*, *62*(12), 4239-4252. https://doi.org/10.1093/jxb/err139

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Improvement ability of male parent by gibberellic acid application to enhancing the outcrossing of cytoplasmic male sterility rice lines

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Improvement ability of male parent by gibberellic acid application to enhancing the outcrossing of cytoplasmic male sterility rice lines

Abstract: The study quantified the effect of gibberellic acid (GA_3) as a pre-flowering treatment for male parent Giza 178 R and the influence of male to female ratio (2R:10A, 2R:12A, 2R:14A and 2R:16A) between male (R) to female (A) for two Cytoplasmic Male Sterility (CMS) lines ('IR69625' and 'G46') on hybrid rice seed production. The main plots were occupied by CMS lines while; GA3 application for male parent Giza 178R were arranged in the sub plots and male to female ratio was arranged in the sub-sub plots. The results indicated that, the, duration of floret opening, angle of floret opening, filaments exsertion, filaments length**,** anther length, plant height and number of tiller hill^{-1} of male parent Giza 178R were significantly at 300 g GA_3 ha^{-1} concentration. Plant height, panicle exsertion, panicle length, flag leaf angle and 1000-grain mass of CMS were not significantly affected by the GA_3 application for male parent and male to female ratio, while, number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index of CMS lines were highly significantly affected. The highest seed yield (2.880 and 2.950 t ha^{-1}) was obtained by CMS line IR69625A using 300 g GA_3 ha^{-1} of male parent Giza 178R with male to female ratio of 2R:14A during both seasons.

Key words: hybrid rice production; cytoplasmic male sterile lines; gibberellic acid (GA_3); male to female ratio; panicle exsertion

Izboljševanje sposobnosti moških staršev z giberilinsko kislino za pospeševanje navskrižnega križanja citoplazmatsko moško sterilnih linij riža

Izvleček: Preučevan je bil učinek dodajanja giberilinske kisline (GA_3) kot obravnavanja pred cvetenjem na moškega starša 'Giza 178' (R) in vpliv razmerja med moškimi (R) in ženskimi rastlinami (A) (2R:10A, 2R:12A, 2R:14A in 2R:16A) za dve citoplazmatsko moško sterilni liniji (CMS), 'IR69625' in 'G46', na pridelek semena hibridnega riža. Raziskava je potrdila razlike v lastnostih navzkrižnega križanja in pridelku zrnja dveh CMS linij (IR69625A in G46A) pri uporabi štirih koncentracij GA_3 uporabljenih dvakrat, pri 15-20 % in 35-40 % latenju. CMS linije so bile na podploskvah, razmerje med moškimi (R) in ženskimi (A) rastlinami pa na njihovih podploskvah (2R:10A, 2R:12A, 2R:14A in 2R:16A). Rezultati so pokazali, da so se trajanje odpiranja cvetov (min), kot odprtega cveta (^{0}), odstotek podaljšanih filamentov (%), dolžina filamentov (mm)**,** dolžina prašnic (mm), višina rastlin (cm) in število poganjkov na sadilno mesto pri moškem staršu 'Giza 178' značilno povečali pri uporabi 300 g GA_3 ha^{-1}.

Ključne besede: pridelovanje hibridnega riža; citoplazmatsko sterilne moške linije; giberilinska kislina (GA_3); razmerje moških in ženskih rastlin; latenje

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1 INTRODUCTION

Hybrid rice breeding, which was initiated in Egypt has led to great improvement in rice production (Zaman et al., 2002; Hamad, 2018). Breeding high-yielding hybrid rice is one of the promising potential strategies in Egypt for increasing rice production. The hybrid rice technology exploits the phenomenon of heterosis or hybrid vigor. The heterosis can be defined as the superiority of F1 when two genetically dissimilar parents are crossed (Sindhua and Kumar, 2002). The three-line rice breeding system which uses cytoplasmic male sterile (CMS) lines (A), maintainer lines (B) and restorer lines (R) has been proven to be the most useful genetic tool in producing F1 hybrid in rice.

The content of endogenous gibberellic acid (GA_3) in male p lines with pollen abortive wild rice cytoplasm (wild abortive [WA] type male sterile [MS] line) is generally lower than that of fertile plants, therefore, resulting in spikelets unavailable for cross-pollination and producing lower seed yield (Lu, 1994; Pan et al., 2013). Exogenous application of GA_3 was done to cause the panicle base of the CMS line to emerge out of the leaf sheath (Gaballah, 2004; Gaballah et al., 2021). In addition, lower heading characteristics such as small spikelet openings, poor panicle layer carriage and poor stigma exsertion can severely reduce cross-pollination and limit seed yield production. Hence, hybrid rice seed production techniques should be improved to increase seed yields and reduce the cost of seeds (Virmani, 2002; Virmani et al., 2002).

Egypt is currently using a number of CMS lines for the hybrid rice-breeding programs. However, no information is available on how these CMS lines will respond to GA_3 application with reference to their heading characteristics. Such data are very important to generate baseline information whether genotypic variations exist among CMS lines in response to GA_3 pre-flowering treatment and whether such responses follow similar trends. This will also help in identifying CMS lines which are responsive to GA_3 application to maximize their utilization in the development of new hybrid rice varieties with higher seed yield potential.

Therefore, the objective of this investigation was to study the performance of Giza178R male parents as affected by GA_3 application rates and male to female ratio on the growth characteristics and hybrid seed yield production of two CMS lines (IR69625A and G46A).

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE DESCRIPTION AND SOIL SAMPLES

The field experiment was conducted during 2019 and 2020 rice growing seasons in Rice Research and Training Center (RRTC) experimental farm, Sakha, Kafr El-Sheikh, Egypt. Representative soil sample was taken from 0-20 cm depth before the growing season. The soil samples were air-dried, ground and passed through 2mm sieve. Composite soil samples were taken and analyzed for physical and chemical characteristics of the soil namely, electrical conductivity (EC,) pH, organic matter (OM), texture, cations and anions following the standard methods as described by (Page et al., 1982). The physico-chemical characteristics of the soil are presented in Table (1).

2.2 EXPERIMENTAL LAYOUT

The experiment was set up as split split-plot design with three replications. The main plots were devoted to two CMS lines (IR69625A and G46A) for male parent male parent. The GA_3 application rates (0, 150, 200 and 300 g GA_3 ha^{-1}) for male parent Giza 178R was allocated to subplots and male to female ratio (2R:10A, 2R:12A, 2R:14A and 2R:16A) between male (R) to female (A) was arranged in the sub-sub plots.

2.3 PLANT MATERIALS

They were obtained from international rice research institute (IRRI) and China and contain the wild rice with abortive pollen CMS (Table 2).

2.4 CULTURAL PRACTICES

Phosphorus fertilizer was applied @ 36 kg P_2O_5 ha^{-1} as superphosphate (15.5 % P_2O_5) as soil basal application. Nitrogen fertilizer was applied @ 165 kg ha^{-1} as urea. Two thirds of the recommended N fertilizer were added as soil basal application, and the other one third was applied at panicle initiation. Zinc sulphate at the rate of 50 kg ha^{-1} was added during soil preparation.

Rice seeds @ 15 kg of the CMS Lines (IR69625A and G46A) and 5 kg for the male parent (Giza 178 R) were soaked in fresh water for 24 hours, then incubated for 48 hours to hasten early germination. To get a proper synchronization of flowering, the CMS line IR69625A (as female parent) was sown on May ^{1st} which is six

Table 1: The physical and chemical characteristics of the soil during 2019 and 2020 growing seasons

Season	pH	EC (dS m^{-1})	NPK (mg kg^{-1})			Clay (%)	Silt (%)	Sand (%)	OM (%)
2019	7.84	1.50	339	13.7	329	56.4	28.3	15.3	1.35
2020	7.89	1.59	368	14.7	359	58.6	27.1	14.3	1.40

EC; Electrical conductivity, OM; Organic matter

Table 2: Cytoplasmic male sterile (CMS) lines used for evaluation

CMS Lines	Cytoplasmic source	Origin
IR 69625A	Wild abortive (WA) CMS line	IRRI
G46A	Gambiaca CMS line	China

days earlier than the male parent 'Giza 178 R' while, the CMS line G46A (as female parent) was sown on 16 May. Thirty days old seedlings (3-4) per hill of R and A lines were transplanted by 3-4 and 2 seedlings per hill, respectively). Row direction was perpendicular to wind direction. The row spacing maintained for R-R, R-A and A-A lines were 20, 30, and 15 cm, respectively. Hill spacing for both R and A lines was maintained at 15 cm. Isolation space of 100 m was considered for CMS seed production. Moreover, the experimental field was surrounded by an additional 20 rows of R line to avoid any possibility of cross pollination. Every main plot was isolated by plastic barrier (2.5 m height) to avoid any pollen grain movement from treatment to another.

2.5 GIBBERELLIC ACID PREPARATION AND APPLICATION

Gibberellic Acid (GA_3) powder with 90.7 % purity was used. Since GA_3 cannot be completely dissolved in distilled water. In 100 ml of ethanol alcohol (70 %), was used to dissolve the GA_3 powder before it was mixed with water. Application of GA_3 was done in two splits. The first split consisted of 40 % of the total amount of GA_3 applied at 15-20 % heading. The second split in which the remaining 60 % of the total amount of GA_3 was applied at 35-40 % heading. Supplementary pollination was done by shaking the pollen parent (R line) with bamboo sticks. This operation was done 3-4 times between 9.30 am to 12.30 am for a period of 10 days.

2.6 TRAIT EVALUATION

At complete heading, duration of floret opening (min), angle of floret opening (^{0}), filaments exertion (%), filaments length (mm) and anther length (mm) of male parent 'Giza 178' were recorded. Ten panicles of male parent 'Giza 178' from each plot were randomly collected to estimate the panicle length (cm). Also, five hills of male parent 'Giza 178' were randomly identified from each plot to estimate the plant height (cm) and number of tillers $hill^{-1}$. Data was collected for CMS lines where it was days to heading 50 %, plant height (cm), panicle exsertion (%), flag leaf angle (^{0}), 1000-grain mass (g), panicle length (cm), number of fertile panicles $hill^{-1}$, panicle mass (g), seed set (%), seed yield (t ha^{-1}), and harvest index (%). After harvesting, rice grain yield was estimated in each plot, and grain yield was adjusted to 14 % moisture content and converted to tons ha^{-1}.

Panicle exsertion % was estimated according to the following equation:

$$\text{Panicle exsertion \%} = \frac{\text{Exserted panicle length (cm)}}{\text{Panicle length (cm)}} \times 100$$

Seed set % was calculated according to the following equation:

$$\text{Seed set \%} = \frac{\text{Number of filled grains/panicle}}{\text{Total Spikelet number/panicle}} \times 100$$

2.7 STATISTICAL ANALYSIS

All data collected were subjected to standard statistical analysis of variance following the method described by Gomez and Gomez (1984). Different means were compared by Duncan's multiple range test (DMRT) with a 5 % probability level.

3 RESULTS AND DISCUSSION

3.1 EFFECT OF GA_3 APPLICATION RATES ON GROWTH TRAITS OF MALE PARENT

The effect of different GA_3 application rates on male parent traits such as duration of floret opening, angle of floret opening, filaments exertion, filaments length, anther length, plant height, number of tiller hill^{-1}and panicle length, are presented in (Table 3). The results indicated that, GA_3 applied for male parent up to 300 g GA_3 ha^{-1} recorded a significant increase in duration of floret opening, angle of floret opening, filaments exertion, filaments length, anther length, plant height, number of tiller hill^{-1} and panicle length as compared with GA_3 0 g ha^{-1} treatment in both seasons. Application of 300 g GA_3 ha^{-1} on male parent gave the highest duration of floret opening (130.59 and 129.94 min), the maximum angle of floret opening (41.96 and 44.30 ^{0}), the highest values of filaments exertion (80.81 and 90.15 %), filaments length (9.34 and 9.78 mm), anther length (3.35 and 3.55 mm), the tallest plant (126.49 and 127.36 cm), the highest number of tiller hill^{-1} (24.83 and 25.19) and longest panicle (25.71 and 25.90), during 2019 and 2020 seasons, respectively. A significant increase in panicle exsertion was observed on GA_3 application. The highest value of panicle exsertion was observed at the rate of 300 g GA_3 ha^{-1}, regardless of CMS lines used, indicating that CMS lines were sensitive to exogenous GA_3 application, hence, the problem of the leaf sheath enclosing the panicle could be alleviated by GA_3 application. Panicle exsertion influenced the percentage of exposed spikelets available for pollination, as higher panicle exsertion means a greater number of exposed spikelets. It also tended to scatter the panicle branches providing more space for each spikelet to trap airborne pollen. The increase in panicle exsertion was mainly a function of the elongating topmost internode in response to GA_3 application that consequently pushes the panicle out of the flag leaf sheath. Therefore, poor panicle exsertion of CMS lines was due to the inability of the topmost internodes to elongate during heading stage. The lowest values of above-mentioned traits were obtained with GA_3 0 g ha^{-1} application rate (Table 3). The improved floral traits of male parent were due to increased activity of cell division, enlargement and elongation. Gibberellins are plant hormones that regulate various processes of plant growth and development, which are particularly important in cell elongation (Hedden and Phillips, 2000).

Table 3: Floral traits and growth characters of male parent (Giza 178) as affected by GA_3 application rates during 2019 and 2020 growing seasons

GA_3 application rate (g ha^{-1})	Duration of floret opening (min)		Angle of floret opening (^{0})		Filaments exertion (%)		Filaments length (mm)		Anther length (mm)		Plant height (cm)		Number of tillers hill^{-1}		Panicle length (cm)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
0	46.75d	48.09d	24.30d	25.03d	44.31c	43.52d	4.32d	4.55d	1.35d	1.65d	100.2d	98.93d	16.05d	15.43d	19.55d	19.33d
150	68.86c	67.91c	31.56c	32.36c	69.54b	70.07c	6.76c	7.19c	2.28c	2.62c	109.6c	110.47c	19.21c	20.32c	21.87c	20.87c
200	90.37b	90.62b	36.70b	38.40b	80.47a	79.82b	8.02b	8.12b	2.68b	2.97b	120.0b	120.88b	22.17b	22.38b	23.84b	23.82b
300	130.5a	129.9a	41.96a	44.30a	80.81a	90.15a	9.34a	9.78a	3.35a	3.55a	126.4a	127.36a	24.83a	25.19a	25.71a	25.90a
F- Test	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**

* = Significant at 0.05 level, ** = Significant at 0.01 level and NS = Not significant. Means in the same column designated by the same letter are not significantly different at 5 % level

3.2 F_1 SEEDS OF CMS LINES CHARACTERISTIC AS AFFECTED BY GA_3 APPLICATION FOR MALE PARENT AND MALE TO FEMALE RATIO

Days to 50 % heading, plant height, panicle exsertion, flag leaf angle, panicle length and 1000-grain mass were not significantly affected by GA_3 application rates for male parent and male to female ratio (Table 4). The results showed that there were significant differences between the two CMS lines IR69625A and G46A. Where CMS line IR69625A gave the longest duration to 50 % heading, produced the tallest plants, the longest panicle exsertion, the highest panicle length and the increased flag leaf angle during both seasons. On the other hand, the CMS line G46A recorded the highest 1000-grain mass during the both seasons. The variation between the CMS lines could be attributed to the difference in genetic background. The results are in agreement with those reported by (Hamad et al., 2015). They founded that, the different doses of GA_3 showed highly significantly influence on panicle length and panicle exsertion when 2:4 row ratio. Similar results agreement with those were reported by Ehsan and Robert (2019). Results in Table (4) also showed that, the all of interactions were not significantly affected on days to 50 % heading, plant height, panicle exsertion, panicle length, flag leaf angle and 1000-grain mass in both growing seasons.

Number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index % of two CMS lines as affected by doses of GA_3 application rates for male parent and male to female ratio as well as their interactions are shown in (Table 5). The results indicated that, the CMS line IR 69625A recorded the highest values of number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index % in both seasons. On the other hand, the CMS line G46A recorded the lowest values of number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index % in both seasons (Table 5). The variation between the CMS lines could be attributed to the difference in genetic background. The results are in agreement with those reported by Gaballah, (2004). Results in Table (5) also showed that application GA_3 on the male parent had a high significant effect on the number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index %. The dose of GA_3 application at 300 g ha^{-1} for male parent recorded the highest values of number of fertile panicle hill^{-1}, panicle mass, seed set, seed yield and harvest index % 2019 and 2020 seasons, while the lowest values recoded by control (GA_3 0 g ha^{-1}) in both seasons. The increase in plant height is due to increased activity of cell division, enlargement and elongation. Gibberellins are plant hormones that regulate various processes of plant growth and development, which are particularly important in stem elongation which enhances the cross pollination between both parents. The results are in agreement with those reported by (Hedden and Phillips, 2000; Sakamoto et al., 2004; Sun, 2004; Tiwari et al., 2011). Male to female ratio significantly influenced number of fertile panicle hill^{-1}, panicle mass, seed set, seed yield and harvest index %. The male to female ratio 2R:14A recorded the highest values number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index% during both seasons. This might be due to that the application of GA_3 for male parent led to a noticeable improvement in the characteristics) plant height, panicle exsertion, flag leaf angle, panicle length) of the male parent, which made it able to pollinate the highest number of male lines consequently, increase the number of fertile panicle hill^{-1} and seed yield. On the other hand, the male to female ratio 2R:10A recorded the lowest values of number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index % during the both seasons.

3.3 INTERACTION EFFECT

All types of interactions among CMS lines, doses of GA_3 application rates for male parent and male to female ratio had highly significant effect on number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index during 2019 and 2020 seasons (Table 5).

The results in Table (6) indicated that, the interaction between the CMS lines and GA_3 different application rates for male parent were highly significantly affected on number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index % in both seasons. The CMS line IR69625A, with dose of GA_3 at the rate of 300 g ha^{-1} for male parent recorded the highest values of number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index % in both seasons. On the contrary, the CMS line G46A with 0 g GA_3 ha^{-1} application rates for male parent recoded the lowest values of number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index % in 2019 and 2020 seasons. The results are in agreement with those reported by Sirajul et al. (2005); Gavino et al. (2008).

Table 4: Genotypic variations in panicle exsertion and other morphological traits between CMS lines in response to GA_3 pre-flowering treatment

	Days to 50 % heading		Plant height (cm)		Panicle exsertion (%)		Panicle length (cm)		Flag leaf angle (^{0})		1000-grain mass (g)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
CMS lines(L)												
IR69625A	102.60a	103.46a	117.26a	117.97a	77.25a	78.16a	23.31a	24.25a	38.90a	40.29a	24.30a	24.24a
G46A	85.50b	87.36b	96.38b	97.00b	75.41b	77.32b	22.42b	23.25b	37.21b	39.60b	25.77b	25.45b
F-test	**	**	**	**	**	**	**	**	**	**	**	**
GA_3 doses for male parent(G)												
0	94.15	95.60	106.79	107.47	76.34	78.70	22.76	23.81	37.71	39.35	25.06	24.88
150	94.13	95.36	106.87	107.51	76.28	78.59	22.84	23.76	38.18	39.35	25.02	24.85
200	94.95	95.45	106.79	107.44	76.40	78.33	22.97	23.77	38.19	39.23	25.00	24.84
300	93.72	95.22	106.85	107.49	76.31	78.48	22.88	23.65	38.15	39.62	25.04	24.77
F-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Male to female ratio (W)												
2R:10A	93.99	95.34	106.93	107.52	76.33	78.71	22.95	23.65	38.16	39.25	25.04	24.83
2R:12A	94.25	95.18	106.84	107.62	76.09	78.52	22.67	23.82	38.71	39.27	25.03	24.87
2R:14A	93.82	95.41	106.89	107.30	76.38	78.33	22.93	23.75	37.75	39.23	25.01	24.80
2R:16A	94.14	95.72	106.64	107.46	76.53	78.58	22.91	23.70	38.27	39.24	25.04	24.84
F-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
L × G	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
L × W	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
G × W	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
L × G × W	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

* = Significant at 0.05 level, ** = Significant at 0.01 level and NS = Not significant. Means in the same column designated by the same letter are not significantly different at 5% level

Table 5: Effect of CMS lines, doses of GA_3 application for male parent and male to female ratio as well as their interactions on seed yield and other morphological traits

	Number of fertile panicles $hill^{-1}$		Panicle mass (g)		Seed set (%)		Seed yield (t ha^{1})		Harvest index (%)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
CMS lines (L)										
IR69625A	17.80a	19.65a	2.67a	2.71a	34.00a	34.90a	1.90a	2.08a	19.26a	20.05a
G46A	15.92b	16.94b	2.49b	2.51b	31.14b	33.58b	1.69b	1.84b	17.82b	18.50b
F-test	**	**	**	**	**	**	**	**	**	**
GA_3 doses for male parent (G)										
0	14.29d	15.39d	1.91d	1.89d	25.60d	26.84d	1.19d	1.36d	16.02d	16.75d
150	16.16c	17.06c	2.47c	2.51c	31.28c	32.69c	1.58c	1.72c	17.91c	18.27c
200	17.40d	19.12b	2.83b	2.86b	33.97b	37.91b	2.02b	2.20b	19.30b	20.00b
300	19.58a	21.62a	3.10a	3.15a	35.41a	39.52a	2.32a	2.56a	20.92a	21.96a
F-test	**	**	**	**	**	**	**	**	**	**
Male to female ratio (W)			2.23c			31.87c				
2R:10A	15.66d	17.16d	2.51b	2.25d	28.85d	33.76b	1.57d	1.71c	17.16d	17.79d
2R:12A	16.93c	18.13c	2.84a	2.50c	31.49c	36.30a	1.76c	1.90bc	18.05c	18.90c
2R:14A	17.64a	19.12a	2.73ab	2.96a	33.59a	35.03a	2.03a	2.19a	19.88a	20.62a
2R:16A	17.20b	18.78b		2.74b	32.33b		1.82b	2.03ab	19.07b	19.77b
F-test	**	**	**	**	**	**	**	**	**	**
L × G	**	**	**	**	**	**	**	**	**	**
L × W	*	*	**	**	**	**	**	**	**	**
G × W	**	**	**	**	**	**	*	*	*	*
L × G × W	**	**	**	**	**	**	**	**	**	**

* = Significant at 0.05 level, ** = Significant at 0.01 level and NS = Not significant. Means in the same column designated by the same letter are not significantly different at 5% level

Table 6: Effect of interaction between CMS liens and doses of GA_3 application for male parent on panicle characteristics and yield during 2019 and 2020 seasons

CMS Lines (L)	GA_3 doses for male parent (G)	Number of fertile panicles hill^{-1} 2019	2020	Panicle mass (g) 2019	2020	Seed Set (%) 2019	2020	Seed yield (t ha^{-1}) 2019	2020	Harvest Index (%) 2019	2020
IR6962A	0	15.50d	16.21d	1.95d	1.92d	25.69f	27.19f	1.35e	1.48de	16.48e	17.23de
	150	16.75c	18.14c	2.50c	2.62bc	31.93d	33.4d	1.72d	1.85c	18.43c	19.21c
	200	18.36b	20. 5b	2.90ab	3.03ab	34.5bc	38.7b	2.10bc	2.27b	20.03b	20.68b
	300	20.55a	23.71a	3.22a	3.27a	35.82a	40.2a	2.43a	2.71a	22.11a	22.99a
G46A	0	13.07f	14.57e	1.87d	1.87d	25.51f	26.49i	1.14f	1.24e	15.57e	16.18e
	150	15.57d	15.98d	2.42c	2.46c	30.64e	31.9e	1.45e	1.58d	17.40d	17.54d
	200	16. 4c	17.67c	2.76b	2.70bc	33.40c	37.0c	1.97c	2.12bc	18.58c	19.23c
	300	18.61b	19.54b	2.88ab	3.03ab	34.9ab	38.8b	2.21b	2.41b	19.73b	20.94b

Means in the same column designated by the same letter are not significantly different at 5 % level

The results in Table (7) showed that the interaction between CMS lines and male to female ratio were significantly affected number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index % in both seasons. The male to female ratio of 2R:14A with CMS line IR69625A, recorded the highest values of number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index % in both seasons. This may be due to the optimum availability of pollen that led to the highest effective grain formation. On the other hand, the lowest values of number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index % were obtained by CMS line G46A when male to female ratio of 2R:10A during 2019 and 2020 seasons. The results are in agreement with those reported by Abo-Youssef (2009).

The results in Table (8) showed that the interaction between doses of GA_3 application for male parent and male to female ratio was significantly affected number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index (%) in both seasons. The male to female ratio 2R:14A with applied 300 g GA_3 ha^{-1} re-

Table 7: Effect of interaction between CMS lines and male to female ratio on panicle characteristics and yield during 2019 and 2020 seasons

CMS Lines	Male to Female ratio	Number of fertile panicles hill^{-1} 2019	2020	Panicle mass (g) 2019	2020	Seed Set (%) 2019	2020	Seed yield (t ha^{-1}) 2019	2020	Harvest Index (%) 2019	2020
IR69625A	2R:10A	16.84c	18.18c	2.31d	2.30d	29.17d	32.3cd	1.71cd	1.84c	18.0de	18.84c
	2R:12A	17.79b	19.70b	2.61bc	2.59bc	31.81b	34.23b	1.85bc	2.03b	18.8cd	19.67b
	2R:14A	18.60a	20.62a	2.93a	3.11a	34.17a	37.29a	2.15a	2.32a	20.57a	21.33a
	2R:16A	17.92b	20.12a	2.8ab	2.84ab	32.8ab	35.69b	1.88bc	2.12ab	19.59b	20.3ab
G46A	2R:10A	14.47e	16.14e	2.16d	2.20d	28.54d	31.38d	1.42e	1.59d	16.29f	16.74d
	2R:12A	16.07d	16.5cd	2.41cd	2.41cd	31.17c	33.29c	1.66d	1.76cd	17.26e	18.14c
	2R:14A	16.6cd	17.6cd	2.75ab	2.80ab	33.02a	35.3b	1.92b	2.07ab	19.1bc	19.92b
	2R:16A	16.4cd	17.4d	2.64b	2.64bc	31.8bc	34.36b	1.77cd	1.9bc	18.55c	19.1bc

Means in the same column designated by the same letter are not significantly different at 5 % level

Table 8: Effect of interaction between doses of GA_3 application for male parent and male to female row ratio on panicle characteristics and yield during 2019 and 2020 seasons

GA_3 doses for male parent (g ha^{-1})	Male to female ratio	Number of fertile panicles hill^{-1}		Panicle mass (g)		Seed set (%)		Seed yield (t ha^{-1})		Harvest Index (%)	
		2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
0	2R:10A	14.15f	14.61h	1.84d	1.78e	25.50e	26.57d	1.20g	1.28g	15.59g	16.51f
	2R:12A	14.53f	15.29gh	1.92d	1.88e	25.46e	26.92d	1.29fg	1.37f	16.0fg	16.66f
	2R:14A	14.25f	15.92g	1.94d	1.99e	25.7de	27.11d	1.25fg	1.46f	16.2fg	17.14ef
	2R:16A	14.22f	15.74g	1.94d	1.91e	25.6de	26.75d	1.24g	1.34f	16.2fg	16.72f
150	2R:10A	15.57e	16.00g	1.93d	1.98e	28.28d	28.68d	1.39fg	1.48f	16.6fg	16.67f
	2R:12A	16.17de	16.67f	2.27c	2.35d	31.26c	32.01c	1.55ef	1.57f	17.51e	18.19e
	2R:14A	16.60d	17.91e	2.92b	3.00c	33.60b	36.10b	1.78de	1.99d	19.04c	19.67d
	2R:16A	16.30de	17.67e	2.76b	2.86c	32.00c	33.99c	1.59de	1.82e	18.45e	18.98d
200	2R:10A	16.01de	17.62ef	2.48c	2.48d	29.6cd	34.45c	1.70de	1.86d	17.45e	18.10e
	2R:12A	17.27c	19.30d	2.79b	2.74cd	34.31b	37.50b	1.95c	2.09c	18.62e	19.61d
	2R:14A	18.48b	20.00cd	3.11a	3.36ab	37.00a	40.40a	2.33b	2.50b	21.0bc	21.71b
	2R:16A	17.85c	19.56d	2.94b	2.88c	34.97b	39.2ab	2.15c	2.34bc	20.07c	20.59cd
300	2R:10A	16.90d	20.43cd	2.69b	2.77c	32.03c	37.81b	1.92c	2.25c	18.9de	19.90d
	2R:12A	19.77b	21.27bc	3.07ab	3.03bc	34.94b	38.6ab	2.20b	2.57ab	20.0cd	21.17c
	2R:14A	21.23a	22.65a	3.39a	3.49a	38.00a	41.59a	2.78a	2.83a	23.16a	23.99a
	2R:16A	20.43a	22.1ab	3.27a	3.31ab	36.67a	40.09a	2.39b	2.61a	21.53b	22.80a

Means in the same column designated by the same letter are not significantly different at 5 % level

corded the highest values of number of fertile panicles hill^{-1}, panicle mass, seed set (%), seed yield and harvest index (%) in both seasons. On the other hand, the male to female row ratio 2R:10A without GA_3 application gave the lowest values number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index % in both seasons. The results are in agreement with those reported by Rahman et al. (2010) and Abo-Youssef et al. (2017).

The results in Table (9) showed that the interaction among CMS lines, doses of GA_3 application for male parent and male to female ratio was significantly affected number of fertile panicles hill^{-1}, panicle mass, seed set, seed yield and harvest index in both seasons. The highest values of number of fertile panicles hill^{-1}, panicle mass, seed set %, seed yield and harvest index (%) were obtained with the CMS line IR69625A by 300 g GA_3 ha^{-1} for male parent when male to female ratio was 2R:14A during both seasons. While the lowest values of number of fertile panicles hill $^{-1}$, panicle mass, seed set, seed yield and harvest index (%) produced by CMS line G46A when using male to female ratio 2R:10A without GA_3 application in both seasons. The results are in agreement with those reported by Riaz et al. (2019); Ghoneim (2020).

Table 9: Effect of interaction among CMS lines, doses of GA_3 application for male parent and male to female ratio on panicle characteristics and yield during 2019 and 2020 seasons

CMS Lines (L)	GA_3 doses for male parent (G)	Male to female ratio	Number of fertile panicles hill^{-1}		Panicle mass (g)		Seed set (%)		Seed yield (t ha^{-1})		Harvest Index (%)	
			2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
IR69625A	0	2R:10A	15.54ef	15.20e	1.87fg	1.79g	25.60h	26.89hi	1.31fg	1.36g	16.01fg	17.01h
		2R:12A	15.65ef	16.08de	1.98fg	1.90efg	25.50h	27.01hi	1.39fg	1.49g	16.54fg	17.20h
		2R:14A	15.40ef	16.87cde	1.96fg	2.01efg	25.86h	27.70hi	1.32fg	1.62fg	16.72fg	17.96gh
		2R:16A	15.42ef	16.67cde	1.98fg	1.96efg	25.79h	27.15hi	1.36fg	1.45g	16.65fg	17.15h
	150	2R:10A	16.53de	17.00cde	1.89fg	2.00efg	28.76g	29.31gh	1.53f	1.64fg	17.42f	18.04gh
		2R:12A	16.70de	18.13c	2.21f	2.41ef	32.01de	32.80f	1.67ef	1.78ef	18.00ef	19.10fg
		2R:14A	17.02cd	18.91bc	3.03b	3.15bc	34.40b	36.80de	1.95cde	2.12cde	19.40de	20.03ef
		2R:16A	16.76d	18.53c	2.87b	2.97bc	32.54d	34.70ef	1.72def	1.85ef	18.90e	19.65f
	200	2R:10A	16.88d	18.03cd	2.61de	2.55de	30.02ef	35.40e	1.77def	1.97ef	18.20ef	19.01fg
		2R:12A	18.27c	21. 00b	2.90b	2.92bc	34.47cd	38.10cd	2.02d	2.16de	19.25de	20.20ef
		2R:14A	19.66bc	21.98ab	3.16b	3.62a	37.80a	41.50a	2.44bc	2.60bc	21.87b	22.39c
		2R:16A	18.68c	21.24b	2.92bc	3.02bc	35.90b	40.03bc	2.15cd	2.36cd	20.79bc	21.10de
	300	2R:10A	18.50c	22.50a	2.86c	2.87bcd	32.29de	37.88de	2.11cd	2.38bc	20.50bc	21.30d
		2R:12A	20.55a	23.60a	3.35a	3.11b	35.27b	39.02c	2.27bc	2.70ab	21.60bc	22.19c
		2R:14A	22.31a	24.70a	3.57a	3.67a	38.60a	43.15a	2.88a	2.95a	24.30a	24.95a
		2R:16A	20.83ab	24.02a	3.50a	3.42ab	37.13a	40.88ab	2.45bc	2.81a	22.03bc	23.50b
G46A	0	2R:10A	12.76g	14.41e	1.80g	1.77g	25.40h	26.24i	1.08g	1.20g	15.17g	16.00i
		2R:12A	13.40fg	14.50e	1.85g	1.86fg	25.42h	26.83hi	1.19fg	1.24g	15.55fg	16.11hi
		2R:14A	13.10g	14.56e	1.92fg	1.97efg	25.70h	26.52hi	1.17fg	1.30g	15.73fg	16.31hi
		2R:16A	13.02g	14.42e	1.90fg	1.86fg	25.55h	26.35i	1.12fg	1.23g	15.81fg	16.29hi
	150	2R:10A	14.60ef	15.00e	1.96fg	1.95ef	27.80gh	28.04h	1.24fg	1.31g	15.89fg	15.29i
		2R:12A	15.63e	15.20e	2.32ef	2.28ef	30.50ef	31.21g	1.46ef	1.36g	17.02f	17.27h
		2R:14A	16.20d	16.90cde	2.80cd	2.84bc	32.80de	35.40ef	1.61de	1.85ef	18.67ef	19.30fg
		2R:16A	15.84e	16.80cde	2.65de	2.75cd	31.46ef	33.28f	1.47ef	1.78ef	18.00ef	18.30g
	200	2R:10A	15.22e	17.20cd	2.35e	2.40ef	29.20fg	33.50f	1.62de	1.74efg	16.69fg	17.18h
		2R:12A	16.26d	17.60cd	2.67cde	2.56de	34.15c	36.90de	1.88d	2.02ef	17.98ef	19.02fg
		2R:14A	17.30d	18.01c	3.06b	3.10b	36.20a	39.30cd	2.21bc	2.40bc	20.29c	21.03de
		2R:16A	17.02d	17.88c	2.96b	2.74cd	34.03cd	38.51cd	2.15cd	2.32cde	19.35cd	20.07ef
	300	2R:10A	15.30e	18.36c	2.51d	2.66cd	31.76e	37.73de	1.73de	2.11cde	17.39ef	18.50g
		2R:12A	18.98bc	18.94bc	2.79cd	2.95bc	34.60cd	38.20cd	2.12cd	2.43bc	18.47e	20.14ef
		2R:14A	20.14ab	20.59ab	3.20b	3.30ab	37.39a	40.02bc	2.68a	2.71ab	22.02b	23.02b
		2R:16A	20.02b	20.26b	3.03b	3.20b	36.20ab	39.30cd	2.32b	2.40bc	21.02bc	22.09c

* = Significant at 0.05 level, ** = Significant at 0.01 level and NS = Not significant. Means in the same column designated by the same letter are not significantly different at 5 % level

4 CONCLUSION

The study was conducted to assess the optimal GA_3 dose on male parent and row ratio between male to female for two CMS lines. The results indicated that, the foliar application of GA_3 significantly increased panicle exsertion, seed set and seed yield of CMS lines at 300 g ha^{-1} concentration. Increase in seed yield was highly influenced by the increase in of seed set % presumably as a result of higher panicle exsertion, wider flag leaf angle, higher degree of spikelet openings. The application of GA_3 on male parent Giza 178R led to a noticeable improvement in its characteristics such as duration of floret opening, angle of floret opening, filaments exsertion, filaments length, anther length, plant height, number of tillers hill^{-1}, panicle length, plant height and panicle exsertion. The CMS line IR69625A produced the highest seed yield with application of 300 g GA_3 ha^{-1} on male parent when male to female ratio was 2R:14A. The highest values of seed yield (2.880 and 2.950 t ha^{-1}) in 2019 and 2020 seasons were obtained by CMS line IR69625A with the application rate of 300 g GA_3 ha^{-1} on male parent when male to female ratio was 2R:14A.

5 REFERENCES

Abo-Youssef, M. I. (2009). The optimum row ratio and doses of GA_3 for two rice CMS lines multiplication. *Proceedings of 6th International Plant Breeding Conference*, Ismailia, Egypt: 326-338.

Abo-Youssef, M., Youssef, M., A., El Sabagh, G., Abo-Gendy, G., & Mohamed, A. (2017). Enhancing seed yield of hybrid rice by maintaining row ratio and dosages of gibberellic acid. *Cercetări Agronomice în Moldova*, *1*(169), 31-45. https://doi.org/10.1515/cerce-2017-0003

Ehsan, Sehsan, S., & Robert, C. S. (2019). *Hybrid Rice Technology*. University of Arkansas Agricultural Experiment Station Research, USA. p. 16-20.

Gaballah, M. M. (2004). *Studies on hybrid rice seed production*. M.Sc. Thesis, Fac. Agric., Kafr El-Sheikh, Tanta Univ, Egypt.

Gaballah, M. M., El-Ezz, A.A., Ghoneim, A. M., Yang, B., & Xiao, L. (2021). Exploiting heterosis and combining ability in two-line hybrid rice. *Acta Agriculturae Slovenica*, *117*(1), 1-16. https://doi.org/10.14720/aas.2021.117.1.1847

Gavino, B. R., Pi, Y., & Abonjr, C.C (2008). Application of gibberellic acid (GA_3) in dosage for three hybrid rice seed production in the Philippines. *Journal of Agricultural Tech nology*, *4*(1), 183-192.

Gomez, K.A., & Gomez, A. A. (1984). *Statistical Procedures for Agricultural Research*. 2nd Ed. John Wiley and Sons, Inc. New York, USA.

Ghoneim, A. M. (2020). Soil nutrients availability, rice productivity and water saving under deficit irrigation conditions. *Journal of Plant Production, Mansoura University*, *11*(1), 7-16. https://doi.org/10.21608/jpp.2020.77983

Hamad, H. Sh. (2018). Impact of male to female ratio, flag leaf clipping and time of GA_3 application on hybrid rice seed productivity. *Egyptian Journal of Plant Breeding, 22*(2), 277-290.

Hamad, H. Sh., Gaballah, M.M., El Sayed, A.A.& El Shamey, E. A.Z. (2015). Effect of GA_3 doses and row ratio on cytoplasmic male sterile line seed production in rice. The 9th Plant Breed Intern Conf, 7-8 Sep., Banha. *Egyptian Journal of Plant Breeding*, *19*(3), 155-167.

Hedden, P. & Phillips, A.L. (2000). Gibberellin metabolism: New insights revealed by the genes. *Trends in Plant Science*, *5*(12), 523-530. https://doi.org/10.1016/S1360-1385(00)01790-8

Lu, Z.M. (1994). Studies on Hybrid Rice Seed Production system. *Hybrid Rice*, *3-4*, 52-54.

Page, A.L., Miler, R. H., & Keeney, D. R. (1982). Methods of Soil Analysis, part 2. Chemical and microbiological properties. *Agronomy monograph No. 9*, pp. 539-624.

Pan, S., Rasul, F., Li, W., Tian, H., Mo, Z., Duan, M., & Tang, X. (2013). Roles of plant growth regulators on yield, grain qualities and antioxidant enzyme activities in super hybrid rice (*Oryza sativa* L.). *Rice*, *16*(6), 9-14. https://doi.org/10.1186/1939-8433-6-9

Rahman, M. H., Ali, M.H., Hasan, M. J., Kulsum, M. U., & Khatun, M. M. (2010). Outcrossing rate in row ratio of restorer and CMS lines for hybrid rice seed production. *Eco-friendly Agriculture Journal*, *3*(5), 233-236.

Riaz, M., Muhammad, I., Tahir, T., Muhammad, S., & Ahsan, R. (2019). Influence of GA_3 on seed multiplication of CMS lines used for hybrid rice development. *African Journal of Plant Science*, *13*(7), 195-200. https://doi.org/10.5897/AJPS2019.1762

Sakamoto, T., Miura K., Tatsumi, T., Ueguchitanaka, M. & Ishiyama, K. (2004). An overview of gibberellins metabolism enzyme genes and their related mutants in rice. *Plant Physiology*, *134*(4), 1642-1653. https://doi.org/10.1104/pp.103.033696

Sindhua, J. S. & Kumar, I. (2002). Quality seed production in hybrid rice. Hyderabad, India. *Proceedings of the 20th Session of the International Rice Commission*.

Sirajul, M., Ahmed, G. J. U., & Julfiquar, A.W. (2005). Effect of flag leaf clipping and GA_3 application on hybrid rice seed yield. *Bangladesh Rice Research Institute (BRRI)*, *30*(1), 46-47.

Sun, T. (2004). Gibberellin signal transduction in stem elongation and leaf growth. In: *Plant Hormones, Biosynthesis, Signal transduction, Action*, Davies P.J. (ends). Kluwer Academic Publ. Dordrecth. Netherlands. Pp: 304-320.

Tiwari, D. K., Pandey, P., Giri, S.P., & Dwivedi, J. L. (2011). Effect of GA3 and other plant growth regulators on hybrid rice seed production. *Asian Journal of Plant Sciences, 10* (2), 133-139. https://doi.org/10.3923/ajps.2011.133.139

Virmani, S.S. (2002). Advances in hybrid rice research and development in the tropics. *Proceedings of the 4th International Symposium on Hybrid Rice*. 14-17 May 2002, Hanoi, Vietnam, 7-20.

Virmani, S.S., CX, X., Mao, R.S. Toledo, M., Hossain, H., & Janaiah, A. (2002). *Hybrid rice seed production technology and its impact on seed industries and rural employment opportunities in Asia*. International Rice Research Institute, Metro Manila, Philippines.

Zaman, F. U., Bastawisi, A.O., Draz, A.D., El-Mowafy, H. M. & Abo-Youssef, M. I. (2002). Hybrid rice technology in Egypt: present status and future strategies. *FAO, RRTC*, *I*, 1-17.

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Sustainable effective use of brackish and canal water for rice-wheat crop production and soil health

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Sustainable effective use of brackish and canal water for rice-wheat crop production and soil health

Abstract: A pot study was conducted to develop reasonable irrigation scheduling methods for rice-wheat crop rotation by conjunctive use of low-quality brackish water and good quality canal water. Treatments tested were; T_1 (canal water), T_2 (brackish water), T_3 (brackish water for rice and canal water for wheat), T_4 (last two irrigations to rice, and initial two irrigations to wheat with canal water), T_5 (last two irrigations to rice but two initial and one last irrigation to wheat with canal water). Results revealed that irrigation with canal water resulted in the maximum mean biomass and grain yield of rice and wheat crops followed by cyclic use of brackish and canal water. While continuous irrigation with brackish water resulted the lowest mean biomass and grain yield. The different modes of irrigations also influenced chemical properties of soil, brackish water adversely affected the soil properties, and maximum pH of soil saturated paste (pH_s), electrical conductivity of soil extract (EC_e) and sodium adsorption ratio (SAR) were recorded where brackish water was used continuously. Therefore, it was concluded that when water is valuable and freshwater resources are limited, cyclic use of the canal and brackish water is also profitable with marginal effect on biomass and grain yield and proves least detrimental for soil health.

Key words: canal water; brackish water; rice; wheat; soil health

Trajnostna in učinkovita raba brakične in vodovodne vode za pridelavo riža in pšenice in ohranjanje zdravja tal

Izvleček: Izveden je bil lončni poskus za razvoj načrta smiselnega namakanja v kolobarju riža in pšenice s hkratno uporabo brakične vode slabe kakovosti in kakovostno vodo iz vodovoda. Obravnavanja so obsegala: T_1 (voda iz vodovoda), T_2 (brakična voda), T_3 (brakična voda za riž in voda iz vodovoda za pšenico), T_4 (dve zadnji namakanji riža in začetno namakanjem pšenice z vodo iz vodovoda), T_5 (dve zadnji namakanji riža, dve začetni in zadnje namakanje pšenice z vodo iz vodovoda). Rezultati so pokazali, da je namakanje z vodo iz vodovoda dalo največjo poprečno biomaso in največji pridelek zrnja riža in enake rezultate pri pšenici pri izmenični rabi brakične in vodovodne vode. Stalno namakanje z brakično vodo je dalo najmanjšo poprečno biomaso in najmanjši pridelek zrnja. Različni načini namakanja so vplivali tudi na kemijske lastnosti tal. Brakična voda je nanje vplivala negativno. Pri njeni stalni uporabi je bil zabeležen najvišji pH tal (pH_s), največja električna prevodnost izvlečka tal (EC_e) in največja adsorpcija natrija (SAR). Na osnovi tega lahko zaključimo, da je tam, kjer so viri sladke vode omejeni, izmenična uporaba brakične in vodovodne vode donosna saj ima majhen učinek na biomaso in pridelek zrnja in se izkaže manj škodljiva za zdravje tal.

Ključne besede: vodovodna voda; brakična voda; riž; pšenica; zdravje tal

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1 INTRODUCTION

Due to Pakistan's arid and semi-arid climate, the agriculture sector of the country is heavily dependent on irrigated farming. However, a considerable gap exists between increasing demand and water supply and farmers are forced to pump the groundwater, which is about 70-80 % brackish (Latif and Beg 2004). Drought prevailing conditions and decreased the surface water supply may intensify the practice of irrigation with brackish water that may results in problem of salinity in irrigated lands (Qadir et al., 2007). Hence, farmers' poor knowledge to manage the brackish water for irrigation is one of the major reasons for the land deterioration.

Soil sodicity is generally described as presence of relative amounts of sodium in the soil solution or on the cation exchange sites. Sodium adsorption ratio (SAR) represents the soluble Na^+ concentration relative to the soluble divalent cation concentrations in the soil solution (Qadir et al., 2008). Soils with SAR more than13 are dispersive and suffer from serious physical problems e.g. permeability to water and air is restricted (Biswas et al., 2014). Further, water with high sodium content results in dispersion of clay particles and clogging of soil pores (Levy et al., 2003); Na-saturation of clay complex (Minhas et al., 2019); impedes aeration and loss in soil permeability (Choudhary et al., 2011); thereby negatively impacting crop productivity through toxicity of Na^+, nutritional imbalances and adverse osmotic effect (Sharma et al., 2016; Murtaza et al., 2017).

Several researchers decided to designate the strategies for optimal use of different quality waters to attain secure and predictable yields on a long-term sustainable basis. Nevertheless, safe and successful use of poor-quality water will require careful planning, stringent monitoring procedures, and efficient management practices to avoid further land degradation (FAO, 2011). Two different strategies can be employed to use the fresh water and poor-quality groundwater, i.e., I) a cyclic mode, in which subsurface poor-quality water and canal water are used separately, and II) a blending model, in which good and poor-quality water are used simultaneously (Qureshi et al., 2004).

The cyclic mode involves brackish and good quality water in different crop rotations comprising salt-tolerant and salt-sensitive crops. In general, canal water or good quality water is used before planting and at early growth stages, while brackish water is used after seedling establishment (Latteef, 2010). In Pakistan, the rice-wheat cropping pattern covers 2.3 X 10^{-6} ha (Qureshi and Barrett-Lennard, 1998). Rice is relatively tolerant to sodicity, while wheat is tolerant to salinity (Qadir et al. 2001). It is a very well-established fact that germination and seedling stages are categorized as the most sensitive growth stages in most crops. Subsequently, irrigation with good quality water has been advocated at early growth stages and then switching over to brackish water at later growth stages when the plant can tolerate high salt stress (Minhas and Gupta, 1993). Efforts have been made to counteract brackish water's detrimental effects through blended and cyclic approaches (Rhoades, 1998). Furthermore, conjunctive use of brackish water with surface water can generate double agricultural revenues, and that profits may be more during drought periods (Bredehoeft and Young, 1983).

In a pot experiment, Gandahi et al. (2017) studied the response of different cotton varieties against conjunctive use of non-saline and saline water. They concluded that cotton genotypes performed better when six irrigations were provided with fresh water and six irrigations with salty water in a conjunctive manner. Similarly, in a field experiment, Chen et al. (2018) observed that shoot dry mass and cotton yield decreased significantly when irrigated with brackish water than freshwater. They stated that an optimal mix of alternating non-saline and saline water may be an effective strategy for cotton production and avoiding secondary salinization when using saline water. Minhas et al. (2007) evaluated the effect of fresh water and alkali water on rice-wheat crop rotation. The yield of rice and wheat crops, was affected negatively when irrigated with alkali water; however, rice was more sensitive to alkali water irrigation. They concluded that cyclic use of alkali and good quality water could be a preferable irrigation mode to avoid the build up of salts in soils.

In a field experiment, Murad et al. (2018) applied the fresh water and brackish water at different maize crop growth stages. They stated that freshwater application yielded the highest grain and straw yield of maize. They concluded that freshwater irrigation at an early sensitive stage while conjunctive use of saline water with fresh water at later growth stages may minimize the yield losses. Therefore, the present research work was carried out to develop reasonable irrigation scheduling methods for rice-wheat crop rotation by conjunctive use of the low-quality brackish water and limited freshwater resources.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SETUP

A pot study was conducted in the wirehouse of Soil Salinity Research Institute Pindi Bhattian, Hafizabad. A normal soil {pH_s = 7.98, EC_e = 2.22 (dS m^{-1}), SAR = 36.50 and texture = sandy clay loam} was filled in glazed pots at the rate of 16 kg/pot. Pots were arranged in Completely Randomized Design (CRD) with three replications to give a total of 15 pots. During the experiment, the average weather conditions were: 13.2 ± 2.8 °C minimum temperature, 41.4 ± 3.8 °C maximum temperature, 35.6 ± 3.4 % minimum relative humidity, 72.6 ± 4.6 % maximum relative humidity, maximum sunshine hours, 14 h and 8 min and minimum sunshine hours, 7 h and 36 min.

2.2 TREATMENTS DETAILS AND CROP ROTATION

Treatments tested were comprised of T_1 (canal water), T_2 (consistence use of brackish water), T_3 (brackish water for rice and canal water for wheat, seasonal cyclic use), T_4 (last two irrigations to rice, and initial two irrigations to wheat with canal water, supplementation of canal water at sensitive stages), T_5 (last two irrigations to rice but two initial and one last irrigation to wheat with canal water). Rice-wheat crop rotation was used for three years (2013 to 2016). Thirty days old seedlings of rice ('Shaheen Basmati') were transplanted in the 2nd week of July 2013, 2014, 2015 at the rate of three seedlings per pot. Fertilizers dose viz. 110-90-60 NPK kg ha^{-1} was used for rice crop. Half of the recommended nitrogen (urea) and full dose of P (single super phosphate) and K (sulphate of potash) were applied at transplanting while the remaining half dose of nitrogen was applied thirty days after transplanting. The pots were irrigated as per crop requirement and approximately 2 liters pot^{-1} $irrigation^{-1}$ were given, a total of 20 irrigations were applied in each season for rice crop. All the plant protection and agronomical practices were carried out uniformly. Rice crop was harvested in the 2nd week of November, and data about biomass and grain yield was documented. After the harvest of rice crop, in the same layout, fertilizers dose viz. 120-110-70 NPK kg ha^{-1} was applied. Half of the recommended nitrogen (urea) and full dose of P (single super phosphate) and K (sulphate of potash) were applied at sowing while the remaining half dose of nitrogen was applied thirty days after sowing. Ten seeds of wheat ('Inqlab-91') were sown in each pot in the 3rd week of November 2013, 2014 and 2015. Thirty days after the germination, plants were thinned, and three seedlings/pot were maintained. The pots were irrigated as per crop requirement and approximately 2 liters pot^{-1} $irrigation^{-1}$ were given, a total of 6 irrigations were applied in each season for wheat crop. The crop was raised to maturity and harvested in the 2nd week of April, and data about biomass and grain yield were recorded.

2.3 SOIL AND WATER ANALYSIS

Before the start of study and after the harvest of 3rd wheat crop soil samples were air dried, passed through 2 mm sieve and analyzed for pH_s, EC_e and SAR (U.S. Salinity Laboratory Staff, 1954). Soil pH of the saturated paste was measured by using pH meter (Microcomputer pH-vision cole parmer model 05669-20). Electrical conductivity of the irrigation water and soil saturated paste extract was measured with the help of conductivity meter (WTW conduktometer LF 191). The Na^+ contents were determined by flame photometer (digiflame code DV 710) while Ca^{2+} and Mg^{2+} were determined titrimetrically. Sodium adsorption ratio (SAR) was calculated as follows where ionic concentration of the saturation extracts is given in $mmol_e\ l^{-1}$. SAR = $Na^+ / [(Ca^{2+}+ Mg^{2+})/2]^{1/2}$. Soil texture was determined by hydrometer method (Bedaiwy, 2012). Carbonate contents (CO_3^{2-} and HCO_3^-) was determined via titration with standard H_2SO_4. Residual sodium carbonate (RSC) was calculated by (Eaton, 1950) as follows:

RSC = (CO_3^{2-} and HCO_3^-) - [($Ca^{2+} + Mg^{2+}$)

2.4 STATISTICAL ANALYSES

The collected crop data were subjected to analysis of variance. The treatment means comparison was made using the Least Significant Difference Test at 5 % probability level (Steel et al., 1997) using STATISTIX 8.1 package software.

Table 1: Analysis of irrigation waters used in study

Parameters	Units	Brackish water	Canal water
Electrical conductivity of irrigation water (EC_{iw})	($dS\ m^{-1}$)	3.29	0.32
Sodium adsorption ratio (SAR)	$(mmol_e\ l^{-1})^{1/2}$	25.52	0.53
Residual sodium carbonate (RSC)	($me\ l^{-1}$)	2.54	Nil

3 RESULTS

3.1 RICE CROP

Data in Table 2 showed that different irrigation modes had a significant effect ($p < 0.05$) on rice biomass yield. Irrigation with canal water produced the maximum biomass yield during all three seasons, while continuous irrigation with brackish water negatively affected rice crop biomass yield. Based on the mean value of three seasons, irrigation with canal water (T_1) produced the maximum biomass yield of 254.53 g/pot followed by (T_3) (214.54 g/pot), where canal water and brackish water was used in a cyclic mode. Whereas continuous irrigation with brackish water produced the minimum biomass yield of 180.63 g/pot. A similar trend was also observed in the case of grain yield, based on average data of three seasons, maximum grain yield (55.40 g/pot) was documented where canal water was used for irrigation followed by cyclic mode of irrigation (brackish water for rice and canal water for wheat) which yielded the grain yield of 42.81 g/pot (Table 3). However, it was statistically ($p < 0.05$) similar to all the other treatments. Continuous irrigation with brackish water negatively impacted grain yield, and the lowest mean grain yield (36.64 g/pot) was divulged with this mode of irrigation.

3.2 WHEAT CROP

Growth characteristics like biomass and grain yield of the wheat crop were also significantly influenced by different irrigation modes. Data presented in Table 4 showed that the highest mean value for biomass yield (84.35 g/pot) was documented in T_1 (canal water irrigation) followed by T_3 with biomass yield of (78.55 g/pot), and both the treatments were significant ($p < 0.05$) from each other. On average, the minimum biomass yield of 69.02 g/pot was recorded in T_2, indicating that continuous irrigation with brackish water significantly reduced the biomass yield. Grain yield also responded significantly to different treatments of irrigation during all three seasons. Data in Table 5 illustrated that the maximum grain yield (35.92 g/pot) was observed with canal irrigation followed by cyclic mode of irrigation (32.09 g/pot). On the other hand, the lowest grain yield (27.25) was observed in T_2, a treatment where brackish irrigation water was used continuously to irrigate the pots.

3.3 SOIL PROPERTIES

Chemical properties of surface soil were also influenced by the different modes of irrigations and pH_s, EC_e and SAR gradually increased during the three years of experimentation. Soil pH_s steadily increased by continuous irrigation with brackish water as compared to other modes of irrigation. At the end of the study maximum increase of 11.52 % over its initial value in soil, pH_s was recorded with brackish water irrigation (Table 6). On the contrary, a minimum increase in soil pH_s (1.12 %) was recorded in canal water irrigation, while in the cyclic mode of irrigation, this increase was (4.38 %) over its initial value. A similar tendency was observed in soil EC_e; different modes of irrigation resulted in the buildup of salts in the soil; however, accumulation of salts was more with brackish water. At the end of the study, a maximum increase in EC_e (234.23 %) was observed in T_2 (brackish water), whereas, minimum increase (5.85 %) was observed in T_1 (canal water) (Table 7). Soil sodicity was also increased remarkably by various modes of irrigation. Maximum sodicity was developed where brackish water was used continuously for three years, and an increase of 648.90 % in SAR over its

Table 2: Effect of conjunctive use of brackish and canal water on rice biomass yield (g/pot)

Treatments	1st crop	2nd crop	3rd crop	Mean
T_1 Canal water	256.98 A	263.38 A	243.22 A	254.53 A
T_2 Consistence use of brackish water	236.14 B	164.80 A	140.96 D	180.63 C
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	234.88 B	216.55 B	192.20 B	214.54 B
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	240.13 B	190.11 C	162.78 C	197.67 BC
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	237.86 B	194.36	170.28 C	200.83 BC

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

Table 3: Effect of conjunctive use of brackish and canal water on rice grain yield (g/pot)

Treatments	1st crop	2nd crop	3rd crop	Mean
T_1 Canal water	55.23 A	57.48 A	53.50 A	55.40 A
T_2 Consistence use of brackish water	48.13 B	32.18 D	29.62 D	36.64 B
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	46.64 B	41.91 B	39.88 B	42.81 B
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	47.89 B	36.04 C	33.41 C	39.11 B
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	50.09 B	36.76 C	32.98 C	39.94 B

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

initial value was observed (Table 8). In contrast, a minimum increase (27.39 %) in SAR over its initial value was recorded, canal water was used for irrigation.

4 DISCUSSION

Due to Pakistan's arid to semi-arid climate, about 70-75 % of the country's tube wells withdraw the brackish groundwater (Ghafoor et al., 1991). Furthermore, many areas of the country with freshwater resources are endangered with contamination due to this excessive withdrawal of brackish groundwater. Under most situations, subsurface brackish water and canal water can be applied in different modes of irrigations (cyclic, blending) to meet the crop water demands. Allocation of these two different quality waters can be done depending upon season, type of crop, and crop growth stage so that salt stress is minimized. For this purpose, we designed an irrigation schedule for rice wheat-crop rotation, where both waters were used in seasonal cyclic mode, and canal (non-saline) water was used at the salt-sensitive stage of crop growth, switching over to brackish water at the tolerant stage. Results of the study showed that pH_s, EC_e, and SAR of soil increased gradually during three years; however, the rate of increase was more where brackish water alone with {EC_{iw} = 3.29 (dS m^{-1}), SAR = 25.52, and RSC = 2.54 (me l^{-1})} was used continuously for three years. This high pH_s, EC_e, and SAR due to brackish water may be explained that salt solution concentrated when water loss through evapotranspiration and induces the salinity/sodicity (Minhas et al., 2007). Different researchers reported similar findings that irrigation with brackish water resulted the residual in salinity and sodicity build up in soils (Avais et al., 2018; Zaka et al., 2018; Qadir et al., 2019). Continuous irrigation with brackish water having SAR 10.4 (mmol $l^{-1})^{1/2}$ may reduced rice and wheat productivity by 16 and 14 %, respectively and resulted in buildup of exchangeable sodium (Sheoran et al., 2021). Similarly in a pot study, Hussain et al. (2016) reported that saline irrigation (5.7 dS m^{-1}) impaired growth of wheat plants and adversely affected the grain and dry matter yield. Therefore, it emerges that high water demanding rotations like rice-wheat are even more prone to sodicity problem when irrigated with sodic waters (Minhas et

Table 4: Effect of conjunctive use of brackish and canal water on wheat biomass yield (g/pot)

Treatments	1st crop	2nd crop	3rd crop	Mean
T_1 Canal water	90.31 A	78.40 A	84.36 A	84.35 A
T_2 Consistence use of brackish water	73.22 C	67.92 D	65.94 D	69.02 D
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	84.86 AB	74.18 B	76.62 B	78.55 B
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	80.17 BC	71.41 C	72.78 C	74.78 C
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	81.60 AB	71.98 BC	73.24 C	75.60 BC

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

Table 5: Effect of conjunctive use of brackish and canal water on wheat grain yield (g/pot)

Treatments	1st crop	2nd crop	3rd crop	Mean
T_1 Canal water	38.63 A	32.96 A	36.18 A	35.92 A
T_2 Consistence use of brackish water	30.76 C	26.47 D	24.52 D	27.25 D
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	35.77 AB	29.65 B	30.86 B	32.09 B
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	33.80 BC	27.65 CD	27.92 C	29.79 C
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	34.30 BC	28.14 BC	28.56 C	30.33 BC

Different letters in the same column indicate significant differences by LSD at $p \leq 0.05$

al., 2019). This development of sodicity and salinity was also correlated to proportions of brackish water used in different irrigation modes. At the end of the study, EC_e value was 4.17, where brackish and canal waters were used in cyclic mode (T_3) while a little variation was observed between T_4 (6.08) and T_5 (5.98) where canal water was supplied at the sensitive stages of crop growth. Similarly, at the end of the study, corresponding final values of SAR were 18.64 with cyclic mode (T_3) and 35.15 and 35.14 in (T_4) and (T_5), respectively, where canal water was used at salt-sensitive stages. In contrast, brackish water was used at tolerant stages of crop growth. Our results are supported by earlier findings of (Minhas et al., 2007) that cyclic use or mixing of good quality water with higher alkaline water resulted in lower exchangeable sodiu percentage (ESP) value (sodicity).

Salinity induced reduction in biomass and grain yield of rice and wheat crop was observed, and maximum reduction was documented in treatment where brackish water alone was used for irrigation, whereas, application of canal water alone or at sensitive growth stages and cyclic mode of irrigation showed less reduction in these attributes. This reduced biomass and grain yield of rice and wheat with brackish water were due to sodicity/salinity in the soil as we discussed earlier that pH_s, EC_e, and SAR of soil increased gradually with brackish water. This accumulation of toxic salts in root zone asserted physiological stress and negatively affected the physical and morphological characters of plants and consequently, crop growth is reduced (De Oliveira et al., 2013; Pessarakli, 2016). Under salt stress, the plant experiences osmotic and ionic stresses, leading to leaf senescence, reduced water uptake, photosynthetic activity, transpiration rate, and promoted metabolic alterations (Munns, 2002; Amirjani, 2011).

According to Zeng and Shannon (2003), salt stress in rice crop before the heading reduces the number and mass of panicles during the three-leaf stages until booting. Further, at the flowering stage, salt stress adversely affected photosynthesis, which resulted in unfilled spikelet formation and ultimately the number of filled grains in the panicle decreased (Moradi, 2002; Zhang et al., 2015). Brackish water salinity resulted in the reduce biomass, leaf area, number of tillers, delay in flowering

Table 6: Effect of conjunctive use of brackish and canal water on soil pH_s

Treatments	1st year	2nd year	3rd year	% increase over initial the value
T_1 Canal water	8.00	8.05	8.07	1.12
T_2 Consistence use of brackish water	8.41	8.59	8.90	11.52
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	8.17	8.25	8.33	4.38
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	8.35	8.45	8.65	8.39
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	8.30	8.42	8.63	8.14

Table 7: Effect of conjunctive use of brackish and canal water on soil EC_e

Treatments	1st year	2nd year	3rd year	% increase over the initial value
T_1 Canal water	2.27	2.32	2.35	5.85
T_2 Consistence use of brackish water	3.30	5.08	7.42	234.23
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	2.58	3.72	4.17	87.83
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	2.92	4.46	6.08	173.87
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	2.86	4.36	5.98	169.36

and ripening in rice crop (Kavosi, 1995; Castillo et al., 2007).

The basic principle of sustainable irrigation using brackish water is that the concentration of toxic salts in the rhizosphere must be below a specific crop threshold (Maas and Hoffman, 1977; Munns and Tester, 2008). Some reports showed that rice is tolerant to salinity at germination and sensitive during reproductive stages (Lafitte et al., 2004; Rad et al., 2011). The current study also indicated that application of canal water at the sensitive (reproductive) stages of rice and wheat growth was also more effective than consistent use of brackish water, and biomass and grain yield were significantly higher in T_4 and T_5 than T_2. Comparatively higher values of growth attributes in T_4 and T_5 demonstrated that farmers can wisely manage the brackish water for irrigation when fresh water resources are limited. The sensitivity of any crop to salt stress often changes from one growth stage to the other growth stage (Mojid et al., 2014) therefore brackish water can be used for irrigation at growth stage where crops have better resistance ability (Munns and Tester, 2008). Our results are supported by previous studies that brackish water could be used for irrigation without significant crop yield loss if managed intelligently (Al Khamisi et al., 2013; Singh, 2014; Murad et al., 2018).

If properly managed, alternate irrigation with brackish and freshwater, minimize the negative impacts on plant growth and displays better soil salt control (Huang et al., 2019). Similarly, Xue and Ren (2017) reported that conjunctive use of fresh and brackish water significantly increased the yield of sunflower, maize, and wheat crop compared with brackish water irrigation. Our results are also in harmony with Minhas (1996), who stated that the conjunctive use of non-saline and saline water improved maize crop yield. Similarly, Gandahi et al.)2017) stated that cotton growth and yield attributes were significantly reduced with brackish water. The maximum values of these attributes were recorded where non-saline water was used to irrigate the crop.

5 CONCLUSION

Based on the current study results, it was concluded that:

Table 8: Effect of conjunctive use of brackish and canal water on soil SAR

Treatments	1st year	2nd year	3rd year	% increase over initial the value
T_1 Canal water	5.96	7.50	7.58	27.39
T_2 Consistence use of brackish water	19.72	29.37	44.56	648.90
T_3 Brackish water for rice and canal water for wheat seasonal cyclic use	12.16	15.02	18.64	213.27
T_4 Last two irrigations to rice and initial two irrigations to wheat with canal water (supplementation of canal water at sensitive stages)	17.29	25.72	35.15	490.75
T_5 Last two irrigations to rice but two initial and one last irrigation to wheat with canal water	15.82	22.68	35.14	490.58

- Continuous use of brackish water caused the salt accumulation in soil and induced severe salt stress on crop growth and yield. Whereas, supplementation of a canal or non-saline water at sensitive growth stages can improve the rice-wheat yield significantly rather than using brackish water alone during all growth stages. Further, the cyclic mode of irrigation can be applied successfully with negligible or no negative impacts on both crop yield and soil health.

- When freshwater resources are finite and the use of brackish water is inevitable, cyclic use of brackish and canal water can ensure the reasonable and sustainable use of brackish water in agricultural production.

- Farmers in Pakistan mostly rely on tube wells that are pumping poor quality water. Alternate irrigation with brackish and canal water for major crops, like rice and wheat is an effective practice for alleviating the shortage of freshwater in agricultural production.

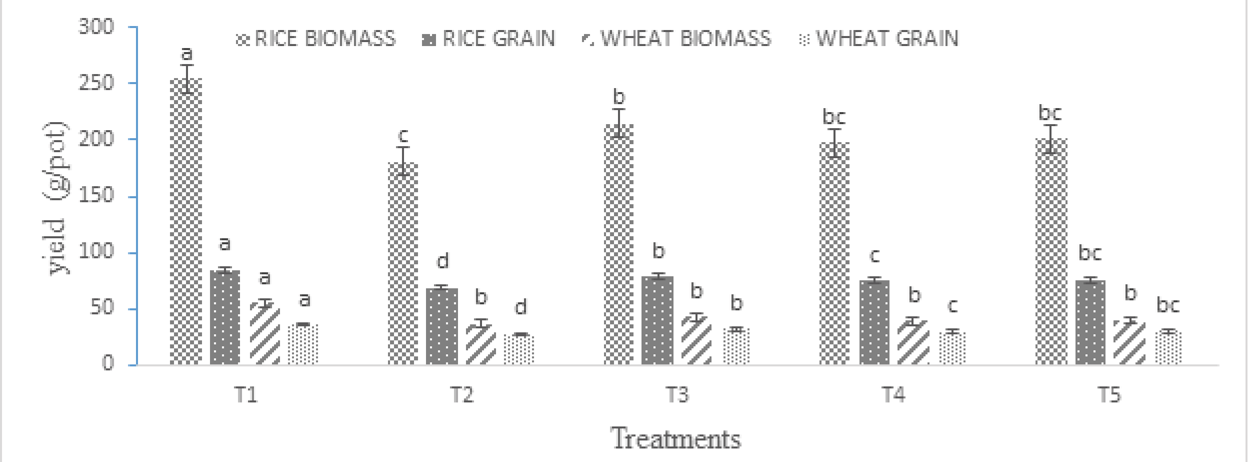

Fig.1: Effect of conjunctive use of brackish and canal water on biomass and grain yield of rice-wheat (average of three seasons). T_1 (Canal water), T_2 (Consistence use of brackish water), T_3 (Brackish water for rice and canal water for wheat seasonal cyclic use), T_4 (Last two irrigations to rice and initial two irrigations to wheat with canal water, supplementation of canal water at sensitive stages), T_5 (Last two irrigations to rice but two initial and one last irrigation to wheat with canal water)

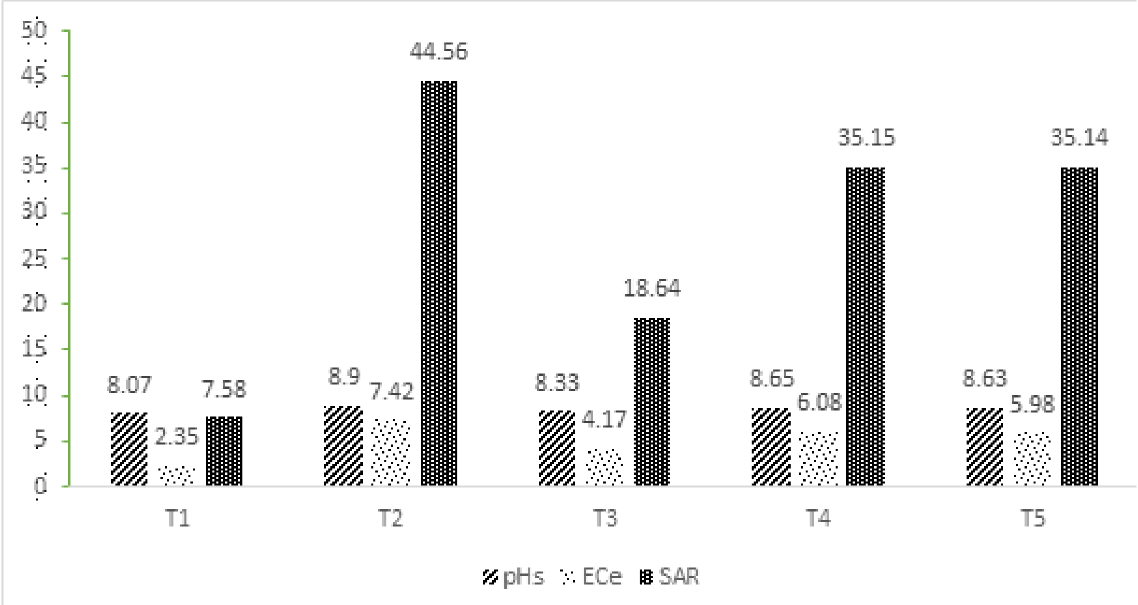

Fig.2: Effect of conjunctive use of brackish and canal water on soil pH_s, EC_e and SAR at the end of study. T_1 (Canal water), T_2 (Consistence use of brackish water), T_3 (Brackish water for rice and canal water for wheat seasonal cyclic use), T_4 (Last two irrigations to rice and initial two irrigations to wheat with canal water, supplementation of canal water at sensitive stages), T_5 (Last two irrigations to rice but two initial and one last irrigation to wheat with canal water)

Therefore, societal awareness among farming community to wisely use groundwater and canal water in cyclic mode for high-valued crops can potentially be helpful to avoid soil salinization and production losses.

- Current study was a pot experiment conducted in a wire house. Therefore, an additional field studies are recommended to gain a better understanding of long-term effects of brackish water and cyclic use of brackish and canal water on production of major crops and soil health.

6 REFERENCES

Al Khamisi, S.A., Prathapar, S.A., Ahmed, M. (2013). Conjunctive use of reclaimed water and groundwater in crop rotations. Agricultural Water Management, 116, 228-234. https://doi.org/10.1016/j.agwat.2012.07.013

Amirjani, M.R. (2011). Effect of salinity stress on growth, sugar content, pigments and enzyme activity of rice. International Journal of Botany, 7, 73-81. https://doi.org/10.3923/ijb.2011.73.81

Avais, M.A., Ghulam, Q., Khalil, A., Muhammad, I., Amar, I.S., Imtiaz, A.W., Muhammad, Q.N., Muhammad, S., Muhammad, A. (2018). Role of inorganic and organic amendments in ameliorating the effects of brackish water for raya-sunflower production. International Journal of Biosciences, 12, 117-122.

Bedaiwy, M.N.A. (2012). A simplified approach for determining the hydrometer's dynamic settling depth in particle-size analysis. Catena, 97, 95-103. https://doi.org/10.1016/j.catena.2012.05.010

Biswas, A., Amiya, B. (2014). Comprehensive approaches in rehabilitating salt affected soils: a review on Indian perspective. Open Transactions on Geosciences, 1, 13-24. https://doi.org/10.15764/GEOS.2014.01003

Bredehoeft, J.D., Young, R. A. (1983). Conjunctive use of groundwater and surface water: Risk aversion, Water Resource Research, 19, 1111-1121. https://doi.org/10.1029/WR019i005p01111

Castillo, E.G., To Phuc, Abdelbaghi, M.A., Kazuyuki, I. (2007). Response to salinity in rice: comparative effects of osmotic and ionic stress. Plant Production Science, 10(2), 159-170. https://doi.org/10.1626/pps.10.159

Chen, W., Menggui, J., Ty, P.A.F., Yanfeng, L., Yang, X., Tianrui, S., Xue, P. (2018). Spatial distribution of soil moisture, soil salinity, and root density beneath a cotton field under mulched drip irrigation with brackish and fresh water. Field Crops Research, 215, 207-221. https://doi.org/10.1016/j.fcr.2017.10.019

Choudhary, O.P., Ghuman, B.S., Singh, B., Thuy, N., Buresh, R.J. (2011). Effects of long-term use of sodic water irrigation, amendments and crop residues on soil properties and crop yields in rice-wheat cropping system in a calcareous soil. Field Crops Research, 121, 363-372. https://doi.org/10.1016/j.fcr.2011.01.004

De Oliveira, A.B., Alencar, N.L.M., Gomes-Filho, E. (2013). Comparison between the water and salt stress effects on plant growth and development. In: Sener Akıncı, S. (Ed.), Responses of Organisms to Water Stress, (Publisher, Intechopen, 2013, published: January 16, 2013 under CC BY 3.0 license. 10.5772/54223). https://doi.org/10.5772/54223

Eaton, F.M. (1950). Significance of carbonate in irrigation waters. Soil Science, 67, 123-133. https://doi.org/10.1097/00010694-195002000-00004

FAO. (2011). Agriculture and water quality interactions: a global overview. SOLAW Background Thematic Report - TR08. http://www.fao.org/3/bl092e/bl092e.pdf.

Gandahi, A.W., Kubar, A., Sarki, M.S., Talpur, N., Gandahi, M. (2017). Response of conjunctive use of fresh and saline water on growth and biomass of cotton genotypes. Journal of Basic & Applied Sciences, 13, 326-334. https://doi.org/10.6000/1927-5129.2017.13.54

Ghafoor, A., Qadir, M., Qureshi, R.H. (1991). Using brackish water on normal and salt affected soil in Pakistan: A review. Pakistan Journal of Agricultural Sciences, 28, 273-288.

Huang, M., Zhang, Z., Sheng, Z., Zhu, C., Zhai, Y., Lu, P. (2019). Effect on soil properties and maize growth by alternate irrigation with brackish water. Transactions of the ASABE, 62(2), 1-9. https://doi.org/10.13031/trans.13046

Hussain, Z., Khattak, R.A., Irshad, M., Mahmood, Q., An, P. (2016). Effect of saline irrigation water on the leachability of salts, growth and chemical composition of wheat (Triticum aestivum L.) in saline-sodic soil supplemented with phosphorus and potassium. Journal of Soil Science and Plant Nutrition, 16(3), 604-620. https://doi.org/10.4067/S0718-95162016005000031

Kavosi, M. (1995). The best model to rice yield prediction in salinity condition. Dissertation of MSc. Tabriz University.

Lafitte, H.R., Ismail, A., Bennett, J. (2004). Abiotic stress tolerance in rice Fore Asia progress and the future. International Rice Research Institute, DAPO 7777, Metro Manila, Philippines.

Latif, M., Beg, A. (2004). Hydrosalinity issues, challenges and options in OIC member states. In: M. Latif, S. Mahmood, and M.M. Saeed, eds. Proceedings of the International Training Workshop on Hydrosalinity Abatement and Advance Techniques for Sustainable Irrigated Agriculture, pp. 1-14. September 20-25, 2004. PCRWR, Islamabad.

Latteef, E.M.A. (2010). Saline irrigation water and its effect on N use efficiency, growth and yield of sorghum plant using 15N. MSC thesis. Al-Azhar University, Cairo. p. 46.

Levy, G.H., Mamedov, A.I., Goldstein, D. (2003). Sodicity and water quality effects on slaking of aggregates from semi-arid soils. Soil Science, 168, 552-562. https://doi.org/10.1097/01.ss.0000085050.25696.52

Maas, E.V., Hoffman, G.J. (1977). Crop salt tolerance-current assessment. Journal of the Irrigation and Drainage Division, 103, 115-134. https://doi.org/10.1061/JRCEA4.0001137

Minhas, P.S. (1996). Saline water management for irrigation in India. Agricultural Water Management, 30(1), 1-24. http://dx.doi.org/10.1016/0378-3774(95)01211-7. https://doi.org/10.1016/0378-3774(95)01211-7

Minhas, P.S., Dubey, S.K., Sharma. D.R. (2007). Comparative effects of blending, intera/inter-seasonal cyclic uses of alkali and good quality waters on soil properties and yields of paddy and wheat. Agricultural Water Management, 87, 83-90. https://doi.org/10.1016/j.agwat.2006.06.003

Minhas, P.S., Gupta, R.K. (1993). Conjunctive use of saline and non-saline waters. I. Response of wheat to initially variable salinity profiles and modes of salinization. Agricultural Water Management, 23, 125-137. https://doi.org/10.1016/0378-3774(93)90036-A

Minhas, P.S., Qadir, M., Yadav, R.K. (2019). Groundwater irrigation induced soil sodification and response options. Agricultural Water Management, 215, 74-85. https://doi.org/10.1016/j.agwat.2018.12.030

Mojid, M.A., Mia, M.S., Saha, A.K., Tabriz, S.S. (2014). Growth stage sensitivity of wheat to irrigation water salinity. Journal of the Bangladesh Agricultural University, 11, 147-152. https://doi.org/10.3329/jbau.v11i1.18226

Moradi, F. (2002). Physiological characterization of rice cultivars for salinity tolerance during vegetative and reproductive stages. Ph.D Thesis. University of philippines, Los Banos. Philippines

Munns, R. (2002). Comparative physiology of salt and water stress. Plant Cell Environment, 25, 239-250. https://doi.org/10.1046/j.0016-8025.2001.00808.x

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

Murad, K.F. Akbar, H., Oli, A.F., Sujit, K.B., Khokan, K.S., Rahena, P.R., Jagadish, T. (2018). Conjunctive use of saline and fresh water increases the productivity of maize in saline coastal region of Bangladesh. Agricultural Water Management, 204, 262-270. https://doi.org/10.1016/j.agwat.2018.04.019

Murtaza, B., Ghulam, M., Muhammad, S., Gary, O., Ghulam, A., Muhammad, I., Ghulam, M.S. (2017). Amelioration of saline-sodic soil with gypsum can increase yield and nitrogen use efficiency in rice-wheat cropping system. Archives of Agronomy and Soil Science, 6, 1267-1280. https://doi.org/10.1080/03650340.2016.1276285

Pessarakli, M. (2016). Handbook of Photosynthesis, third ed. CRC Press Florida, Taylor & Francis Publishing Group p. 846. https://doi.org/10.1201/b19498

Qadir, G., Khalil, A., Amar, I.S., Muhammad, I., Muhammad, Q.N., Muhammad, S., Zaheen, M. (2019). Sustainable use of brackish water for cotton wheat rotation. Asian Journal of Agriculture and Biology, 7(4), 593-601

Qadir, M., Ghafoor, A. Murtaza, G. (2001). Use of saline sodic waters through phytoremediation of calcareous saline sodic soils. Agricultural Water Management, 50, 197-210. https://doi.org/10.1016/S0378-3774(01)00101-9

Qadir, M., Oster, J.D., Schuber S., Noble, A.D., Sahrawatk, K.L. (2007). Phytoremediation of sodic and saline-sodic soils. Advances in Agronomy, 96, 197-247. https://doi.org/10.1016/S0065-2113(07)96006-X

Qadir, M., Sharma, B.R., Bruggeman, A., Choukr-Allah, R., Karajeh, F. (2007). Non-conventional water resources and opportunities for water augmentation to achieve food security in water scarce countries. Agricultural Water Management, 87, 2-22. https://doi.org/10.1016/j.agwat.2006.03.018

Qureshi, A.S., Turral, H., Masih, I. (2004). Strategies for the management of conjunctive use of surface water and groundwater resources in semi-arid areas: A case study from Pakistan. Research Report 86. Colombo, Sri Lanka: IWMI.

Qureshi, R.H., Barrett-Lennard, E.G. (1998). Saline Agriculture for Irrigated Land in Pakistan: A handbook. Australian Centre for International Agriculture Research, Canberra.

Rad, H.E., Farshid, A., Rezaei, M., Amiri, E., Khaledian, M.R. (2011). The effects of salinity at different growth stage on rice yield. Ecology, Environment and Conservation, 17(2), 111-117.

Rhoades, J.D. (1998). Use of saline and brackish waters for irrigation: implications and role in increasing food production, conserving water, sustaining irrigation and controlling soil and water degradation. In: R. Ragab, and G. Pearce, eds. Proceedings of the International Workshop on the Use of Saline and Brackish Water for Irrigation, pp. 261-304. July 23-24, 1998, National ICID Committee, Bali, Indonesia.

Sharma, D.K., Singh, A., Sharma, P.C., Dagar, J.C., Chaudhari, S.K. (2016). Sustainable management of sodic soils for crop production: opportunities and challenges. Journal of Soil Salinity and Water Quality, 8, 109-130.

Sheoran, P., Basak, N., Ashwani Kumar, A., Yadav, R.K., Randhir, S., Raman, S., Satyendra, K., Ranjay, K., Sharma, P.C. (2021). Ameliorants and salt tolerant varieties improve rice-wheat production in soils undergoing sodification with alkali water irrigation in Indo-Gangetic Plains of India. Agricultural Water Management, 243, 1-13. https://doi.org/10.1016/j.agwat.2020.106492

Singh, A. (2014). Conjunctive use of water resources for sustainable irrigated agriculture. Journal of Hydrology, 519, 1688-1697. https://doi.org/10.1016/j.jhydrol.2014.09.049

Steel, R.G.D., Torrie, J.H., Dickey, D.A. (1997). Principles and Procedures of Statistic: A Biometrical Approach. 3rd edition, pp, 400-428. Mc Graw Hill book Co. Inc. New York.

U.S. Salinity Lab. Staff. (1954). Diagnosis and Improvement of Saline and Alkali Soils. USDA Handbook 60, Washington DC, USA.

Xue, J., Ren, L. (2017). Conjunctive use of saline and non-saline water in an irrigation district of the Yellow River Basin. Irrigation and Drainage, 66, 147-162. https://doi.org/10.1002/ird.2102

Zaka, M.A., Helge, S., Hafeezullah, R., Muhammad, S, Khalil, A. (2018). Utilization of brackish and canal water for reclamation and crop production. International Journal of Biosciences, 12, 7-17. https://doi.org/10.12692/ijb/12.3.7-17

Zeng, L., Shannon, M.C. (2003). Salinity effects on seedling growth and yield components of rice. Crop Science, 40, 996-1003. https://doi.org/10.2135/cropsci2000.404996x

Zhang, J., Lin, Y.J., Zhu, L.F., Yu, S.M., Sanjoy, K.K., Jin, Q.Y. (2015). Effects of 1-methylcyclopropene on function of flag leaf and development of superior and inferior spikelets in rice cultivars differing in panicle types. Field Crops Research, 177, 64-74. https://doi.org/10.1016/j.fcr.2015.03.003

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Zatiranje plevelov v vinogradu z alternativnimi metodami v primerjavi s herbicidom glifosat

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Vineyard weed control using alternative methods compared to glyphosate-based herbicide

Abstract: In a two-year field experiment, six different weed control methods were studied. The methods were: use of the herbicide glyphosate (GL), use of herbicides based on acetic acid (AA), pelargonic acid (PA) and citrus essential oil (EO), mowing weeds with a thread trimmer (TT) and flaming of weeds with fire (FL). Alternative methods of weed control were significantly less effective than the use of herbicide glyphosate. Due to the lower efficiency of alternative methods, large yield losses have occurred, on average, 31 % at AA, 30.6 % at PA, 22.7 % at EO, 5.4 % at TT and 12.9 % at FL in two years. The cost of carrying out controls with alternative methods was significantly higher than the cost of GL. AA it was higher by 3.2-times, in PA by 7.1-times, in EO by 3.8-times, in TT by 3.8- times and in FL by 5.8-times on average in two years. To achieve a comparable control efficiency of GL, five applications of alternative preparations per year have to be performed, or four times mowing of weeds or five weed flaming operations per year.

Key words: weed; vineyard; herbicides; mowing; flaming; cost

Zatiranje plevelov v vinogradu z alternativnimi metodami v primerjavi s herbicidom glifosat

Izvleček: V dveletnem poskusu smo preučevali šest različnih metod zatiranja plevelov. Preučevane metode so bile: uporaba herbicida glifosat (GL), uporaba pripravkov na podlagi ocetne kisline (OK), pelargonske kisline (PK) ter eteričnega olja agrumov (EO), košnja plevelov s kosilnico na nit (KO) in ožiganje plevelov z ognjem (OG). Alternativne metode zatiranja plevelov so bile značilno manj učinkovite od uporabe herbicida glifosat. Zaradi manjše učinkovitosti alternativnih metod so nastale obsežne izgube pridelka, in sicer pri OK 31 %, PK 30,6 %, EO 22,7 %, KO 5,4 % in pri OG za 12,9 %. Strošek izvedbe zatiranja z alternativnimi metodami je bil značilno večji od stroškov pri GL, pri OK za 3,2-krat, pri PK za 7,1-krat, pri EO za 3,8-krat, pri KO za 3,8-krat in pri OG za 5,8-krat večji. Če bi z uporabo alternativnih metod želeli doseči primerljivo učinkovitost zatiranja kot pri obravnavanju GL, bi morali izvesti 5 aplikacij alternativnih pripravkov letno, oziroma izvesti štiri košnje ali pet ožiganj plevelov letno.

Ključne besede: pleveli; vinograd; herbicidi; košnja; ožiganje; stroški

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1 UVOD

V agronomski praksi smo v pričakovanju prepovedi rabe herbicidov na podlagi aktivne snovi glifosat (Antier in sod., 2020). V EU se izvajajo raziskave o alternativnih pripravkih in metodah, ki omogočajo temeljito zatiranje plevelov brez uporabe glifosata (Kehlenbeck in sod., 2015; Steinkellner, 2019). Trenutno kaže, da z alternativnimi metodami ne moremo doseči povsem primerljive ekonomske učinkovitosti zatiranja plevelov kot z uporabo herbicida glifosat (Allegri, 2019; Pergher in sod., 2019; Manzone in sod., 2020). Prepoved uporabe snovi glifosat ima širše družbene posledice in vpliva tudi na vse manjšo družbeno sprejemljivost rabe drugih vrst herbicidov. Iz omenjenega razloga se preskušajo pripravki, ki temeljijo na organskih kislinah (npr. ocetna in pelargonska kislina), na podlagi olj (npr. eterična olja agrumov) ali mikroorganizmov (npr. bakterije rodu *Pseudomonas*) (Shrestha in sod., 2013).

Vinogradniki so že dalj čas pod velikim ekonomskim pritiskom. Konstantno zniževanje stroškov pridelave je ena od prioritet poleg izboljšanja marketinga vina. Povečevanje stroškov zatiranja plevelov v takšnih okoliščinah zelo težko sprejmejo. V analize ekonomske učinkovitosti alternativnih metod zatiranja plevelov je potrebno vnesti vrednotenje ekosistemskih učinkov, in dokazati, da lahko povečanje stroškov zatiranja z alternativnimi metodami kompenziramo s povečano ekosistemsko učinkovitostjo (manjše izgube rodovitnosti tal, boljši izkoristek vode, uspešnejše zatiranje škodljivcev in podobno) (Mainardis in sod., 2020). Seveda kot standardno sredstvo kompenzacije ostanejo različne proizvodne in okoljske podpore. Izkušnje iz tujih raziskav (npr. Irrslinger in Wetzel, 2017; Martelloni in sod., 2020; Manzone in sod., 2020) skušamo prenesti v naše okolje, a moramo biti previdni, saj so rezultati raziskav zelo variabilni in vezani na lokalne biotične, edafske in ekonomske razmere.

Integriran sistem zatiranja plevelov v vinogradu je takšen, da kombiniramo različne tehnike in pripravke. V primeru prenehanja uporabe snovi glifosat pričakujemo uravnoteženo povečanje rabe ostalih dovoljenih herbicidov, povečanje uporabe mehanskih metod in tudi delno povečanje porabe alternativnih pripravkov. Z ekosistemskega stališča je v sodobnem vinogradništvu cilj v vinogradu imeti pestro rastlinje negovane ledine, ne samo v medvrstnem prostoru, ampak tudi delno pod trtami. Vse več je študij, ki izpostavljajo pomen pestrosti rastlinstva za rodnost vinske trte in za celovito delovanje vinograda kot visoko produktivnega ekosistema (Mainardis in sod., 2020). Morda bo odpovedovanje uporabi snovi glifosat lažje, če upoštevamo vse manjšo učinkovitost, saj število tolerantnih plevelov, ki jih glifosat ne zatira učinkovito pri standardnih odmerkih hitro narašča (Heap in Duke, 2018; Vidotto, 2018). Vse več je objav, ki kažejo, da ima glifosat negativen učinek na talne organizme in trto (Mandl in sod., 2018). Ti učinki se odražajo tako v količini kot kakovosti pridelka. Tudi to dejstvo prispeva k zmanjšani porabi glifosata v vinogradih že v obdobju pred morebitno prepovedjo.

Namen naše raziskave je bil primerjati učinek zatiranja plevelov v vinogradu z alternativnimi metodami na pridelek trte v primerjavi z učinkom, ki ga dosežemo ob zatiranju z uporabo herbicida na podlagi snovi glifosat. Preučiti smo želeli tudi neposredne stroške izvedbe zatiranja plevelov pri različnih metodah.

2 MATERIAL IN METODE DELA

2.1 ZASNOVA POLJSKEGA POSKUSA

Poskus je bil izveden v letih 2019 in 2020, v vinogradu na raziskovalni postaji Meranovo (UKC, FKBV UM), na lokaciji Prinčev vrh, v severovzhodni Sloveniji, v 10 let starem vinogradu s sorto ‚Beli pinot' (GIS: 46°32′17.01″N, 15 °33′23.45″E (n. v. 475 nm)). Trte so cepljene na podlago ‚Kober 5BB'. Tla vinograda so srednje dobro založena s hranili (org. snov 1,7 %, pH (KCl) 6,5; P_2O_5 13,5 mg/100 g; K_2O 18,8 mg/100 g). Sistem vzdrževanja je običajni integriran sistem z mulčenjem negovane ledine v medvrstnem prostoru in uporaba herbicidov pod trtami v vrsti. Gojitvena oblika je bila enokraki guyot (en reznik / en šparon z 8-10 očesi). Za izračun pridelka smo uporabili gostoto 4550 trt na ha (sajenje 2,4 m x 0,9 m). Vinograd je bil zelo temeljito varovan proti boleznim in škodljivcem, tako da le-ti niso imeli vpliva na maso pridelka, oziroma le-ti niso povzročali dodatne variabilnosti pri rezultatih. Statistična zasnova poskusa je bil poljski poskus v naključnih blokih s parcelicami v štirih ponovitvah. Posamezna parcelica je obsegala 10 zaporednih trt v vrsti. Za statistično analizo razlik med povprečji obravnavanj smo izvedli ANOVA test in Tukey HSD test ($\alpha < 0,05$). Uporabili smo statistični program Statgraphics for Windovs Centurion 15.1 (Statgraphics Technologies Inc., Virginia, ZDA).

2.2 POSKUSNA OBRAVNAVANJA, TESTIRANI PRIPRAVKI IN IZVEDBA NEKEMIČNEGA ZATIRANJA PLEVELOV

V poskusu smo imeli osem različnih obravnavanj (Preglednica 2). Poleg parcelic, kjer smo plevele zatirali s pripravki, s košnjo ali z uporabo ognja, smo imeli še dve vrsti kontrolnih parcelic. Kontrola A - celotno obdobje zapleveljena parcela za ugotavljanje izgube pridelka zaradi plevelov in kontrola B - parcelice, kjer smo plevele vse leto ročno odstranjevali, da ni bilo nikakršnega učinka na pridelek trte. Kontrola B je služila kot izhodišče za izračun izgube pridelka v vseh drugih obravnavanjih. Uporabljeni pripravki, odmerki in čas izvedbe zatiranja so prikazani v preglednicah 1, 2 in 3. Aplikacija pripravkov je bila izvedena z ročno nahrbtno škropilnico Solo 425 pri uporabi šobe Hypro VP 110 – 03 pri porabi vode 300 l ha^{-1}. Kapljice so imele premer 200 in 300 µm (podatki iz kataloga proizvajalca šob). Poškropili smo 50 cm širok pas pod trtami. Košnja plevelov je bila izvedena z nahrbtno nošeno kosilnico na nitko Kawasaki KR53. Nitka je bila dolga 20 cm. Zelo temeljito do tal smo pokosili plevele v 0,5 m širokem pasu pod trtami. Zatiranje plevelov z ognjem smo izvedli z uporabo ročnega gorilnika s 15 cm širokim plamenom s temperaturo med 420 do 480 °C (izmerjeno z laserskim merilnikom). Porabo gospodinjskega plina butan/propan smo izračunali tako, da smo jeklenko stehtali pred ožiganjem plevelov in ob zaključku dela na znani površini. Potem smo iz razlike v masi jeklenke pred in po uporabi na znani površini izračunali porabo plina. Položaj gorilnika smo počasi premikali sem in tja, da smo sistematično prešli preko celotne površine parcelice.

2.3 OCENA STROŠKOV IZVEDBE ZATIRANJA PLEVELOV

Izvedli smo preprost izračun stroškov izvedbe različnih metod zatiranja s standardno vinogradniško strojno tehniko. Pri ponudnikih opreme in pripravkov smo se pozanimali glede cen. Upoštevali smo povprečno ceno pripravkov pri različnih ponudnikih za veliko embalažo za profesionalne uporabnike. Iz praktičnih izkušenj in poizvedb pri vinogradnikih smo pridobili podatke o storilnosti strojne tehnike v vinogradih s podobno konfiguracijo terena in medvrstnimi razdaljami, kot jih imamo v poskusnem vinogradu. Dodatno smo podatke glede storilnosti poiskali v nekaterih virih literature (Elmore in sod., 1997; Hembree 2002; Balsari in sod., 2006; Shrestha in sod., 2013; Irrslinger in Wetzel 2017; Fahey in Englefield, 2019; Allegri, 2019; Manzone in sod., 2020). Upoštevali smo povprečno storilnost iz omenjenih virov. Kljub temu, da je bil poskus izveden ročno, smo izračune naredili za standardno strojno tehniko – za traktorske priključke. To pomeni, da smo upoštevali, da se nanos herbicida izvrši ob mulčenju s škropilnico pritrjeno na mulčer. Prav tako smo pri stroškovni analizi predvidevali, da se ožiganje plevelov izvede z ožigalnikom, pritrjenim na mulčer, ki ima bočno montirana dva gorilnika. Storilnost priključkov lahko glede na zahtevnost terena variira ± 25 %. Priključitev na mulčer navadno poceni izvedbo zatiranja. V naši raziskavi smo predvideli, da je učinkovitost zatiranja pri ročnem delu primerljiva učinkovitosti pri strojnem delu. Verjetno smo pri ročnem delu bolj natančni in je pri ročnem delu učinkovitost zatiranja nekoliko večja. To tukaj zanemarimo.

2.4 ANALIZA PRIDELKA

Za analizo količine pridelka smo izvedli ročno obiranje grozdja z naključno izbranih trt, na vsaki poskusni parcelici. Izvedli smo tudi osnovno analizo mošta. Na vsaki parcelici vseh obravnavanj smo nabrali 150 jagod v različnih delih krošnje trte in iz njih iztisnili sok

Preglednica 1: Sestava preučevanih pripravkov in cena za liter pripravka

Komercialno ime	Aktivna snov	Okrajšava obravnavanj	Delež aktivne snovi	Cena za liter v (€)
Finalsan (Neudorf)	Pelargonska kislina	PK	186,7 g l^{-1}	11,0
Beloukha (Belchim)	Pelargonska kislina	PK	68 %	18,0
Kis za vlaganje (Leclerc)	Ocetna kislina	OK	9 %	0,49
Tajfun (Karsia)	Glifosat v obliki amino soli	GL	511 g l^{-1}	4,89
Oranol (Samson Kamnik)	Eterično olje agrumov	EO	96 %	10,5
Ocetna kislina (Agronet)	Ocetna kislina	OK	80 %	3,5
LDC Detergent (Golden)	Amidi kokosovega olja		60 %	11,0
Wetcit (Metrob)	Omočilo iz olja agrumov	EO	85 %	28,0
Plin v jeklenki	Butan / propan	OG		1,65 kg^{-1}

Preglednica 2: Uporabljeni pripravki in termini izvedbe zatiranja plevelov v letu 2019. Tretirana površina je bila 20 % celotne površine vinograda

Obravnavanje	Odmerek na 1 ha tretirane površine	Odmerek na 1 ha površine vinograda	Datum
1 – košnja z nitko	Košnja s kosilnico na nitko	1,4 h ha^{-1}	T1, T2, T3
2 – ožiganje plevelov	Plin butan/propan 40 kg ha^{-1}	8 kg ha^{-1}	T1, T2, T3
3 – glifosat	Tajfun 5 l ha^{-1}	1 l ha^{-1}	T1, T3
4 – pelargonska kislina	Finalsan 20 l ha^{-1}	4 l ha^{-1}	T1
	Finalsan 40 l ha^{-1}	8 l ha^{-1}	T2, T3
5 – ocetna kislina	Kis za vlaganje 100 l ha^{-1}	20 l ha^{-1}	T1
	Kis za vlaganje 200 l ha^{-1}	40 l ha^{-1}	T2
	Kis za vlaganje 300 l ha^{-1}	60 l ha^{-1}	T3
6 – eterično olje	Oranol 20 l ha^{-1} + Wetcit 3 l ha^{-1}	4 l ha^{-1} + 0,6 l ha^{-1}	T1, T3
	Oranol 30 l ha^{-1} + Wetcit 3 l ha^{-1}	6 l ha^{-1} + 0,6 l ha^{-1}	T2
7 – kontrola A	Zapleveljeno – vse leto brez zatiranja plevelov		
8 – kontrola B	Nezapleveljeno - vse leto ročno odstranjevanje plevelov		

Legenda: Termini zatiranja plevelov: T1 – 24. 5., T2 – 2. 7. in T3 – 6. 8

Preglednica 3: Uporabljeni pripravki in termini izvedbe zatiranja plevelov v letu 2020. Tretirana površina je bila 20 % celotne površine vinograda

Obravnavanje	Odmerek na 1 ha tretirane površine	Odmerek na 1 ha površine vinograda	Datum
1 – košnja z nitko	Košnja s kosilnico na nitko	1,4 h ha^{-1}	T1, T2, T3
2 – ožiganje plevelov	Plin butan/propan 40 kg ha^{-1}	8 kg ha^{-1}	T1,
	Plin butan/propan 60 kg ha^{-1}	12 kg ha^{-1}	T2, T3
3 – glifosat	Tajfun 5 l ha^{-1}	1 l ha^{-1}	T1
	Tajfun 6 l ha^{-1}	1,2 l ha^{-1}	T3
4 – pelargonska kislina	Beloukha 18 l ha^{-1}	3,6 l ha^{-1}	T1
	Beloukha 40 l ha^{-1}	8 l ha^{-1}	T2, T3
5 – ocetna kislina	Kis za vlaganje 80 l ha^{-1}	16 l ha^{-1}	T1
	Ocetna kislina 75 l ha^{-1}	15 l ha^{-1}	T2, T3
6 – eterično olje	Oranol 15 l ha^{-1}	3 l ha^{-1}	T1
	Oranol 30 l ha^{-1} + LDC 2 l ha^{-1}	6 l ha^{-1} + 0,4 l ha^{-1}	T2
	Oranol 40 l ha^{-1} + LDC 2 l ha^{-1}	8 l ha^{-1} + 0,4 l ha^{-1}	T3
7 – kontrola A	Nezapleveljeno - vse leto ročno odstranjevanje plevelov		
8 – kontrola B	Zapleveljeno – vse leto brez zatiranja plevelov		

Legenda: Termini zatiranja plevelov: T1 – 5. 5., T2 – 28. 5. in T3– 10. 7

ter pridobili homogen vzorec za laboratorijsko analizo. Vsebnost suhe topne snovi (STS) in titracijskih kislin (STK) smo potem izmerili z uporabo digitalnega refraktometra in standardne titracijske metode pri sobni temperaturi (Košmerl in Kač, 2009).

Preglednica 4: Neposredni stroški izvedbe strojnih operacij za zatiranje plevelov

Orodje - postopek	Letna raba (h)	Delovna hitrost (km h^{-1})	Storilnost (ha h^{-1})	Strošek (€ h^{-1})
Delo traktorista				9,0
Vinogradniški traktor 4 x 4 55 kW	350	2-8	0,4-3,0	22,0
Sistem za dvostransko aplikacijo herbicidov pritrjen na mulčer	100	4-5	1,5	2,5
Mulčer pletvenik na nitko za dvostransko mehansko zatiranje plevelov pod trtami	130	2,2-3	0,7	9,0
Sistem za dvostransko ožiganje plevelov z dvema gorilnikoma pritrjen na mulčer	150	2-2,5	0,5	11,5

Predstavljeni stroški so povprečen rezultat primerjav s podatki iz nekaterih objav naštetih v metodah dela in poizvedb pri vinogradnikih in ponudnikih strojne opreme. Amortizacijska doba za traktor je 15 let in za priključke 10 let. Letna raba je prilagojena za kmetijo, ki ima 10 ha vinograda

2.5 PODATKI O BOTANIČNI SESTAVI PLEVELNE POPULACIJE

Vinograd na poskusni lokaciji je bil povprečno zapleveljen s pleveli. Zadnjih deset let so za zatiranje plevelov pod trtami enkrat do dvakrat letno uporabili herbicide na podlagi aktivne snovi glifosat v registriranih odmerkih. Dominantne plevelne vrste so bile *Aegopodium podagraria* L., *Convolvulus arvensis* L., *Elymus repens* (L.) Gould, *Epilobium parviflorum* (Schreb.) Schreb., *Erigeron annuus* (L.) Pers., *Festuca pratensis* Huds., *Glechoma hederacea* L., *Lolium perenne* L., *Poa annua* L., *Ranunculus repens* L., *Setaria glauca* (L.) Morrone, *Urtica dioica* L. in *Veronica persica* Poir.

Manj številčne rastlinske vrste so bile: *Achillea millefolium* L., *Carex hirta* L., *Cirsium arvense* (L.) Scop., *Conyza canadensis* L., *Digitara sanguinalis* (L.) Scop., *Equisetum arvense* L., *Linaria vulgaris* Mill., *Lysimachia nommularia* L., *Medicago lupulina* L., *Polygonum aviculare* L., *Potentilla reptans* L., *Taraxacum officionale* L., *Trifolium repens* L. in *Vicia cracca* L.

3 REZULTATI IN RAZPRAVA

3.1 PRIDELEK GROZDJA

V poskusu smo imeli zelo zapleveljen vinograd z veliko različnimi vrstami plevelov. V prvem letu so v kontrolnem obravnavanju brez zatiranja pleveli povzročili 37,2% izgubo pridelka, medtem ko v drugem letu za kar 53 % izgubo. To je bila posledica kumulativnega učinka zapleveljenosti skozi dve rastni dobi. Kontrolne zapleveljene parcelice in vse druge parcelice so bile v obeh letih na istem mestu. Pridelek v obravnavanju, kjer ni bilo plevelov je v letu 2019 znašal 8301 kg ha^{-1} in v letu 2020 13680 kg ha^{-1}. Količina pridelka kaže, da smo v poskusu imeli povsem običajen povprečno roden vinograd. Uporaba herbicida na podlagi snovi glifosat (Tajfun) je predstavljala standard proti kateremu primerjamo vsa druga obravnavanja. V obeh sezonah nam tudi pri dveh aplikacijah snovi glifosat letno, plevelov ni uspelo popolnoma zatreti. Zmanjšano učinkovitost (pod 90 %) smo imeli pri ljuljki, vrbovcu, suholetnici, zlatici, pirnici, šašu, regačici in pijavčnici. Posledica tega je bila, da smo tudi pri uporabi glifosata v letu 2019 v primerjavi s kontrolnim obravnavanjem, kjer plevelov ni bilo, izgubili 1,8 % pridelka, v letu 2020 pa 9,9 % pridelka.

Glede na količino pridelka sta bili naslednji najbolj učinkoviti obravnavanji uporaba ognja in košnja z nitko trikrat letno. Pri košnji s kosilnico na nit je izguba pridelka v letu 2019 znašala 3,4 % in v letu 2020 7,5 %. Glede na rezultat iz leta 2020 ugotavljamo, da bi morali izvesti še četrto košnjo in v obeh letih s košnjo začeti malo bolj zgodaj. Poleti 2020 je bilo veliko padavin in pleveli so se po zatiranju dobro obnovili, ker niso bili izpostavljeni sušnemu stresu. Nekaj slabši rezultat smo dosegli pri uporabi ognja. V letu 2019 je izguba pridelka znašala 4,6 % in v letu 2020 kar 21,1 %. Tudi za uporabo ognja velja, da bi s prvim ožiganjem morali začeti bolj zgodaj in da bi gotovo morali izvesti še eno zatiranje. Učinkovitost zatiranja plevelov z alternativnimi pripravki na podlagi ocetne in pelargonske kisline ali pa olja agrumov ni primerljiva z učinkovitostjo, ki jo dosežemo z uporabo snovi glifosat in posledično smo izmerili velike izgube pridelka. Pri uporabi pelargonske kisline je izguba pridelka v 2019 zanašala 34,2 % in v letu 2020 malo manj, 27 %, kar kaže, da nudi uporaba te snovi za več kot 50 % manjšo učinkovitost, kot uporaba glifosata. Značilno primerljive izgube so nastale pri uporabi ocetne kisline (v 2019 30,3 % in v 2020 31,7 %) in eteričnega olja agrumov (v 2019 16,1 % in v 2020 29,3 %). Če bi hoteli doseči učinkovitost, primerljivo tisti ob uporabi glifosata, bi v obeh letih zelo verjetno morali izvesti pet aplikacij, kar bi bilo stroškovno zelo neugodno.

Podatki v preglednici 5 kažejo, da razlike glede vsebnosti topne suhe snovi (TSS) in titracijskih kislin, ki sta osnova parametra kakovosti mošta, niso statistično značilne.

Deloma je evidentno, da se pri manjši učinkovitosti zatiranja plevelov ne zmanjša TSS, vendar moramo upoštevati interaktivni učinek s količino pridelka. Pri večjem pridelku je vsebnost TSS manjša. Tako se kaže, da je vsebnost TTS v obeh letih pri uporabi glifosata manjša, kot v ostalih bolj zapleveljenih obravnavanjih. V literaturi smo našli študijo glede zatiranja plevelov v vinogradu z zelo podobno botanično sestavo plevelov (Karl, 2015). Praktično je bilo v njihovem poskusu 90 % vrst plevelov, ki so bili tudi v našem vinogradu. V njegovem poskusu v obdobju 2011-2013 so pleveli povzročili med 30 in 50 % izgubo pridelka. Primerjali so uporabo glifosata, mehansko obdelavo pasu pod tratami in setev mešanice rastja iz metuljnic pod trtami. Strošek zatiranja z glifosatom je znašal 548 \$ ha^{-1}, mehansko zatiranje je stalo 1036 \$ ha^{-1} (Karl, 2015). V zapleveljeni kontroli se je prihodek na ha v primerjavi z obravnavanjem, kjer so plevele zatirali z glifosatom zmanjšal med 26 in 40 %, pri mehanskem zatiranju plevelov pa med 18 in 50 %. Izguba pridelka in prihodka je bila podobna, kot v naši raziskavi.

Preglednica 5: Pridelek grozdja v letu 2019 in 2020 v odvisnosti od načina tretiranja podlage

Obravnavanje	Pridelek grozdja (kg ha^{-1})	Izguba (%) pridelka proti V7	Topna suha snov (TSS) (°Oe, 25 °C)	Titracijske kisline (g l^{-1})
Podatki za leto 2019				
1 Košnja z nitko 3 x letno	8021 a	3,4 c	93,5 a	7,15 a
2 Uporaba ognja 3 x letno	7902 a	4,8 c	91,0 a	6,45 a
3 Glifosat 2 x letno	8150 a	1,8 c	88,5 a	6,45 a
4 Pelargonska k. 3 x letno	5709 ab	34,2 b	88,8 a	6,48 a
5 Ocetna kislina 3 x letno	5786 ab	30,3 ab	90,5 a	6,13 a
6 Olje agrumov 3 x letno	6965 a	16,1 b	89,8 a	6,70 a
7 BREZ PLEVELOV	8301 a	/	91,3 a	5,90 a
8 Vse leto zapleveljeno	5214 b	37,2 a	86,8 a	6,78 a
Podatki za leto 2020				
1 Košnja z nitko 3 x letno	12807 ab	7,5 d	97,0 a	7,03 a
2 Uporaba ognja 3 x letno	11376 abc	21,1 bcd	93,0 a	6,90 a
3 Glifosat 2 x letno	12695 ab	9,9 cd	89,0 a	7,05 a
4 Pelargonska k. 3 x letno	9994 bc	27,0 bc	91,0 a	7,30 a
5 Ocetna kislina 3 x letno	9314 cd	31,7 b	93,3 a	7,10 a
6 Olje agrumov 3 x letno	9526 c	29,3 b	92,8 a	7,00 a
7 BREZ PLEVELOV	13680 a	/	93,8 a	7,30 a
8 Vse leto zapleveljeno	6278 d	53,5 a	91,3 a	7,60 a

Povprečja označena z enako črko znotraj posameznega parametra za posamezno leto se med seboj ne razlikujejo glede na rezultate Tukey HSD testa pri ($\alpha < 0{,}05$)

3.2 OCENA STROŠKOV ZATIRANJA PLEVELOV

Preglednica 6 kaže, da so stroški zatiranja plevelov veliki in da so vse alternativne metode zatiranja bistveno dražje od uporabe snovi glifosat. Faktor povečanja stroškov je od tri do osemkrat (preglednica 6, desna kolona). Prav tako se vidi, da pleveli lahko povzročijo velike izgube pridelka gledano finančno (tudi občutno več kot 1000 € h^{-1}). Kljub temu, da so alternativne metode drage, so stroški pri njihovi uporabi še vedno manjši od vrednosti izgubljenega pridelka. Med obema letoma je nekaj manjših razlik. V obeh letih je košnja in uporaba ognja dala boljši rezultat, kot uporaba alternativnih pripravkov. Hektarski odmerki alternativnih pripravkov so veliki in posledično so tudi stroški veliki, kljub temu, da cena za liter pripravka ni visoka. V literaturi lahko najdemo nekaj podatkov o stroških različnih metod zatiranja, a so primerjave zelo težke, ker so cene pripravkov in strojnih uslug zelo variabilne. Neposredna ekonomska primerjava stroškov mehanskega zatiranja plevelov v vinogradih v različnih sosednjih državah je zelo težka. Razpon cene istega priključka za mehansko zatiranje je vsaj ± 40 %, razpon vrednosti delovne ure upravljalcev strojev pa je več kot ± 200 % (od 3,5 do 25 € h^{-1}). Razlike med našimi rezultati in rezultati drugih raziskovalcev (npr. Shrestha in sod., 2013; Webber in sod. 2018; Pergher in sod. 2019; Manzone in sod. 2020; Martelloni in sod., 2020) so velike, kar kaže da njihovih izkušenj nebi mogli neposredno prenesti v naš pridelovalni sistem.

Preglednica 6: Izgube pridelka in primerjava stroškov zatiranja plevelov trikrat letno, v rastnih dobah 2019 in 2020. Za en kilogram grozdja smo upoštevali ceno 0,5 €

Obravnavanje	Vrednost pridelka (€ ha^{-1})	Vrednost izgubljenega pridelka (€ ha^{-1}) primerjano proti V7	Strošek zatiranja plevelov (€ ha^{-1})	Indeks povečanja stroškov zatiranja proti V3 (n-krat)
Podatki za leto 2019				
1 Košnja z nitko 3 x letno	4010,5 a	140 c	168	3,3
2 Uporaba ognja 3 x letno	3951 a	199,5 c	281,3	5,6
3 Glifosat 2 x letno	4075 a	75,5 c	50,2	/
4 Pelargonska k. 3 x letno	2854,5 ab	1296 a	290,4	5,8
5 Ocetna kislina 3 x letno	2893 ab	1257,5 a	139,2	2,8
6 Olje agrumov 3 x letno	3482,5 a	668 b	130,8	2,6
7 BREZ PLEVELOV	4150,5 a	/	/	/
8 Vse leto zapleveljeno	2607 b	1543,5 a	/	/
Podatki za leto 2020				
1 Košnja z nitko 3 x letno	6403,5 ab	436,5 b	168	3,3
2 Uporaba ognja 3 x letno	5688 abc	1152 ab	318,2	6,1
3 Glifosat 2 x letno	6347,5 ab	492,5 b	52,2	/
4 Pelargonska k. 3 x letno	4997 bc	1843 a	440,4	8,4
5 Ocetna kislina 3 x letno	4657 cd	2183 a	193,2	3,7
6 Olje agrumov 3 x letno	4763 c	2077 a	260,9	5,0
7 BREZ PLEVELOV	6840 a	/	/	/
8 Vse leto zapleveljeno	3139 d	3701 a	/	/

Povprečja označena z enako črko znotraj posameznega parametra vrednosti pridelka za posamezno leto se med seboj ne razlikujejo glede na rezultate Tukey HSD testa pri ($\alpha < 0,05$)

Pri uporabi pelargonske kisline nismo dosegli učinkovitosti, kot smo jo pričakovali glede na vire iz literature. V letu 2019 smo uporabili pripravek Finalsan z majhno koncentracijo pelargonske kisline. Dosežena kratkotrajna učinkovitost je bila okrog 50 %. Kljub temu, da smo v letu 2020 povečali odmerek in smo imeli večjo koncentracijo pelargonske kisline (pripravek Beloukha), zatiranje plevelov ni bilo uspešno. Izguba pridelka je znašala 27 % in strošek zatiranja je znašal 440 € ha^{-1}. Glede na oba rezultata sklepamo, da zatiranje plevelov s pelargonsko kislino gotovo ni ekonomsko zanimivo za naše vinogradnike. Do podobnih zaključkov so prišli Crmaric in sod. (2018).

Glede na podatke iz literature (Campiglia in sod., 2007; Dayan in Duke 2010) smo pri uporabi eteričnih olj pričakovali večjo učinkovitost zatiranja. Verjetno smo v našem poskusu uporabili premajhne odmerke eteričnega olja (olje agrumov). Dodajanje omočila Wetcit in detergenta LDC ni povečalo učinkovitosti testiranega eteričnega olja. Učinek je bil večji v poletnem času. Trave so na eterična olja precej odporne. Stroški nad 200 € ha^{-1} so visoki glede na doseženo učinkovitosti in malo verjetno je, da bi naši vinogradniki bili pripravljeni plevele zatirati z uporabo eteričnih olj.

Uporaba ognja se je v našem poskusu izkazala kot primerna metoda, upoštevajoč količino pridelka, primerjalno proti drugim obravnavanjem. Tako v letu 2019, kot v letu 2020 razlika v količini pridelka glede na obravnavanje glifosat ni bila značilna, so pa žal bili izrazito povečani stroški zatiranja plevelov (za 5- do 6-krat), v primerjavi z obravnavanjem glifosat. S stališča biotične učinkovitosti zatiranja je metoda uporabna, s finančnega stališča pa manj uporabna.

4 ZAKLJUČEK

Z uporabo preučevanih alternativnih metod trikrat v rastni dobi v vinogradu ne dosežemo učinkovitosti zatiranja plevelov, ki jo nudi dvakratna uporaba glifosata. Verjetno bi primerljivo učinkovitost lahko dosegli s petkratno uporabo alternativnih pripravkov ali s štirikratno košnjo oziroma z 4-5-kratnim ožiganjem plevelov. Stroški zatiranja z alternativnimi pripravki so zelo visoki in malo verjetno je, da bi vinogradniki sprejeli med 4- do 8-krat višje stroške, kot jih imajo pri uporabi glifosata. Kot najbolj realna alternativna opcija se kaže uporaba različnih mulčerjev na nit. Pri mehanskih metodah ima velik vpliv letna raba priključkov, hitrost dela in ustrezna moč traktorja. Če imamo veliko storilnost in letno rabo mulčerja na nit nad 150 ur lahko dosežemo, da je strošek mehanskega zatiranja med 2,5- do 3,5-krat večji od stroška uporabe glifosata. Pri uspešnem marketingu vina pri pridelkih nad 10 ton grozdja na ha je tolikšno povišanje stroškov možno prenesti. Izgube pridelkov grozdja v poskusu so bile velike (nad 30 %) in to ekonomsko povečuje sprejemljivost povečanja stroškov zatiranja plevelov z alternativnimi metodami, če glifosata ne bomo več uporabljali.

5 ZAHVALA

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6 VIRI

Allegri, A. (2019). Gestione del diserbo e possibili alternative all uso del glifosate nel vigneto. *Bilancio difesa vite*, 1–31.

Antier, C., Kudsk, P., Reboud, X., Ulber, L., Baret, P. V., Messéan, A. (2020). Glyphosate use in the European agricultural sector and a framework for its further monitoring. *Sustainability*, *12*(14), 5682. https://doi.org/10.3390/su12145682

Balsari, P., Marucco, P., Vidotto, F., Tesio, F. (2006). Assessment of different techniques for weed control in vineyard. *In Proceedings of Giornate Fitopatologiche*, 529–534.

Campiglia, E., Mancinelli, R., Cavalieri, A., Caporali, F. (2007). Use of essential oils of cinnamon, lavender and peppermint for weed control. *Italian Journal of Agronomy*, *2*, 171–175. https://doi.org/10.4081/ija.2007.171

Crmaric, I., Keller, M., Krauss, J., Delabays, N. (2018). Efficacy of natural fatty acid based herbicides on mixed weed stands - Wirksamkeit von naturlichen, auf Fettsauren basierten Herbiziden auf Unkrautbestande. 28. Deutsche Arbeitsbesprechung uber Fragen der Unkrautbiologie und -Bekampfung, 27.02. – 01.03.2018 in Braunschweig. *Julius-Kuhn-Archiv J. K.*, *458*, 327–332.

Dayan, F.E., Duke, S.O. (2010). Natural products for weed management in organic farming in the USA. *Outlooks on Pest Management*, *21*(4), 156–160. https://doi.org/10.1564/21aug02

Elmore, C.L., Roncoroni, R., Wade, L., Verdegaal, P. (1997). Mulch plus herbicides effectively control vineyard weeds. *Californian Agriculture*, *51*(2), 14–8. https://doi.org/10.3733/ca.v051n02p14

Fahey D., Englefield A. (2019). Alternative weed control measures for vineyards. *Development Officer, Viticulture*, 54–58. https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0006/1158315/Alternative-weed-control-measures-for-vineyards.pdf

Heap, I., Duke, S.O. (2018). Overview of glyphosate-resistant weeds worldwide. *Pest Management Science*, *74*(5), 1040–1049. https://doi.org/10.1002/ps.4760

Hembree, K. (2002). *Cost-Effective Vineyard Weed Management*. UCCE, Fresno County. 1–4.

Irrslinger, R., Wetzel, D. (2017). *Kosten der herbizidfreien Unterstockpflege*. https://obstwein-technik.eu/Core?aktiveNavigationsID=879&fachbetraegeID=279. Accessed 12/02/2019.

Karl, A. D. (2015). *Impact of under-vine management in a finger lakes cabernet franc vineyard*. A thesis, presented to the Faculty of the Graduate School of Cornell University. 112.

Kehlenbeck, H., Saltzmann, J., Schwarz, J., Zwerger, P., Nordmeyer, H., Roßberg, D., Karpinski, I., Strassemeyer, J., Golla, B., Freier, B. (2015). Folgenabschätzung für die Landwirtschaft zum teilweisen oder vollständigen Verzicht auf die Anwendung von glyphosathaltigen Herbiziden in Deutschland. Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, *Julius-Kühn-Archiv*, *451*, 1–150.

Košmerl, T., Kač, M. (2009). Osnovne kemijske in senzorične analize mošta in vina; *Laboratorijske vaje pri predmetu Tehnologije predelave rastlinskih živil – vino*. UL, Biotehniška fakulteta. Ljubljana.

Mainardis, M., Boscutti, F., Rubio Cebolla,, M.M., Pergher, G. (2020). Comparison between flaming, mowing and tillage weed control in the vineyard: Effects on plant community, diversity andabundance. *Plos One, 15*(8). https:// doi.org/10.1371/journal.pone.0238396

Mandl, K., Cantzelmo, C., Gruber, E., Faber, F., Friedrich, B., Zaller, J.G. (2018). Effects of glyphosate, glufosinate and flazasulfuron based herbicides on soil microorganisms in a vineyard. *Bulletin of Environmental Contamination and Toxicology*, *101*, 562 – 569. https://doi.org/10.1007/s00128-018-2438-x

Manzone, M., Demeneghi, M., Marucco, P., Grella, M., Balsari, P. (2020). Technical solutions for under-row weed control in vineyards: Efficacy, costs and environmental aspects analysis. *Journal of Agricultural Engineering*, *51*(1), 36–42. https://doi.org/10.4081/jae.2020.991

Martelloni, L., Frasconi, C., Sportelli, M., Fontanelli, M., Raffaelli, M., Peruzzi, A. (2020). Flaming, glyphosate, hot foam and nonanoic acid for weed control: A Comparison. *Agronomy*, *10*(1), 129. https://doi.org/10.3390/agronomy10010129

Pergher, G., Gubiani, R., Mainardis, M. (2019). Field testing of a biomass-fueled flamer for in-row weed control in the vineyards. *Agriculture*, *9*(10), 210. https://doi.org/10.3390/agriculture9100210

Shrestha, A., Kurtural, S.K., Fidelibus, M.W., Dervishian, G., Konduru, S. (2013). Efficacy and cost of cultivators, steam, or an organic herbicide for weed control in organic vineyards in the San Joaquin Valley of California. *HortTehnology*, *23*(1), 99–108. https://doi.org/10.21273/HORTTECH.23.1.99

Steinkellner, S. (2019). *Nationale machbarkeitsstudie zum glyphosatausstieg, Endbericht zum forschungsprojekt nummer 101347*. Universität für Bodenkultur Wien, 1–257. (https://www.bmlrt.gv.at/land/land-bbf/Forschung/machbarkeitsstudie.html).

Vidotto, F. (2018). *Il caso glifosate e altri arcani agricoli - Perché una soluzione viene scambiata per un problema? 1–46* http://www.confagricoltura.org/piacenza/wp-content/uploads/sites/3/2018/04/180413-ACCLS-VidottoRelazione.pdf).

Webber, C.L., Whit,e P.M. Jr., Shrefler, J.W., Spaunhorst, D.J. (2018). Impact of acetic acid concentration, application volume, and adjuvants on weed control efficacy sugarcane research unit, USDA, Agriculture Research Service, Houma, LA, USA. *Journal of Agricultural Science*; *10*(8). https://doi.org/10.5539/jas.v10n8p1

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The role of exogenous glycinebetaine on some antioxidant activity of non-T and T tobacco (*Nicotiana tabacum* L.) under *in vitro* salt stress

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The role of exogenous glycinebetaine on some antioxidant activity of non-T and T tobacco (*Nicotiana tabacum* L.) under *in vitro* salt stress

Abstract: Glycine betaine is an osmoprotectant compound which enhances cell tolerance in plant species in response to environmental stresses. This study aimed to investigate the effect of exogenous application of glycine betaine on some antioxidant activities of tobacco plants overexpressing *P5CS* gene. Sterile tobacco seedlings with four to six leaves were transferred to MS medium containing 0, 100, and 200 mM NaCl, after which glycine betaine (20 and 40 mg l^{-1}) were foliar sprayed on the surface of the plants. After four weeks, glycine betaine treatment enhanced the antioxidant capacity of the plant through activation of catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX). In contrast, H_2O_2 content and MDA level were reduced by glycine betaine under similar conditions. Therefore, application of exogenous glycine betaine under salt stress improved stress tolerance in T and non-T plants. Meanwhile, our results indicated the positive effect of glycine betaine in T plants was greater than in non-T plants. On the other hand, this result suggested that the synergistic effects of glycine betaine and proline in plants enhanced the antioxidant defense system in T plants overexpressing *P5CS* gene.

Key words: glycine betaine; proline; antioxidant enzyme; tobacco plants; salt tolerance

Vloga dodajanja glicin betaina na nekatere antioksidacijske aktivnosti transformiranega in navadnega tobaka (N*icotiana tabacum* L.) v razmerah *in vitro* solnega stresa

Izvleček: Glicin betain je ozmoprotektant, ki vzpodbuja toleranco rastlinskih celic na okoljski stres. V tej raziskavi so bili preučevani učinki dodajanja glicin betaina na nekatere antioksidacijske aktivnosti tobaka, ki ima prekomerno izražanje *P5CS* gena. Sterilne sejanke tobaka s štirimi do šestimi listi so bile premeščene v MS gojišče, ki je vsebovalo 0, 100, in 200 mM NaCl, nakar je bil dodan s pršenjem nadzemnih delov glicin betain (20 in 40 mg l^{-1}). Po štirih tednih je obravnavanje z glicin betainom pospešilo antioksidacijsko sposobnost rastlin z aktivacijo katalaze (CAT), superoksid dizmutaze (SOD) in askorbat peroksidaze (APX). Nasprotno sta se vsebnosti H_2O_2 in MDA zmanjšali po obravnavanju z glicin betainom v podobnih razmerah. Dodajanje glicin betaina lahko v razmerah solnega stresa izboljša toleranco na stres transformiranih in netransformiranih rastlin. Rezultati raziskave so še pokazali, da je bil pozitivni učinek glicin betaina večji pri transformiranih rastlinah. Po drugi strani ti rezultati nakazujejo, da sinergistični učinki glicin betaina in prolina v rastlinah pospešujejo antioksidacijski obrambni sistem v transformiranih rastlinah, ki imajo prekomerno izraženo delovanje *P5CS* gena.

Ključne besede: glicin betain; prolin; antioksidacijski encim; tobak; toleranca na solni stres

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1 INTRODUCTION

Salt stress is one of the most important factors, which limits and reduces the growth and development of plants worldwide. Generally, plant metabolism changes under salinity stress by ion toxicity and osmotic pressure (Mittler, 2002). There are many cellular mechanisms decreasing the adverse effects of environmental stresses such as salinity. Understanding salt tolerance mechanisms is useful to develop novel strategies for salt-tolerant crops. It is known that accumulation of compatible osmolytes such as proline (Pro) and glycine betaine (GB) (Díaz et al., 2005) as well as reactive oxygen species (ROS) detoxification in plants increases salt tolerance. Salinity, drought, and heavy metal stresses induce generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Rajaeian & Ehsanpour, 2015). Under abiotic stresses, plants always improve enzymatic and non-enzymatic antioxidant defense systems to remove ROS content (Hasanuzzaman et al., 2011). The enzymatic antioxidants include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). In this system, superoxide radical is converted to hydrogen peroxide (H_2O_2) by SOD enzyme (Bowler et al., 1992). Then, H_2O_2 is converted to water by catalase and peroxidase enzymes (Fridovich, 1983).

Further, the accumulation of organic solutes in the cytoplasm is a typical reaction of plants to osmotic stress resulting in osmotic adjustment. Compatible solutes such as Pro and GB are general osmoregulators, which are accumulated during salt stress in plants and play a critical role in osmotic adjustment (Szabados & Savoure, 2010). Pro biosynthesis and signaling contribute to the redox balance of cells under normal and stressful conditions (Per et al., 2017).It seems that Pro has an important role in salinity tolerance by preserving the metabolism and protein synthesis, osmotic balance, protecting cellular enzymes and proteins, protecting intracellular structures, maintaining carbon and nitrogen reserves, and regulating cellular pH. Furthermore, GB and Pro protect the protein structure and cellular membrane integrity under salt stress (Iqbal et al., 2014; Per et al., 2017). It has been well documented that, GB is an osmolyte in plants and bacteria. Nevertheless, recently many studies have indicated its role as a methyl donor in homocysteine metabolism and a protein stabilizer (Figueroa-Soto & Valenzuela-Soto, 2018). It has also been demonstrated that, Pro and GB have also an important role in the removal of free radical by activating antioxidant defense systems (Banu et al., 2010).

The GB content has been increased in many crop plants such as spinach (Parida & Das, 2005), barley (Chen et al., 2007), wheat (Wang et al., 2010), and sorghum (Forghani et al., 2018; Neto et al., 2009) in response to various stresses. However, all plants are not capable to produce enough amounts of GB under abiotic stress. There is extensive evidence suggesting the positive effects of exogenous application of GB on plant growth and crop yield under salt stress (Ashraf & Foolad, 2007; Figueroa-Soto & Valenzuela-Soto, 2018). One of the key enzymes in Pro biosynthesis pathway is 1-pyrroline-5-carboxylate synthase (*P5CS*). This enzyme phosphorylates glutamate to form glutamyl phosphate, which is reduced to an intermediate glutamic-5-semialdehyde. It has been documented that, accumulated Pro by overexpressing the *P5CS* gene can improve salt tolerance in plants under salt stress (Kishor et al., 1995). Although there are several reports about the positive effect of GB and Pro on growth and induction of antioxidant defense systems of plants under salt stress, the effect of GB on salinity tolerance of T plants overexpressing *P5CS* gene under salinity is still unknown. Therefore, it was hypothesized that, increasing proline in transgenic tobacco plants and application of glycine betaine in the medium may offer positive effects on the response of tobacco plants under salinity stress? Accordingly, the present study investigated the protective effect of GB on the antioxidant defense systems of transgenic (T) tobacco plants overexpressing *P5CS* gene to determine the relationship between osmoprotectant properties of GB and its antioxidant capacity under salt stress.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND TREATMENTS

The transgenic *Nicotiana tabacum*eeds cv. Wisconsin (T) carrying *P5CS* gene and non-transgenic seeds (NT) were supplied from Laboratory of Plant Physiology, Univerity of Isfahan, Iran. Our previous study confirmed that, regenerated plants from transgenic seeds produced more proline than wild type seeds (Razavizadeh & Ehsanpour, 2009). The seeds were surface sterilized in ethanol 70 % and were then grown on MS medium (Murashige & Skoog, 1962) and kept in the growth room (16/8 h light and dark respectively, with approximately 40 µmol photon $m^{-2}s^{-1}$ light density) at 25 °C. After 20 days, seedlings (T and non-T) were transferred to MS medium containing 0, 100, and 200 mM NaCl. Then, foliar application of glycine betaine (0, 20, and 40 mg. l^{-1}) was sprayed on top of the seedling with four to six leaves. After 4 weeks, plants were harvested with H_2O_2 contents, malondialdehyde (MDA) level, ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) activities measured in the leaves. The non-T plants not treated with salt and GB were used as control

2.2 DETERMINATION OF H_2O_2 CONCENTRATION

The H_2O_2 content was measured according to the method of Velikova et al. (2000). Fresh leaves (200 mg) were homogenized in trichloroacetic acid (TCA) 0.1 % (w/v) on an ice bath. The homogenates were then centrifuged at 10000 × g for 15 min at 4 °C. Then, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide. The absorbance was recorded at 390 nm using a spectrophotometer (Shimadzu UV-160, Japan). The H_2O_2 content was quantified by a calibration curve using H_2O_2 solutions.

2.3 DETERMINATION OF LIPID PEROXIDATION

Fresh leaf samples (200 mg) were homogenized with 5ml of TCA0.1 % (w/v). They were then centrifuged at 10000 × g for 5 min and supernatant was mixed with 0.5 % thiobarbituric acid (TBA) in TCA 20 % (w/v). Next, the samples were heated at 95 C for 30 min in a water bath, and the reaction was stopped in an ice bath. The absorbance of the extract was measured at 532 and 600 nm using a spectrophotometer. The concentration of MDA was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$and expressed as nmol MDA g^{-1} fresh mass (Heath & Packer, 1968).

2.4 ENZYME EXTRACTION

Approximately, 100 mg of fresh leaves was homogenized with 1 ml of 100 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA, 4 mM dithiothreitol (DTT), polyvinylpyrrolidone (PVP)1 % (w/v). The homogenate was centrifuged at 14000 rpm at 4 °C for 20 min. The supernatants were then used for protein and enzyme activity assays. Protein concentration was determined by Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.5 SUPEROXIDE DISMUTASE ACTIVITY (SOD, EC 1.15.1.1)

Total SOD activity was measured using nitro blue tetrazolium (NBT) method with some modifications (Beauchamp & Fridovich, 1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitro blue tetrazolium, 2 µM riboflavin, 0.1 mM EDTA, and 50 µl enzyme extract. The samples were shaken and exposed under light intensity (5000 lux) at 25 °C for 30 min, after which they were transferred to the dark room. Absorbance was measured by spectrophotometer at 560 nm. The activity of SOD was recorded as NBT reduction in light compared with the samples in the dark. One unit of SOD activity refers to the amount of protein required to inhibit 50 % of initial reduction of NBT under light.

2.6 CATALASE ACTIVITY (CAT, EC 1.11.1.6)

The CAT activity was determined by 1 ml of the reaction mixture (50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2, and 0.09 ml of the enzyme extract). The decrease in the absorbance of H_2O_2 was recorded at 240 nm for 1 min. The CAT activity was calculated using the coefficient of absorbance of 0.0394 mM^{-1} cm^{-1} (Aebi, 1984).

2.7 ASCORBATE PEROXIDASE ACTIVITY (APX)

The reaction mixture (1 ml) for the APX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.2 mM H_2O_2, and 50 µl of the enzyme extract. The activity was recorded by decreasing the absorbance at 290 nm for 1 min (extinction coefficient 2.8 $mM^{-1}cm^{-1}$) (Nakano & Asada, 1987).

2.8 STATISTICAL ANALYSIS

All experiments were performed with three replicates per treatment. Analysis of the data was carried out by three-way ANOVA and mean data were compared using Duncan's test at the level of $p \leq 0.05$. SPSS software (version 21) was utilized for statistical analysis of the data and the results were expressed as the mean ± standard deviation (SD).

3 RESULTS AND DISCUSSION

3.1 H_2O_2 CONTENT AND LIPID PEROXIDATION

Analysis of variance showed that salt, GB and plant effect were significant on H_2O_2 and MDA content (Table1). Also, there was a significant interaction in two-way analysis between salt and GB on H_2O_2 and MDA content. Furthermore, the two-way interaction between salt and plant were significant for H_2O_2 and MDA. The two-way interaction between GB and lines as well as three-way interaction of salt × GB× lines were only significant for MDA content. The H_2O_2 content of T and non-T plants was increased in response to salinity. The results indicated that GB treatment significantly reduced H_2O_2 content in both T and NT plants under salt stress (Fig.1). Notably, the H_2O_2 content in T plants either with or without GB was significantly lower than in non-T plants in the medium with similar NaCl concentration. Specifically, the H_2O_2 content of T plants treated with 40 mg l^{-1} GB and 0, 100, and 200 mM NaCl was 12, 25, and 13 % lower than in NT plants with the similar treatment of GB and salinity respectively. Therefore, the positive impact of GB for decreasing the H_2O_2 content in T plants was higher than in non-T plants.

Based on the data illustrated in Fig. 2, the MDA content was elevated by increasing the salinity in T and NT plants. The results showed that application of GB significantly reduced lipid peroxidation under salt stress in both types of plants especially in 200 mM salt. Hence, the MDA content in NT plants treated with 200 mM NaCl and 40 mg l^{-1}GB was reduced by about 45 % compared to NT plants treated only with 200 mM NaCl. Furthermore, the MDA content of T plants was lower than that of non-T plant under salt stress. Indeed, the MDA content of T plants treated with 100 and 200 mM salt was 29 and 28% lower than that of non-T plants treated with similar concentrations of NaCl, respectively. The major changes in biochemical and physiological processes such as antioxidant capacity under salt stress trigger generation of ROS, tissue destruction, and oxidative damages. It has been proposed that the MDA content is a reliable indicator for extended oxidative damage. Indeed, an increase in MDA content is correlated with intensified oxidative damage caused by biotic and abiotic stress such as salinity (Garg & Manchanda, 2009). Therefore, accumulation of H_2O_2, which is toxic for the cell, has been suggested as an indicator of oxidative stress (Hasanuzzaman et al., 2011). According to previous reports (Seckin et al., 2009), an increase in both MDA and H_2O_2 content was observed in plants in the research under salt stress. Similar to our findings, the reduction of MDA and H_2O_2 contents were also observed in some plants using GB(El-Samad et al., 2011; Nawaz & Ashraf, 2007; Yamada et al., 2009). It was observed that compatible solutes such as Pro and GB had an important role in osmotic adjustment and protection of proteins, mitochondria, and chloroplast membranes under salt stress (Dawood, 2016; Takabe et al., 2006). Therefore, modification of negative effects of salinity by GB might be due to its protective role and antioxidant capacity against oxidative stress (Park et al., 2004). Further, we found that overexpression of *P5CS* gene and consequently Pro overproduction had a remarkable role in scavenging free radicals and lipid peroxidation under salinity. Our findings indicated that exogenous GB reduced the negative effect of salt stress by decreasing of MDA and H_2O_2 content and improved antioxidant defense system in transgenic tobacco plants.

3.2 ANTIOXIDANT ENZYMES

Analysis of variance showed that salt, GB and lines effect were significant on SOD and APX activity. Also, salt and GB effect were significant on CAT activ-

Table 1: Analysis of variance (mean squares) for the main and interaction effects of salt, GB and line on H_2O_2, MDA, SOD, CAT and APX (* Significant at the 0.05 probability levels. ** Significant at the 0.01 probability levels. ns = not significant)

Source	df	H_2O_2	MDA	SOD	CAT	APX
Salt	2	168.403**	156.448**	.472**	612.870**	.007**
GB	2	103.218**	99.401**	.049**	86.484**	.0003**
line	1	161.317**	75.219**	.032**	450^{ns}	.0003**
Salt * GB	4	11.681**	24.267**	.007**	23.552**	$.00013^{ns}$
Salt * line	2	15.934**	27.015**	.007**	3.45^{n}	.00012**
GB * line	2	$.996^{ns}$	25.964**	$.001^{ns}$	7.190^{ns}	$.000012^{ns}$
Salt * GB * line	4	2.335^{ns}	6.970*	$.00032^{ns}$	2.827^{ns}	$.000002^{ns}$
Error	36	1.370	2.289	.001	2.621	.00001

ity. Moreover, there was a significant two-way interaction between salt and GB on SOD and CAT activity. Furthermore, the two-way interaction between salt and lines were significant for SOD and APX (Table1). The SOD activity of T and NT plants was enhanced by salt stress (Fig.3). Although the application of GB significantly improved SOD activity in both type of plants, the SOD activity of T plants either with or without GB was significantly higher than that of NT plants under salt stress. For instance, the SOD activity of T plants treated with 200 mM NaCl and 40 mg l^{-1} GB grew by 4 times in T plant, while NT plants treated with the same concentration of salt and GB increased 3 times compared to the control. Like SOD activity, the CAT activity of both types of plants was enhanced in response to salinity (Fig.4). When 200 mM NaCl was added to the medium, CAT activity of NT plants treated with 20 and 40 mg l^{-1} GB rose by 44 and 20 % compared to NT plants without GB respectively. On the other hand, only T plants treated with 20 mg l^{-1} GB and 200 mM salt improved CAT activity compared to T plants treated with same salinity and 0 mg l^{-1} GB. Notably, the level of CAT activity in T plants treated only with 200 mM NaCl was higher than in NT plants. Elevation of NaCl concentration enhanced APX activity in both types of plants (Fig. 5). Our results indicated that GB application improved APX activity in NT and T plants under salt stress. Meanwhile, the APX activity in T plants either with or without GB was higher than in NT plants under salinity.

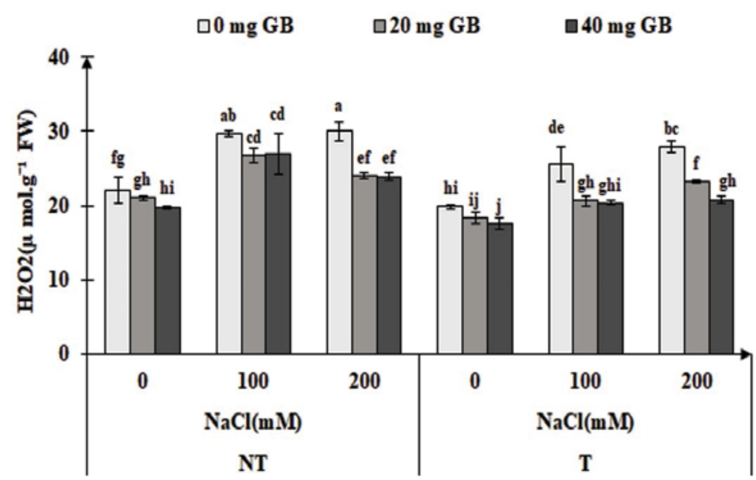

Figure 1: The effect of GB treatment on the H_2O_2 content of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

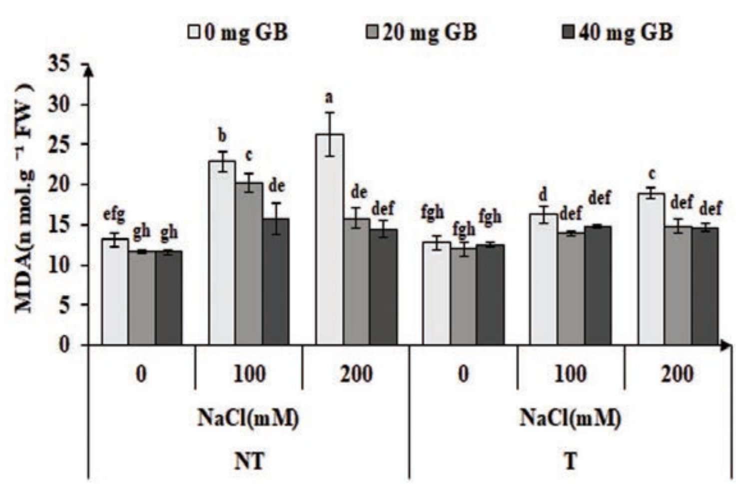

Figure 2: The effect of GB treatment on MDA content of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

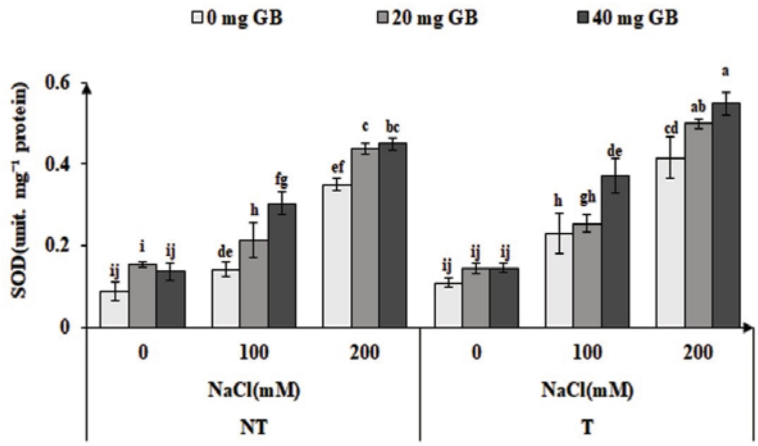

Figure 3: The effect of GB treatment on SOD activity of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

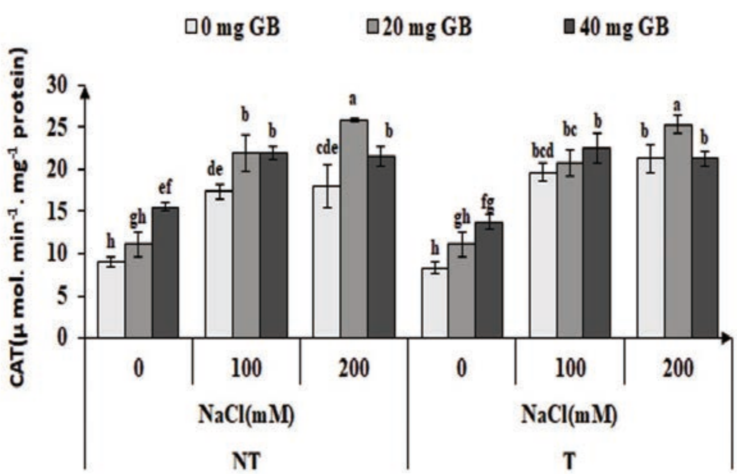

Figure 4: The effect of GB treatment on CAT activity of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

Under salinity stress, balance between production and destruction of ROS is crucial for plant survival. Generation of ROS induced by salt stress is a common response in plants (Fahad et al., 2015). Subsequently, an increase in the activity of many antioxidant enzymes such as SOD, CAT, and APX were supposed to be activated for detoxification of ROS in many plant species (Fahad et al., 2015; Hasanuzzaman et al., 2014). The first enzyme in detoxification pathway is SOD. This enzyme converts superoxide molecules to H_2O_2 (Foyer & Noctor, 2003). The activity of SOD was enhanced by GB in the present study as also reported in rice under salt stress (Raza et al., 2007). Hasanuzzaman et al. (2014) showed that SOD activity grew considerably by GB under salt stress. Then, H_2O_2 as a product of SOD is converted to H_2O and O_2 by CAT and APX enzymes (Gossett et al., 1996). Therefore, the high activity of CAT and APX enzymes in transgenic and non-transgenic tobacco plants treated with GB might be due to the high amount of H_2O_2 produced by SOD under salinity. It is important to note that APX has high affinity for the substrate (H_2O_2), while CAT is known an enzyme with lower affinity for H_2O_2. Thus, CAT is suggested to be involved in mass scavenging of H_2O_2 (Abogadallah, 2010) as we observed in our study. Accordingly, the main H_2O_2 scavenger in plants treated by GB was CAT. Indeed, the high activity of SOD is correlated with high activity of CAT in plants treated with GB. These results were in accordance with the findings obtained by Nounjan et al. (2012) on rice seedlings. It has been suggested that GB might be in-

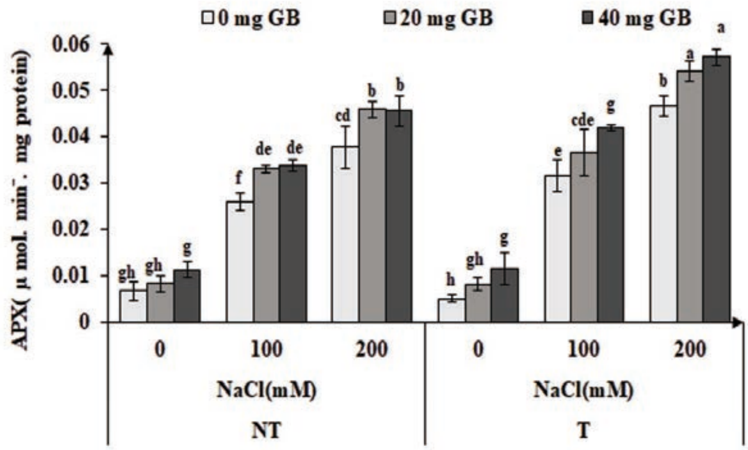

Figure 5: The effect of GB treatment on APX activity of non- transgenic (NT) and transgenic (T) tobacco plants under salt stress. The values are means of three replicates, ± SD. Common letters are not significant ($p < 0.05$) based on the Duncan test

volved indirectly in the regulation of gene expression (Al Hassan et al., 2015; Ben Ahmed et al., 2010; Nounjan et al., 2012).

Different functions have been considered for Pro such as protection of enzymes and proteins as well as antioxidant for ROS scavenging (Bellinger, 1987). In accordance with the function of Pro, our findings clearly suggested that transgenic tobacco plants treated by GB maybe had greater scavenging power under salt stress. The previous study on transgenic tobacco plants with overexpression *P5CS* gene indicated improved activity of antioxidant enzymes and proline content (Razavizadeh & Ehsanpour, 2009).

4 CONCLUSION

Although some evidences indicated that use of GB has not been effective to improve salt tolerance in plants (Figueroa-Soto & Valenzuela-Soto, 2018), the present data revealed transgenic (T) plants overexpressing *P5CS* gene treated with GB reduced H_2O_2 and MDA (in particular 100 mM NaCl) more than non-transgene plant (NT) suggesting better adaptation to salinity could be related to proline production due to overexpressing *P5CS* gene and enhanced levels of proline biosynthesis. Moreover, the synergistic effects of glycine betaine and proline in tobacco plants enhanced antioxidant defense system and resulted in increasing salt tolerance of tobacco plants overexpressing *P5CS* gene.

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5 REFERENCES

Abogadallah, G. M. (2010). Antioxidative defense under salt stress. *Plant Signaling & Behavior, 5*(4), 369-374. https://doi.org/10.4161/psb.5.4.10873

Aebi, H. (1984). [13] Catalase in vitro. *Methods in Enzymology, 105*, 121-126. https://doi.org/10.1016/S0076-6879(84)05016-3

Al Hassan, M., Fuertes, M. M., Sánchez, F. J. R., Vicente, O., & Boscaiu, M. (2015). Effects of salt and water stress on plant growth and on accumulation of osmolytes and antioxidant compounds in cherry tomato. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 43*(1), 1-11. https://doi.org/10.15835/nbha4319793

Ashraf, M., & Foolad, M. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany, 59*(2), 206-216. https://doi.org/10.1016/j.envexpbot.2005.12.006

Banu, M. N. A., Hoque, M. A., Watanabe-Sugimoto, M., Islam, M. M., Uraji, M., Matsuoka, K., . . . Murata, Y. (2010). Proline and glycinebetaine ameliorated NaCl stress via scavenging of hydrogen peroxide and methylglyoxal but not superoxide or nitric oxide in tobacco cultured cells. *Bioscience, Biotechnology, and Biochemistry, 74*(10), 2043-2049. https://doi.org/10.1271/bbb.100334

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry, 44*(1), 276-287. https://doi.org/10.1016/0003-2697(71)90370-8

Bellinger, Y. (1987). Proline accumulation in higher plants: a redox buffer? *Plant Physiology(Life Sci. Adv.), 6*, 23-27.

Ben Ahmed, C., Ben Rouina, B., Sensoy, S., Boukhriss, M., & Ben Abdullah, F. (2010). Exogenous proline effects on photosynthetic performance and antioxidant defense system of young olive tree. *Journal of Agricultural and Food Chemistry, 58*(7), 4216-4222. https://doi.org/10.1021/jf9041479

Bowler, C., Montagu, M. v., & Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Biology, 43*(1), 83-116. https://doi.org/10.1146/annurev.pp.43.060192.000503

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Chen, Z., Cuin, T. A., Zhou, M., Twomey, A., Naidu, B. P., & Shabala, S. (2007). Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *Journal of Experimental Botany, 58*(15-16), 4245-4255. https://doi.org/10.1093/jxb/erm284

Dawood, M. G. (2016). Influence of osmoregulators on plant tolerance to water stress. *Sci Agric, 13*(1), 42-58. https://doi.org/10.15192/PSCP.SA.2016.13.1.4258

Díaz, P., Borsani, O., Márquez, A., & Monza, J. (2005). Osmotically induced proline accumulation in *Lotus corniculatus* leaves is affected by light and nitrogen source. *Plant Growth Regulation, 46*(3), 223-232. https://doi.org/10.1007/s10725-005-0860-7

El-Samad, H. A., Shaddad, M., & Barakat, N. (2011). Improvement of plants salt tolerance by exogenous application of amino acids. *Journal of Medicinal Plants Research, 5*(24), 5692-5699.

Fahad, S., Hussain, S., Matloob, A., Khan, F. A., Khaliq, A., Saud, S., . . . Ullah, N. (2015). Phytohormones and plant responses to salinity stress: a review. *Plant Growth Regulation, 75*(2), 391-404. https://doi.org/10.1007/s10725-014-0013-y

Figueroa-Soto, C. G., & Valenzuela-Soto, E. M. (2018). Glycine betaine rather than acting only as an osmolyte also plays a role as regulator in cellular metabolism. *Biochimie, 147*, 89-97. https://doi.org/10.1016/j.biochi.2018.01.002

Forghani, A. H., Almodares, A., & Ehsanpour, A. A. (2018). Potential objectives for gibberellic acid and paclobutrazol under salt stress in sweet sorghum (*Sorghum bicolor* [L.] Moench cv. Sofra). *Applied Biological Chemistry, 61*(1), 113-124. https://doi.org/10.1007/s13765-017-0329-1

Foyer, C. H., & Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum, 119*(3), 355-364. https://doi.org/10.1034/j.1399-3054.2003.00223.x

Fridovich, I. (1983). Superoxide radical: an endogenous toxicant. *Annual Review of Pharmacology and Toxicology, 23*(1), 239-257. https://doi.org/10.1146/annurev.pa.23.040183.001323

Garg, N., & Manchanda, G. (2009). ROS generation in plants: boon or bane? *Plant Biosystems, 143*(1), 81-96. https://doi.org/10.1080/11263500802633626

Gossett, D. R., Banks, S. W., Millhollon, E. P., & Lucas, M. C. (1996). Antioxidant response to NaCl stress in a control and an NaCl-tolerant cotton cell line grown in the presence of paraquat, buthionine sulfoximine, and exogenous glutathione. *Plant Physiology, 112*(2), 803-809. https://doi.org/10.1104/pp.112.2.803

Hasanuzzaman, M., Alam, M., Rahman, A., Hasanuzzaman, M., Nahar, K., & Fujita, M. (2014). Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxalase systems provides better protection against salt-induced oxidative stress in two rice (*Oryza sativa* L.) varieties. *BioMed Research International, 2014*. https://doi.org/10.1155/2014/757219

Hasanuzzaman, M., Hossain, M. A., & Fujita, M. (2011). Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. *Plant Biotechnology Reports, 5*(4), 353. https://doi.org/10.1007/s11816-011-0189-9

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics, 125*(1), 189-198. https://doi.org/10.1016/0003-9861(68)90654-1

Iqbal, N., Umar, S., Khan, N. A., & Khan, M. I. R. (2014). A new perspective of phytohormones in salinity tolerance: regulation of proline metabolism. *Environmental and Experimental Botany, 100*, 34-42. https://doi.org/10.1016/j.envexpbot.2013.12.006

Kishor, P. K., Hong, Z., Miao, G.-H., Hu, C.-A. A., & Verma, D. P. S. (1995). Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology, 108*(4), 1387-1394. https://doi.org/10.1104/pp.108.4.1387

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science, 7*(9), 405-410. https://doi.org/10.1016/S1360-1385(02)02312-9

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nakano, Y., & Asada, K. (1987). Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant and Cell Physiology, 28*(1), 131-140.

Nawaz, K., & Ashraf, M. (2007). Improvement in salt tolerance of maize by exogenous application of glycinebetaine: growth and water relations. *Pakistan Journal of Botany, 39*(5), 1647-1653.

Neto, C. O., Lobato, A., Costa, R., Maia, W., Filho, B. S., Alves, G., . . . Cruz, F. (2009). Nitrogen compounds and enzyme activities in *sorghum* induced to water deficit during three stages. *Plant Soil Environ, 55*, 238-244. https://doi.org/10.17221/84/2009-PSE

Nounjan, N., Nghia, P. T., & Theerakulpisut, P. (2012). Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes. *Journal of Plant Physiology, 169*(6), 596-604. https://doi.org/10.1016/j.jplph.2012.01.004

Parida, A. K., & Das, A. B. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety, 60*(3), 324-349. https://doi.org/10.1016/j.ecoenv.2004.06.010

Park, E. J., Jeknić, Z., Sakamoto, A., DeNoma, J., Yuwansiri, R., Murata, N., & Chen, T. H. (2004). Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *The Plant Journal, 40*(4), 474-487. https://doi.org/10.1111/j.1365-313X.2004.02237.x

Per, T. S., Khan, N. A., Reddy, P. S., Masood, A., Hasanuzzaman, M., Khan, M. I. R., & Anjum, N. A. (2017). Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: phytohormones, mineral nutrients and transgenics. Plant Physiology and Biochemistry, 115, 126-140. https://doi.org/10.1016/j.plaphy.2017.03.018

Rajaeian, S., & Ehsanpour, A. (2015). Physiological responses of tobacco plants (Nicotiana rustica) pretreated with ethanolamine to salt stress. Russian Journal of Plant Physiology, 62(2), 246-252. https://doi.org/10.1134/S1021443715020156

Raza, S. H., Athar, H. R., Ashraf, M., & Hameed, A. (2007). Glycinebetaine-induced modulation of antioxidant enzymes activities and ion accumulation in two wheat cultivars differing in salt tolerance. Environmental and Experimental Botany, 60(3), 368-376. https://doi.org/10.1016/j.envexpbot.2006.12.009

Razavizadeh, R., & Ehsanpour, A. (2009). Effects of salt stress on proline content, expression of delta-1-pyrroline-5-carboxylate synthetase, and activities of catalase and ascorbate peroxidase in transgenic tobacco plants. Biological Letters, 46(2), 63-75. https://doi.org/10.2478/v10120-009-0002-4

Seckin, B., Sekmen, A. H., & Türkan, I. (2009). An enhancing effect of exogenous mannitol on the antioxidant enzyme activities in roots of wheat under salt stress. Journal of Plant Growth Regulation, 28(1), 12. https://doi.org/10.1007/s00344-008-9068-1

Szabados, L., & Savoure, A. (2010). Proline: a multifunctional amino acid. Trends in Plant Science, 15(2), 89-97. https://doi.org/10.1016/j.tplants.2009.11.009

Takabe, T., Rai, V., & Hibino, T. (2006). Metabolic engineering of glycinebetaine Abiotic stress tolerance in plants (pp. 137-151): Springer. https://doi.org/10.1007/1-4020-4389-9_9

Velikova, V., Yordanov, I., & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Science, 151(1), 59-66. https://doi.org/10.1016/S0168-9452(99)00197-1

Wang, G., Zhang, X., Li, F., Luo, Y., & Wang, W. (2010). Overaccumulation of glycine betaine enhances tolerance to drought and heat stress in wheat leaves in the protection of photosynthesis. Photosynthetica, 48(1), 117-126. https://doi.org/10.1007/s11099-010-0016-5

Yamada, N., Promden, W., Yamane, K., Tamagake, H., Hibino, T., Tanaka, Y., & Takabe, T. (2009). Preferential accumulation of betaine uncoupled to choline monooxygenase in young leaves of sugar beet–importance of long-distance translocation of betaine under normal and salt-stressed conditions. Journal of Plant Physiology, 166(18), 2058-2070. https://doi.org/10.1016/j.jplph.2009.06.016

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Potassium mobilization and plant growth promotion by soil bacteria isolated from different agroclimatic zones of Odisha, India

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Potassium mobilization and plant growth promotion by soil bacteria isolated from different agroclimatic zones of Odisha, India

Abstract: Potassium is essential for plant metabolism; improves immunity to stress and increase crop productivity. Soil contains insoluble form of potassium, which is unavailable for plant absorption. Potash mobilizing bacteria (KMB) solubilise complex potassium and make it available to plant. KMB with plant growth promoting (PGP) traits could enhance growth and crop productivity. Here we attempt to screen KMBs with PGP traits from different agroclimatic zones of Odisha and study dynamics of potassium in soil. Isolation of KMB and determination of PGP traits was performed with standard protocols. Pot culture experiment was aimed to study their effect on sunflower crop. Available soil potassium was quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES). Thirty KMBs were isolated from different agro-climatic zones of Odisha, out of which 6 isolates exhibited maximum PGP traits. Moreover, after adding inoculums the available soil potassium decreased over 0 to 30 days as compared to control, with increase in shoot length. T7 (consortium) reported maximum (144 %) increase in shoot length. Available soil potassium content decreased with increase in time. A maximum decrease was reported in T7 (26.31 %), suggesting potassium accumulation by plant.

Key words: soil; potash mobilizing bacteria; plant growth promoting rhizobacteria; soil potassium; potassium availability

Mobilizacija kalija in pospeševanje rasti rastlin s talnimi bakterijami, izoliranimi iz različnih agroklimatskih območij Odishe, Indija

Izvleček: Kalij je nujno potreben element v presnovi rastlin. Izboljšuje odpornost na stres in povečuje pridelek. Tla vsebujejo netopne oblike kalija, ki ni dostopen rastlinam. Kalij sproščajoče bakterije (KMB) raztapljajo vezan kalij in ga naredijo dostopnega rastlinam. KMB bi skupno s snovmi, ki izboljšujejo rast lahko povečale rast in pridelek. V raziskavi poskušajo preveriti KMB z PGP lastnostni iz različnih agroklimatskih območij Odishe in preučiti dinamiko kalija v tleh. Izolacija KMB in določitev njihovih PGP lastnosti sta bili izvedeni s strandardnimi protokoli. Izveden je bil lončni poskus za preučitev njihovega vpliva na pridelek sončnic. Razpoložljiv kalij v tleh je bil določen z ICP-OES protokolom. V različnih agroklimatskih območjih Odishe je bilo izoliranih 30 KBM, od katerih je 6 izolatov pokazalo največje vrednosti PGP. Po dodatku teh inokulumov se je razpoložljiv kalij v tleh zmanšal v 30 dneh v primerjavi s kontrolo s hkratnim povečanjem dolžine poganjkov. T7 inokulum (consortium) je dal največje povečanje (144 %) v dolžini poganjkov. Vsebnost razpoložljivega kalija v tleh se je zmanjševala s časom poskusa. Največje zmanjašanje je bilo zabeleženo pri uporabi inokuluma T7 (26,31 %), kar kaže na kopičenje kalija v rastlinah.

Ključne besede: tla; kalij sproščajoče bakterije; rast vzpodbujajoče rizobakterije; talni kalij; razpoložljivost kalija

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1 INTRODUCTION

Potassium (K), a vital macro-nutrient absorbed by plants from the soil, which enters into the food chain to meet requirement of animals including human (Morgan and Connolly, 2013). Soil mostly contains complex insoluble forms of potash like biotite, feldspar, mica, sylvite, etc. and unavailable for plant uptake. Deficiency of potassium in plants leads to scorching and curling of leaf tips, chlorosis between leaf veins, reduced root, leaf size and seed & fruit development (Uchida, 2000). Generally, Potassium is considered the most abundant of the major soil nutrient elements. In soil the total K content ranges from 0.01 % to 4 %, usually about 1 % (Sparks, 1987; Blake et al., 1999). The average soil K value for production of corn is 71-130 ppm and alfalfa 71-140 ppm. The average soil test K content of coarse-textured soil is 103 ppm and for medium and fine textured soil is 128 ppm (Peters, 2011), needs application of potassium fertilizer. Due to imbalanced use of NPK fertilizers and intensive cropping system, widespread deficiency of K is observed in Indian soil (Naidu et al., 2011). At national level, potassium depletion in Indian soil was approx. 10.2 t year^{-1}. In Odisha, soil K content was 36.7- 458.3 kgha^{-1} against the required amount of 110-280 kgha^{-1}. Although soil is rich with potassium, about 90-98 % is chemically bound in the crystal lattice structure of the minerals and unavailable for plant uptake (Gurav et al., 2019). Odisha has 10 agroclimatic zones (Mishra and Mishra, 2016). Total K content of Odisha soil ranges between 0.3 to 3.0 % of which non exchangeable K comprises 21-61 % and exchangeable K constitue 12.5-35.7 % (Jena et al., 2009).

Replacement of potassium through chemical fertilizers significantly imposes threat to environmental safety and sustainability by leaching, soil & water pollution, susceptibility of crop to diseases (Perez-Lucas et al., 2018). High percentage of K in chemical fertilizers also causes longterm imbalance in soil pH affecting fertility. Soil is a habitat for multitudinous microbial population (Nayak et al., 2020). Microbial conversion of insoluble K into soluble form by potassium mobilizing bacteria (KMB) can enhance availability of soluble K for plant uptake. Many predominantly soil bacteria such as, *Bacillus circulans* (Jordan, 1890), *B. mucilaginous* (Avakyan et al., 1986), *Paenibacillus* spp. (Ash et al., 1994) etc. are known to readily solubilise K minerals. Potassium mobilizers dissolve the complex silicate minerals to release soluble K through various mechanisms like chelation, production of inorganic and organic acids, acidolysis, polysaccharides, exchange reactions, also produce exopolymeric substances (Bhattacharyya and Jha, 2012) to solubilise element. These potential bacteria can be better utilized as biofertilizer in recycling and biofortification of potash in crop fields. Raghavendra et al. (2016) and Zahedi (2016) opined, biofortification of essential nutrients through microbes is an effective measure to overcome macro & micro-nutrient deficiency in growing cereal crops; with maximum levels of bioavailable nutrient concentration and subsequently eliminating nutrient deficiency in plants and animals (Saravanan et al., 2011).

Plant growth promoting rhizobacteria are the group of microbes having the ability to promote growth of the plants by several direct mechanisms (nitrogen fixation, phytohormones production, phosphate solubilisation, siderophore production etc.) and indirect mechanisms (antibiotic production, lytic enzymes production, induced systemic resistance (ISR), HCN production, etc.) (Nazir et al., 2019; Lakra and Mishra, 2018). Application of potassium mobilizing bacteria (KMB) exhibiting different plant growth promoting (PGP) traits would not only deal with the nutrient deficiency but also improve crop yield and soil fertility. In view of this, the present work is designed to isolate potent KMBs from different agro climatic zones of Odisha, India, dynamics of soil potassium and validation of PGPR traits with its effect on the oil producing crop, sunflower.

2 MATERIALS AND METHODS

2.1 COLLECTION OF SOIL SAMPLE

Rhizospheric soil samples were collected aseptically from various crops at different agroclimatic locations of Bargarh, Kakatpur, Jharsuguda, Nimapara, ICAR-NRRI, Cuttack, Rourkela, Sonepur, Sambalpur, Sundergarh, Bhubaneswar and OUAT. Top layer of the soil (about 1 cm) was removed and 3 samples of about 10 g of each were collected at the depth of 10-20 cm, mixed thoroughly and aseptically put in polythene packets with proper labels.

2.2 ISOLATION OF RHIZOBACTERIA

Standard isolation method was followed taking specimens randomly, without any prior knowledge of the microbial composition at the source under investigation (Donaldio et al., 2002). The rhizospheric samples were processed in the laboratory and microorganisms were isolated *in vitro* in basal medium. Enumerations of heterotrophic bacteria were conducted through serial dilution method and spread plate technique. One g of soil sample was suspended in 10 ml sterile distilled water, logarithmic dilutions were made upto 10^{-4}level and 100 µl suspensions was spread on nutrient agar plate (NA). The plates were incubated at 37 ± 1 ˚C for 24 h. The CFUs of different morphology were then selected and sub cultured on NA slants, incubated for 24 h at 37 ± 1 ˚C and the slants were numbered and preserved at 4 ºC. The slant cultures were periodically subculture and used for different experiments. The isolated bacteria were coded with number for further experiment.

2.3 QUALITATIVE TEST FOR KMB

The potassium mobilization test was done on Alexandrow's medium. Isolates were spot inoculated in agar plates and incubated for 72 h at 37 ˚C (Zhang and Kong, 2014). A clear zone around the colony was taken as positive for potassium metabolism.

2.4 VALIDATION OF PLANT GROWTH PROMOTING TRAITS(PGP)

Plant growth promoting activities of bacteria were tested *in vitro*. The PGPR traits viz. IAA production, phosphate solubilization, ammonia production, nitrate reduction test, antibiosis and siderophore production were determined with following standard methods (Pahari and Mishra, 2017).

2.4.1 IAA production

The qualitative test for IAA was carried out following Bric et al. (1991). Rhizobacterial isolates were inoculated in 5 ml peptone water amended with 0.1 % tryptophan and incubated at 30 ºC for 48-72 h in dark. Salkowski reagent (2 % 0.5M $FeCl_3$ in 35 % (v/v) perchloric acid) was added in tubes and observed for pink colour development in the concerned tubes.

2.4.2 Phosphatesolubilization

The phosphate solubilization test was done on Pikovaskaya medium (Pikovaskaya, 1948). Agar plates were prepared and the isolates were spot inoculated on it and incubated for 5-6 days 37 ºC. A clear zone around the colony was taken as positive for phosphate solubilization.

2.4.3 Ammonia production

All the isolates were tested for qualitative production of ammonia. Peptone water was prepared by the method of Dye (1962). All the isolates were inoculated in 1 % (v/v) peptone water and incubated at 30 ºC for 3 days. After the incubation period, 1 ml of Nessler's reagent was added into each of the tubes. The presence of faint yellow colour indicated small amount of ammonia production and deep yellow to deep reddish brown colour indicated maximum production of ammonia.

2.4.4 Nitrate reduction test

The ability of the microorganisms to reduce nitrate to nitrite is detected through the test (Knapp and Clark, 1984). All ten isolates were inoculated into nitrate broth, incubation at 30 ˚C for 96 hours. After inoculation sulphanillic acid and α-naphthylamine mixture (1:1) was added. Appearance of deep pink colour indicated positive result.

2.4.5 Antibiosis

This test was carried out for isolates against the pathogenic fungi *Fusarium* sp. (Link, 1809) All screenings were carried out on PDA plates for fungal pathogens (Zhao et al., 2018). An actively growing fungal agar plug (3 mm diameter) was placed at centre of PDA plates. Bacterial isolates were inoculated and the plates were incubated for four days at 28 ˚C.

2.4.6 Siderophoreproduction

Production of siderophore was assayed by growing them on Chrome azurol S (CAS) agar plates at 28 ± 2 ºC for 5 days incubation (Schwyn and Neilands, 1987). Appearance of yellow or brown zone around the colony indicated positive result for siderophore production.

2.5 MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

Six isolates showing more number of PGP traits were selected for further characterization and application. Gram's reaction was conducted on the isolated microorganisms to study their morphology. The organisms were taken and inoculated in freshly prepared and sterilized peptone water. A basic biochemical test that includes IMViC was performed following indole test, MR test, Voges-Proskauer test and citrate utilization test. In addition to this, manitol motility test, ONPG test and TSI test was also conducted (Pahari and Mishra, 2017).

2.5.1 Enzymatic test

Isolates were tested for oxidase, urease, catalase, starch hydrolysis, casein hydrolysis, esculin hydrolysis, DNAse, coagulase and decarboxylation test reaction (Gupta et al., 2000).

2.5.2 Sugar utilization test (O-F Test)

All isolates were inoculated in the freshly prepared carbohydrate fermentative O-F medium with the sugars. The sugars used for this test were xylose, sorbitol, cellobiose, salicin, raffinose and inositol. Colour change to yellow indicated positive results.

2.6 GROWTH IN DIFFERENT PHYSICAL PARAMETERS

Nutrient broth was used for this test. The pH was maintained to 5.6. The isolates were inoculated into it and incubated 24 hours at 37 ˚C. Growth in 7 % NaCl was done by adding 7 % NaCl to the medium and sterilized (Tank and Saraf, 2009). The isolates were inoculated and incubated for 24 hours at 37 ˚C. Growth of isolates at different temperature was also tested by inoculating the isolates in nutrient broth, which were further incubated at 10 ˚C, 45 ˚C and 65 ˚C for 24 hours (Getahun et al., 2020). The growth in medium indicated positive result.

2.7 ANAEROBIC GROWTH

Thioglycolate slant plus butt was prepared according to Chandler (2013). The isolates were inoculated into it by stabbing followed by streaking method. A sterile cotton plug was inserted into the tube followed by the addition of pyrogallol powder and NaOH. Paraffin tape was wrapped around the tube and incubated at 37 ˚C for 72 h in an inverted manner. Growth in the medium indicated a positive result.

2.8 ANTAGONISTIC EFFECT BETWEEN ORGANISMS

To determine negative interactions amongst the isolates in consortium application, inhibiting growth of each other was studied. The experiment was carried out on nutrient agar plates. One of organism was lawn cultured and five other organisms were inoculated on the wells made on the agar. Similar method was followed for all the selected isolates.

The plates were observed for the production of halo-zones around the wells which would a negative interaction between the test organisms.

2.9 EVALUTION OF EFFECTIVE BACTERIAL ISOLATES ON SEED GERMINATION

The potent bacterial isolates were further tried with sunflower seeds for determination of effect on germination under laboratory condition.

2.10 POT CULTURE METHOD

Sunflower seeds were sown in different pots based on randomized block design. The inoculums were centrifuged and added in single and consortium to the pots. Nomenclature of the organisms was done as T1 to T6, consortium was T7 and control was C.

2.11 RESIDUAL SOIL POTASSIUM

The available potassium in the soil was estimated by following the ammonium acetate extract protocol of Malathi and Stalin (2018). The estimation of potassium in soil was done on 10 to 30 days at an interval of 10 days post addition of inoculums. Soil and ammonium acetate were mixed in the ratio of 1:10. It was incubated in the shaker incubator for 30 minutes at 90 RPM and 25 degree Celsius. After digestion, the solution was filtered out and further analysis was carried out in ICP-OES, from which the available potassium was estimated.

3 RESULTS

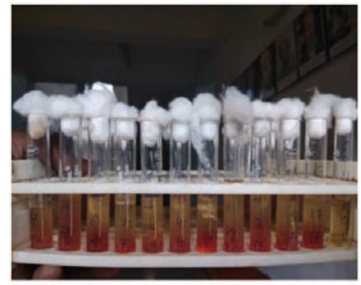
3.1 ISOLATION OF POTASH MOBILIZING BACTERIA FROM THE RHIZOPHERIC SOIL

A total of 84 morphologically distinct colonies isolated from different rhizospheric soil sample and were screened for potassium mobilization using Alexandrow's medium agar plates. KMB positive bacteria were observed by the formation of clear zone in the agar plates. Out of the 84 isolated bacteria, a total of 30 potent KMBs were positive (Table 1; Fig. 1).

Fig. 1: Potassium solubilisation by the isolates

3.2 PLANT GROWTH PROMOTING TRAITS OF THE POTENT KMB ISOLATES

The plant growth promoting traits like IAA production, phosphate solubilization and ammonia production are presented in Table 1, Figure 2. Nitrate reduction test, antibiosis and siderophore production exhibited by the isolates along with potassium mobilization. These traits are effective for enhancement in growth and productivity of the crop. The isolates showing maximum plant growth promoting traits in addition to potassium mobilisation were selected from the 30 test organisms. The highest



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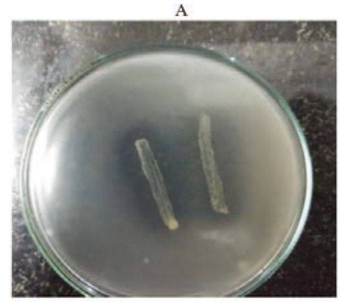

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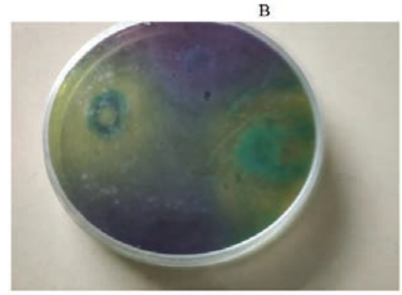

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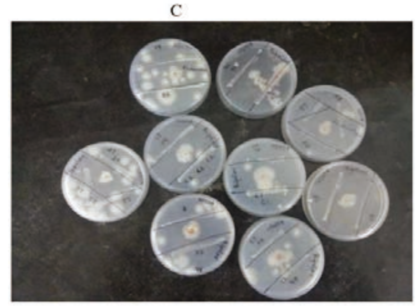

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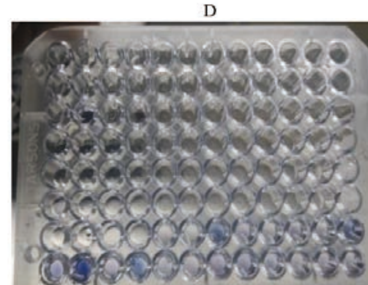

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Fig. 2: Plant growth promoting traits exhibited by the isolates (A: Indole acetic acid production; B-Phosphate solubilizing bacteria test; C-Siderophore production; D-Antibiosis; E-Nitrate reduction test.)

Table 1: KMB and plant growth promoting characteristics of the potential isolates

Isolates	KMB	PSB	IAA	SIDO	AMM	NIT	ANTI	TOTAL
26	+	-	-	-	-	+	+	4
27	+	+	-	+	-	+	+	6
33	+	-	-	+	-	-	-	3
39	+	-	+	+	-	-	-	4
43	+	+	-	+	-	-	-	4
44	+	-	-	+	-	-	-	3
46	+	+	+	+	-	+	+	7
47	+	-	-	+	-	-	+	4
54	-	+	-	+	-	-	+	4
55	+	-	-	+	-	-	-	3
56	+	+	+	+	+	+	+	8
58	+	-	-	+	-	-	-	3
59	+	-	-	-	-	-	-	2
61	+	+	+	+	-	-	-	5
62	+	+	-	+	-	-	+	5
63	+	-	+	-	-	-	-	3
69	+	-	-	+	-	-	-	3
72	+	-	-	+	-	+	+	5
74	+	+	-	+	-	-	-	4
76	-	-	+	-	+	-	-	3
77	+	+	+	+	+	+	+	8
78	+	+	+	+	+	+	+	8
79	+	-	-	+	-	-	+	4
81	+	+	-	+	-	-	-	4
83	+	+	-	+	+	-	-	5
84	+	+	+	-	+	+	+	7

+:Positive for the trait, -: negative for the trait; KMB: Potassium mobilizing bacteria, PSB: Phosphate Solubilizing bacteria, IAA: Indoleacetic acid, SIDO: Siderophore, AMM: Ammonification, NIT: Nitrification, ANTI: Antibiosis

Table 2: Physical-chemical parameters of soil samples having potash mobilizing isolates

Isolates	Sampling Site	pH (1:2, w/v)	Soil Organic Carbon (SOC) (g kg^{-1})	Electrical conductivity (EC) (dS m^{-1})
27	Nimapada	6.38	7.3	0.093
46	Rourkela	6.71	8.25	0.038
56	Sonepur	8.1	9.81	0.167
77,78,84	OUAT Field	6.52	6.9	0.088

eight number of PGP traits were depicted by isolate number 56, 77 and 78. Isolate number 46 and 84 were positive for seven traits. Isolate number 27 exhibited positive results for six PGP traits. Soil from four agroclimatic zones having bacteria with potash mobilizing and plant growth promoting properties were analysed (Table. 2) These six isolates were then used for further biochemical characterization and application in crop.

3.3 MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

From the colony morphology and gram's reaction of all the six isolates, it was observed that the colonies were small, smooth, irregular and raised with Gram positive rods and cocci (Table3; Fig. 3). Furthermore basic biochemical tests revealed, all the six isolates were indole negative, Vokes Proskeur positive and besides 44 and 77, all the isolates were positive for citrate utilization. Other than 78, mannitol motility was positive in other isolates. The isolates had positive growth at 45 ˚C and negative growth at 65 ˚C and 10 ˚C. Growth at pH 5.7 and anaerobic medium was found to be positive in all. All the isolates were positive for esculin hydrolysis, catalase, oxidase, urease, arginine dehydrolase, ONPG and negative for starch & casein hydrolysis, DNAse, ornithine decarboxylase and coagulase. Each of the isolates except 77, showed negative result in nitrate reductase and in case of lysine decarboxylase 27, 46, 56 were negative and 77, 78, 84 were positive (Table. 4). Moreover all the isolates depicted positive result in triple sugar iron test (Table 5). Gas production was observed in all but 77 and 84. As for sugar utilization, all the isolates could utilize cellobiose, raffinose, sorbitol and xylose (Table 6). En route to further experimentation, nomenclature was done as; 27-T1, 46-T2, 56-T3, 77-T4, 78-T5, 84-T6, the consortium of all the isolates was T7 and control as C.

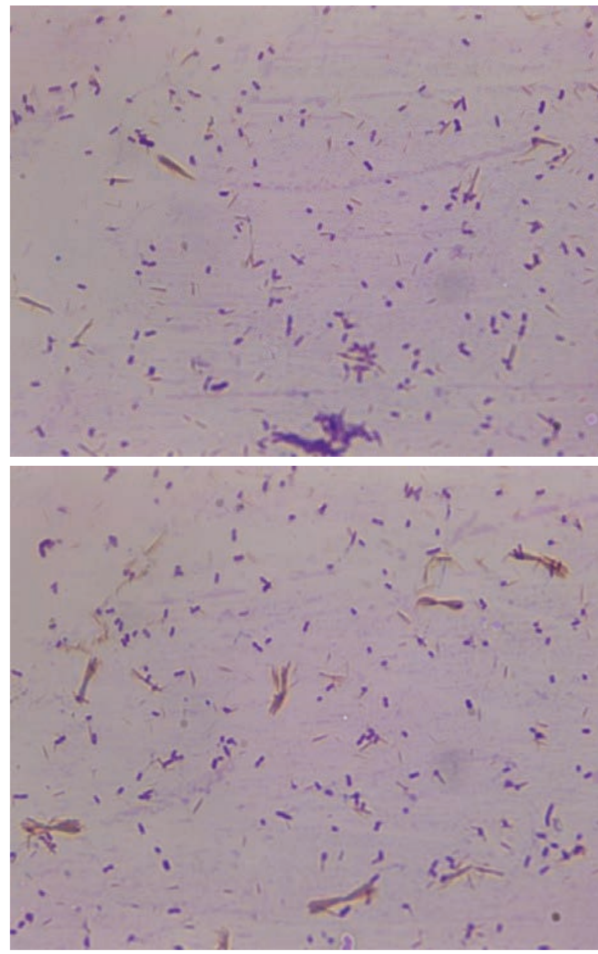

Fig. 3: Gram's staining

Table 3: Colony morphology and Gram's variability of the isolates

characteristics	27	46	56	77	78	84
Shape	Circular	Irregular	Irregular	Irregular	Irregular	Irregular
Elevation	Raised	Crateriform	Convex	Raised	Umbonate	Raised
Margin	Entire	Curled	Undulate	Undulate	Lobate	Undulate
Size	Small	Large	Small	Medium	Small	Medium
Surface	Smooth	Rough	Rough	Smooth	Smooth	Smooth
Colour	White	White	White	White	White	white
Opacity	Opaque	Opaque	Opaque	Opaque	Translucent	opaque
Gram	Gram +ve	Gram	Gram+ve	Gram +ve	Gram	Gram+ve
staining	Rod	+verod	rod	rod	+ve cocci	cocci

Table 4: Biochemical characterization of the isolates

SlNo	Biochemical test	27	46	56	77	78	84
1	Indole	-	-	-	-	-	-
2	Vogeus- Proskaur	+	+	+	+	+	+
3	Citrate utilization	+	-	+	-	+	+
4	Mannitol motility test	+	+	+	+	-	+
5	Growth at 45 °C	+	+	+	+	+	+
6	Growth at 65 °C	-	-	-	-	-	-
7	Growth at 10 °C	-	-	-	-	-	-
8	Growth at pH5.7	+	+	+	+	+	+
9	Anaerobic Growth	+	+	+	+	+	+
10	Starch hydrolysis	-	-	-	-	-	-
11	Casein hydrolysis	-	-	-	-	-	-
12	Esculin hydrolysis	+	+	+	+	+	+
13	Catalase	+	+	+	+	+	+
14	Oxidase	+	+	+	+	+	+
15	Urease	+	+	+	+	+	+
16	DNase	-	-	-	-	-	-
17	Arginine dihydrolase	+	+	+	+	+	+
18	Lysine decarboxylase	-	-	-	+	+	+
19	Ornithine decarboxylase	-	-	-	-	-	-
20	Nitrate reductase	-	-	-	+	-	-
21	Coagulase	-	-	-	-	-	-
22	ONPG	+	+	+	+	+	+

Table 5: Triple Sugar Iron Test

Isolate no.	Alkaline slant	Acidic butt	H_2S Production	Gas Production
27	+	+	+	+
46	+	+	+	+
56	+	+	+	+
77	+	+	+	-
78	+	+	+	+
84	+	+	+	-

Table 6: Sugar Utilization

Isolate no.	Cellobiose		Inositol		Raffinose		Salicin		Sorbitol		Xylose	
	O	F	O	F	O	F	O	F	O	F	O	F
27	+	+	-	+	+	+	-	+	+	+	+	+
46	+	+	-	-	+	+	-	-	+	+	+	+
56	+	+	-	-	+	+	-	-	+	+	+	+
77	+	+	-	-	+	+	-	+	+	+	+	+
78	+	+	-	+	+	+	+	+	+	+	+	+
84	+	+	-	+	+	+	+	+	+	+	+	+

3.6 INTERACTION BETWEEN ISOLATES

No inhibition zone was reported around the wells indicating that the organisms were not antagonistic to each other and can be used in a consortium (Fig. 4).

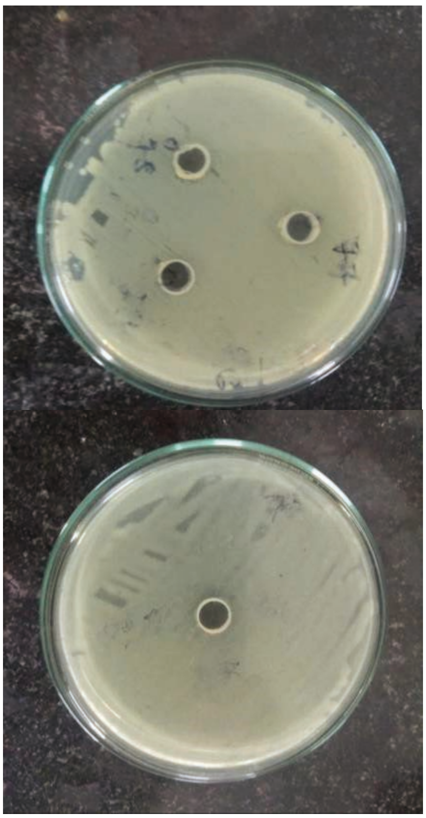

Fig. 4: Interaction between the isolates

3.7 EFFECT OF THE ISOLATES ON THE GERMINATION OF SUNFLOWER SEEDS

Seed germination was studied by using germination paper following the roll towel method under laboratory conditions. It was found that in all the isolates except T4, the percentage of germination was significantly higher (40-50 %) as compared to the control (Table.7; Fig. 5).

Fig. 5: Seed germination of sunflower by the isolates

3.8 EFFECT OF THE ISOLATES ON SUNFLOWER CROPS IN POT CULTURE METHOD

The shoot length of sunflower increased with inoculation of the organisms and in consortia (Fig. 6(a)). The percentage of increase ranged between 54.2 % with the organism T6 to 124.1 % with T1 in 30days over that of the 10 days. Maximum increase of 144 % in shoot length with consortium (T7) was reported. The control

Table 7: Effect of the isolates on germination of Sunflower seed

Isolate	C	T1	T2	T3	T4	T5	T6	T7
Germination percentage (%)	52	98	92	92	40	100	95	98

Table 8: Changes in shoot length of sunflower (cm) with application of the isolates and in consortia

Isolate	10 days	20 days	30 days
T1	8.7	11.8 (+ 35.63 %)	19.5 (+124.1 %)
T2	12.8	16.5 (+ 22.42 %)	25 (+95.312 %)
T3	14.36	15.7 (+9.33 %)	26.1 (+81.75 %)
T4	15.96	17.33 (+8.58 %)	25.6 (+60.40 %)
T5	13.9	15.4 (+10.79 %)	24.5 (+76.25 %)
T6	15.43	17.76 (+15.1 %)	23.8 (+54.24 %)
T7	10	11.8 (+18 %)	24.4 (+144 %)
C	12.63	15.9 (+25.89 %)	20.23 (+60.17 %)

set (C) there was an increase of 60.17 % in 30 days over that of the 10 days (Table 8; Fig. 6(b)).

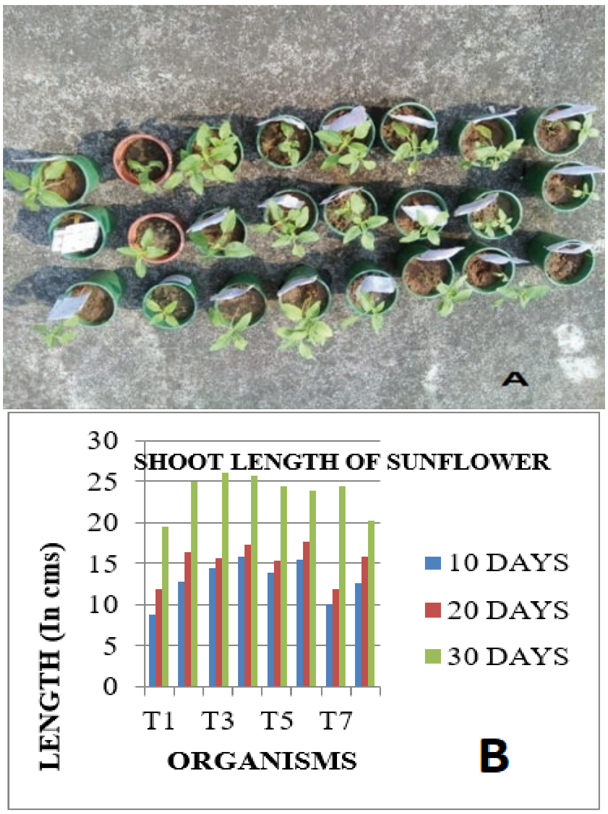

Fig. 6: (A) Sunflower plants in pot culture method; (B) Shoot length of sunflower (graph)

3.9 AVAILABLE POTASSIUM IN SOIL

The amount of soil available potassium decreased with increase in time from 10 days to 30 days (Table 9; Fig. 7). It decreased 2.85 % with isolate T1, 5.26 % with T2, 20.04 % with isolate T3, 19.62 %, with T4, 10.45 % with T5 and 9.94 %, decrease of with isolate T6. Percent decrease in soil potassium content was maximum 26.31 % with consortium (T7). In case of control, an increase of 7.61 % available soil potassium content was reported.The decrease in soil potassium content with time was statistically significant ($p \leq 0.5$).

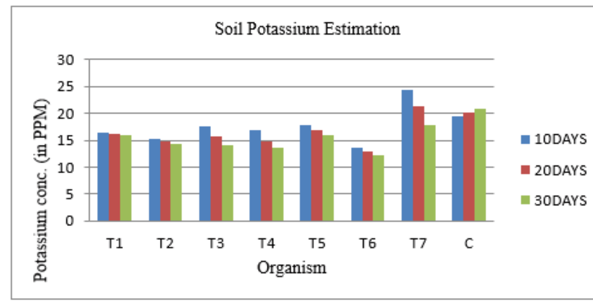

Fig.7: Soil potassium estimation in sunflower

4 DISCUSSION

4.1 ISOLATION OF POTASH MOBILIZING BACTERIA

In view of the adverse effects of chemical fertilizers and agrochemical application in crop fields, organic farming is advocated in Green Revolution-II. Soil is a rich source of organic matter, mostly released through root exudates. Forty percent of the photosynthate, released through the root, provides an ideal environment for the microbes to inhabit in the rhizospheric region (Bramhaprakash et al., 2017). These organic matters also contain many macro and micro nutrient in combined form which is unavailable to plants for its growth and metabolism. Soil microbes including PGPR have the potential to solubilise these elements including potassium through various mechanisms and make it available to plants (Pradhan and Mishra, 2015; Dhaked et al., 2017).Archana et al. (2013) isolated 4 potent KMBs from crop field soil. In the present investigation, 30 out

Table 9: Soil potassium content (ppm) with application of the potential organisms and in consortia

Isolate	10 days	20 days	30 days
T1	16.46	16.25 (-1.21 %)	15.99 (-2.85 %)
T2	15.2	14.71 (-3.22 %)	14.4 (-5.26 %)
T3	17.56	15.792 (-10.06 %)	14.04 (-20.04 %)
T4	16.97	14.82 (-12.66 %)	13.64 (-19.62 %)
T5	17.89	16.98 (-5.08 %)	16.02 (-10.45 %)
T6	13.58	12.89 (-5.081 %)	12.23 (-9.94 %)
T7	24.28	21.33 (-12.14 %)	17.89 (-26.31 %)
C	19.43	20.11 (+3.4 %)	20.91 (+7.61 %)

of 84 organisms isolated from the soil samples of various agroclimatic regions of Odisha exhibited potassium mobilization.

4.2 PLANT GROWTH PROMOTING TRAITS OF THE KMB ISOLATES

The 30 potential isolates with KMB potential exhibited PGP traits like IAA production, phosphate solubilization, ammonia production, nitrate reduction, antibiosis and siderophore production. Dinesh et al. (2018) reported that soil bacteria and isolates from industrial effluent (Lakra et al., 2019) exhibited various PGP traits as reported in the present investigation. Pahari and Mishra (2017) isolated siderophore producing bacteria from different regions of Odisha, showing various PGP traits like IAA production, phosphate solubilization, ammonia production, nitrate reduction, antibiosis. On application in crop fields, these PGP microbes increased productivity of rice, mungbean and ground nut (Pradhan et al., 2016). In the present investigation, 6 out of 30 isolates exhibited maximum number of PGP traits; 56, 77, 78 showed eight PGP traits, 46 and 84 were positive for seven and 27 for six PGP traits.

4.3 MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE POTENTIAL ISOLATES

The colony morphology, Gram's variability and biochemical characteristics of the six isolates carried out in accordance with ABIS software for bacterial identification showed the organisms to be species of *Bacillus* and *Coccus* which corroborates with the findings of Pahari et al. (2017) who isolated species of *Bacillus* from the coastal soil samples and halotolerant Enterobacteriaceae, *Clostridium* (Prazmowski, 1880) and *Corynebacterium* spp. (Lehmann & Neumann, 1896) from soil confirmed after biochemical characterization followed by using ABIS software (Rahman et al., 2017).

4.4 EFFECT OF THE ISOLATES ON SUNFLOWER IN POT CULTURE METHOD

A significant increase in shoot length of the sunflower was reported with application of the organisms in isolation and in consortia as compared to the control. Concomitant to this, Pradhan and Mishra (2015) reported a significant increase in shoot length of rice, mung bean and groundnut with the application of rhizospheric bacteria. The potential six isolates exhibiting many PGP traits are effective in increasing the growth of crop with enhanced nutrients availability and plant growth promoting traits. Similar increase in shoot length of brinjal, tomato and okra was reported by Pahari and Mishra (2017) with the application of siderophore producing bacteria isolated from soil of Ganjam and Khurda district of Odisha with reduced application of chemical fertilizer.

4.5 MOBILIZATION OF SOIL POTASSIUM

Park et al. (2003) reported that bacterial inoculation could improve phosphorus and potassium availability in the soils by producing organic acid like oxalic acid, tartaric acids and also due to the production of capsular polysaccharides which helps in dissolution of minerals to release potassium (Sheng and He, 2006; Prajapati et al., 2013) in addition to other growth stimulating chemicals facilitating plant mineral uptake. A significant decrease in sunflower soil potassium content, quantitatively analysed through ICP-OES with application of KMBs could be due to its accumulation of potassium by the plant. Bhattacharyya et al. (2016) reported increase in potash content following application of KMBs to tea soil. The decrease in K content in this finding is due to accumulation of solubilized potassium by sunflower plants (Dash, 2019).

It is evident from the present investigation that the six isolates have a potential for potassium mobilization with various PGP traits which increased growth of the oil seed crop sunflower.

5 CONCLUSION

Six of the 84 isolates mobilized K in addition to different PGP traits. The 6 isolates along with the consortium when applied on sunflower seeds in pot culture showed increased absorption of potassium in the soil. Among the 6 bacterial isolates, T7 showed 144 % increase in shoot length in sunflower plant and 26.31 % decrease in available soil potassium suggests absorption of potassium by the crop plant. The six organisms in consortia, reported better growth of the crop. It is evident that they can supplement potassium to the plants by solubilizing complex forms. Moreover, the organism showing higher PGP traits along with the potassium solubilizing have a greater agricultural and environmental significance and can be a replacement for chemical fertilizers.

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7 REFERENCES

Archana, D., Nandish, M., Savalagi, V., Alagawadi, A. (2013). Characterization of potassium solubilizing bacteria (KSB) from rhizosphere soil. *BIOINFOLET-A Quarterly Journal of Life Science*, *10*, 248-257. https://doi.org/10.9734/JALSI/2017/36848

Ash, C., Priest, F.G., Collins, M.D. (1994). *Paenibacillus* gen. nov. and *Paenibacillus polymyxa* comb. Nov. In Validation of the publication of new names and new combinations previously effectively published outside the IJSB, List no. 51. *International Journal of Systemic Bacteriology*, *44*, 852. https://doi.org/10.1099/00207713-44-4-852

Avakyan, Z.A., Pivovarova, T.A., Karavaiko, G.I. (1986). Characteristics of a new *Bacillus mucilaginosus* species. *Mikrobiologiia, 55*, 477-482.

Bhattacharyya, P.N. & Jha, D.K. (2012).Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*(4), 1327-50. https://doi.org/10.1007/s11274-011-0979-9

Bhattacharrya, P., Dutta, P., Madhab, M., Phukan, I.K. (2016). Isolation of potash mobilizing microorganisms in tea soil and evaluation of their efficiency in potash nutrition in tea: a novel approach. *Two and a Bud, 63,* 8-12.

Bric, J., Bostoc, R., Silverstone, S. (1991). Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Applied Environmental Microbiology*, *57,* 535-538. https://doi.org/10.1128/aem.57.2.535-538.1991

Blake, L., Mercik, S., Koerschens, M., Goulding, S., Stempen, S., Weigel, A., Poulton, P.R., Powlson, D.S. (1999). Potassium content in soil, uptake in plants and the potassium balance in three European long-term field experiments. *Plant and Soil*, *216*, 1–14. https://doi.org/10.1023/A:1004730023746

Brahmaprakash, G.P., Sahu, P., Lavanya, G., Nair, S., Gangaraddi, V., Gupta, A., (2017). Microbial functions of the rhizosphere. In D. Singh, H. Singh, R. Prabha (eds.) *Plant-Microbe Interactions in Agro-Ecological Perspectives*. (pp. 177-210), Springer, Singapore. https://doi.org/10.1007/978-981-10-5813-4_10

Chandler, L. (2013). Challenges in clinical microbiology testing. In: Dasgupta, A., Sepulveda, J.L. (eds.) *Accurate Results in Clinical Laboratory*, Elsevier, pp-315-326. https://doi.org/10.1016/B978-0-12-415783-5.00020-7

Dash, A. (2019). *Biofortification of zinc & potassium by plant growth promoting rhizobacteria on oilseed crops with special reference to growth performance*. M.Sc. Thesis, Odisha University of Agriculture and Technology, BBSR, Odisha, India.

Dhaked, B.S., Triveni, S., Reddy, R.S., Padmaja, G. (2017). Isolation and screening of potassium and zinc solubilizing bacteria from different rhizosphere soil. *International Journal of Current Microbiology and Applied Sciences, 6*(8), 1271-1281. https://doi.org/10.20546/ijcmas.2017.608.154

Dinesh,R.,Srinivasan,V.,Hamza,S., Sarathambal, C., Gowda, S.J., Ganeshamurthy, A, Gupta, S.B., Nair, V., Subila, K., Lijina, A., Divya, V.C. (2018). Isolation andcharacterization of potential zinc solubilizing bacteria from soil and its effect on soil Zn release rates, soil available Zn and plant Zn content. *Geoderma, 321*, 173-186. https://doi.org/10.1016/j.geoderma.2018.02.013

Donaldio, S., Carrano, L., Brandi, L., et al. (2002). Targets and assays for discovering novel antibacterial agents. *Journal of Biotechnology, 99,* 175-185. https://doi.org/10.1016/S0168-1656(02)00208-0

Dye, D.W. (1962). The inadequacy of the usual determinative tests for identification of *Xanthomonas* spp. *New Zealand Journal of Science, 5,* 393-416.

Malathi, P. & Stalin, P. (2018). Evaluation of AB-DTPA extractant for multinutrients extraction in soils. *International Journal of Current Microbiology and Applied Sciences, 7*(3), https://doi.org/10.20546/ijcmas.2018.703.141

Getahun, A., Muleta, D., Aseefa, F., Kiros, S. (2020). Plant growth promoting rhizobacteria isolated from degraded habitat enhance drought tolerance of Acacia (*Acacia abyssinica* Hochst. ex Benth.) seedlings. *International Journal of Microbiology.* ID 8897998. https://doi.org/10.1155/2020/8897998

Gupta, A., Gopal, M., Tilak, K.V. (2000). Mechanism of plant growth promotion by rhizobacteria. *Indian Journal of Experimental Biology*, *38*, 856-862.

Gurav, P.P., Choudhari, P.L., Srivastava, S. (2019). Role of clay minerals in potassium availability of black soils in India. Harit Dhara 2(1): Jan-June. Web link: http://iiss.nic.in/eMagazine/v2i1/10.pdf

Jena, D., Pal, A.K., Rout, K.K. (2009). Potassium management for crops in soils of Orissa. *Proceedings IPI-OUAT-IPNI International Symposium*. Pp: 417-435.

Jordan, E.O. (1890). A report on certain species of bacteria observed in sewage. In Sedgewick, A report of biological work of the Lawrence experiment station, including an account of methods employed and results obtained in the microscopical and bacteriological investigation of sewage and water. *Report on water supply and sewage,* part 2. (pp. 821-844). Massachusetts State Board of Health, Boston.

Knapp, J.S. & Clark, V.L. (1984). Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. *Infection and Immunity, 46,* 176-181. https://doi.org/10.1128/iai.46.1.176-181.1984

Lakra, P. & Mishra, B.B. (2018). Plant growth promoting traits exhibited b metal tolerant bacterial isolates of industrial effluent. *International Journal of Current Microbiology and Applied Science, 7*(5), 3458-3471. https://doi.org/10.20546/ijcmas.2018.705.400

Lakra, P., Pahari, A., Mishra, B.B. (2019). Biocontrol activity of metal tolerant plant growth promoting bacteria isolated from industrial effluent. *Journal of Pharmacognosy and Phytochemistry, 8*(6), 1617-1620.

Lehmann, K.B. & Neumann, R. (1896*). Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen*

Diagnostik (Atlas and outline of bacteriology and textbook of special bacteriological diagnostics). First edition, Munchen:J.F. Lehmann.

Link, J.H.F. (1809). Observationes in ordines plantarum naturals. Dissertation I. *Magazin der Gesellschaft Naturforschenden Freunde Berlin* (in Latin), *3*(1), 10.

Mishra, S.K. & Mishra, P. (2016). Do adverse ecological consequences cause resistance against land acquisition? The experience of mining regions in Odisha, India. *The Extractive Industries and Society, 4*(2017), 140-150. https://doi.org/10.1016/j.exis.2016.11.004

Morgan, J.B. & Connolly, E.L. (2013). Plant-soil interactions: Nutrient uptake. *Nature Education Knowledge, 4*(8), 2.

Naidu, L.G.K., Ramamurthy, V., Sidhu, G.S., Sarkar, D. (2011). Emerging deficiency of potassium in soils and crops of India. *Karnataka Journal of Agricultural Sciences, 24*(1), 12-19.

Nayak, S., Dash, B., Mishra, S., Mishra, B.B. (2020). Chitinase producing soil bacteria: Prospects and applications. *Frontiers in Soil and Environmental Microbiology,* 289-298. https://doi.org/10.1201/9780429485794-30

Nazir, N., Kamili, A., Shah, D. (2019). Mechanism of plant growth promoting rhizobacteria (PGPR) in enhancing plant growth - A Review. *International Journal of Management, Technology and Engineering, 8*(7), 709-721.

Pahari, A., Pradhan, A., Priyadarshini, S., Nayak, S., Mishra, B.B. (2017). Isolation and characterization of plant growth promoting rhizobacteria from coastal region and their effect on different vegetables. *International Journal of Science, Environment and Technology, 6,* 3002-3010.

Pahari, A. & Mishra, B.B. (2017). Antibiosis of siderophore producing bacterial isolates against phytopathogens and their effect on growth of okra. *International Journal of Current Microbiology and Applied Sciences, 6*(8), 1925-1929. https://doi.org/10.20546/ijcmas.2017.608.227

Park, M., Singvilay,O., Seok, Y., Chung, J., Ahn, K., Sa, T. (2003). Effect of phosphate solubilising fungi on P uptake and growth to tobacco in rock phosphate applied soil. *Korean Journal of Soil Science and Fertilizers, 36,* 233–238.

Perez-Lucas, G., Nuria, V., Atik, A.E., Navarro, S. (2018). Environmental risk of groundwater pollution by pesticide leaching through the soil profile, pesticide-use and misuse and their impact in the environment, In M. Larramendy & S. Soloneski, (eds), *IntechOpen*, https://doi.org/10.5772/intechopen.82418

Peters, J. (2011). Average soil test phosphorous and potassium levels decline in Wisconsin. Department of soil science, *Integrated pest and crop management*. Web link: https://ipcm.wisc.edu/blog/2011/01/average-soil-test-phosphorus-and-potassium-levels-decline-in-wisconsin/

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial pecies. *Mikrobiologiya, 17*, 362-370.

Pradhan, A. & Mishra, B.B. (2015). Effect of plant growth promoting rhizobacteria on germination and growth of rice (*Oryza sativa* L.). *The Ecoscan, 9*(1 & 2), 213-216.

Pradhan, A., Mohapatra, S., Samantaray, D., Mishra, B.B. (2016). A note on agricultural importance of PHAs producing *Bacillus* sp. on plant growth promoting activities. *Journal of Advanced Microbiology, 2*, 159-63.

Prajapati, K., Sharma, M.C., Modi, H.A. (2013). Growth promoting effect of potassium solubilizing microorganisms on okra (*Abelmoscus esculentus*). *International Journal of Agriculture Science and Research, 1,* 181-188.

Prazmowski, A. (1880). *Untersuchung uber die Entwickelungsgeschichte und Fermentwirking einiger Bacterin-Arten Inaugural Dissertation*. Hugo Voigt Leipiz, Germany.

Rahman, S.S., Siddique, R., Tabassum, N. (2017). Isolation and identification of halotolerant soil bacteria from coastal Patenga area. *BMC Research Notes, 10*, 531. https://doi.org/10.1186/s13104-017-2855-7

Raghavendra, M.P., Nayaka, N.C., Nuthan, B.R. (2016). Role of rhizosphere microflora in potassium solubilization. In V.S. Meena et al., (eds) *Potassium solubilizing microorganisms for sustainable agriculture*. (pp. 43–59). https://doi.org/10.1007/978-81-322-2776-2_4

Saravanan, V.S., Kumar, M.R., Sa, T.M. (2011). Microbial zinc solubilization and their role on plants. In D.K. Maheshwari (eds) *Bacteria in Agrobiology: Plant Nutrient Management*, (pp. 47–63). https://doi.org/10.1007/978-3-642-21061-7_3

Schwyn, B. & Neilands, J.B. (1987). Universal CAS assay for the detection and determination of siderophores. *Analytical Biochemistry, 160,* 47-56. https://doi.org/10.1016/0003-2697(87)90612-9

Sheng, X.F. & He, L.Y. (2006). Solubilization of potassium-bearing minerals by a wild-type strain of *Bacillus edaphicus* and its mutants and increased potassium uptake by wheat. *Canadian Journal of Microbiology, 52,* 66-72. https://doi.org/10.1139/w05-117

Shrivastava, U.P. & Kumar, A. (2011). A simple and rapid plate assay for the screening of indole 3-acetic acid (IAA) producing organisms. *International Journal of Applied Biology and Pharmaceutical Technology, 2*, 120-123.

Sparks, D.L. (1987). Potassium dynamics in soil. *Advances in Soil Science, 6,* 1-63. https://doi.org/10.1007/978-1-4612-4682-4_1

Sugumaran, P. & Janarthanam, B. (2007). Solubilization of potassium containing minerals by bacteria and their effect on plant growth. *World Journal of Agricultural Science, 3*(3), 350-355.

Tank, N. & Saraf, M. (2010). Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants, *Journal of Plant Interactions, 5*(1), 51-58. https://doi.org/10.1080/17429140903125848

Uchida, R. (2000). Essential nutrients for plant growth: Nutrient functions and deficiency symptoms. In J.A. Silva, and R. Uchida (eds.) *Plant Nutrient Management in Hawaii's soils, Approaches for Tropical and Subtropical Agriculture,* (pp: 31-55).

Zahedi, H. (2016). Growth-promoting effect of potassium-solubilizing microorganisms on some crop species. In: V.S. Meena et al., (eds.) *Potassium solubilising microorganisms for sustainable agriculture* (pp. 31–42). https://doi.org/10.1007/978-81-322-2776-2_3

Zhao, L., Xu, Y., Lai, X. (2018). Antagonistic endophytic bacteria associated with nodules of soya bean (*Glycine max* L.)

and plant growth-promoting properties. *Brazilian Journal of Microbiology, 49,* 269-278. https://doi.org/10.1016/j.bjm.2017.06.007
Zhang, C. & Kong, F. (2014). Isolation and Identification of potassium solubilising bacteria from tobacco rhizospheric soil and their effect on tobacco plants. *Applied Soil Ecology, 82*, 18-25. https://doi.org/10.1016/j.apsoil.2014.05.002

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Correlation, regression and cluster analyses on yield attributes and popping characteristics of popcorn (*Zea mays* L. *everta*) in derived savanna and rainforest agro-ecologies of Nigeria

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Correlation, regression and cluster analyses on yield attributes and popping characteristics of popcorn (*Zea* mays L. *everta*) in derived savanna and rainforest agro-ecologies of Nigeria

Abstract: Information on the genetic and agronomic relationship among the crop characters is important for the breeding programs. This study aimed at determining the relationship among grain yield, popping expansion and other agronomic characters in 19 popcorn lines evaluated in replicated trials at two locations. Correlation analysis was carried out to determine the relationship between agronomic traits while multiple stepwise regression analyses was used to determine the contribution of other agronomic traits to grain yield. Results showed that plant and ear heights as well as cob length exhibited positive and significant association with grain yield. Popping volume showed negative and significant association (-0.45**) with grain yield while 100-grain mass had a negative and significant correlation (-0.37**) with popping volume. Stepwise multiple regression analysis showed that ear height, cob length, plant aspect and 100-grain mass contributed a total of 53.66 % to variation in grain yield, with ear height contributing the highest portion (22.51 %). Cluster analysis grouped popcorn lines into four different clusters, where 'Small Pearl Shaped' and 'Popcorn 33-1-Y' belonged to cluster II and IV, respectively, showing how divergent they are and possible utilization for hybrid formation. Improvement for popcorn should focus on identifying lines with acceptable level of popping volume and improved on their grain yield and yield attributes.

Key words: correlation; qualitative traits; dendrogram; popcorn yield; popping volume; principal component; popcorn; morphology

Korelacijska, regresijska in klasterska analiza dejavnikov, ki vplivajo na pridelek in ekspanzijske lastnosti pokovke (*Zea mays* L. *everta*) v agroekosistemih prehodne savane in deževnega gozda Nigerije

Izvleček: Informacije o povezanosti genetskih in agronomskih lastnosti poljščin so pomembne za žlahtniteljske programe. Namen te raziskave je bil določiti povezavo med pridelkom zrnja, stopnjo ekspanzije in drugimi agronomskimi lastnostmi 19 linij pokovke, ovrednoteno v poskusih s ponovitvami na dveh lokacijah. Za določitev povezav med agronomskimi lastnostmi je bila uporabljena korelacijska anliza, za določitev prispevka posameznih agronomskih lastnosti na pridelek zrnja je bila uporabljena multipla postopna regresija. Rezultati so pokazali da so imeli višina rastlin, višina izraščanja storžev in dolžina storžev značilno pozitivno povezavo s pridelkom zrnja. Volumen ekspandiranih zrn je pokazal značilno negativno povezavo (-0,45**) s pridelkom zrnja, masa 100 zrn je imela značilno negativno korelacijo (-0,37**) z volumnom ekspandiranih zrn. Analiza s postopno multiplo regresijo je pokazala, da višina storža na rastlini, njegova dolžina, izgled rastline in masa 100 zrn prispevajo 53,66 % variabilnosti v pridelku zrnja, pri čemer višina storža na rastlini prispeva največji delež (22,51 %). Klasterska analiza je združila linije pokovke v štiri različne grozde, kjer sta bili 'Small Pearl Shaped' in 'Popcorn 33-1-Y' uvrščeni v II in IV skupino, kar kaže na njuno veliko različnost in možnost uporabe pri tvorbi križancev. Izboljšave pri pokovki bi se morale osredotočiti na prepoznavanju linij s sprejemljivim volumnon ekspandiranih zrn in izboljšanim pridelkom zrnja ter izboljšanimi lastnostmi povezanimi s pridelkom.

Ključne besede: korelacija; kakovostne lastnosti; dendrogram; pridelek pokovke; volumen ekspadiranih zrn; glavna komponenta; pokovka; morfologija

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1 INTRODUCTION

Success in improvement of grain yield and popping quality of popcorn requires the understanding of the nature of relationships between component traits that determine yield and other quantitative characters. Among the tools that provide such information are correlation and regression analyses. Correlation refers to the degree of measure of association between two characters or degree to which they vary among themselves (Mohanan, 2010). Association among characters can be measured in terms of direction (viz: positive or negative) and/or magnitude of the association. A positive correlation implies that improvement of one trait can lead to the improvement of the other trait through indirect selection while a negative correlation shows that improvement of one trait leads to the declination of another trait. This suggests that the knowledge of association of traits such as grain yield and/or popping expansion with other agronomic traits is very pertinent in putting together selection criteria or indices useful for popcorn (*Zea mays* L. *everta*) improvement.

Popcorn is cultivated for grain yield and popping quality and previous studies have shown that the two traits are negatively correlated. For example, the report of a study conducted by Vijayabharathi et al. (2009) revealed a positive association between popping expansion volume and popping expansion ratio, whereas the relationship between ear length and popping expansion ratio as well as between grain yield and popping expansion ratio was negative. In an earlier study, Dofing et al. (1991) had reported a negative association between volume expansion and yield components (such as ear length, ear diameter, 50-kernel mass), except for number of kernel rows/ear. The information obtained from these study implies that selection for large popping volume will result to reduction of grain yield and vice versa. Moradi and Azarpour (2011) as well as Sreckov et al. (2011) in separate studies also reported that grain yield was significantly and positively correlated with ear length, rows/ear, plant height and ear prolificacy.

While correlations provide information on the nature and magnitude of association between characters, regression analysis determines the significant level of contribution of each independent variable to the dependent variable, such as grain yield. Traits that contribute significantly to dependent variable help to justify the amount of variation observed in the dependent trait, thereby predicting the outcome of the trait. There are scanty information in literature on the grain yield and popping determinants of local popcorn types in Nigeria compared to field corn. However, study conducted by Alikhani et al. (2010) on sweet corn (*Zea mays* convar. *saccharata* Koern.) reported a significant contribution of 1000-grain mass and grain number per square meter to observed grain yield. Similarly, Zhang et al. (2013) reported that ear length, kernel row number, plant height and ear height contributed significantly to average grain yield in field corn. Consequently, studies targeted towards improving popcorn yield should focus on traits that contribute significantly to its yield.

Apart from gaining information on relationships among traits, it is also important to determine the extent of relationship among genotypes for the purpose of hybridization. The use of multivariate approach permits the evaluation of genetic material on a set of traits that combine multiple pieces of information in a way to select the most promising materials, considering the relative importance of the traits for the total existing variance (Cruz et al., 2014). Cluster analysis among other multivariate approaches shows a great deal in providing knowledge of genetic diversity between parents, aiming to identify the hybrid combinations with heterotic effect and high level of heterozygosity, and to identify duplicates in germplasm banks (Cruz et al., 2014). This technique assists breeders in identifying genotype combinations with good heterotic effect, thereby increasing the possibility of coming up with improved cultivars.

Popcorn is a popular and nutritious delicacy, which is primarily utilized for human consumption as freshly popped corn, snacks, confectioneries, biscuits and cornflakes (Iken, 1993). It can be found on almost all the streets, campuses and at cinemas in Nigeria, serving as main or additional source of income for youths and other stakeholders in popcorn industry. This shows the high level of popcorn consumption in Nigeria Popcorn is a consumed by people of different ages and status in Nigeria. Its production is however faced with poor popping expansion with low ratio of popped to unpopped and consequently poor economic return to the processors. The available varieties were also poor in grain yield per hectare with marginal profit to farmers, while some farmers completely abandoned it production because of these production challenges. Some processor therefore resort to buying exotic popcorn varieties with high exchange rate to further deplete the foreign reserve. Crop breeding as a matter of fact requires proper understanding of the agronomic traits of the crop as well as the interactions of the traits for yield. This study was therefore designed to determine (i) the nature of the relationship between grain yield, popping expansion and other agronomic characters, (ii) the percentage contribution of agronomic traits to grain yield in popcorn, and (iii) and the genetic diversity among the tested popcorn lines. This vital information will provide plant breeders with some selection criteria for breeding popcorn for good popping expansion, higher grain yield and consumer acceptability.

2 MATERIALS AND METHODS

The details of the genetic materials used for this study have been provided elsewhere (Olakojo et al., 2019). Briefly, the genetic materials comprised 19 popcorn lines that were collected from various part of southwestern Nigeria and improved for uniformity through intra-population selection prior to evaluation. A commercial variety (Eruwa Local), which is commonly grown by farmers, was used as a check variety as there are no released popcorn varieties in Nigeria so far. The study was conducted at the Institute of Agricultural Research and Training (IAR & T), Moor Plantation, Ibadan (Latitude 3^{0}50'E Longitude 7$^°$ 22' N) and IAR & T Research Station at Ikenne, Ogun State, (Latitude 3$^°$ 42 'E Longitude 6 ^{0} 53' N) representing derived savannah and rainforest agro-ecologies of Nigeria, respectively. The trial was laid out in a randomized complete block design with three replications. Each plot had two five-meter long rows. Two seeds were sown per hole at a spacing 75 × 50 cm to give 44 stands per plot of 3 × 5 meter square with an alley of one meter between one plot and the other to give a total experimental area of 17 × 27 m. A total of 180 kg ha^{-1} of NPK 20:10:10 fertilizer was applied at three weeks after sowing (3 WAS) with 100 kg ha^{-1} of urea six weeks after sowing (6 WAS). Weeds were chemically controlled with the application of pre-emergence herbicides (250 g l^{-1} Metolachlor and 250 g l^{-1} Atrazine a.i.) @ 5 l ha^{-1}. Two supplementary hoeing were carried out 6 WAS and 10 WAS to reduce crop-weed competition.

Data on flowering, diseases, aspects and grain yield were taken on all the plants per plot while agronomic characters were recorded on five random plants per plot in each replication. The data on popping characters were taken after popcorn varieties from each replicate has been bulked. Data were collected on days- to- 50 % tasseling and silking (recorded as the number of days from sowing to when 50 % of the plants had emerged tassel and silks in a plot, respectively), plant and ear height (cm) (measured as the distance (cm) from the base of the plant to the first tassel branch (plant) and node bearing the upper leaf (ear)); plant aspect, ear aspect and, husk cover, using a 1-5 rating scale, where 1 = excellent, 2 = very good, 3 = good, 4 = fair and 5 = poor, according to Badu-Apraku et al. (2012), cob length, cob width, number of rows/cob, number of kernels/row, moisture content was measured with the aid of a moisture meter at harvest, while grain yields were computed at the adjusted moisture content of 12.0 % using the formula (Tandzi and Mutengwa, 2019):

$$GY = \frac{\text{Field mass x } 10\,(100 - \text{MC}) \text{ x } 0.8}{(100 - \text{Adjusted MC}) \text{ x } (5 \text{ x } 1.5)}$$

Where MC = Moisture content (%); 0.8 = shelling coefficient and the harvested area in m^2, GY= Grain yield.

Diseases scored for includes streak (*Maize streak virus*), rust (*Puccina polysora* Underw.), blight (*Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker), curvularia, using a rating of 1-5. Morphological data was also recorded at different stages of growth (seedling, vegetative and flowering stage). Stem color and broadness of leaves were recorded at the seedling stage; leaf color, color of mid-rib, color of leaf blade, leaf orientation at vegetative stage; color of anther, color of silk, nature of anthesis at flowering stage; cob shape, cob circumference, cob length, shape of the tip and kernel row arrangement at harvest while seed characters such as seed color, seed shape, number of seeds per row, number of rows per cob and presence or absence of awn were recorded at post-harvest stage by mere observation of the cobs.

Data collected were subjected to statistical analysis using Statistical Analysis System (SAS) version 9.3 (SAS institute, 2011) Pearson correlation and multiple stepwise regression analyses were carried out to determine the relationship between traits and contribution of other agronomic traits to grain yield, respectively. Principal Component Analysis (PCA) was also conducted to determine the contribution of each trait to variability in the performance of the popcorn lines. Cluster analysis was carried out using ward linkage dendrogram with the aid of Statistical Package for Social Science (SPSS) (IBM Corp, 2011).

3 RESULTS AND DISCUSSION

Morphological characteristics of the 19 popcorn lines are presented in Table 1. Results showed that stem color ranged from purple to brown while 'Popcorn 2-S_0' was the only variety with green stem color. This unique feature of 'Popcorn 2-S_0' for stem color suggests that this line is in a class of its own and this color uniqueness can serve as a distinguishing factor and as a morphological marker that differentiates it from other popcorn lines. Green leaf color was observed for most of the popcorn evaluated except for small pearl shaped and 'Popcorn 36-Y' which had light green leaf color. The dominating nature of green among the lines show the high level of chlorophyll content in them. The lines with light green leaf may possibly share the same parental line. Color of mid-rib ranged from light green to white. However, 'Popcorn 34-Y', 'Popcorn 4-Y' and 'Popcorn 52-Y' had green mid-rib color. With mid-rib color ranging between green, light green and white, the light green mid-rib colored materials are likely to have emanated from the combination between green and white. The leaf broadness of 'Popcorn 9-Y', 'Popcorn 20-Y', large pearl shaped, 'Popcorn 2-S_0', 'Popcorn 37-Y' and 'Popcorn 33-1-Y' is desirable as its wide plant architecture in terms of area covered tend to reduce weed emergence by preventing light penetration into the soil. The wide canopy structure could also promote better assimilation of light during photosynthesis thereby increasing grain filling and consequently grain yield (Sun et al., 2019). Popcorn lines with fairly broad or narrow leaf as well as erect leaf orientation ('Popcorn44-Y' and large pearl shaped) are also desirable as they allow increase in plant population when planted sole. There will be less completion among plants since area covered by these lines of popcorn is minimal. Similarly, the characteristics suggest the lines suitable for better intercropping with other crops. Erect leaf orientation was observed in 'Popcorn 44-Y' and large pearl shaped while other lines were drooping. The color of anther ranged from green to light green while some of the varieties had purple dots on their green anther, which is an indication that it can be used to distinguish their respective popcorn lines from others. 'Popcorn 4-Y' is unique among others with respect to silk color (cream), suggesting that this particular trait can be used as a morphological marker to identify this genotype during simple selection exercise, or to track the line when used in composite varietal development.

Most of the popcorn lines had primary-secondary branching anthesis, while few of them had only primary branching anthesis. Leaf blades of the popcorn lines were mostly green. However, 'Popcorn 44-Y', 'Popcorn 6-Y' and 'Popcorn 52-Y' had brown leaf blade while that of 'Eruwa' local was white.

Table 2 shows the morphological characteristics of cob and kernel of the 19 popcorn lines. Some of the popcorn lines had cylindrical cob shape while others were cylindrical-conical. 'Popcorn 9-Y' and 'Popcorn 3-Y' in particular had conical shape. Conical shaped cob tends to have higher measure of cob width while cylindrical shaped cob gives lesser value of cob width. As earlier reported by Reddy et al. (2003), the degree of popping and reduced cob girth were the only criteria that could be used as selection criteria for improving popping expansion. It may therefore be safe to assume that lesser cob width proportionately give larger popping expansion. Popcorn breeders may therefore be guided by this phenomenon by selecting for cylindrical shaped cob for enhanced popping expansion. These cylindrical shaped popcorn varieties may be good sources of gene for higher popping expansion. Kernel row arrangement for most of the lines was either regular or straight. 'Popcorn 33-1-Y' and 'Popcorn 36-Y' however had an irregular arrangement of kernel row while 'Popcorn 66-Y' and 'Popcorn 37-Y' had a spiral kernel arrangement. 'Popcorn 3-Y' had a strongly pointed kernel shape compared to other popcorn lines with either pointed or round shape, showing its distinctiveness with respect to this trait. The presence of awn on the kernels of all the popcorn suggests that they are closer to the wild type than the domesticated type and they tend to be resistant to many popcorn pests, and can be utilized as source of resistant gene for future breeding effort towards resistance to prevalent popcorn pest especially maize weevil. The deep yellow color observed on the kernels of some of the popcorn lines suggests that they have more number of complementary genes controlling them than those with light yellow coloration. During popping, the flakes are likely to assume the natural color of the kernel, therefore the use of artificial coloring during popping to make it appealing to children by some popcorn processors and sellers may not be necessary.

Pearson correlation coefficients of grain yield and other characters are presented in Table 3. Plant heights (0.41**) and ear heights (0.42**) as well as cob length (0.36**) correlated positively and significantly with grain yield which suggests a strong and positive relationship among these traits probably the two traits are being controlled by the same gene. This may be as a result of linkage or pleiotropy, indicating that an improvement in any of these traits could lead to an improvement in grain yield and vice versa. This invariably focuses on the importance of ear placement as one of the selection criteria for popcorn yield improvement. Hence, ear height and cob length may be considered necessary as traits to be looked out for in any popcorn improvement program relating to grain yield. This observation agrees with previous reports of Gautam et al. (1999) and Singh et al. (2006). However, the association between plant aspect and grain yield, as well as husk cover and grain yield were negative and significant ($\rho \leq 0.05$). Therefore lower rating for husk cover and plant aspect on the field is important for enhanced yield. Loose husk cover predisposes popcorn ears to invasion of bird pests as they find it easy to peck on it because of its small kernel nature. The results further showed thatcob width and 100-grain mass were negatively cor-

Table 1: Morphological characteristics of 19 popcorn lines

Popcorn lines	Stem colour	Leaf colour	Colour of mid-rib	Leaf broadness	Leaf orientation	Colour of anther	Colour of silk	Nature of anthesis	Colour of leaf blade
Popcorn 44-Y	purple	green	light green	fairly broad	erect	green	purple	pry	brown
Popcorn 18-Y	brown	green	white	fairly broad	drooping	light green	purple	pry	green
Popcorn 9-Y	purple	green	light green	broad	drooping	light green	white	pry-sec	green
Popcorn 34-Y	brown	green	green	fairly broad	drooping	GWPD	purple	pry-sec	green
Popcorn 4-Y	purple	green	green	fairly broad	drooping	green	cream	pry-sec	green
Popcorn 66-Y	brown	green	light green	fairly broad	drooping	green	purple	pry-sec	green
Small pearl shaped	purple	light green	light green	fairly broad	drooping	light green	purple	pry-sec	green
Popcorn 40-Y	purple	green	white	broad	drooping	GWPD	purple	pry	green
Popcorn 20-Y	purple	green	white	broad	drooping	light green	white	pry-sec	green
Large pearl shaped	brown	green	light green	broad	erect	GWPD	purple	pry-sec	green
Popcorn 2-So	green	green	white	broad	drooping	GWPD	purple	pry-sec	green
Popcorn 3-Y	purple	green	white	fairly broad	drooping	light green	purple	pry-sec	green
Popcorn 32-Y	purple	green	white	fairly broad	drooping	GWPD	purple	pry-sec	green
Popcorn 37-Y	brown	green	white	broad	drooping	green	purple	pry-sec	green
Popcorn 33-1-Y	purple	green	white	broad	drooping	GWPD	white	pry-sec	green
Popcorn 6-Y	purple	green	white	fairly broad	drooping	GWPD	purple	pry	brown
Popcorn 52-Y	purple	green	green	fairly broad	drooping	green	white	pry-sec	brown
Popcorn 36-Y	brown	light green	light green	narrow	drooping	light green	purple	pry	green
Eruwa local (check)	purple	green	light green	airly broad	drooping	GWPD	purple	pry-sec	white

GWPD = green with purple dots; pry = primary; pry-sec = primary-secondary

Table 2: Morphological characteristics of cob and kernel of 19 popcorn lines

Popcorn lines	Cob shape	Shape of cob tip	Kernel row arrangement	Kernel shape	Presence of awn	Kernel colour
Popcorn 44-Y	cylindrical	normal	regular	round	+	deep-y
Popcorn 18-Y	cylindrical	curved	regular	pointed	+	light-y
Popcorn 9-Y	conical	normal	regular	round	+	deep-y
Popcorn 34-Y	cyl-conical	normal	straight	round	+	deep-y
Popcorn 4-Y	cylindrical	normal	regular	round	+	deep-y
Popcorn 66-Y	cylindrical	normal	spiral	round	+	light-y
Small pearl shaped	cylindrical	normal	straight	pointed	+	deep-y
Popcorn 40-Y	cyl-conical	normal	straight	round	+	light-y
Popcorn 20-Y	cyl-conical	normal	straight	round	+	light-y
Large pearl shaped	cylindrical	normal	regular	round	+	light-y
Popcorn 2-So	cyl-conical	normal	regular	round	+	deep-y
Popcorn 3-Y	conical	curved	regular	strongly pointed	+	light-y
Popcorn 32-Y	cyl-conical	curved	straight	pointed	+	deep-y
Popcorn 37-Y	cylindrical	curved	spiral	pointed	+	deep-y
Popcorn 33-1-Y	cylindrical	normal	irregular	round	+	light-y
Popcorn 6-Y	cylindrical	curved	straight	pointed	+	light-y
Popcorn 52-Y	cylindrical	curved	regular	pointed	+	light-y
Popcorn 36-Y	cylindrical	normal	irregular	round	+	light-y
Eruwa local (check)	cyl-conical	normal	straight	pointed	+	light-y

Cyl-conical - cylindrical-conical + = awn was present; Light-Y = light yellow; Deep-Y = deep yellow

related with popping volume suggesting that these pairs of variables cannot be improved simultaneously. This implies that larger kernels will likely have a reduced popping volume. Small size of grain will bring about large number per mass thereby giving higher popping expansion by volume, knowing fully well that expansion is by volume while increased number of kernels will enhance the mass. Hence, popcorn breeders focusing on enlarged popping volume should select smaller kernels to boost the expansion of popcorn flakes. Guatam et al. (1999) also reported negative and significant correlation between 1000-kernel mass and popping expansion. Findings of Reddy et al. (2003) equally revealed that degree of popping and lesser cob girth were the only criteria which could be used as selection indices for improving popping expansion. Therefore, improvement of popping volume will be effective if selection is based on popcorn individuals with lesser cob width and kernel size. Grain yield on the other hand had a negative but significant association with popping volume. The negative correlation between grain yield and popping volume has been reported by several authors in previous studies (Dofing et al., 1991; Burak and Broccoli, 2001; Vijayabharathi et al., 2009; Rangel et al., 2011). This seems to be one of the major problems in popcorn improvement as these two traits appear to be of great importance in popcorn industry. However, large popping volume is desirable by all stakeholders in popcorn industry being the end-product trait to be reckoned with. Popping expansion of popcorn should therefore be considered as a primary trait and associating traits should be focused on during selection exercise.

Stepwise multiple regression analyses for grain yield indicating the contribution of component traits to popcorn grain yield is shown in Table 4. The results showed that ear height, cob length, plant aspect and 100-grain mass contributed a total of 53.66 % to grain yield, with ear height contributing the highest portion (22.51 %), followed by 17 % contribution by cob length and 10.17 % contribution by plant aspect while 100-grain mass contributed the least portion (3.98 %). The results further explain the level of significance observed in these contributions, which in all cases were at 0.01 level of probability.

$$Yi = a + bX1 + ei$$

Table 3: Pearson correlation coefficient for grain yield related traits of 19 popcorn lines

	DS	PH	EH	HC	PA	EA	EPP	CLT	RCB	KNR	CBM	GMT	PV	YLD
DT	0.95**	-0.26**	-0.21*	0.39**	0.40**	0.10	0.03	-0.14	-0.18	-0.27**	-0.16	0.37	-0.29*	-0.10
DS		-0.26**	-0.19*	0.34**	0.38**	0.10	0.03	-0.14	-0.17	-0.29**	-0.13	0.29*	-0.40**	-0.05
PH			0.96**	-0.66**	-0.65**	-0.59**	0.03	0.51**	0.49**	0.48**	0.45**	-0.13	-0.06	0.41**
EH				-0.66**	-0.58**	-0.56**	0.09	0.48**	0.45**	0.45**	0.38**	-0.07	-0.22	0.42**
HC					0.71**	0.57**	0.05	-0.37**	-0.23**	-0.28**	-0.32**	-0.05	0.34**	-0.37**
PA						0.54**	0.04	-0.39**	-0.25**	-0.29**	-0.35**	-0.01	0.03	-0.45**
EA							0.02	-0.38**	-0.22*	-0.37**	-0.21*	0.26*	-0.002	-0.39**
EPP								0.17	-0.12	0.20*	-0.06	-0.003	-0.14	0.21*
CLT									0.31**	0.71**	0.37**	-0.07	0.04	0.36**
RCB										0.35**	0.39**	-0.11	0.26*	0.07
KNR											0.27**	-0.29*	0.30*	0.17
CBM												0.41**	-0.32*	0.21*
GMT													-0.37**	0.16
PV														-0.45**
YLD														

*, **, significantly different at 0.05 and 0.01 levels of probability, respectively
DT, days to tasseling; DS, days to silking; PH, plant height; EH, ear height; HC, husk cover; PA, plant aspect; EA, ear aspect; EPP, ears/plant; CLT, cob length; RCB, rows/cob; KNR, kernels/row; CBM, cob mass; GMT, 100-grain mass, PV, popping volume; YLD, yield

Where *Yi* is the mean measurement of the dependent variable (Grain yield), *Xi* is the measurement of the independent variables, *b* is the slope of regression line of *Yi* on *Xi*, a is the value of Y when X = 0 and *ei* is the error associated with the *Yi*.

Principal Component Analysis (PCA) identifies the characters that contribute most to the variation within a group of entries (Ogunbodede, 1997). PCA data generated 21 components axes with eigen values ranging from 0.00-3.73, and components with above 9 % proportion of variance were retained The first four principal components cumulatively accounted for about 55 % of total variation (Table 5), while the first principal component (PC1) contributed approximately 20 % of observed variation which was associated with popping volume, grain yield, 100-grain mass and husk cover. The second principal component (PC2) contributed about 16 % of the variation and was associated with plant height, ear height, grain yield, days to tasseling and silking, cob length and kernels/row. The third principal component (PC3) on the other hand contributed about 11 % of the variation which are linked to cob length, kernels/row, rows/cob, streak, ears per plant and plant height while the fourth principal component (PC4) contributed about 9 % of the observed variation and was associated with ear aspect, husk cover, cob width and 100-grain mass. PCA can be estimated from the contribution of different variables to each principal component according to the Eigen vectors (Lezzoni and Pritts, 1991). Therefore, it is considered that the characters grouped into each of the components are associated in one way or the other and are governed by the same type of gene action. Consequently, characters with high loading value within the first four principal components such as ear aspect, cob width, popping volume and cob length are of high relevance and should be considered for selection in improvement program of popcorn.

Cluster analysis refers to a group of multivariate techniques which group individuals or objects with respect to the characters they possess, such that individuals with similar descriptions are gathered into the same cluster (Hair et al., 1995). In this study, popcorn lines in cluster I (popcorn '34-Y' to popcorn '32-Y') are grouped together on the basis of their similarities which can be traced (Olakojo et al., 2019) to some attributes such as medium cob length, grain filling potential (number of kernels/row), tallness of the plant and early maturity. The second cluster comprising of 6 popcorn lines (small pearl shaped - popcorn 20-Y) are mostly low

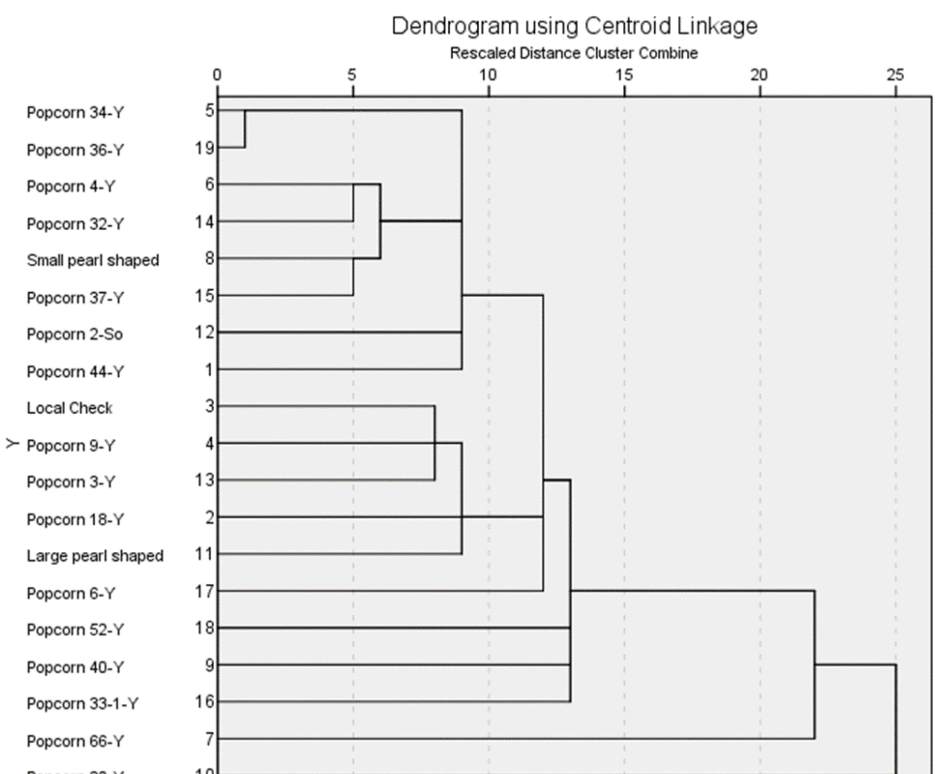

Figure 1: Dendrogram of relationship among 19 popcorn lines

Table 4: Summary of Stepwise multiple regression for grain yield in 19 popcorn lines

Characters	Partial R	% contribution to yield	F-value
Ear height	0.2251	22.51	15.97**
Cob length	0.1700	17.00	15.17**
Plant aspect	0.1017	10.17	10.72**
100 - grain mass	0.0398	3.98	4.46*

*, ** significantly different at 0.05 and 0.01 levels of probability, respectively

Table 5: Eigen values and vectors of observed characters in 19 Popcorn lines and their PCA values

Characters	PC1	PC2	PC3	PC4
Days to tasseling	-0.244	-0.277	-0.038	-0.231
Days to silking	-0.274	-0.284	-0.064	-0.201
Plant height	-0.127	0.360	-0.263	0.142
Ear height	-0.225	0.347	-0.135	-0.056
Husk cover	0.265	-0.045	0.059	0.401
Plant aspect	0.114	-0.255	0.201	-0.097
Ear aspect	0.015	-0.218	-0.048	0.510
Ear per plant	-0.075	0.186	0.363	0.113
Streak	0.144	-0.253	0.374	0.153
Rust	0.188	-0.138	-0.023	-0.018
Blight	0.00	0.00	0.00	0.00
Curvularia	0.00	0.00	0.00	0.00
Army worm	0.051	0.162	-0.117	0.018
Cob length	0.029	0.290	0.440	0.156
Rows/cob	0.135	-0.004	-0.378	0.200
Kernels/row	0.201	0.257	0.402	-0.019
Cob width	-0.194	-0.092	-0.149	0.489
100-Grain mass	-0.248	-0.245	0.112	0.299
Popping volume	0.450	0.056	-0.101	-0.041
Grain Yield	-0.295	0.330	0.149	0.142
Eigen values	3.732	3.073	1.996	1.728
% Variance	19.64	16.17	10.50	9.09
% cumulative	19.64	35.81	46.32	55.41

streak (maize streak virus), rust (*Puccina polysora*), blight (*Bipolaris maydis*), curvularia, army worm (*Spodoptera frugiperda*)

yielding with short height, short cob length and has good popping potential. The 5 lines grouped together in cluster III have the measure of only 100-grain mass in common but closely associated with those in cluster IV with respect to this attribute (100-grain mass). Other attributes found to be similar among the 3 popcorn lines in cluster IV are long cob length, high yield and low popping volume. This confirms the strong relationship that existed between cob length and grain yield and also affirm the contribution of cob length to grain yield among the popcorn lines used in this study. This trait may be considered for yield improvement in popcorn breeding. Moreover, with cluster IV having low popping volume but high yield; it is at par with the result presented in Table 3, which shows the negative association between grain yield and popping volume of the lines.

Popcorn lines grouped within the same cluster are expected to exhibit high internal homogeneity while those between clusters are to exhibit high external heterogeneity. Therefore, each cluster in the dendrogram can be considered as a heterotic group. In other words, crosses made between inbred population developed from lines in cluster II (such as small pearl shaped) and that of cluster IV (such as popcorn '33-1-Y') may likely give high heterosis leading to the development of commercial F_1 hybrids for the popcorn industry.

4 CONCLUSION

Ear height and cob length have demonstrated a great deal in affecting grain yield of the evaluated popcorn lines. They also contribute significantly to grain yield of popcorn. These traits no doubt should be of high consideration as selection criteria for the improvement of popcorn yield. The observed (positive) relationships among these traits will enable simultaneous improvement of these characters, thus saving time and resources. Similarly, lesser grain mass should be the target during improvement for popping expansion. Different heterotic group where the highest yielding popcorn and largest popping popcorn lines belong is an indication of the potential value of these materials for future breeding plans especially for hybrid development.

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6 REFERENCES

Alikhani, M.A., Khazaei, F., Yari, L. and Khandan, A. (2010). Study on the correlation, regression and path coefficient analysis in sweet corn (*Zea mays* var. *saccharata*) under different levels of plant density and nitrogen rate. *ARPN Journal of Agricultural and Biological Science, 5*(6), 14-19.

Badu-Apraku, B., Fakorede, M.A.B., Menkir, A. and Sanogo, D. (editors). (2012). *Conduct and Management of Maize Field Trials*. IITA, Ibadan, Nigeria. 59p.

Burak, R. and Broccoli, A. M. (2001). Genetic and environmental correlations between yield comonents and popping expansion in popcorn hybrids. *Maize Genetics Cooperation Newsletter, 75*, 38-40.

Cruz, C.D., Carneiro, P.C.S. and Regazzi, A.J. (2014). *Modelos biométricos aplicados aomelhoramento genético*. 3rd edn. Ed. UFV, Viçosa.

Dofing, S.M., Croz-Mason, N.D. and Compton, M.A.T. (1991). Inheritance of expansion volume and yield in two popcorn x dent corn crosses. *Crop Science, 31,* 715-718. https://doi.org/10.2135/cropsci1991.0011183X003100030035x

Gautam, A.S., Mittal, R.K and Bhandari, J.C. (1999). Correlations and path analysis in popcorn. *Annals of Biology* (Hissar) *15*(2), 196.

Hair, J. R., Anderson, R. E., Tatham, R. L. and Black, W. C. (1995). *Multivariate data analysis with readings*. 4th edition, Prentice-Hall, Englewood cliffs, NJ.

IBM Crop, Releases (2011). *IBM SPSS Statistics for Windows*, version 20.0. Armonk, NY: IBM Corp.

Iken, J.E. (1993). Popcorn production and utilization. In Fakorede, M.A., Alofe, O.O. and Kim, S.K. (eds). *Maize Improvement Production and Utilization in Nigeria.*

Lezzoni, A.F. and Pritts, M.P. (1991). Application of principal component analysis to horticultural research. *Horticultural Science, 26*(4), 334-338. https://doi.org/10.21273/HORTSCI.26.4.334

Mohanan, K. (2010). *Essentials of Plant Breeding PHI Learning Private*. Ltd. New Delhi.

Moradi, M. and Azarpour E. (2011). Determination of most important part of yield components by path analysis in corn. *Journal of American Science, 7*, 134-147.

Ogunbodede, B.A. (1997). Multivariate analysis of genetic diversity in kenaf (*Hibiscus cannabinus* L.). *African Crops Science Journal, 5*(2), 127-133. https://doi.org/10.4314/acsj.v5i2.27855

Olakojo, O.O., Olaoye, G., Akintunde, A.T. (2019). Performance of popcorn introductions for agronomic characters, grain yield and popping qualities in the forest and derived savannah agro-ecologies of Nigeria. *Acta agriculturae Slovenica, 114*(1), 53-60. https://doi.org/10.14720/aas.2019.114.1.6

Rangel, R.M., Junior, A.T.A and Junior, S.P.F. (2011). Association between agronomical traits and popping expansion in a popcorn population under recurrent selection. *Cienciae agrotechnologia, 35*(2), 225-233. https://doi.org/10.1590/S1413-70542011000200001

Reddy, V.S., Mohan, Y. C., Rao, N.V and Krishna, L. (2003). Character association and path analysis in popcorn (*Zea mays* var. *everta*). *Crop research, 25*(2), 297-300.

Rupak, K., Verma and Singh, T.P. (1979). Interrelations among certain quantitative traits in popcorn. *The Mysore Journal of Agricultural Science, 13*, 15-18.

SAS Institute (2009). *SAS system for Windows v. 9.3*. SAS Inst. Inc., Cary, NC.

Singh, H., Chawla J. and Grewa, M. (2006). Correlation and path coefficient analysis on some elite maize genotypes. *Crop Improvement, 33*, 31-33.

Sun, J., Gao, J., Wang, Z., Hu, S., Zhang, F., Bao, H. and Fan Y. (2019). Maize canopy photosynthetic efficiency, plant growth, and yield responses to tillage depth. *Agronomy*, *9*(3), 1-18. https://doi.org/10.3390/agronomy9010003

Sreckov, Z., Nastasic A., Bocanski J., Djalovic I., Vukosavljev M. and Jockovic B. (2011). Correlation and path analysis of grain yield and morphological traits in test-cross populations of maize. *Pakistan Journal of Botany, 43*, 1729-1731.

Tandzi, N. L. and Mutengwa, C. (2019). Estimation of maize (*Zea mays* L.) yield per harvest area: appropriate methods. *Agronomy. https://doi.org/10.3390/agronomy10010029*

Vijayabharathi, A., Anandakumar, C.R. and Gnanamalar, R.P. (2009). Combining ability analysis for yield and its components in popcorn (*Zea mays* var. *everta* Sturt.). *Electronic Journal of Plant Breeding, 1*, 28-32.

Zhang, H., Wang, X., He, D. and Shui, H. (2013). Regression and correlation analysis between high-yield stability and main agronomic traits in maize. *Journal of Southern Agriculture*, *44*(10), 1625-1628.

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Foliar silicate application improves the tolerance of celery grown under heat stress conditions

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Foliar silicate application improves the tolerance of celery grown under heat stress conditions

Abstract: Temperature rise is one of the most challenging climate change impacts that increase the intensity of heat stress. In this investigated the production of celery (*Apium graveolens* var. *rapaceum* F1 hybrid)) was tested during the late season. The experiment was carried out during the two successive summer seasons of 2019 and 2020 in Giza Governorate, Egypt. The experimental design is a split-plot, the main plots consist of three low tunnel cover treatments, and three spray treatments with three replicates in sub-main plots. Results showed that the use of white net cover gave the highest vegetative growth and yield followed by the black net. Values of plant yield were 951, 765, and 660 g/plant for white, black and without cover, respectively, in the first season. The foliar application of 3 mM of potassium silicate produced the highest vegetative growth and yield compared to the control treatment. Referring to the effect of spray foliar application of potassium silicate on yield 1.5 mM (S1), 3 mM (S2), and control were 892, 795, and 689 g/plant in the first season, respectively. The best combination that delivered the highest vegetative growth and yield was a cover low tunnel with a white net combined with S2 foliar application.

Key words: celery**;** climate measures; physical protection; vegetative growth; chemical analysis

Foliarno dodajanje silikata izboljšuje toleranco zelene, ki raste v razmerah vročinskega stresa

Izvleček: Dvig temperatrure je eden izmed največjih izzivov podnebnih sprememb, katerega učinki povečujejo jakost vročinskega stresa. V tej raziskavi je bil v zvezi s tem preučevan pridelek zelene (*Apium graveolens* var. *rapeceum* F1 hibrid) tekom pozne rastne sezone. Poskus je bil izveden v dveh zaporednih poletnih sezonah 2019 in 2020 na območju upravne enote Giza, Egipt. Poskus je bil izveden kot poskus z deljenkami, kjer so obravnavanja na glavnih ploskvah obsegale tri vrste nizke tunelske kritine in tri obravnavanja s škropljenjem s tremi ponovitvami na podploskvah. Rezultati so pokazali, da je uporaba bele mreže kot kritine dala največji prirast biomase in največji pridelek, čemur je sledila uporaba črne mreže. V prvi rastni sezoni so bili pridelki 951, 765, in 660 g/rastlino za belo, črno kritino in brez nje. V primerjavi s kontrolo je foliarno dodajanje 3 mM kalijevega silikata dalo največjo prirast biomase in največji pridelek. Glede na učinek foliarnega dodajanja kalijevega silikata (1,5 mM (S1), 3 mM (S2), in kontrola) na velikost pridelka so bile njegove vrednosti 892, 795, in 689 g/rastlino v prvi rastni sezoni. Najboljša kombinacija, ki je povzročila najboljšo rast in dala največji pridelek je bil nizek tunel pokrit z belo mrežo in s S2 foliarnim obravnavanjem.

Ključne besede: zelena**;** podnebne razmere; fizikalna zaščita; vegetativna rast; kemijska analiza

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1 INTRODUCTION

The global climate is expected to witness an increase in temperature in the range 2–4 °C by the end of 21st century (IPCC, 2007). More importantly, predictions based on global climate model analysis suggested that the tropical and subtropical regions of the world will be the worst to suffer from the forthcoming heat stress (Battisti and Naylor, 2009). Because of rising temperature, alterations in plant's phenology such as spring and autumn, phenology was noticed across different plant species (Li et al., 2014).

Improving micro-climatic conditions for horticultural plants and its influence on the plant growth and productivity were considered of the critical factors that control the need to continuous production allover the year (El-Gayar et al., 2018). Studies conducted by Zakher and Abdrabbo (2014) showed that shading could increase plant growth and productivity through moderating of the harmful effects of high air temperature during summer season for tomato plants. Plastic screen nets in the form of protected cultivation covering materials were widely used for many purposes in the horticulture sector. For example, it was used to protect crops from different farming negatively influencing factors such as heat waves, strong wind, flying insects, mamals, and birds (Al-Helal and AbdelGhany, 2010). Applying screen net regardless of its color had been proved to protect plant against environmental risks such as high air temperature, excessive solar radiation and wind, which improve microclimate for the grown crops through reduction heat, drought stresses, and moderation of extreme climatic events which led to improving crop yield and quality (Abul-Soud et al., 2014). The application of plastic net covers in crop production was a sufficient way to provide a cheap and reduced energy consuming technology than polyethylene greenhouses (Shahak, 2008). Abdrabbo et al. (2013) stated that open field treatment had a higher air temperature than white net, while black net had the lowest air temperature during summer season. Regarding the effect of cover net on relative humidity Hashem et al. (2011) stated that relative humidity increased under black net by 2–4 % compared with open field conditions. Vegetative growth parameters such as plant height, number of leaves, leaf area and productivity under white net were expressively higher than that under open field (Medany et al., 2009). Treder et al. (2016) proved that covering the greenhouses with screen net increases light scattering without affecting the light spectrum which led to increase light efficiency that reflected on increasing growth and production measures.

One of the most common applications for protecting plants from heat stress is foliar spraying with Si, which is approved to be a good option concerning the food productivity; consequently, using Si application was recommended as one of the acceptable practices to increase of vegetable plants productivity (Bakhat et al., 2018). Therefore, several stress factors such as heat waves, which affect vegetables and its productivity are managed by the foliar application of Si via mitigate the injurious impacts of stressors (Cooke and Leishman, 2016).

Also, Si is considered as a growth regulator, which participates in the regulation of physiological processes in plants including seed germination, stomata closure, ion uptake and transport, membrane permeability, photosynthesis and plant growth rate according to Noura et al. (2019). This research was conducted to study the effect of protection of celery plant using black and white screen net as well as three foliar applications of potassium silicate and their interactions on vegetative growth, and yield of celery during summer season.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE

This study was carried out in Dokki Location, Giza Governorate, Egypt, during the summer seasons of 2019 and 2020. Dokki location is located at latitude 30.03 and longitude 31.20 with an altitude of 23 m above sea level. Describing the climate of the region; it is dry during summer season, while warm and moderate rain during winter season. The soil of the experimental site is clay soil and having bulk density 1.16 g cm^{-3}, pH in soil paste (1:2.5) 7.81, EC_e 2.39 dS m^{-1}, and field capacity 25.77 %.

2.2 EXPERIMENTAL PROCEDURE

Seedlings of celery (*Apium graveolens* L. var. *rapaceum* (Miller) Gaudin F1 hybrid) with one month from seed germination was used in the current applied study. Seeds were obtained from Takii and Co. LTD (Kyoto, Japan). Seedlings were transplanted into substrate system on 16th and 18th of March in the 2019 and 2020 seasons, respectively. The following measurements were performed for five labeled plants per replication for each treatment at the end of growing seasons: plant length, number of leaves per plant, base plant diameter, chlorophyll content as well as celery yield. Total of nitrogen (N), phosphorus (P) and potassium (K) in leaves were measured, ascorbic acid (vitamin C) and soluble sugar were measured in the fresh leaves. Experimental plots were arranged in a split plots design with three replicates. Each experimental plot contained five raised beds (4 m length x 0.8 m width). The distance between each two beds was 0.50 m.

The experimental design consists of main plots and sub-plots. The main plot is comprised of three cover treatments including white screen net, black screen net, and control (without cover). And the sub-plot contains 1.5 mM (S1), 3 mM (S2), and 0 mM (S0) of potassium silicate with S0 serving as control (sprayed with tap water). The silicate foliar applications were sprayed on the plant leaves three times, at 3, 5 and 15 weeks from cultivation, at a rate of 50 ml per plant for each. Three replicates were used in this study. Celery plants were irrigated using drippers with flow rate of 4 l h^{-1} and the distance between each two plants was 0.30 m. Chemical fertilizers $(NH_4)_2SO_4$ (20.6 % N), K_2SO_4 (48 % K_2O) and P_2O_5 (37 % P_2O_5) were injected within irrigation water system at the rate of 80, 40 and 50 kg $acre^{-1}$ respectively for fertigation purpose. The fertigation was programmed to be three times weekly, and the duration of irrigation time depended highly upon the plant needs. All treatments received the same quantity of fertilizers.

2.3 PLANT ANALYSES

Plant samples (outer leaves) were collected after six weeks from transplanting and dried in the oven at 70 °C for one day. Total nitrogen (N) in the dried leaves, digested by H_2SO_4/H_2O_2 mixture, was measured using Kjeldahl method according to the procedure described by Chapman and Pratt (1961). Total phosphorus (P) was measured using spectrophotometer according to Watanabe and Olsen (1965) and total potassium (K) in leaves was measured using flame photometer as described by Jackson (1958). Total chlorophyll was measured using chlorophyll meter SPAD-502Plus. Soluble sugar content was measured by photometer using the anthrone-sulfuric acid method (Yemm & Wills 1954). Ascorbic acid (vitamin C) was measured in the fresh leaves following 2, 6, dichlorophenol indophenol visual titration method (A.O.A.C., 1980).

2.4 CLIMATE MEASURES

Light intensity, maximum and minimum temperature as well as relative humidity were measured under different screen net cover treatments every day using digital climatic sensors. Digital thermo-hygrograph (model: TFA Dostman/Wertheim - Kat. Nr. 5002) was used to measure temperature and relative humidity. The digital thermo-hygrograph was allocated over polystyrene trays in the middle of each treatment above the level of celery plants canopy and the maximum air temperature was recorded at 13:00, while the average relative humidity was calculated by the average of maximum and minimum relative humidity every day. The average weekly maximum temperature and humidity was calculated using the daily climatic data. Light intensity was measured in each treatment daily above the celery plants canopy at mid-day (13:00) by portable Lux-meter (Model FMC- 10M). The average weekly light intensity was calculated from the measured data.

2.5 STATISTICAL ANALYSIS

Analysis of data was done using SAS program (SAS, 2000). The differences among means for all traits were tested for significance at 5 % level using LSD according to Waller and Duncan (1969).

2.6 ECONOMIC ANALYSIS OF APPLIED TREATMENTS

Economic analysis, after considering the cost of cover celery with screen net and potassium silicate, the incomes from celery yield was used (CIMMYT, 1988) according to the formulas:

(Net Income = value of obtained yield – annual cost of screen net and potassium silicate application).

(Relative increase in income (RII) = (net income / income of control) x 100.)

The lifetime of screen net is five years. The cost of spray application of potassium silicate was considered.

3 RESULTS AND DISCUSSION

3.1 CLIMATIC DATA

The average maximum air temperatures for the physical protection treatments showed that the use of screen net influenced maximum and minimum temperature (Figure. 1 and 2). Temperature tended to be lower under the black net cover by almost 3 °C compared to open field conditions. The white net reduced the maximum air temperature by almost 1 °C compared to ambient conditions. The minimum air temperature took the same trend, the lowest minimum air temperature was recorded under the black screen net; while the white slightly lower than the black

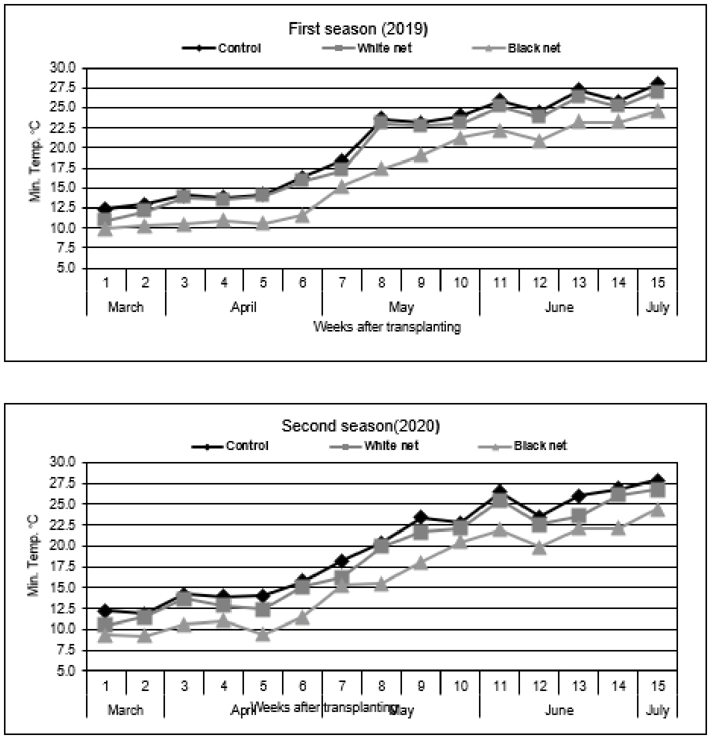

Figure 1: The minimum air temperature under black net and white net compared to the open field of the two studied seasons of 2019 and 2020

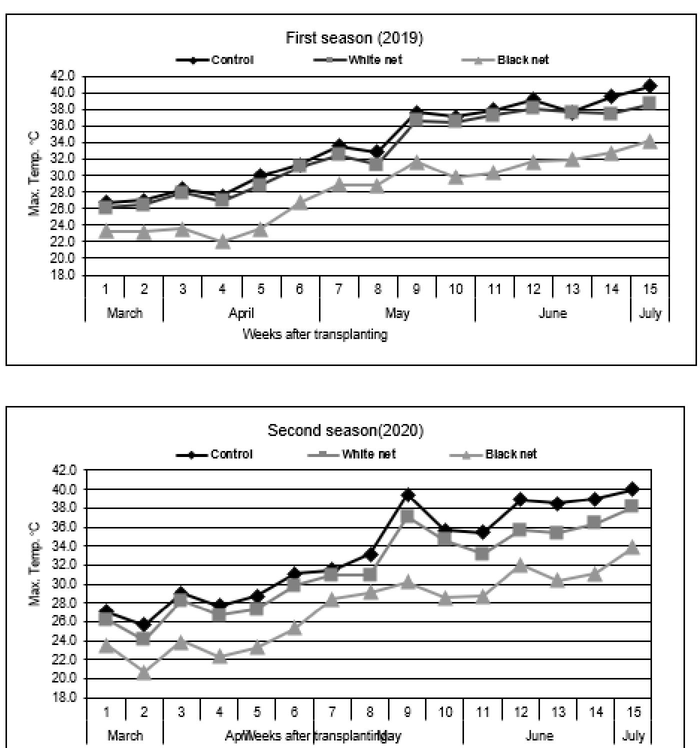

Figure 2: The maximum air temperature under black net and white net compared to the open field of the two studied seasons of 2019 and 2020

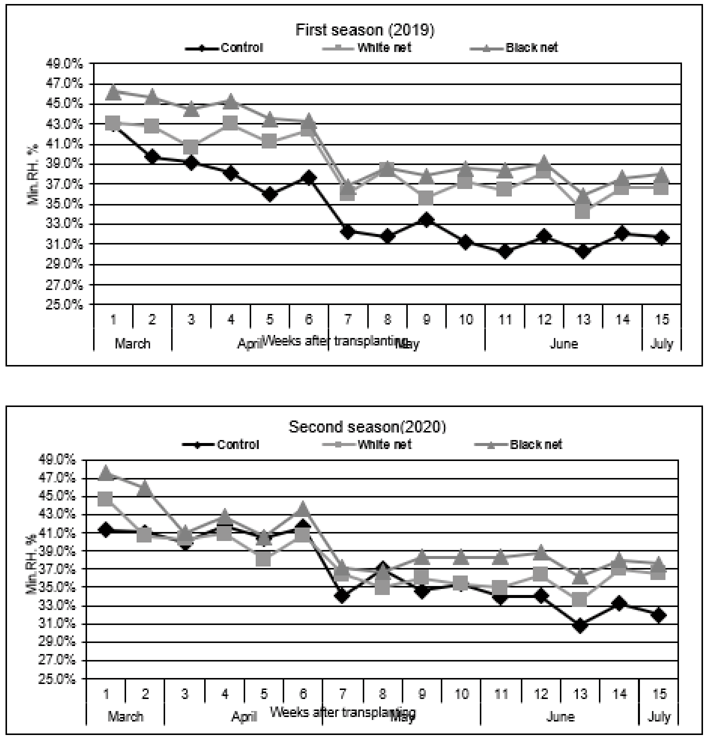

Figure 3: The minimum relative humidity under black net and white net compared to the open field of the two studied seasons of 2019 and 2020

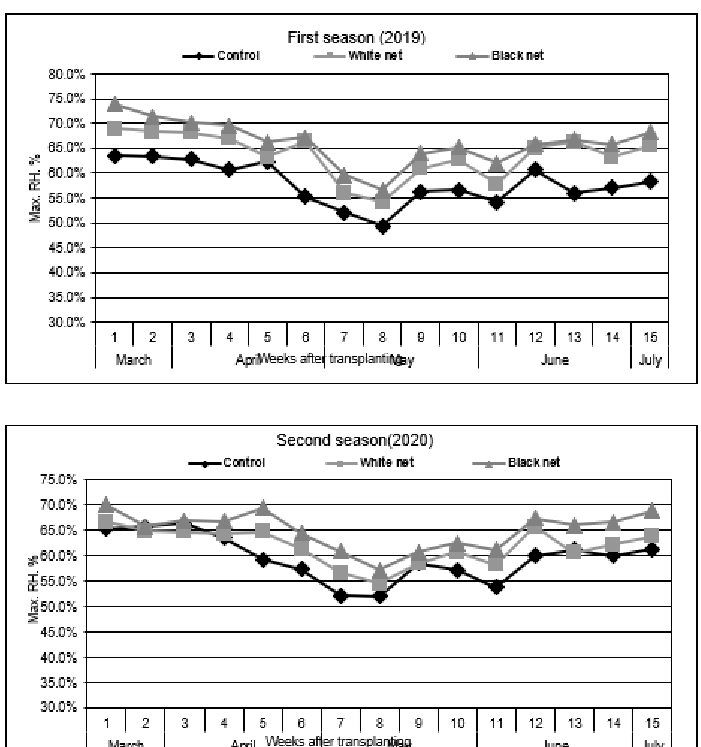

Figure 4: The maximum relative humidity under black net and white net compared to the open field of the two studied seasons of 2019 and 2020

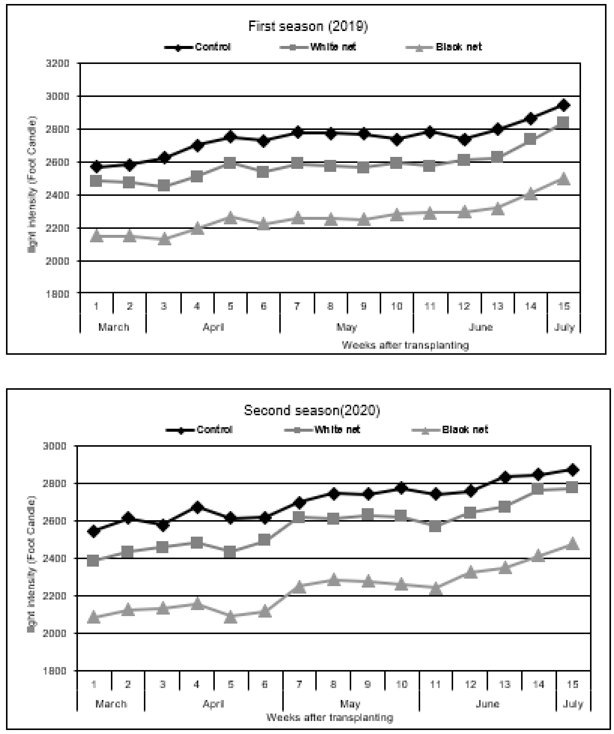

Figure 5: Light intensity under black net and white net compared to the open field of the two studied seasons of 2019 and 2020

considering the minimum temperature (about 0.5 °C), which is lower compared to open field conditions. The same trend of maximum and minimum temperature was obtained during both seasons. The maximum relative humidity took another trend; the open field had the lowest relative humidity during both seasons. Black net cover had the highest average maximum and minimum relative humidity followed by the white net cover during the two studied seasons (Figure. 3). Relatively, humidity under the black net cover was higher by 3 – 5 % than open field. Maximum average weekly light intensity under different physical protection treatments showed that open field conditions recorded the highest light intensity followed by the white net cover while the black net screen net had the lowest light intensity during both seasons (Figure. 4). The obtained results of the low temperature under physical protection treatments according to the observation, achieved lower interception of sun radiation rays under screen cover than ambient conditions. Use of screen net especially black nets penetrated light intensity and increase relative humidity by 2-5 %. Similar results were reported by Al-Helal and Abdel-Ghany (2010) and Abdrabbo et al. (2013) who indicated that covering the plants with black or white net led to the reduction of the temperature because of the lessening of the radiation via reflection or absorption by covered materials. Formerly addressed by Shahak et al. (2008) that using screen net led to decrease in air temperature around the cultivated plants in comparison with open field. In conclusion, the lowest recorded maximum temperature was achieved by black net

3.2 VEGETATIVE GROWTH

The effect of different net covers on celery vegetative growth characteristics, *i.e.,* plant height, number of leaves per plant, base of plant diameter and chlorophyll content, were presented in Table 1 and 2. Data showed that using black net cover significantly increased the celery plant height, followed by a white net cover, while the lowest plant height was obtained without applying net cover, during the two studied seasons. Number of leaves per plant, base plant diameter and chlorophyll took different trends, the highest values were obtained by white net cover followed by black net procedure. The lowest number of leaves per plant, base of plant diameter and chlorophyll were obtained by open field.

Regarding the foliar application using two concentrations of potassium silicate, the highest plant height was obtained by S2 treatment followed by S1. The lowest plant height was obtained by control. Number of leaves per plant, base of plant diameter and chlorophyll took the same trend.

Regarding the interaction between different net covers and foliar application of potassium silicate, data illustrated that the highest plant height were obtained by black net cover combined with S2 foliar application. Number of leaves per plant, base of plant diameter and chlorophyll took another trend. The highest values were obtained by covering with white net combined with S2 foliar application. The lowest vegetative growth of celery plants was obtained by open field treatment combined with control. The same results were obtained by Tubana and Heckman (2015) who stated that application of silicon led to decrease the harmful effects of heat waves and then enhancing the plant growth and productivity. The same result was confirmed by Bakhat et al. (2018), they concluded that application of silicon compound as foliar application led to enhancement of plant growth and improving the ability of plants to combat the abiotic stresses such as heat waves. On the other hand, the protection of celery plants from high temperature using black net led to increase the plant height due to low light intensity under the tunnel, which led to improvement of the plant elongation. Using white net led to decrease the stress from exposure to direct sun radiation without reducing the light intensity such as black net, which also led to increasing the reception of plant leaves to daylight and increasing photosynthesis and then the enhancement of plant growth parameter such as number of leaves and plant diameter (Medany et al., 2009). Moreover, use of screen net led to reduction of the daylight intensity but increasing the light usage efficiency due to sun rays scattered when penetrate the screen later, which led to achieve the sun rays a new angels that led to reach for all plant leaves and then increases the total plant photosynthesis (Abul-soud et al., 2014). The screen net could also increase solar radiation scattering up to 50 %; that enhanced plant growth. On the other hand, dark net reduced radiation reaching crops canopy (Shahak et al., 2004). Low light inten-

Table 1: Different efficiencies of different net covers on celery vegetative growth characteristics during the first growing season of 2019

Treatment		Plant height (cm)	Number of leaves	Base plant diameter	chlorophyll	Yield (g)
			cm	SPAD	g/plant	
			Net covers treatment			
black		59.3	68.8	4.05	30.6	765
white		47.1	73.8	5.01	36.2	951
control		39.5	56.6	3.72	45.2	660
LSD 5%		3.07	2.37	0.37	2.42	5.82
			Potassium Silicate treatment			
S0		35.8	55.9	3.33	42.5	689
S1		49.3	68.5	4.53	36.0	795
S2		60.8	74.8	4.92	33.5	892
LSD 5%		4.18	2.03	0.146	1.05	4.84
			Interaction between cover net and slilicate spray			
black	S0	42.0	58.7	3.66	33.5	653
	S1	59.7	68.4	4.22	31.8	718
	S2	76.1	79.3	4.27	26.4	925
white	S0	33.4	66.9	3.90	42.8	863
	S1	48.9	72.3	5.41	29.6	988
	S2	59.1	82.1	5.70	36.3	1003
control	S0	32.0	41.9	2.42	51.2	553
	S1	39.3	64.9	3.96	46.7	680
	S2	47.1	63.0	4.77	37.8	748
LSD 5%		1.95	2.19	0.256	2.47	7.03

S0 (sprayed with tap water), S1 (1.5 mM of potassium silicate), S2 (3 mM of potassium silicate), SPAD (Unit for determines the amount of chlorophyll present by measuring absorbance two wavelength regions), LSD 5% (Significance at 5 % level)

Table 2: Different efficiencies of different net covers on celery vegetative growth characteristics during the second growing season of 2020

Treatment		Plant height (cm)	Number of leaves cm	plant diameter SPAD	chlorophyll g/plant	Yield (g)
				Net covers treatment		
black		61.0	67.7	5.19	26.9	808
white		56.6	76.4	5.55	34.4	912
control		41.6	49.8	3.50	38.3	677
LSD 5%		2.75	3.42	0.26	2.35	6.04
				Potassium Silicate treatment		
S0		50.4	60.3	4.19	35.8	674
S1		52.8	61.3	4.86	34.4	798
S2		55.9	72.3	5.19	29.5	924
LSD 5%		1.792	1.032	0.253	1.985	5.07
			Interaction between cover net and slilicate spray			
black	S0	56.0	63.0	4.89	27.5	723
	S1	60.3	69.4	5.11	28.4	830
	S2	66.7	70.6	5.56	24.9	870
white	S0	54.7	70.6	5.44	36.5	740
	S1	56.7	76.4	5.43	35.4	933
	S2	58.3	82.3	5.78	31.4	1063
control	S0	40.7	47.3	2.22	43.3	560
	S1	41.3	37.9	4.04	39.5	630
	S2	42.7	64.2	4.22	32.2	840
LSD 5%		1.03	1.84	0.209	1.83	6.93

S0 (sprayed with tap water), S1 (1.5 mM of potassium silicate), S2 (3 mM of potassium silicate), LSD 5 % (Significance at 5 % level)

sity under black net resulting from netting affected the micro-climatic conditions and reduce the plant growth especially in the winter season because of low light intensity (Hashem et al., 2011). Furthermore, silicon application increased the chlorophyll content of plant leaf and enhanced the antioxidant system in plants that were exposed to abiotic stress which led to better photosynthesis (Al-aghabary et al., 2004)

3.3 CELERY YIELD

Presented data in Table (1 and 2) shows that there were significant effects considering the used cover on celery plants wither it is the black or white screen net, the celery yield was improved during both seasons. The indicated data from using white net coverage led to increasing in celery yield significantly; secondly came the black net coverage, while the lowest mass of celery was obtained by control treatment.

Effect of foliar application treatments of potassium silicate on celery yield was significantly noticeable during both seasons. The high concentration of potassium silicate (S2) treatment gave the highest celery yield followed by low concentration of potassium silicate (S1). The lowest celery yield was obtained by control treatment during both seasons.

Concerning the interaction effect of screen net coverage and foliar application of potassium silicate, it was statistically significant; the highest celery yield was obtained by white net cover combined with S2 foliar application during the two season followed by black screen net cover combined with S2 foliar application. The lowest celery yield was obtained by control (without cover) treatment combined by without foliar application. The same results were obtained by Piotr et al. (2009) who mentioned that using cover screen led to enhance celery stalks quality, however the dark screen cover decreased dry matter content and obtained yield. Zakher and Abdrabbo (2014) studied the growing vegetable crops during the summer using shading net; shading led to decrease air temperature, which reduces plant growth and lower yield percentage. Another study was conducted considering the production of celery during summer season by using screen net; the results indicated that white screen net led to increasing the plant growth, celery quality and productivity (Siwek et al., 2009). As of the effect of silicon, it enhanced plant tolerance during summer season. Bakhat et al. (2018) reveled that silicon improved plant growth and productivity under abiotic stresses conditions. Cooke and Leishman (2016) had the same results under heat waves stresses compared to plants without silicon foliar application. Moreover, foliar application of potassium silicate led to the increase of potassium concentration in celery leaves under different screen net coverage; Potassium (K) is necessary for the function of all living cells and is thus present in all plant tissues. K is a vital element for plant growth and productivity as well as the quality of produced vegetables (Marschner, 2012). Shen et al. (2009) concluded that foliar application by silicon compound led to relieve of high-temperature stress in vegetables. In addition, silicon application protects cultivated vegetables against the ultraviolet-B radiation by increasing photosynthesis and antioxidant levels. High level of ultraviolet-B radiation produces a wide physiological damage to plants, which had been implanted during summer season.

3.4 CHEMICAL ANALYSIS OF CELERY OUTER LEAF

Table 3 and 4 shows that the concentration of N, P,

Table 3: Different efficiencies of different net covers on chemical analysis of celery outer leaf during the first growing season of 2019

Treatment		N	P	K	Soluble sugars content	Vitamin C
%		%	%	$mg.kg^{-1}$ fresh	mg / 100g fresh	
				Net covers treatment		
black		1.49	0.27	3.11	6.59	2.52
white		1.78	0.39	3.80	6.97	2.86
control		1.85	0.49	4.48	8.23	3.44
$LSD_{5\%}$		0.11	0.08	0.26	0.39	0.22
				Potassium Silicate treatment		
S0		1.95	0.44	3.30	8.05	3.28
S1		1.62	0.36	3.63	7.20	2.92
S2		1.55	0.34	4.45	6.53	2.62
$LSD_{5\%}$		0.07	0.03	0.19	0.66	0.15
				Interaction between cover net and slilicate spray		
black	S0	1.88	0.32	2.70	7.72	2.98
	S1	1.30	0.24	2.90	6.59	2.64
	S2	1.29	0.23	3.73	5.46	1.93
white	S0	1.84	0.46	3.57	7.30	2.96
	S1	1.86	0.37	3.54	6.93	2.80
	S2	1.63	0.34	4.29	6.67	2.81
control	S0	2.13	0.54	3.65	9.13	3.89
	S1	1.70	0.47	4.46	8.08	3.31
	S2	1.72	0.46	5.33	7.47	3.11
$LSD_{5\%}$		0.06	0.04	0.38	0.21	0.15

S0 (sprayed with tap water), S1 (1.5 mM of potassium silicate), S2 (3 mM of potassium silicate), N (Nitrogen), P (phosphorous), K (potassium). $LSD_{5\%}$ (Significance at 5 % level

K, soluble sugar content and vitamin C in celery leaves cultivated under the tested treatments during the two studied seasons. It indicated that, in general, the treatment of covered celery plants was sufficient to give high values of the studied macronutrient percentages N, P and K as well as soluble sugar content and vitamin C in the celery leaf. Plants that were covered by white net gave the lowest N, P, K, soluble sugar content and vitamin C, while the highest values were obtained by control treatment; due to effect of heat stress for plants at ambient conditions which reduced the photosynthesis and metabolism, leading to storing the nutrient in the plant tissues (Abul-Soud et al., 2014 and Zakher and Abdrabbo, 2014).

On the other hand, the appropriate microclimate under white and black screen net led to enhancement of the plant primary metabolism and then improves the ability of plant roots to absorb water and fertilizer from soil without stress which led to the enhancement of growth parameters (Hashem et al., 2011; Medany et al., 2009).

Regarding the using of potassium silicate foliar application treatments, data in Table 3 and 4 indicated that using 3.0 mM of potassium silicate increased K percentage in celery leaf more than the other treatments. N and P took another trend, as the control treatment had the highest percentages in celery's outer leaves. It may be due to the role of potassium silicate in improving the ability of plants to combat the stresses during summer and enhancing the machinery and metabolism, which reflected on increasing plant mass making dilution effect of nutrients in its tissues (Abd El-Rahman et al., 2018 and Zakher and Abdrabbo, 2014).

Table 4: Different efficiencies of different net covers on chemical analysis of celery outer leaf during the second growing season of 2020

Treatment		N	P	K	Soluble sugars content	Vitamin C
				$mg.kg^{-1}$		
%		%	%	fresh	mg / 100g fresh	
				Net covers treatment		
black		1.41	0.29	3.74	6.18	2.59
white		1.70	0.36	4.47	7.44	3.17
control		1.90	0.37	5.04	8.30	3.45
$LSD_{5\%}$		0.16	0.06	0.29	0.40	0.12
				Potassium Silicate treatment		
S0		1.90	0.39	3.73	8.42	3.52
S1		1.65	0.34	4.41	7.31	3.08
S2		1.46	0.29	5.10	6.19	2.61
$LSD_{5\%}$		0.13	0.03	0.56	0.93	0.22
				Interaction between cover net and slilicate spray		
black	S0	1.76	0.36	3.08	7.68	3.24
	S1	1.16	0.27	3.49	5.76	2.41
	S2	1.31	0.23	4.66	5.10	2.12
white	S0	1.75	0.39	4.02	7.98	3.38
	S1	1.83	0.35	4.59	7.65	3.27
	S2	1.53	0.33	4.80	6.68	2.86
control	S0	2.20	0.41	4.11	9.59	3.95
	S1	1.95	0.40	5.16	8.52	3.56
	S2	1.55	0.32	5.84	6.78	2.85
$LSD_{5\%}$		0.07	0.03	0.204	0.11	0.11

S0 (sprayed with tap water), S1 (1.5 mM of potassium silicate), S2 (3 mM of potassium silicate), N (Nitrogen), P (phosphorous), K (potassium). $LSD_{5\%}$ (Significance at 5 % level)

Table 5: Economic analysis for using cover net and potassium silicate during the first growing season of 2019

Cover	Potassium Silicate	Avge. yield kg/plant	Yield Ton/ Acre	Grass in-come Acre	Cover cost *L. E/ Acre	Annual cover cost *L. E/ Acre	Applied Potassium Silicate cost Liter/ Acre	Potassium Silicate cost Cost/ Acre	Spray cost *LE/ Acre	Total treat-ments cost *LE	Net in-come *L. E/ GH	Incremental income *L. E / GH
Black net	S0	653	15.7	31320	21000	4200	0	0	150	4350	26970	600
	S1	718	17.2	34440	21000	4200	3	360	150	4710	29730	3360
	S2	925	22.2	44400	21000	4200	6	720	0	4920	39480	13110
White net	S0	863	20.7	41400	21000	4200	0	0	150	4350	37050	10680
	S1	988	23.7	47400	21000	4200	3	360	150	4710	42690	16320
	S2	1003	24.1	48120	21000	4200	6	720	0	4920	43200	16830
control	S0	553	13.3	26520	0	0	0	0	150	150	26370	0
	S1	680	16.3	32640	0	0	3	360	150	510	32130	5760
	S2	748	17.9	35880	0	0	6	720	0	720	35160	8790

Average price 2 LE/ kg
cover cost 5 LE/ m^2
The greenhouse applied by one acre net cover
Potassium Silicate cost 120 L.E/Liter
Average currency change rate = (1 USD = 16 L.E.)

Table 6: Economic analysis for using cover net and potassium silicate during the second growing season of 2020

Cover	Potassium Silicate	Avge. yield kg/plant	Yield	Grass income	Cover cost	Annual cover cost	Applied Potassium Silicate cost	Potassium Silicate cost	Spray cost	Total treat-ments	Net income	Incremental income
			Ton/ Acre	Acre	L. E/ Acre	L. E/ Acre	Liter/ Acre	Cost/ Acre	LE/ Acre	cost LE	L. E/ GH	L. E / GH
Black net	S0	723	17.4	34720	21000	4200	0	0	150	4350	30370	3640
	S1	830	19.9	39840	21000	4200	3	360	150	4710	35130	8400
	S2	870	20.9	41760	21000	4200	6	720	0	4920	36840	10110
White net	S0	740	17.8	35520	21000	4200	0	0	150	4350	31170	4440
	S1	933	22.4	44800	21000	4200	3	360	150	4710	40090	13360
	S2	1063	25.5	51040	21000	4200	6	720	0	4920	46120	19390
control	S0	560	13.4	26880	0	0	0	0	150	150	26730	0
	S1	630	15.1	30240	0	0	3	360	150	510	29730	3000
	S2	840	20.2	40320	0	0	6	720	0	720	39600	12870

Average price 2 LE/ kg
cover cost 5 LE/ m^2
The greenhouse applied by one acre net cover
Potassium Silicate cost 120 L.E/Liter
Average currency change rate = (1 USD = 16 L.E.)

3.5 ECONOMIC ANALYSIS

Cost of using nets for protect celery plants were 21000 Egyptian pound (L.E.) per acre, for white or black nets during the two studied seasons (Tables 5 and 6). We consider a lifetime for the cover screen net of 5 years then the annual cost of covering with net was 4200 L.E. The cost of spray potassium silicate was also considered in this analysis. The other costs of production were not considered such as labor, inputs, irrigation, etc., because these are the same for the tested treatments (under white and black screen net as well as open field) on one acre of celery. Compared to control, the benefits (total gross profit) of using the different treatments were higher than cultivate in open field. White net combined with application of potassium silicate S2 was superior in yield of two years, comparing with the other treatments during both seasons; the white net combined with S1 came in the second order; the lowest values was obtained by open field treatment combined with absent potassium silicate application. Regarding the relative increase in income compared to control treatment; the white net with S1 and S2 gave the highest values; use of potassium silicate S2 came in the third option. The lowest relative increase in income was obtained by without screen cover combined with absent potassium silicate treatment. From the above we can conclude that using of physical or chemical protection led to the improvement of the profitability of celery during the early season compared to the control treatment.

4. CONCLUSIONS

This research provided evidence on how to produce winter leafy crops such as celery plants during the early summer season providing high quality production by applying the required physical and chemical protection for plants. The results showed that using of screen net coverage increased plant growth and provided high quality yield compared to the situation without appropriate coverage. Also, the acquired results gave a recommendation for the usage of potassium silicate as foliar application for plant protection from heat waves. This study confirmed that silicon has a beneficial effect of foliar application. Using white screen net combined by foliar application of potassium silicate 3 mM gave the highest yield of celery and enhanced the vegetative growth and yield during the late season.

5. REFERENCES

A.O.A.C., (1980). *Association of Official Methods of Analytical Chemists, Official Methods of Analysis* 13th ed., Washington, D.C., U.S.A.

Abd El-Rahman, N. G., Emam, M. S. A., Farag, A. A. & Abdrabbo M. A. A. (2018). Use of aquagel-polymer as a soil conditioner for celery plants grown in sand culture. Future of food. *Journal on Food, Agriculture and Society, 6*(1), 85-94.

Abdrabbo, M. A., Farag, A. A. & Abul-Soud, M. A. (2013). The intercropping effect on potato under net house as adaption procedure of climate change impacts. *Researcher, 5*(6), 48-60.

Abul-Soud, M. A., Emam, M. S. A., & Abdrabbo, M. A. A. (2014). Intercropping of some brassica crops with mango trees under different net house colour. *Research Journal of Agriculture and Biological Sciences*, *10*(1), 70-79.

Al-aghabary, K., Zhu Z., & Shi, Q. (2004). Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidant enzyme activities in tomato plants under salt stress. *Journal of Plant Nutrition*, *27*, 2101–2115. https://doi.org/10.1081/PLN-200034641

Al-Helal, I. M. & Abdel-Ghany, A. M. (2010). Responses of plastic shading nets to global and diffuse PAR transfer Optical properties and evaluation. *Journal of Life Sciences*, *57*, 125–132. https://doi.org/10.1016/j.njas.2010.02.002

Bakhat, H. F., Bibi, N., Zia, Z., Abbas, S., Hammad, H. M., Fahad, S., Ashraf, M. R.; Shah, G. M., Rabbani, F., & Saeed, S. (2018). Silicon mitigates biotic stresses in crop plants: A review. *Crop Protection Journal, 104*, 21–34. https://doi.org/10.1016/j.cropro.2017.10.008

Battisti, D. S., & Naylor, R. L., (2009). Historical warnings of future food insecurity with unprecedented seasonal heat. *Science, 323*, 240-244. https://doi.org/10.1126/science.1164363

Cimmyt. (1988). *An Economic Training Manual: From Agronomic Data to Farmer Recommendations*. Mexico, 1-25.

Cooke, J., & Leishman, M. R. (2016). Consistent alleviation of abiotic stress with silicon addition: A meta-analysis. *Functional Ecology*, *30*, 1340–1357. https://doi.org/10.1111/1365-2435.12713

Chapman, H. D. & Pratt, P. F. (1961). *Methods of Analysis for Soils, Plants, and Waters*. Division of Agric. Sci. Berkeley, Univ. California, USA, 150-152.

El-Gayar, S., Negm, A., & Abdrabbo, M. (2018). *Greenhouse Operation and Management in Egypt*. In Hazardous Chemicals Associated with Plastics in the Marine Environment; Springer: Cham, Switzerland, pp. 489–560. https://doi.org/10.1007/698_2017_230

Hashem, F. A., Medany, M. A., Abd El-Moniem, E. M., & Abdallah, M. M. F. (2011). Influence of greenhouse cover on potential evapo-transpiration and cucumber water requirements. *Arab Universities Journal of Agricultural Sciences*, *19*(1), 205-215. https://doi.org/10.21608/ajs.2011.14657

Intergovernmental Panel on Climate Change (IPCC). (2007). The physical science basis. *Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change*.

Jackson, M. L. (1958). *Soil Chemical Analysis. USA*. Prentice-Hall, Inc. Englewood Cliffs, NJ Library of Congress, 38-388.

Li, Y., Cheng, R., Spokas, K. A, Palmer, A. A., & Borevitz, J. O. (2014). Genetic variation for life history sensitivity to seasonal warming in Arabidopsis thaliana. *Genetics, 196*, 569-577. https://doi.org/10.1534/genetics.113.157628

Marschner, H. (2012). *Mineral Nutrition of Higher Plants*, 3rd ed. Academic Press: San Diego, CA, USA.

Medany, M. A., Abdrabbo, M. A. A., Awny, A. A., Hassanien, M. K., & Abou-Hadid, A. F. (2009). Growth and productivity of mango grown under greenhouse conditions. *Egyptian Journal of Horticulture*, *36*, 373-382.

Taha, N. M., Abd-Elrahman, S., H. & Hashem, F. A. (2019). Improving yield and quality of garlic (*Allium sativum* L.) under water stress conditions. *Middle East Journal of Agriculture Research*, *8*(1), 330-346.

Siwek, P., Wojciechowska, R., Libik, A. & Kalisz, A. (2009). The effect of different kind of polyethylene film used as a low tunnel cover on celery yield and stalk quality. *Vegetable Crops Research Bulletin, 70*, 91-100. https://doi.org/10.2478/v10032-009-0009-8

Prashant, K. & Saini, D. (2019). Silicon as a vegetable crops modulator: A review. *Plants*, *148*(8), 1-18. https://doi.org/10.3390/plants8060148

SAS. (2000). *Statistical Analysis System*, SAS User's Guide: Statistics. USA: SAS Institute Inc., Cary.

Shahak, Y. (2008). Photoselective netting for improved performance of horticultural crops: A review of ornamental and vegetable studies carried out in Israel. *Acta Horticulturae*, *770*, 161-168. https://doi.org/10.17660/ActaHortic.2008.770.18

Shen, X., Li, J., Duan, L., Li, Z., & Eneji, A. E. (2009). Nutrient acquisition by soybean treated with and without silicon under ultraviolet-B radiation. *Journal of Plant Nutrition*, *32*, 1731–1743. https://doi.org/10.1080/01904160903150966

Treder, W., Mika, A., Buler, Z. & Klamkowski, K. (2016). Effects of hail nets on orchard light microclimate, apple tree growth, fruiting and fruit quality. *Acta Science Pollution Hortorum Cultus, 15*(3), 17-27

Tubana, B. S. & Heckman, J. R. (2015). Silicon in Soils and Plants. In *Silicon and plant diseases*; Springer Int. Publ.: Basel, Switzerland, 7–51. https://doi.org/10.1007/978-3-319-22930-0_2

Waller, R. A. & Duncan, D. B. (1969). A bayes rule for the symmetric multiple comparison problem, *Journal of the American Statistical Association*, *64*, 1484-1504. https://doi.org/10.2307/2286085

Watanabe, F. C., & Olsen, S. R. (1965).Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soils. *Soil Science Society of America Journal*, *29*, 677-678. https://doi.org/10.2136/sssaj1965.03615995002900060025x

Yemm, E.W. & Wills, A. J. (1954). The estimation of carbohydrates in plant extracts by antrone. *Biochem*istry *Journal*, *57*, 508-514. https://doi.org/10.1042/bj0570508

Zakher, A. G., & Abdrabbo, M. A. A. (2014). Reduce the harmful effect of high temperature to improve the productivity of tomato under conditions of newly reclaimed land. *Egyptian Journal of Horticulture*, *4*, 85-97.

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The possible use of scarce soluble materials as a source of phosphorus in *Vicia faba* L. grown in calcareous soils

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The possible use of scarce soluble materials as a source of phosphorus in *Vicia faba* L. grown in calcareous soils

Abstract: Phosphorus (P) is affected by many factors that minimize its solubility especially in calcareous soils. The aim of this work was to conduct laboratory and greenhouse experiments to study the effect of using P solubilizing substances, *i.e.,* compost, humic acid (HA), citric acid and ethylene di-amine tetra acetic acid (EDTA), and rhizobacteria, *Bacillus megaterium* var. *phosphaticum* on solubilizing P from different sources, ordinary superphosphate (OSP), rock phosphate (RP) and basic slag (BS). The effect of these treatments on the P- availability in El-Nubaria calcareous soil and P- uptake by faba bean (*Vicia faba* 'Giza 843') were studied. Obtained results showed that the solubility of P sources differs in their ability to release soluble P in the following order: OSP > RP > BS. The following descending order was appeared of available P in soil with addition of solubilizing agents: citric acid > EDTA > HA > compost for these sources of P, for both experiments. Regarding the interaction between solubilizing agents, the treatments of HA combined with EDTA or citric acid were superior in giving high concentrations in soil, and vigor plant growth. In addition, the solubility of P increased by about 5-6 times for all sources in the presence of P- dissolving bacteria. It seemed that the presence of appreciable amounts of Mg, S, Fe, Mn, B and other elements in BS played a role in enhancing plant growth and increasing yield, especially in the presence of added bacteria. BS could be used in calcareous soils and for soils characterized by low nutrient supply as sandy.

Key words: phosphorus sources; basic slag; organic substances; chelating substances; P availability; P dissolving bacteria; calcareous soils; *Vicia faba*

Možnost rabe slabo topnih snovi kot vir fosforja pri gojenju boba (*Vicia faba* L.) na apnenčastih tleh

Izvleček: Na topnost fosforja (P) vplivajo številni dejavniki, še posebej v apnenčastih tleh. Namen te raziskave je bil izvesti poskus v laboratoriju in rastlinjaku za preučevanje učinkov fosfor sproščajočih snovi kot so kompost, huminska kislina (HA), citronska kislina, etilen diamin tetra ocetna kislina (EDTA) in rizobakterij (*Bacillus megaterium* var. *phosphaticum*) na topnost fosforja iz različnih virov kot so navaden superfosfat (OSP), fosfat v kamnini (RP) in tomaževa žlindra (BS). Preučevani so bili učinki teh obravnavanj na dostopnost fosforja v apnenčastih tleh v El-Nubaria, Egipt in privzem fosforja v bob (*Vicia faba* 'Giza 843'). Rezultati so pokazali, da se topnost fosforja iz različnih virov razlikuje glede na njegovo sposobnost sproščanja v naslednjem vrstnem redu: OSP > RP > BS. Po dodatku agensov za topnost se je v tleh pojavil naslednji padajoči redosled razpoložljivega P: citronska kislina > EDTA > HA > kompost, za vse vire fosforja v obeh poskusih. Glede na interakcije med agensi za topljenje se je obravnavanje HA v kombinaciji z EDTA ali citronsko kislino izkazalo kot najboljše, z največjo koncentracijo topnega P v tleh in najboljšo rastjo rastlin. Dodatno se je vsebnost P povečala za okrog 5-6 krat pri vseh virih P v prisotnosti fosfor sproščajočih bakterij. Zdi se, da je je prisotnost precejšnih količin Mg, S, Fe, Mn, B in drugih elementov v tomaževi žlindri vplivala na pospešeno rast rastlin in povečanje pridelka, še posebej ob dodatku bakterij. Tomaževa žlindra bi se torej lahko uporabljala na apnečastih tleh in v peščenih tleh, ki jih označuje majhna vsebnost hranil.

Ključne besede: viri fosforja; tomaževa žlindra; organske snovi; helatirajoče snovi, razpoložljivost P; P raztapljajoče bakterije; apnenčasta tla; *Vicia faba*

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1 INTRODUCTION

In Egypt, phosphorus (P) is the second major fertilizer comes after nitrogen and it is added to the soil mainly as an ordinary superphosphate (OSP). Phosphorus is an insoluble element in alkaline soil especially in soils containing high calcium carbonate, e.g., calcareous soils, which causes rapid precipitation to insoluble phosphate forms (Elgala & Amberger, 2017). The definition of calcareous soils, as reported by Hopkins & Ellsworth (2005), that are having significant quantities of calcium or magnesium carbonate (2-12 % depending on their particle size). These salts dissolve in neutral to acid soil pH (7-6.5), but not readily dissolve in alkaline soil (at about pH ≥ 8) and, instead, serves as a sink for surface adsorbed calcium phosphate precipitation. In other words, calcareous soils with high pH resulting from high content of salts or Na^+ and OH^- ions, made P is a limiting factor, causing nutritional stress conditions.

Many factors affect the solubility of P in soil and its availability to growing plants, particularly under P-stressed conditions: with using rock phosphate or other untraditional components as a source of P; such as acidifying the root medium (Houassine, 2020) and adding organic acids, amino acids and other chelating substances (Grover, 2003; Taskin et al., 2019; Elhag et al., 2019). Accordingly, getting benefit of factors that help in increasing solubility and availability of P from insoluble sources may encourage the use of rock phosphate (RP) or recycling untraditional sources *i.e.*, basic slag (BS), even under alkaline conditions, with preserving the environment from contamination. Basic slag or steel slag, as common, contains calcium oxide (CaO, 40-50 %) and silica (SiO_2, 10-28 %). Also, it includes alumina (Al_2O_3, 1-3.5 %) and magnesium oxide (MgO, 2.5-10 %), as well as iron oxide (FeO, 14-22 %) and manganese oxide (MnO, 1.5-6 %), total Fe (17-27 %), and appreciable amounts of P, K, S, and micronutrients (Tsakiridis et al., 2008; Yildirim & Prezzi, 2011; Bing et al., 2019). BS could be used in agricultural fertilizers, and environmental protection (Bing et al., 2019). Negim et al. (2010) found that the BS additions increased soil pH and conductivity, while immobilized Cu, Zn, Cr and Cd in the studied contaminated acid soil, which reflected on *Phaseolus vulgaris* L. growth. Also, Ning et al. (2016) reported that BS was an effective amendment for soil acidity adjustment, plant Si nutrition and stabilization of Cd in acidic soils.

Humic acid (HA) is a common fertilizer containing most elements that improve soil fertility and increase nutrients availability, thus enhances plant growth, and yield as well as decreases the harmful effect of stresses (Doran et al., 2003). The effect of HA on the availability of P and micronutrients in calcareous soils have been given especial attention because of observed increases in uptake rates of these nutrients following application of HA (Satisha & Devarajan, 2005; Elhag et al., 2019). Also, compost is seen to be beneficial in improving soil fertility and crop productivity (Adugna, 2016), remediating polluted environment, recycling agricultural wastes (Taiwo, 2011), reducing the phytotoxicity of heavy metals (Huang et al., 2016), increasing water use efficiency (Adugna, 2016), and microbial activity (Huang et al., 2016; Lee et al., 2019). In addition, organic chelating agents such as EDTA and citric acid, significantly enhance element solubility and uptake by plants (Afshan et al., 2015), and are commonly used as they are more effective in chelating elements and increasing their concentrations in the upper plant organs (Kanwal et al., 2014).

Bio-fertilizers are playing a vital role in sustainable agricultural management to reduce environmental contamination (Bulut, 2013). *Bacillus megaterium* var. *phosphaticum*, which is considered a rhizobacteria, can exert a positive effect on plant growth through solubilizing inorganic phosphate and mineralizing organic phosphate, helping P to be readily available to plants with time (Abd-Elrahman, 2016; Saxena et al., 2020). Due to the P solubilization capacity, *B. megaterium* var. *phosphaticum* could be used along with RP or any other natural source to raise their efficiency in the soil. These cells can produce amino acids, vitamins, indole acetic acid (IAA), gibberellic acids, antibiotics, siderophore, as well as organic and inorganic acids that mobilize P and other nutrients and encourage the plant growth (Cakmakci et al., 1999; Amalraj et al., 2012). In addition, for the mineralization of organic P compounds, it could be due to the release of phosphatase enzymes (Illmer et al., 1995; Płaza et al., 2021).

So, the aim of this work was to conduct laboratory and greenhouse experiments to study the effect of using P solubilizing substances and rhizobacteria to solubilize P from different sources. The effect of these treatments on the P- availability in calcareous soils and P- uptake by faba bean plants (*Vicia faba* 'Giza 843') were also studied.

2 MATERIALS AND METHODS

The current study involves two trial types:

2.1 INCUBATION EXPERIMENT

To assess P content in a pure media (any salts,

$CaCO_3$ and P were removed) treated by several scarce soluble materials, 200 g of acid (HCl 10^{-4} M) washed quartz sand were placed in a plastic bowl, kept at the laboratory conditions (24 ± 2.5 °C). Thirty combinations generated from application of fifteen treatments either without adding bacteria or in the presence of dissolving bacteria (*Bacillus megaterium* var. *phosphaticum*) as follows were tested with three replications:

1- Ordinary Superphosphate (OSP)
2- OSP + Compost 1 %
3- OSP + Humic acid 1 %
4- OSP + Citric acid 1 %
5- OSP + EDTA 1 %
6- Rock Phosphate (RP)
7- RP + Compost 1 %
8- RP + Humic acid 1 %
9- RP + Citric acid 1 %
10- RP + EDTA 1 %
11- Basic Slag (BS)
12- BS + Compost 1 %
13- BS + Humic acid 1 %
14- BS + Citric acid 1 %
15- BS + EDTA 1 %

Extractable P concentration in each treatment and total element concentrations in basic slag were measured before adding to the soil. The P sources, *i.e.,* ordinary superphosphate (OSP), rock phosphate (RP) and basic slag (BS), were added at a rate of 4.0 g kg^{-1} sand (equal to 9.6 t ha^{-1}). To meet the proper requirements as recommended by the Egyptian Ministry of Agriculture for faba bean cultivation in newly reclaimed soils (55.8 kg P_2O_5 ha^{-1} in the form of ordinary superphosphate). Each of rock phosphate granules fertilizer (obtained from Abou Zaabal Company) and basic slag (obtained from Iron and Steel Company in Helwan) were ground to pass through a 2.0 mm sieve. According to the treatment, 20 ml $bowl^{-1}$ of *Bacillus megaterium* var. *phosphaticum* bacterial suspension, 1 x 10^9 cells ml^{-1}, (supplied by the Department of Microbiology, Faculty of Agriculture, Ain Shams University) were added. Tap water was added to keep the moisture of the medium at the field capacity till the end of the incubation period.

Sand samples (20 g $bowl^{-1}$) were taken 3 times; after 2, 4 and 8 weeks. The collected samples were air dried, crushed, sieved to pass through a 2.0 mm sieve, and prepared to determine available P spectrophotometrically using Olsen extract (0.5 M $NaHCO_3$ at pH 8.5) according to the method described by Watanabe & Olsen (1965).

2.2 POT EXPERIMENT

A pot experiment was carried out in autumn season of 2019 at the greenhouse of Soil Science Department, Faculty of Agriculture, Ain Shams University, Qalubia governorate, Egypt. The experiment was kept in air temperature (21.9 ± 3.8 °C). Representative soil samples were collected from the surface layers (0-20 cm) of a calcareous soil, *Typic Torripsamments* (according to Soil Survey Staff, 2010), sandy loam soil from El-Nubaria district (30º39′55″ N and 30º41′49″ E), Beheira governorate, Egypt. The polythene lined pots (18 cm in diameter and 15 cm in height) were packed uniformly with 3.0 kg of the investigated soil which was already air dried and ground to pass through a 2.0 mm sieve. Some initial physical and chemical properties of the studied soil were tested before plant cultivation according to the standard methods outlined by Page et al. (1982) and Klute (1986). The abovementioned 15 treatments plus 4 solubilizing agents' treatments (compost, humic acid, citric acid and EDTA) and their 6 combinations (compost+ humic acid, citric acid+ EDTA, compost+ citric acid, compost+ EDTA, humic acid+ citric acid and humic acid+ EDTA) were added to the pots and mixed well with the soil during packing, with the same doses. Two control (check) treatments were also applied (one for soil free of P sources and without adding bacteria, and the other for soil free of P sources in the presence of dissolving bacteria). Tap water was used to keep the moisture of the soil before and after plant cultivation at the field capacity till the end of the experimental work.

After one week from adding the treatments, pots were cultivated with faba bean seeds (*Vicia faba* 'Giza 843', 5 seeds pot^{-1}) on 16^{th} of October 2019. At the same time, according to the treatment, 20 ml pot^{-1} of *Bacillus megaterium* var. *phosphaticum* bacterial suspension (1 x 10^9 cells ml^{-1}) were added. After seeds germination, plants were thinned to one plant pot^{-1}. Nitrogen fertilizer in the form of $(NH_4)_2SO_4$ and potassium in the form of K_2SO_4 were applied, at a rate of 1.0 g kg^{-1} soil (equal to 2.4 t ha^{-1}) for each, in two batches the first one at the vegetative growth stage, 60 days after sowing (DAS), and the other one at the flowering stage (90 DAS).

2.3 MEASUREMENTS

2.3.1 Soil P content

Soil in the investigated pots was sampled 4 times: (i) after seeds germination (14 DAS), (ii) at the vegetative growth stage (60 DAS), (iii) at the flowering stage (90 DAS), and (iv) at plant harvest (145 DAS). The collected samples were air dried, crushed, sieved through a 2.0 mm sieve, and prepared to determine available P spectrophotometrically using Olsen extract, as described by Watanabe & Olsen (1965).

2.3.2 Crop traits

Plants were harvested on the second week of March 2020, to assess plant height, plant fresh and dry mass, as well as number of pods plant^{-1}, fresh mass of pods and seeds plant^{-1}. Also, samples of plant leaves were oven dried at 70 °C for 48 h and digested by H_2SO_4/H_2O_2 mixture according to the method described by Chapman & Pratt (1961). Total nitrogen in leaves was determined using Kjeldahl method according to the procedure described by Chapman & Pratt (1961), total phosphorus was determined using Spectrophotometer according to Watanabe & Olsen (1965) and total potassium in plant leaves was determined using Flame photometer as described by Chapman & Pratt (1961).

2.4 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The two experiments (incubation and pot experiments) were designed in a completely randomized design and each treatment was replicated three times. The obtained data were then statistically analyzed using SAS software package (SAS, 2000). Values expressed as mean and were compared for each other using Duncan's multiple range test (at $p \leq 0.05$ considered significant) ± standard error of the mean (SEM, n = 3).

Table 1a: Extractable- P in some of the studied treatments

Treatment	P, μg g^{-1}
Available form in:	
OSP	60.4
RP	21.6
BS	11.2
Compost	19.5
Humic acid	32.0
Mixtures (1:1, *v/v*)	
Citric acid 1 % + OSP (1:5)	133
Citric acid 1 % + RP (1:5)	47.0
Citric acid 1 % + BS (1:5)	30.7
EDTA 1 % + OSP (1:5)	125
EDTA 1 % + RP (1:5)	42.5
EDTA 1 % + BS (1:5)	32.0

3 RESULTS AND DISCUSSION

3.1 INITIAL CHARACTERISTICS OF SOIL AND TREATMENTS

Extractable P concentration in each treatment before adding to the investigated soil are shown in Table 1a. The treatment of OSP is rich with P, followed by humic acid, RP, compost and BS respectively. Regarding the mixtures between treatments (Table 1a), the treatment of citric acid+ OSP gave high concentration of soluble P, followed by EDTA+ OSP, citric acid+ RP, EDTA+ RP, EDTA+ BS and citric acid+ BS respectively. Total element concentrations in basic slag were measured before adding to the soil (Table 1b). It seems good that finding appreciable amounts of P, Mg, S, Fe, Mn, B and other elements in BS. Some initial physical (soil texture, field capacity, wilting point, and saturation percent) and chemical ($CaCO_3$ fractions content, organic matter content, soil cation exchange capacity, pH, electrical conductivity of salts, soluble ions concentration, total and available concentration of macronutrients NPK) properties of the studied soil before plant cultivation are presented in Table 2. The studied soil is calcareous sandy loam with no saline hazards and low macronutrients concentration.

3.2 INCUBATION EXPERIMENT

Data in Table 3 shows the availability of P concentrations in acid washed sand with time; after applying different P- sources and solubilizing agents, with or without adding P dissolving bacteria. As P fertilizers (OSP, RP and BS) which vary in their P contents were added to washed sand at equal rates (4 g kg^{-1}), the extractable amounts in washed sand were significantly different between all sources after 2 weeks. With time, the soluble amounts of P increased to about 4 times at 4 weeks then dropped to about the values of the first pe-

Table 1b: Total elements concentration in the basic slag sample

Element	P	K	Ca	Mg	S	Fe	Mn	Si	Al	Cr	B	Mo
Concentration, %	0.55	0.08	32.1	5.40	0.06	19.5	2.32	7.02	1.32	0.06	0.02	<0.01

Table 2: Some initial physical and chemical characteristics of the surface layer of the experimental soil (0-20 cm) before plant cultivation

Particle size distribution, %		Soluble cations, $mmol_c\ l^{-1}$	
Sand	65.8	Ca^{2+}	10.2
Silt	20.3	Mg^{2+}	6.34
Clay	13.9	Na^{+}	1.11
Textural class	Sandy loam	K^{+}	0.52
FC, %	12.3	Soluble anions, $mmol_c\ l^{-1}$	
WP, %	4.20	CO_3^{2-}	n.d.*
SP, %	31.5	HCO_3^{-}	4.27
$CaCO_3$ fractions, %		Cl^{-}	2.49
Coarse sand	16.8	SO_4^{2-}	6.28
Fine sand	8.30	Total macronutrients, %	
Silt	6.10	N	0.01
Clay	5.30	P	0.01
$CaCO_3$, g kg^{-1}	365	K	0.02
OM, g kg^{-1}	1.10	Available macronutrients, μg g^{-1}	
CEC, $cmol_c\ kg^{-1}$	11.7	N	12.3
pH (1:2.5 soil:water suspension)	8.06	P	1.00
EC_e, dS m^{-1}	0.99	K	92.6

*n.d. means not detected, field capacity (FC), wilting point (WP), saturation percent (SP), organic matter content in soil (OM), cation exchange capacity (CEC), and electrical conductivity of salts in soil extract (EC_e)

riod for the various P- sources. It also appears that the P solubility increased to about 5-6 times for all sources in the presence of the added bacteria compared to the treatments without adding P dissolving bacteria.

With respect to the effect of the natural compounds (compost and humic acid), there was a slight increase in P solubility of the three sources when compost was added. Also, the same results were found when humic acid was added, but for OSP the P solubility increased more than the double. These results agreed with those obtained by Elhag et al. (2019) concerning the effect of humic acid on increasing P availability in washed sand. On the other hand, the effect of these natural organic sources was different when the dissolving bacteria was added, as the values of P solubility increased with compost or humic acid in these sources with more P solubility for OSP than for RP or BS. This could be related to increasing the activity of the dissolving bacteria in the presence of organic sources. The bacteria decompose these compounds to simple materials beside the materials excreted by the bacteria. All these new compounds act in dissolving the P- sources. The effect was almost double in the OSP treatment than with RP or BS treatments. These results agreed with those obtained by Abd-Elrahman (2016) about the effect of P dissolving bacteria on the solubility of P from OSP and RP fertilizers.

Results of the ability of humic acid (HA) added or formed from the decomposition of compost or any simple or complex organic compounds resulted from the action of the bacteria added could be explained on the bases that these compounds may have positive or negative charges. The positive charges bind PO_4^{3-} groups, so help in releasing the phosphate from the insoluble sources. On the other hand, the negative charge can bind Ca^{2+} ion or any cation, so, also, helps in releasing the phosphate group from the insoluble sources (Campitelli et al., 2003).

Regarding to the action of citric acid and EDTA, results show that in the absence of dissolving bacteria these compounds were superior to the compost and humic acid as they play their role directly without the action of the bacteria. This was clear with the insoluble sources RP and BS. The following descending order generally appeared in the chemically extractable mount of P with addition of solubilizing agents: citric acid > EDTA > HA > compost for these sources of P. Mihoub et al. (2018) reported that after a period of 960 h from incubation of highly calcareous soil samples (50 % $CaCO_3$) fertilized with triple superphosphate and mono-ammonium phosphate and treated with citric acid and oxalic acid solutions, treatments showed a significant decrease in extractable P with time, however, applying these solutions exerted a very favorable effect on P solubility in soil. Also, in our study, with the addition of dissolving bacteria to the studied treatments activated the bacteria in dissolving the insoluble sources beside binding phosphate groups. Results also indicate that in the absence of dissolving bacteria, the soluble phosphates decrease by increasing the time of incubation at 8 weeks. But in the presence of dissolving bacteria the values in most treatments remain stable at 8 weeks. This clearly indicates that the compound formed in the presence of bacteria were more stable than that in the absence of bacteria. Abd-Elrahman (2016) reported that P dissolving bacteria increases P availability from OSP, and RP fertilizers added to a calcareous soil, with time.

3.3 POT EXPERIMENT

3.3.1 Available P in soil

Table 4 shows the effect of different P sources and solubilizing agents on available P in calcareous soil in the presence or absence of P dissolving bacteria, during the physiological stages of faba bean growth. Results indicate that the values ranged from 1.2 to 10.4 μg g^{-1} in the absence of bacteria, and from 3.4 to 29.8 μg g^{-1} in the presence of bacteria. The solubility of P sources differs in their ability to release soluble P in the following order: OSP > RP > BS. In fact, this is related to their difference in their content of total- and extractable- P amounts. With respect to the ability of solubilizing agents in releasing P in the soil, it appears that they differ in the following descending order: citric acid > EDTA > HA > compost, similar to that found in the incubation experiment. As these agents were applied at the rate of 1 % (w/w), so it is expected that the active material of citric acid will be more than in compost and humic acid, as humus is composed of simple and complex compost as lignin (Taiwo, 2011). The superiority of citric acid compared to EDTA, could be explained on the basis that citric acid is smaller molecule compared to EDTA, so the active molecule well be more in 1 % of the material added compared to EDTA (Kanwal et al., 2014; Afshan et al., 2015). Besides the ability of EDTA to react with Ca to release P in the soil, it can chelate elements as Mg, Fe, Mn and Pb with higher stability (Hamed & Gamal, 2014; Kanwal et al., 2014). This may be the reason

Table 3: Effect of the studied treatments on chemically available P (μg g^{-1}) in washed sand with time, in the presence or absence of P- dissolving bacteria

	Available P in washed sand (μg g^{-1})					
	Without adding bacteria			In the presence of dissolving bacteria		
Treatment	after 2 weeks	4 weeks	8 weeks	after 2 weeks	4 weeks	8 weeks
OSP	1.00 ± 0.03hi	4.20 ± 0.12hi	0.80 ± 0.04i	6.40 ± 0.11f	24.4 ± 1.92d	25.0 ± 2.14d
OSP + Compost 1 %	1.80 ± 0.04g	5.80 ± 0.15f	1.60 ± 0.06g	7.60 ± 0.13d	24.6 ± 2.02cd	25.6 ± 2.19c
OSP + Humic Acid 1 %	4.00 ± 0.14c	10.6 ± 0.26c	1.80 ± 0.06f	8.20 ± 0.16c	24.8 ± 2.03bc	25.0 ± 2.10d
OSP + Citric Acid 1 %	9.40 ± 0.21a	12.8 ± 0.28a	13.2 ± 0.17a	13.8 ± 0.23a	54.6 ± 2.54a	57.6 ± 2.64a
OSP + EDTA 1 %	6.80 ± 0.19b	11.0 ± 0.26c	4.60 ± 0.09b	11.4 ± 0.17b	25.0 ± 2.11b	27.4 ± 2.23b
RP	0.80 ± 0.03i	3.80 ± 0.10ij	0.60 ± 0.05j	6.20 ± 0.10f	10.2 ± 0.21j	10.2 ± 0.19j
RP + Compost 1 %	1.00 ± 0.03hi	5.20 ± 0.14g	1.40 ± 0.06h	6.40 ± 0.12f	11.4 ± 0.25h	10.8 ± 0.22hi
RP + Humic Acid 1 %	1.60 ± 0.04g	5.60 ± 0.15f	1.40 ± 0.07h	6.80 ± 0.12e	12.2 ± 0.26f	12.6 ± 0.29f
RP + Citric Acid 1 %	3.60 ± 0.11d	12.0 ± 0.27b	3.80 ± 0.08c	7.00 ± 0.13e	12.6 ± 0.27e	11.6 ± 0.23g
RP + EDTA 1 %	3.00 ± 0.12e	6.80 ± 0.17e	3.60 ± 0.07d	7.60 ± 0.15d	11.8 ± 0.26g	13.0 ± 0.26e
BS	0.40 ± 0.02j	3.40 ± 0.11j	0.40 ± 0.03k	4.40 ± 0.08k	8.60 ± 0.14k	9.00 ± 0.08k
BS + Compost 1 %	1.00 ± 0.03hi	4.60 ± 0.13h	0.60 ± 0.04j	4.80 ± 0.08j	10.3 ± 0.22ij	10.6 ± 0.21i
BS + Humic Acid 1 %	1.20 ± 0.03h	5.20 ± 0.15g	0.80 ± 0.06i	5.20 ± 0.09i	10.4 ± 0.22i	11.4 ± 0.23g
BS + Citric Acid 1 %	3.40 ± 0.14d	9.60 ± 0.25d	3.60 ± 0.08d	5.40 ± 0.09hi	10.4 ± 0.24i	11.0 ± 0.22h
BS + EDTA 1 %	2.60 ± 0.09f	6.60 ± 0.16e	3.40 ± 0.07e	5.60 ± 0.11h	10.2 ± 0.23j	10.8 ± 0.20hi

Ordinary Superphosphate (OSP), Rock Phosphate (RP), Basic Slag (BS). Values expressed as mean ± SE, the significant value was set at $p \leq 0.05$. Different letters indicate significant difference between treatments.

why extractable P from the treated soil with EDTA was less than citric acid. Mihoub et al. (2016) found that organic acids, i.e., citric acid and oxalic acid, decreased P sorption capacity on the investigated calcareous soil whereas increased Gibbs free energy (ΔG) of P which reflected on increasing its solubility in soil, however, with citric acid more than oxalic acid.

It appears that, as a function of time with growing the faba bean plants, extractable P decreased with time for the three P sources added alone and when compost and humic acid were added. This is related to activity of plant roots to utilize and withdraw the soluble P to fulfill the plant requirements. Also, it probably due to refixation of soluble P in soil by released Ca or another cation. In addition, data of extractable P in the presence of bacteria were significantly higher compared when bacteria were not added, and still the sequence was as follow: OSP > RP > BS. The reason for remaining BS in the last order may be due to its low P content. Yildirim & Prezzi (2011) reported that BS is containing small amounts of total P in the form of P_2O_5 ranged from 0.01 to 3.3 % in all different types.

Regarding the combinations between organic substances and chelating agents, the treatment of HA+ citric acid was superior in giving high extractable amount of P in soil, followed by HA+ EDTA in most physiological stages of growing bean plants, with significant differences in the presence of added bacteria. Humic acid plays a vital role in increasing P availability in soil (Doran et al., 2003; Sahin et al., 2014), plus its considerable content of P (Table 1a). Citric acid, in addition to decrease soil pH, it makes complexes with Ca forming calcium citrate and releasing P in soluble form in the soil (Drouillon & Merckx, 2003). EDTA may solubilize the insoluble P forms in calcareous soils by chelating Ca^{2+} and Mg^{2+} cations, lowering soil pH and/ or the partial occupation of active anionic groups on the surface of $CaCO_3$ and clay minerals (Hamed & Gamal, 2014). It appears that the presence of bacteria played a protective role against P- fixation (Abd-Elrahman, 2016; Płaza et al., 2021).

3.3.2 Vegetative growth parameters

Data in Table 5 shows the effect of the studied P-treatments on faba bean plant height, and plant fresh and dry mass, in the presence or absence of P- dissolving bacteria. The data of plant dry mass indicate that the addition of P- sources increased the dry mass yield more than the control and the rate of increase was in the same sequence mentioned for P availability in the soil: OSP > RP > BS (Table 4). The role of P in enhancing roots growth and their absorption efficiency in the soil was observed, which reflected on the plant growth and its yield. Razaq et al. (2017) found that applying P fertilizer increased root surface area, specific root length and root-shoot ratio. Fouda (2017) confirmed the effect of P fertilizer on increasing faba bean productivity.

With respect to the effect of solubility agents, the sequence was different: EDTA > citric acid > HA > compost for OSP and RP, but the values were almost the same for BS. This indicates that despite relatively low total P content in BS (Table 1b), the P is found in a form easily released by the solubilizing agents. The addition of solubilizing bacteria increased the yield of dry mass to the extend to record slight significant difference between the studied P- sources. This was also found when comparing the effect of solubilizing agents on yield in case of RP and BS were almost the same. Such soil with its high content of $CaCO_3$ is characterized by deficiency problems with some elements, particularly the micronutrients. The presence of appreciable amounts of Fe, Mn, Mg, S, B and other elements in BS had an effect of plant growth despite the low P content added (Table 1b).

It is interesting to note that the highest yield recorded for this experiment was when EDTA was added to OSP treatment, or mixed with HA, in the presence of P- dissolving bacteria. The above results indicate that solubilizing agents and dissolving bacteria not only act in solubilizing P from added materials and from soil, but also act in solubilizing other elements as Fe, Mn and Mg which are essential for plant growth. EDTA is known to chelate elements as Fe, Mn, Zn and Mg with higher stability as compared to citric acid or natural compounds as humic acid (Hamed & Gamal, 2014).

Similar trend was observed with the other vegetative growth parameters of faba bean plants as affected by the studied P treatments, with significant effect in the presence of P- dissolving bacteria as compared to not adding bacteria. Plant height ranged from 25 cm (in control treatment, without adding P sources and dissolving bacteria) to 70 cm (with applying the treatment of HA combined with EDTA, in the presence of dissolving bacteria). Also, the plant fresh mass ranged from 22.8 g $plant^{-1}$ in control treatment (without any additions) to 60.5 g $plant^{-1}$ with applying the treatment of HA combined with EDTA, in the presence of P- dissolving bacteria.

3.3.3 Numbers of pods $plant^{-1}$, fresh mass of pods $plant^{-1}$ and fresh mass of faba bean seeds $plant^{-1}$

Results of mass of seeds (g $plant^{-1}$) shown in Table

Table 4: Effect of the applied P sources on chemically available P (µg g^{-1}) in El-Nubaria calcareous soil, in the presence or absence of P- dissolving bacteria, during the physiological stages of growing faba bean plants

	Available P in soil (µg g^{-1})							
	After seeds germination (14 days after sowing)		At the vegetative growth stage (60 days after sowing)		At the flowering stage (90 days after sowing)		At plant harvest (145 days after sowing)	
Treatment	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**
Control (-P)	0.80 ± 0.04q	2.40 ± 0.12o	1.00 ± 0.04r	3.60 ± 0.26v	0.80 ± 0.04r	3.40 ± 0.18s	0.80 ± 0.03p	2.40 ± 0.12q
OSP	2.64 ± 0.07k	6.40 ± 0.51h	4.20 ± 0.26jk	17.0 ± 1.16f	3.20 ± 0.13j	18.2 ± 1.26e	2.10 ± 0.08jk	9.20 ± 0.71l
OSP + Compost 1 %	3.60 ± 0.10g	7.80 ± 0.80f	5.20 ± 0.34g	18.6 ± 1.21d	3.80 ± 0.15i	19.0 ± 1.33d	2.60 ± 0.09h	10.0 ± 0.83k
OSP + Humic Acid 1 %	5.20 ± 0.23d	9.20 ± 0.86c	8.00 ± 0.62c	19.8 ± 1.28c	5.60 ± 0.34d	19.5 ± 1.35c	3.20 ± 0.17f	11.0 ± 0.92hi
OSP + Citric Acid 1 %	9.83 ± 0.49a	15.6 ± 1.02a	10.4 ± 0.81a	29.0 ± 2.06a	10.2 ± 0.83a	29.8 ± 2.31a	4.80 ± 0.21a	18.4 ± 1.27a
OSP + EDTA 1 %	7.80 ± 0.33b	12.4 ± 0.97b	8.80 ± 0.64b	21.2 ± 1.78b	8.20 ± 0.60b	22.0 ± 2.01b	4.20 ± 0.20c	13.6 ± 1.16c
RP	2.00 ± 0.06m	5.60 ± 0.29i	3.80 ± 0.15l	10.6 ± 0.84mn	2.40 ± 0.11n	10.7 ± 0.80m	1.40 ± 0.07n	10.0 ± 0.85k
RP + Compost 1 %	2.41 ± 0.09k	6.80 ± 0.48g	4.00 ± 0.23kl	11.4 ± 0.90l	2.44 ± 0.12n	11.2 ± 0.92kl	2.01 ± 0.09k	10.8 ± 0.89ij
RP + Humic Acid 1 %	3.20 ± 0.12h	8.00 ± 0.85ef	5.20 ± 0.30g	12.2 ± 0.95k	4.00 ± 0.23h	12.8 ± 0.96j	2.40 ± 0.09i	11.6 ± 0.91g
RP + Citric Acid 1 %	5.60 ± 0.26c	8.41 ± 0.84d	6.80 ± 0.42d	13.4 ± 0.98j	6.40 ± 0.41c	13.6 ± 0.098i	4.40 ± 0.23b	13.4 ± 1.15cd
RP + EDTA 1 %	4.20 ± 0.18f	8.20 ± 0.80de	6.40 ± 0.40e	12.5 ± 0.93k	4.80 ± 0.20f	12.8 ± 0.95j	4.03 ± 0.21c	12.8 ± 1.06e
BS	1.80 ± 0.09n	4.40 ± 0.23l	3.20 ± 0.17n	8.23 ± 0.61s	2.00 ± 0.10o	8.40 ± 0.61q	1.00 ± 0.06o	9.20 ± 0.72l
BS + Compost 1 %	2.20 ± 0.10l	5.20 ± 0.28j	4.00 ± 0.22kl	9.20 ± 0.74q	2.40 ± 0.12n	9.00 ± 0.73op	1.61 ± 0.07m	10.6 ± 0.85j
BS + Humic Acid 1 %	2.80 ± 0.13j	6.20 ± 0.47i	4.63 ± 0.25hi	9.60 ± 0.76p	3.00 ± 0.16k	10.0 ± 0.80n	1.80 ± 0.07l	12.2 ± 1.02f
BS + Citric Acid 1 %	4.60 ± 0.17e	6.80 ± 0.50g	5.80 ± 0.35f	10.8 ± 0.81m	5.20 ± 0.29e	11.6 ± 0.94k	3.80 ± 0.17d	13.2 ± 1.11de
BS + EDTA 1 %	3.60 ± 0.15g	6.40 ± 0.49h	4.86 ± 0.26h	10.4 ± 0.80n	4.20 ± 0.23g	10.8 ± 0.84lm	3.61 ± 0.16e	12.6 ± 1.06e
OSP+ RP+ BS (50 % for everyone)	2.40 ± 0.11k	7.02 ± 0.68g	4.40 ± 0.24ij	18.0 ± 1.27e	3.20 ± 0.16j	18.8 ± 1.20d	1.80 ± 0.08l	9.00 ± 0.78l
Compost 1 %	1.80 ± 0.07n	4.60 ± 0.28kl	2.40 ± 0.08p	8.81 ± 0.62r	2.40 ± 0.13n	8.60 ± 0.67pq	2.00 ± 0.09k	4.82 ± 0.21o
Humic Acid 1 %	2.20 ± 0.013l	5.60 ± 0.30i	3.20 ± 0.18n	10.0 ± 0.81o	3.00 ± 0.15k	10.2 ± 0.83n	2.20 ± 0.09j	5.70 ± 0.23n
Citric Acid 1 %	1.40 ± 0.08o	3.80 ± 0.19m	1.80 ± 0.06q	7.40 ± 0.54t	2.00 ± 0.11o	7.80 ± 0.55r	2.00 ± 0.08k	4.80 ± 0.22o
EDTA 1 %	1.20 ± 0.06p	3.40 ± 0.17n	1.60 ± 0.06q	6.80 ± 0.44u	1.60 ± 0.08q	7.40 ± 0.53r	1.42 ± 0.07n	4.00 ± 0.19p
Compost 1 %+ Humic Acid 1 % (50 % for both)	2.20 ± 0.10l	5.20 ± 0.26j	2.80 ± 0.09o	14.6 ± 1.10h	2.60 ± 0.13m	14.7 ± 1.15hi	2.20 ± 0.10j	11.1 ± 0.90h
Citric Acid 1 %+ EDTA 1 % (50 % for both)	1.40 ± 0.07o	3.60 ± 0.18mn	1.89 ± 0.07q	9.87 ± 0.71op	1.80 ± 0.08p	9.20 ± 0.72o	1.60 ± 0.08mn	6.60 ± 0.42m
Compost 1 %+ Citric Acid 1 % (50 % for both)	2.20 ± 0.12l	5.20 ± 0.29j	3.00 ± 0.23no	15.0 ± 1.18j	2.80 ± 0.15l	15.8 ± 1.24f	2.60 ± 0.12h	10.8 ± 0.89ij
Compost 1 %+ EDTA 1 % (50 % for both)	2.02 ± 0.09m	4.80 ± 0.25k	3.00 ± 0.25no	14.0 ± 1.05i	2.60 ± 0.14m	14.6 ± 1.13hi	2.60 ± 0.11h	10.0 ± 0.90k
Humic Acid 1 %+ Citric Acid 1 % (50 % for both)	3.00 ± 0.15i	6.60 ± 0.54h	4.02 ± 0.26kl	17.0 ± 1.17f	3.80 ± 0.17i	19.2 ± 1.38cd	3.00 ± 0.18g	15.6 ± 1.18b
Humic Acid 1 %+ EDTA 1 % (50 % for both)	2.40 ± 0.11k	5.40 ± 0.32i	3.40 ± 0.25m	14.2 ± 1.12i	3.00 ± 0.18k	15.2 ± 1.20g	2.65 ± 0.13h	11.2 ± 0.97h

*(-PDB) means without adding P dissolving bacteria, **(+PDB) means in the presence of P dissolving bacteria. Ordinary Superphosphate (OSP), Rock Phosphate (RP), Basic Slag (BS). Values expressed as mean ± SE, the significant value was set at $p \leq 0.05$. Different letters indicate significant difference between treatments.

Table 5: Effect of the applied P sources on vegetative growth parameters of faba bean plants cultivated on El-Nubaria calcareous soil, in the presence or absence of P- dissolving bacteria

	Plant height, cm		Plant fresh mass, g		Plant dry mass, g	
Treatments	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**
Control (-P)	25.0 ± 2.21q	35.5 ± 2.44s	22.8 ± 2.13s	28.4 ± 2.38u	4.94 ± 0.23s	7.48 ± 0.54s
OSP	32.5 ± 2.80k	39.0 ± 2.68o	26.2 ± 2.25m	34.9 ± 3.29o	7.12 ± 0.50l	10.9 ± 0.81no
OSP + Compost 1 %	34.0 ± 2.87i	44.0 ± 3.15j	28.1 ± 2.31j	37.8 ± 3.40l	8.34 ± 0.57k	12.1 ± 0.98k
OSP + Humic Acid 1 %	36.3 ± 3.01g	47.0 ± 3.21g	29.8 ± 2.45i	41.7 ± 4.04ij	9.18 ± 0.65gh	13.5 ± 1.07h
OSP + Citric Acid 1 %	50.0 ± 4.43b	58.4 ± 4.01d	33.3 ± 2.70e	49.1 ± 4.25d	9.90 ± 0.72d	16.1 ± 1.29c
OSP + EDTA 1 %	53.0 ± 4.50a	60.1 ± 4.29c	34.6 ± 2.76c	58.6 ± 4.46b	11.3 ± 0.86a	18.0 ± 1.33b
RP	28.0 ± 2.52o	36.5 ± 2.35r	23.4 ± 2.22q	32.2 ± 2.89qrs	6.69 ± 0.43p	9.83 ± 0.76r
RP + Compost 1 %	30.5 ± 3.22l	40.7 ± 2.80n	27.1 ± 2.29l	35.4 ± 2.93n	7.19 ± 0.52l	11.1 ± 0.91no
RP + Humic Acid 1 %	32.0 ± 2.34k	43.0 ± 3.39k	28.3 ± 2.36j	38.5 ± 3.45k	8.90 ± 0.64i	11.4 ± 0.90m
RP + Citric Acid 1 %	40.0 ± 3.11f	46.0 ± 3.50h	31.8 ± 2.83g	45.5 ± 4.11f	9.30 ± 0.73f	14.8 ± 1.12f
RP + EDTA 1 %	41.0 ± 3.23e	48.0 ± 3.55f	32.3 ± 2.87f	47.8 ± 4.19e	9.82 ± 0.79d	15.8 ± 1.18d
BS	26.3 ± 2.25p	35.9 ± 2.37rs	23.1 ± 2.26r	30.9 ± 2.47t	5.93 ± 0.32r	9.65 ± 0.76r
BS + Compost 1 %	29.1 ± 2.53n	38.0 ± 2.41pq	24.1 ± 2.32o	32.5 ± 2.90qr	6.89 ± 0.39no	10.4 ± 0.87pq
BS + Humic Acid 1 %	30.5 ± 2.87l	38.2 ± 2.38p	24.4 ± 2.34n	32.7 ± 2.89p	6.93 ± 0.45mno	10.5 ± 0.88p
BS + Citric Acid 1 %	36.5 ± 2.91g	43.5 ± 3.60jk	27.6 ± 2.78k	37.7 ± 3.37l	8.90 ± 0.62i	11.8 ± 0.94l
BS + EDTA 1 %	35.4 ± 2.76h	45.0 ± 3.62i	27.8 ± 2.76k	41.9 ± 4.12i	9.11 ± 0.71h	12.6 ± 0.98ij
OSP+ RP+ BS (50 % for everyone)	32.0 ± 2.79k	41.3 ± 3.22lm	27.2 ± 2.69l	37.1 ± 3.32m	8.63 ± 0.59j	12.5 ± 0.97j
Compost 1 %	28.0 ± 2.23o	37.8 ± 2.43q	23.4 ± 2.23q	32.0 ± 3.45s	6.33 ± 0.44q	10.3 ± 0.86q
Humic Acid 1 %	30.0 ± 2.69m	38.0 ± 2.47pq	23.9 ± 2.29p	32.6 ± 3.51pq	6.81 ± 0.47o	10.4 ± 0.87pq
Citric Acid 1 %	33.4 ± 2.80j	41.1 ± 3.26mn	24.0 ± 2.38op	33.1 ± 3.60p	6.97 ± 0.48mn	10.8 ± 0.92o
EDTA 1 %	34.0 ± 2.85i	41.7 ± 3.35l	24.0 ± 2.37op	35.4 ± 3.66n	6.98 ± 0.51m	11.9 ± 0.98kl
Compost 1 %+ Humic Acid 1 % (50 % for both)	35.4 ± 2.49h	47.0 ± 3.56g	30.5 ± 2.44h	41.3 ± 4.13j	9.23 ± 0.42fg	12.7 ± 1.02i
Citric Acid 1 %+ EDTA 1 % (50 % for both)	42.5 ± 3.44d	45.0 ± 3.41i	34.1 ± 2.51d	44.0 ± 4.20g	10.1 ± 0.78c	14.8 ± 1.13f
Compost 1 %+ Citric Acid 1 % (50 % for both)	40.0 ± 3.39f	54.3 ± 4.52e	32.3 ± 2.49f	43.1 ± 4.18h	9.10 ± 0.69h	12.9 ± 1.05i
Compost 1 %+ EDTA 1 % (50 % for both)	42.0 ± 3.38d	60.0 ± 4.58c	32.5 ± 2.47f	48.7 ± 4.70d	9.31 ± 0.73f	14.3 ± 1.08g
Humic Acid 1 %+ Citric Acid 1 % (50 % for both)	46.0 ± 2.56c	64.0 ± 4.71b	36.1 ± 2.72b	53.9 ± 4.93c	9.50 ± 0.75e	15.3 ± 1.17e
Humic Acid 1 %+ EDTA 1 % (50 % for both)	50.0 ± 2.70b	70.0 ± 4.93a	39.4 ± 3.14a	60.5 ± 5.03a	10.9 ± 0.82b	18.7 ± 1.38a

*(-PDB) means without adding P dissolving bacteria, **(+PDB) means in the presence of P dissolving bacteria. Ordinary Superphosphate (OSP), Rock Phosphate (RP), Basic Slag (BS). Values expressed as mean ± SE, the significant value was set at $p \leq 0.05$. Different letters indicate significant difference between treatments

6 indicates the values ranged between 1.21 g to 11.5 g with lower values for treatments without bacteria addition. The addition of P fertilizers significantly increased the seed yield by about 5 times with OSP and only about 3 times for RP and BS compared to the control without bacteria addition. When solubilizing bacteria was added the magnitude of increase was only about the double in case of OSP and slightly less than double in case of RP and BS. This means that the solubilizing bacteria play a major role than the P- sources. *Bacillus megaterium* var. *phosphaticum* produced acids that enhanced the availability of phosphates and increased the uptake of other nutrients, leading to increased yields (Cakmakci et al., 1999; Saxena et al., 2020; Płaza et al., 2021).

With respect to solubilizing agents, there are slight significant difference among solubilizing agents in the absence of bacteria. However, when solubilizing bacteria was added the effect follows the sequence: EDTA > citric acid > HA > compost in case of OSP and RP, but the effect was almost the same when BS was used. This means there are factors other than P in BS contributed to the production of seeds yield. It is possible that the presence of appreciable amounts of Mg, S, Fe, Mn, B and other elements played a role in plant growth, although P percentage was relatively low compared to RP and OSP (Yildirim & Prezzi, 2011).

Regarding the interaction among the studied treatments, the treatment of HA+ EDTA gave the highest seeds yield, recording 8.57 g $plant^{-1}$ without adding bacteria. While recorded 11.5 g $plant^{-1}$ in the presence of added bacteria. Similar trend was found with the other yield parameters, with high significant difference in the presence of P- dissolving bacteria. Number of pods $plant^{-1}$ varied from 1 in control treatment (without any additions) to 6.67 that recorded by many treatments, OSP in combination with citric acid or EDTA and the treatment of HA combined with EDTA, in the presence of P- dissolving bacteria. Regarding the fresh weight of pods, ranged from 2.59 g $plant^{-1}$ (in control treatment, without any additions) to 22.3 g $plant^{-1}$ with applying the treatment of HA combined with EDTA, in the presence of P- dissolving bacteria (Table 6).

3.3.4 N, P and K concentrations in faba bean leaves

Results of P concentration in leaves of faba bean plants (Table 7) indicated that by addition of P sources, P contents increased with remarkable increase with applying OSP treatment than applying RP or BS treatments. This was found in case of without adding or with adding solubilizing bacteria, but the values were generally higher with the later. Elhag et al. (2019) reported that, increasing available P in soil by addition of P sources was reflected on increasing P concentration in bean roots and shoots, with high concentrations in shoots more than roots.

However, no remarkable difference in concentration among solubilizing agents, except when EDTA or citric acid was added with BS. This again clearly indicate the role of these relatively small compounds in solubilizing not only insoluble P, but also other elements as Mg, Fe, Mn in BS which may activate plant roots to absorb nutrients. The enhanced role of EDTA or citric acid in calcareous soils is not only due to acidification of the plant rhizosphere, but also to its Ca and Mg complexing capacity (Drouillon & Merckx, 2003; Hamed & Gamal, 2014). Mihoub et al. (2019) found that P uptake by wheat plants grown in alkaline calcareous soil was 0.493 mg P pot^{-1} in the control treatment, however, it reached 0.701 and 0.785 mg P pot^{-1} in the amended pots with pigeon manure juice and citric acid, respectively.

Regarding the interaction between solubilizing agents, the treatment of HA+ EDTA, followed by that plus citric acid gave the highest concentration of P in plant leaves, with significant difference as compared to the other treatments. Sahin et al. (2014) found that humic substances in interaction with P in the soil could decrease the P- fixation and increase the P- uptake by plants.

With respect to the effect of the studied treatments on N and K concentrations in plant leaves (Table 7), it was clear that the addition of P increased N and K content in all treatments. Also, there was a remarkable difference recorded with respect to the solubilizing agents and bacterial additions. Although HA and compost enhanced the plant uptake from N and K, due to their considerable content of the macro elements; the treatments of EDTA and citric acid were superior in increasing plant uptake of both. These may be due to enhancing root efficiency in absorbing nutrients from soil and added fertilizers, lowering soil pH, and their high Ca and Mg complexing capacity (Drouillon & Merckx, 2003; Hamed & Gamal, 2014).

The main problem of the investigated soil is its high content of $CaCO_3$ (36.5 %, Table 2), and this is influence on nutrients availability and uptake by plants. So, the soil moisture content should be kept up to field capacity till the end of the experimental work. Also, the role of EDTA and citric acid in enhancing root growth and absorption, besides their interaction with $CaCO_3$ in soil as well as their effects on lowering soil pH; reflected on enhancing plant growth and productivity, as compared to the effect of compost or HA (Campitelli et al., 2003; Drouillon & Merckx, 2003). The interac-

Table 6: Numbers of pods $plant^{-1}$, fresh mass of pods $plant^{-1}$ and fresh mass of faba bean seeds $plant^{-1}$ cultivated on El-Nubaria calcareous soil as affected by the different P sources, in the presence or absence of P- dissolving bacteria

	No. of pods $plant^{-1}$		F. mass of pods (g $plant^{-1}$)		F. mass of seeds (g $plant^{-1}$)	
Treatments	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**
Control (-P)	1.00 ± 0.03k	2.00 ± 0.05l	2.59 ± 0.10r	5.93 ± 0.33t	1.21 ± 0.05q	2.58 ± 0.09p
OSP	3.00 ± 0.07g	5.00 ± 0.27e	7.42 ± 0.50m	11.6 ± 0.90n	4.19 ± 0.12k	6.43 ± 0.39i
OSP + Compost 1 %	3.67 ± 0.10e	6.00 ± 0.30c	8.23 ± 0.62j	13.7 ± 0.98j	4.56 ± 0.18h	7.61 ± 0.47g
OSP + Humic Acid 1 %	4.00 ± 0.10d	6.33 ± 0.30b	8.55 ± 0.65i	15.7 ± 1.02h	4.72 ± 0.20g	8.06 ± 0.49f
OSP + Citric Acid 1 %	4.33 ± 0.13c	6.67 ± 0.33a	9.72 ± 0.70h	17.8 ± 1.13f	5.23 ± 0.24e	9.78 ± 0.56d
OSP + EDTA 1 %	4.67 ± 0.17b	6.67 ± 0.35a	9.84 ± 0.74g	19.9 ± 1.29c	5.50 ± 0.27d	10.1 ± 0.70c
RP	2.33 ± 0.05i	4.00 ± 0.25h	6.87 ± 0.43o	10.1 ± 0.81q	3.53 ± 0.08o	5.11 ± 0.21o
RP + Compost 1 %	2.67 ± 0.07h	4.33 ± 0.30g	6.92 ± 0.47o	10.3 ± 0.82q	3.87 ± 0.09m	5.56 ± 0.23m
RP + Humic Acid 1 %	3.00 ± 0.07g	4.67 ± 0.30f	7.61 ± 0.52l	12.6 ± 0.86l	4.31 ± 0.14j	6.53 ± 0.30i
RP + Citric Acid 1 %	3.67 ± 0.10e	5.00 ± 0.33e	7.98 ± 0.53jk	13.6 ± 0.94j	4.43 ± 0.16i	7.32 ± 0.43h
RP + EDTA 1 %	3.67 ± 0.10e	5.33 ± 0.35d	9.76 ± 0.73gh	14.8 ± 1.05i	5.42 ± 0.21d	8.12 ± 0.49f
BS	2.00 ± 0.05j	3.00 ± 0.20k	6.10 ± 0.43q	9.38 ± 0.70s	3.51 ± 0.08o	5.17 ± 0.21n
BS + Compost 1 %	2.67 ± 0.07h	3.67 ± 0.25i	6.55 ± 0.45p	10.1 ± 0.82q	3.75 ± 0.10n	5.15 ± 0.20n
BS + Humic Acid 1 %	3.00 ± 0.10g	4.00 ± 0.30h	7.27 ± 0.57n	11.5 ± 0.93n	4.07 ± 0.13l	6.20 ± 0.33k
BS + Citric Acid 1 %	3.00 ± 0.07g	4.33 ± 0.35g	7.88 ± 0.56k	12.2 ± 0.96m	4.39 ± 0.15ij	6.41 ± 0.35ij
BS + EDTA 1 %	3.67 ± 0.13e	4.67 ± 0.30f	8.11 ± 0.60j	12.5 ± 0.97l	4.58 ± 0.16h	7.30 ± 0.41h
OSP+ RP+ BS (50 % for everyone)	4.33 ± 0.20c	6.00 ± 0.37c	9.76 ± 0.74gh	13.1 ± 1.04k	5.45 ± 0.25d	7.60 ± 0.49g
Compost 1 %	2.33 ± 0.07i	3.33 ± 0.17j	6.50 ± 0.45p	9.81 ± 0.72r	3.23 ± 0.10p	5.13 ± 0.24no
Humic Acid 1 %	2.67 ± 0.10h	4.00 ± 0.30h	6.67 ± 0.46p	10.3 ± 0.85q	3.86 ± 0.12m	5.47 ± 0.27m
Citric Acid 1 %	3.00 ± 0.07g	4.33 ± 0.30g	7.01 ± 0.49o	10.7 ± 0.87p	3.98 ± 0.15l	5.83 ± 0.30l
EDTA 1 %	3.33 ± 0.15f	4.33 ± 0.25g	7.29 ± 0.51mn	11.1 ± 0.95o	4.03 ± 0.15l	6.25 ± 0.38ijk
Compost 1 %+ Humic Acid 1 % (50 % for both)	4.33 ± 0.20c	5.00 ± 0.23e	10.6 ± 0.78d	16.0 ± 1.15g	5.27 ± 0.23e	8.53 ± 0.46e
Citric Acid 1 %+ EDTA 1 % (50 % for both)	5.00 ± 0.27a	6.00 ± 0.27c	13.7 ± 0.99b	19.5 ± 1.28d	7.15 ± 0.45b	10.3 ± 0.53c
Compost 1 %+ Citric Acid 1 % (50 % for both)	4.00 ± 0.20d	6.00 ± 0.30c	10.1 ± 0.80f	18.8 ± 1.16e	5.08 ± 0.28f	9.51 ± 0.49d
Compost 1 %+ EDTA 1 % (50 % for both)	4.00 ± 0.23d	6.33 ± 0.30b	10.4 ± 0.87e	19.7 ± 1.27cd	5.31 ± 0.29e	10.2 ± 0.54c
Humic Acid 1 %+ Citric Acid 1 % (50 % for both)	4.67 ± 0.25b	6.33 ± 0.35b	12.2 ± 0.91c	21.0 ± 1.33b	6.94 ± 0.32c	11.2 ± 0.63b
Humic Acid 1 %+ EDTA 1 % (50 % for both)	5.00 ± 0.27a	6.67 ± 0.37a	16.8 ± 1.12a	22.3 ± 1.35a	8.57 ± 0.50a	11.5 ± 0.64a

*(-PDB) means without adding P dissolving bacteria, **(+PDB) means in the presence of P dissolving bacteria. Ordinary Superphosphate (OSP), Rock Phosphate (RP), Basic Slag (BS). Values expressed as mean ± SE, the significant value was set at $p \le 0.05$. Different letters indicate significant difference between treatments

Table 7: N, P and K concentrations in faba bean leaves cultivated on El-Nubaria calcareous soil as affected by the different P sources, in the presence or absence of P- dissolving bacteria

	N, %		P, %		K, %	
Treatments	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**	(-PDB)*	(+PDB)**
Control (-P)	2.10 ± 0.07s	2.34 ± 0.09t	0.16 ± 0.02m	0.22 ± 0.03p	1.69 ± 0.08r	1.95 ± 0.08v
OSP	2.39 ± 0.09o	2.81 ± 0.12o	0.27 ± 0.05h	0.34 ± 0.09j	2.04 ± 0.10kl	2.16 ± 0.12q
OSP + Compost 1 %	2.43 ± 0.09jkl	2.92 ± 0.14l	0.30 ± 0.07e	0.37 ± 0.09g	2.13 ± 0.10i	2.27 ± 0.23jkl
OSP + Humic Acid 1 %	2.49 ± 0.10h	2.97 ± 0.15hi	0.33 ± 0.07c	0.39 ± 0.08e	2.17 ± 0.12g	2.31 ± 0.22i
OSP + Citric Acid 1 %	2.65 ± 0.12c	3.11 ± 0.18f	0.37 ± 0.08b	0.42 ± 0.10c	2.21 ± 0.15e	2.39 ± 0.28g
OSP + EDTA 1 %	2.72 ± 0.13b	3.26 ± 0.21e	0.39 ± 0.08a	0.46 ± 0.14a	2.30 ± 0.19b	2.45 ± 0.30f
RP	2.31 ± 0.09q	2.74 ± 0.10r	0.24 ± 0.04j	0.30 ± 0.06m	1.92 ± 0.09p	2.11 ± 0.09s
RP + Compost 1 %	2.40 ± 0.10no	2.84 ± 0.10n	0.27 ± 0.05h	0.32 ± 0.06l	2.03 ± 0.09l	2.18 ± 0.10op
RP + Humic Acid 1 %	2.46 ± 0.10i	2.88 ± 0.12m	0.28 ± 0.05g	0.35 ± 0.06i	2.12 ± 0.10ij	2.25 ± 0.18l
RP + Citric Acid 1 %	2.50 ± 0.12gh	2.96 ± 0.13ij	0.30 ± 0.07e	0.37 ± 0.07g	2.17 ± 0.13g	2.28 ± 0.20j
RP + EDTA 1 %	2.59 ± 0.13d	3.09 ± 0.15g	0.31 ± 0.07d	0.40 ± 0.07d	2.25 ± 0.18c	2.32 ± 0.21i
BS	2.28 ± 0.08r	2.69 ± 0.10s	0.21 ± 0.03l	0.26 ± 0.04o	1.87 ± 0.08q	2.08 ± 0.10t
BS + Compost 1 %	2.36 ± 0.09p	2.75 ± 0.11qr	0.24 ± 0.04j	0.30 ± 0.07m	1.97 ± 0.09n	2.14 ± 0.11r
BS + Humic Acid 1 %	2.41 ± 0.10mn	2.76 ± 0.11q	0.26 ± 0.06i	0.33 ± 0.07k	2.01 ± 0.09m	2.19 ± 0.11o
BS + Citric Acid 1 %	2.45 ± 0.10ij	2.87 ± 0.12m	0.28 ± 0.07g	0.36 ± 0.08h	2.13 ± 0.11i	2.23 ± 0.15m
BS + EDTA 1 %	2.55 ± 0.12e	2.98 ± 0.14h	0.29 ± 0.07f	0.39 ± 0.09e	2.21 ± 0.16e	2.26 ± 0.15kl
OSP+ RP+ BS (50 % for everyone)	2.35 ± 0.08p	2.83 ± 0.12n	0.30 ± 0.08e	0.35 ± 0.06i	2.05 ± 0.10k	2.05 ± 0.11u
Compost 1 %	2.32 ± 0.08q	2.70 ± 0.11s	0.22 ± 0.04k	0.28 ± 0.03n	1.94 ± 0.09o	2.10 ± 0.12t
Humic Acid 1 %	2.39 ± 0.09o	2.74 ± 0.11r	0.24 ± 0.04j	0.29 ± 0.03n	1.98 ± 0.09n	2.16 ± 0.14q
Citric Acid 1 %	2.42 ± 0.11lm	2.81 ± 0.12o	0.27 ± 0.05h	0.32 ± 0.07l	2.11 ± 0.012j	2.17 ± 0.15pq
EDTA 1 %	2.51 ± 0.12fg	2.94 ± 0.15k	0.28 ± 0.07g	0.34 ± 0.08j	2.16 ± 0.13gh	2.21 ± 0.18n
Compost 1 %+ Humic Acid 1 % (50 % for both)	2.43 ± 0.12jkl	2.79 ± 0.12p	0.26 ± 0.06i	0.32 ± 0.07l	2.15 ± 0.15h	2.36 ± 0.23h
Citric Acid 1 %+ EDTA 1 % (50 % for both)	2.55 ± 0.13e	3.28 ± 0.19d	0.28 ± 0.08g	0.38 ± 0.08f	2.23 ± 0.19d	2.48 ± 0.26e
Compost 1 %+ Citric Acid 1 % (50 % for both)	2.52 ± 0.14f	3.27 ± 0.20de	0.27 ± 0.06h	0.39 ± 0.09e	2.19 ± 0.17f	2.59 ± 0.27d
Compost 1 %+ EDTA 1 % (50 % for both)	2.56 ± 0.13c	3.36 ± 0.22c	0.28 ± 0.08g	0.40 ± 0.10d	2.21 ± 0.19e	2.67 ± 0.30c
Humic Acid 1 %+ Citric Acid 1 % (50 % for both)	2.74 ± 0.18b	3.45 ± 0.25b	0.28 ± 0.09g	0.43 ± 0.11b	2.29 ± 0.21b	2.81 ± 0.30b
Humic Acid 1 %+ EDTA 1 % (50 % for both)	2.90 ± 0.19a	3.58 ± 0.26a	0.30 ± 0.08e	0.46 ± 0.12a	2.34 ± 0.25a	2.94 ± 0.33a

*(-PDB) means without adding P dissolving bacteria, **(+PDB) means in the presence of P dissolving bacteria. Ordinary Superphosphate (OSP), Rock Phosphate (RP), Basic Slag (BS). Values expressed as mean ± SE, the significant value was set at $p \leq 0.05$. Different letters indicate significant difference between treatments

tion among the studied treatments gave better results, especially between chelating agents and organic compounds. In addition, the P dissolving bacteria produce organic and inorganic acids that mobilize P and other nutrients and encourage plant growth, as well as releasing phosphatase enzymes to mineralize organic P (Illmer et al., 1995; Cakmakci et al., 1999; Amalraj et al., 2012; Płaza et al., 2021).

4 CONCLUSION

Many factors are affecting the solubility of P in calcareous soils. In our study we tried to use some sources of P as well as organic substances and chelating agents, the interaction between them being the best. BS gave promising results especially when combined with citric acid and EDTA not only in calcareous soil, but possibly in soils poor in nutrient elements as sandy soils, even under the alkaline conditions. Using BS gained many benefits such as recycling of wastes, protecting the environment from contamination, and being a source of P fertilizer. Also, application of citric acid and EDTA enhanced faba bean growth in the investigated soil, particularly with addition of organic substances. In addition, adding solubilizing bacteria played a major role than the P- sources in enhancing the availability of P and increasing the uptake of other nutrients, leading to increased yield.

5 RECOMMENDATIONS

The use of RP as a source of P in calcareous soil at the time of applying the organic and bio fertilizers and close to plant roots is so beneficial. Applying RP or BS during the preparation of compost will enrich the compost with P. Application of BS along with organic fertilizers and chelating agents can be recommended in low fertile soil as sandy soil.

6 REFERENCES

Abd-Elrahman, Shaimaa H. (2016). Effect of unconventional phosphorus sources and phosphate solubilizing bacteria on fractions of phosphorus in a calcareous soil cultivated with wheat plants. *International Journal of Plant and Soil Science, 12*, 1-11. http://dx.doi.org/10.9734/IJPSS/2016/28375

Adugna, G. (2016). A review on impact of compost on soil properties, water use and crop productivity. *Academic Research Journal of Agricultural Science and Research, 4*, 93-104.http://dx.doi.org/10.14662/ARJASR2016.010

Afshan, S., Ali, Sh., Bharwana, S., Rizwan, M., Farid, M., & Abbas, F., et al. (2015). Citric acid enhances the phytoextraction of chromium, plant growth, and photosynthesis by alleviating the oxidative damages in *Brassica napus* L. *Environmental Science and Pollution Research, 22*, 11679-89. http://dx.doi.org/10.1007/s11356-015-4396-8

Amalraj, E.L.D., Maiyappan, S., & Peter, A.J. (2012). *In vivo* and *In vitro* studies of *Bacillus megaterium* var. *phosphaticum* on nutrient mobilization, antagonism and plant growth promoting traits. *Journal of Ecobiotechnology, 4*, 35-42.

Bing, L., Biao, T., Zhen, M., Hanchi, Ch., & Hongbo, L. (2019). Physical and chemical properties of steel slag and utilization technology of steel slag at home and abroad. *IOP Conf. Series: Earth and Environmental Science*, 242, 1-6. http://dx.doi.org/10.1088/17551315/242/3/032012

Bulut, S. (2013). Evaluation of yield and quality parameters of phosphorous-solubilizing and N-fixing bacteria inoculated in wheat (*Triticum aestivum* L.). *Turkish Journal of Agriculture and Forestry, 37*, 545-554. http://dx.doi.org/10.3906/tar-1212-96

Cakmakci, R., Kantar, F., & Algur, F. (1999). Sugar beet and barley yields in relation to *Bacillus polymyxa* and *Bacillus megaterium* var. *phosphaticum* inoculation. *Journal of Plant Nutrition and Soil Science, 162*, 437-442. https://doi.org/10.1002/(SICI)1522-2624(199908)162:4%3C437::AID-JPLN437%3E3.0.CO;2-W

Campitelli, P.A., Velasco, M.I., & Ceppi, S.B. (2003). Charge development and acid-base characteristics of soil and compost humic acids. *Journal of the Chilean Chemical Society, 48*(3). http://dx.doi.org/10.4067/S0717-97072003000300018

Chapman, H.D., & Pratt, P.F. (1961). *Methods of Analysis for Soils, Plants, and Waters*. Division of Agric. Sci. Berkeley, Univ. California, USA, pp. 150-152.

Doran, I., Akinci, C., & Yildirim, M. (2003). Effects of delta humate applied with different doses and methods on yield and yield components of diyarbakir-81 wheat cultivar. *5th Field Crops Congress,* Diyarbakir, Turkey, 2, 530-534.

Drouillon, M., & Merckx, R. (2003). The role of citric acid as a phosphorus mobilization mechanism in highly P-fixing soils. *Gayana Botanica, 60*(1), 55-62. http://dx.doi.org/10.4067/S0717-66432003000100009

Elgala, A.M., & Amberger, A. (2017). Factors affecting solubilization of rock phosphates in soils. *International Journal of Plant and Soil Science, 14*, 1-8. http://dx.doi.org/10.9734/IJPSS/2017/28526

Elhag, R.S., Elgala, A.M., Elsharawy, M.O., & Eid, M.A. (2019). Evaluate the effect of some factors affecting solubilization of phosphorus in rhizosphere. *Arab Universities Journal of Agricultural Sciences, Ain Shams University, 27*, 913-923. https://doi.org/10.21608/ajs.2019.43847

Fouda, K.F. (2017). Effect of phosphorus level and some growth regulators on productivity of faba bean (*Vicia faba* L.). *Egyptian Journal of Soil Science, 57*, 73-87.http://dx.doi.org/10.21608/ejss.2017.3593

Grover, R. (2003). Rock phosphate and phosphate solubilizing microbes as a source of nutrients for crops. *M.Sc. Thesis*, Patiala.

Hamed, M.H., & Gamal, M.M. (2014). Effect of incubation periods and some organic materials on phosphorus forms in calcareous soils. *International Journal of Technology Enhancements and Emerging Engineering Research (IJTEEE), 2*, 108-118.

Hopkins, B., & Ellsworth, J. (2005). Phosphorus availability with alkaline/ calcareous soil. *Western Nutrient Management Conference*, Vol. 6. Salt Lake City, UT, pp. 88-93.

Houassine, D., Latati, M., Rebouh, N.Y., & Gérard, F. (2020). Phosphorus acquisition processes in the field: Study of faba bean cultivated on calcareous soils in Algeria. *Archives of Agronomy and Soil Science, 66*, 168-181. https://doi.org/10.1080/03650340.2019.1605166

Huang, M., Zhu, Y., Li, Z., Huang, B., Luo, N., Liu, Ch., & Zeng, G. (2016). Compost as a soil amendment to remediate heavy metal-contaminated agricultural soil: Mechanisms, efficacy, problems, and strategies. *Water, Air and Soil Pollution,* 227-359. https://doi.org/10.1007/s11270-016-3068-8

Illmer, P., Barbato, A., & Schinner, F. (1995). Solubilization of hardly soluble $AlPO_4$ with P- solubilizing microorganisms. *Soil Biology and Biochemistry, 27*, 260-270. https://doi.org/10.1016/0038-0717(94)00205-f

Kanwal, U., Ali, S., Shakoor, M.B., Farid, M., Hussain, S., & Yasmeen, T., et al. (2014). EDTA ameliorates phytoextraction of lead and plant growth by reducing morphological and biochemical injuries in *Brassica napus* L. under lead stress. *Environmental Science and Pollution Research, 21*, 9899-9910. https://doi.org/10.1007/s11356-014-3001-x

Klute, A. (1986). *Methods of Soil Analysis*, part I, 2nd ed. Madison, Wisconsin, USA. https://doi.org/10.2136/sssabookser5.1.2ed

Lee, Ch., Park, S., Hwang, H., Kim, M., Jung, H., & Luyima, D., et al. (2019). Effects of food waste compost on the shift of microbial community in water saturated and unsaturated soil condition. *Applied Biological Chemistry, 62*, 1-7. https://doi.org/10.1186/s13765-019-0445-1

Mihoub, A., Daddi Bouhoun, M., Asif, N., & Saker, M.L. (2016). Low-molecular weight organic acids improve plant availability of phosphorus in different textured calcareous soils. *Archives of Agronomy and Soil Science*, *63*, 1023-1034. http://dx.doi.org/10.1080/03650340.2016.1249477

Mihoub, A., Daddi Bouhoun, M., & Naeem, A. (2018). Short-term effects of phosphate fertilizer enriched with low-molecular-weight organic acids on phosphorus release kinetics and its availability under calcareous conditions in arid region. *Journal of Scientific Agriculture, 2*, 66-70. http://dx.doi.org/10.25081/jsa.2018.v2.884

Mihoub, A., Amin, A.A., Asif, N., & Daddi Bouhoun, M. (2019) Improvement in phosphorus nutrition of wheat plants grown in a calcareous sandy soil by incorporating chemical phosphorus fertilizer with some selected organic substances. *Acta Agriculturae Slovenica, 113*(2), 263-272. https://doi.org/10.14720/aas.2019.113.2.7

Negim, O., Eloifi, B., Mench, M., Bes, C., Gaste, H., Motelica-Heino, M., & Le Coustumer, P. (2010). Effect of basic slag addition on soil properties, growth and leaf mineral composition of beans in a Cu-contaminated soil. *Journal of Soil and Sediment Contamination, 19*, 174-187. https://doi.org/10.1080/15320380903548508

Ning, D., Liang, Y., Liu, Z., Xiao, J., & Duan, A. (2016). Impacts of steel-slag-based silicate fertilizer on soil acidity and silicon availability and metals-immobilization in a paddy soil. *PLoS ONE, 11*, 1-15. https://doi.org/10.1371/journal.pone.0168163

Page, A.L., Miller, R.H., & Keeney, D.R. (1982). *Methods of Soil Analysis*, part II, 2nd ed. Wisconsin, USA.

Płaza, A., Rzążewska, E., & Gąsiorowska, B. (2021). Effect of *Bacillus megaterium* var. *phosphaticum* bacteria and L-Alpha proline amino acid on iron content in soil and *Triticum aestivum* L. plants in sustainable agriculture system. *Agronomy, 11*, 511. doi:10.3390/agronomy11030511

Razaq, M., Zhang, P., Shen, H., & Salahuddin (2017). Influence of nitrogen and phosphorous on the growth and root morphology of *Acer mono*. *PLoS ONE, 12*, 1-13. https://doi.org/10.1371/journal.pone.0171321

Sahin, S., Karaman, M.R., & Gebologlu, N. (2014). The effect of humic acid application upon the phosphorus uptake of the tomato plant (*Lycopersicum esculentum* L.). *Scientific Research and Essays, 9*, 586-590. http://dx.doi.org/10.5897/SRE2014.581

SAS. (2000). *Statistical analysis system, SAS User's Guide: Statistics*. SAS Institute Inc., Cary, USA.

Satisha, G., & Devarajan, L. (2005). Humic substances and their complexation with phosphorus and calcium during composting of press mud and other biodegradables. *Communications in Soil Science and Plant Analysis, 36*, 805-818. https://doi.org/10.1081/CSS-200049454

Saxena, A.K., Kumar, M., Chakdar, H., Anuroopa, N., & Bagyaraj, D.J. (2020). Bacillus species in soil as a natural resource for plant health and nutrition. *Journal of Applied Microbiology, 128*, 1583-1594. doi:10.1111/jam.14506

Soil Survey Staff. (2010). *Keys to Soil Taxonomy* (11th ed.). Washington, DC: U.S. Department of Agriculture, Natural Resources Conservation Service, U.S. Government Printing Office.

Taiwo, A.M. (2011). Composting as a sustainable waste management technique in developing countries. *Journal of Environmental Science and Technology, 4*, 93-102. http://dx.doi.org/10.3923/jest.2011.93.102

Taskin, M.B., Kadioglu, Y.K., Sahin, O., Inal, A., & Gunes, A. (2019). Effect of acid modified biochar on the growth and essential and non-essential element content of bean, chickpea, soybean, and maize grown in calcareous soil. *Communications in Soil Science and Plant Analysis, 50*, 1604-1613. https://doi.org/10.1080/00103624.2019.1631326

Tsakiridis, P.E., Papadimitriou, G.D., Tsivilis, S., & Koroneos, C. (2008). Utilization of steel slag for Portland cement clinker production. *Journal of Hazardous Materials, 152*, 805-811. https://doi.org/10.1016/j.jhazmat.2007.07.093

Watanabe, F.C., & Olsen, S.R. (1965). Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soils. *Soil Science Society of America Proceedings, 29*, 677-678. https://doi.org/10.2136/sssaj1965.03615995002900060025x

Yildirim, I.Z., & Prezzi, M. (2011). Chemical, mineralogical, and morphological properties of steel slag. *Advances in Civil Engineering, 2011*, 1-13. https://doi.org/10.1155/2011/463638

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Phenotypic variation and traits interrelationships in bread wheat (*Triticum aestivum* L.) genotypes in Northern Ethiopia

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Phenotypic variation and traits interrelationships in bread wheat (*Triticum aestivum* L.) genotypes in Northern Ethiopia

Abstract: Information on phenotypic variation helps to breed better varieties. Forty-nine bread wheat genotypes were evaluated in simple lattice design at Jamma and Geregera to determine the extent of variation and association among 11 traits. Analysis of variance showed significant differences ($p < 0.01$) among the genotypes for all traits, indicating the presence of adequate variability. Maximum values of genotypic coefficients of variation were recorded for spike length (8.66 %), number of productive tillers (8.4 %), number of grains per spike (6.4 %) and thousand-seed mass (6.15 %); this also shows the presence of substantial variability for these traits. Genetic parameters of the study revealed that days to heading, plant height, spike length, number of grains per spike and thousand-seed mass had moderate to high heritability and genetic advance as percent of the mean. Therefore, direct selection could be practiced to improve bread wheat for these traits. Moreover, selection of early-cycle lines which can escape the negative effects of climate change will be possible. Grain yield had strong and significant positive correlation with thousand-seed mass (r_g = 0.395**), biological yield (r_{gv}= 0.617**) and harvest index (r_g = 0.731**); selection based on these traits will be most effective in future bread wheat yield improvement programs as they also exerted strong positive direct effects on grain yield.

Key words: bread wheat; coefficient of variation; correlation; genetic advance; path coefficients

Fenotipska variabilnost in medsebojna povezanost lastnosti genotipov krušne pšenice (*Triticum aestivum* L.) v severni Etiopiji

Izvleček: Informacije o fenotipski variabilnosti pomagajo pri vzgoji boljših sort. V poskusu z dvema ponovitvama je bilo ovrednotenih 49 genotipov krušne pšenice na območjih Jamma in Geregera z namenom določitve obsega spremenljivosti in medsebojne povezanosti enajstih lastnosti. Analiza variance je pokazala značilne razlike med genotipi ($p < 0,01$) za vse lastnosti, kar kaže, da je prisotna primerna variabilnost. Največje vrednosti genotipskega koeficienta variabilnosti so bile ugotovljene za dolžino klasa (8,66 %), število cvetočih poganjkov na rastlino (8,4 %), število zrn na klas (6,4 %) in maso1000 semen (6,15 %), kar nakazuje tudi prisotnost precejšnje spremenljivosti teh lastnostih. Raziskava genetskih parametrov je odkrila, da imajo parametri kot so dnevi do klasenja, višina rastlin, dolžina klasa, število zrn na klas in masa 1000 semen zmerno do veliko dednost in genetsko prednost v odstotku poprečja. Zaradi tega bi lahko bila izvedena neposredna selekcija za izboljšanje krušne pšenice na osnovi teh lastnosti. Še več, možen bi bil izbor zgodnejših linij, ki bi pobegnile učinkom podnebnih sprememb. Pridelek zrnja je imel močno in značilno pozitivno korelacijo z maso 1000 semen (r_g = 0,395**), biološkim pridelkom (r_g = 0,617**) in žetvenim indeksom (r_g = 0,731**). Izbor na osnovi teh lastnosti bo najučinkovitejši v bodočih žlahtniteljski programih za izboljšanje pridelka krušne pšenice, ker ima neposredni pozitivni učinek na pridelek zrnja.

Ključne besede: krušna pšenica; koeficient variabilnosti; korelacija; genetska prednost; koeficienti povezanih lastnosti

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1 INTRODUCTION

The two wheat types, both bread (*Triticum aestivum* L.) and durum (*T. durum* Desf.), are among very important cereal crops in the world in terms of production and area coverage. In 2014, about 723.4 million tons of wheat was produced on 222.3 million hectares (ha) of land, with average yield of 3.25 t ha^{-1} worldwide (FAO, 2015). Very successful wheat producing countries in the world, like Germany and France, attained average wheat yields of 7.4 and 7.2 t ha^{-1}, respectively (Yao et al., 2012). However, in Ethiopia, the national wheat cultivated area was about 1.66 million ha in 2014, and the share in production was 4.23 million metric ton, with average yield of 2.54 t ha^{-1}. It was ranked third in total production among cereals behind maize and tef [*Eragrostis tef* (Zucc.) Trotter], and forth in area coverage after tef [*E. tef* (Zucc.) Trotter], maize and sorghum (CSA, 2015).

Bread wheat productivity in Ethiopia is much lower as compared to other countries. Among other things, lack of high yielding varieties is the most important bottle neck. As varieties under production may become susceptible to diseases and insects, and eventually become obsolete, continuous screening and selection of bread wheat genotypes is vital for breeders to develop new varieties. For such a purpose, a sufficiently high variability within the pools of germplasm is needed.

Variation in plant genetic resources for traits of agronomic importance provides the basis and the raw material that plays a fundamental role in crop improvement programs (Dwivedi et al., 2015). Assessment of the amount of this variation is useful to allow more effective genetic improvement (Haussmann et al., 2004). The effectiveness of selection, however, depends on the relative importance of genetic and non-genetic factors in the expression of phenotypic differences among genotypes, which is known as heritability (Fehr, 1987). Unless it is used together with genetic advance, heritability value by itself provides no indication of the amount of genetic progress (Johnson et al., 1955). Quantitative traits, like yield, are more difficult to select in a breeding program because they are influenced to a greater degree by the environment (Acquaah, 2007). For such traits, indirect selection through correlated traits becomes effective.

In bread wheat, some reports are available on phenotypic variability and traits interrelationship studies (Moghaddam et al., 1997; Ali et al., 2008; Tesfaye et al., 2014). However, the information generated so far is insufficient. The objectives of the present study, therefore, are to assess the nature and extent of phenotypic variability, to study interrelationship of traits as well as direct and indirect effects of yield attributing traits on bread wheat grain yield.

2 MATERIALS AND METHODS

2.1 DESCRIPTION OF THE STUDY AREA

The field experiment was conducted at Jamma and Geregera, experimental sites of Sirinka Agricultural Research Center, in 2015. Jamma lies between the geographical coordinates of 10^{o} 38’ N latitude and 39^{0} 20’ E longitude, at an altitude of 2600 m. a. s. l.; the soil type is *vertisol* with pH of 6.0, and has total rainfall of 720.5 mm. Geregera is located at an altitude of 2650 m. a. s. l, which lies between 11^{o} 46’ N latitude and 38^{o} 45’ E longitude; it has annual rainfall of 1105 mm, the soil type is *lithosol* with pH of 5.6.

2.2 PLANTING MATERIALS

Forty-nine bread wheat genotypes, 22 released varieties and 27 elite materials were used in the study. The genotypes are believed to be adapted to the tropical condition of Ethiopia, hence spring wheat types. Variety ‘Alidoro’ was sourced from Holeta Agricultural Research Center; ‘Gassay’ and ‘TAY’ from Adet; ‘Mada-Wolabu’, ‘Sofumar’, ‘UTQUE96/3/PYN/BAU//MILLAN’ and ‘WORRAKATTA/PASTOR’ from Sinana; ‘Mekelle-3’ and ‘Mekelle-4’ from Mekelle; and the rest were sourced from Kulumsa Agricultural Research Center (Table 1).

2.3 EXPERIMENTAL DESIGN AND TRIAL MANAGEMENT

The experiment was laid out in 7 × 7 simple lattice design with two replications. The dimension of an individual plot area was 1.2 m × 2.5 m (3 m^2) with six rows for each entry. The spacing between blocks, plots and rows were 1.5 m, 0.4 m and 0.2 m, respectively. Planting was done with the seed rate of 150 kg ha^{-1} (45 g $plot^{-1}$). Diammonium phosphate (DAP) and urea fertilizers were applied at the rate of 100 kg ha^{-1}. The total dose of DAP was applied at planting, while urea was splitted $^{1}/_{3}$ at planting and $^{2}/_{3}$ at mid-tillering stages. All the other recommended agronomic practices were applied uniformly to all plots.

2.4 DATA COLLECTION

Data on phenological, agronomic, yield and yield components were recorded. For plant height (cm), number of productive tillers per plant, spike length (cm), number of spikelets per spike and number of grains per spike, data were collected from ten randomly selected plants from the central four rows and the mean values were computed. The data for days to heading (number of days from sowing till flowering) and maturity (number of days from sowing till maturity), thousand-seed mass (g), biological yield (kg m^{-2}), grain yield (qt ha^{-1}) and harvest index (%) were collected on plot basis from the central four rows (2 m^2).

2.5 STATISTICAL ANALYSES

The data collected were subjected to the analysis of variance (ANOVA) for simple lattice design using SAS version 9.2 (SAS Institute, 2008). Test of homogeneity of error variance of each character for the two locations was done by using F-max ratio (Hartley, 1950) before combining the data. Following the analysis of variance, phenotypic and genotypic coefficients of variation (PCV and GCV) were estimated from the corresponding genotypic and phenotypic components of variances as suggested by Burton and DeVane (1953). Heritability (h^2) in the broad sense and genetic advance (GA) were calculated with the method suggested by Johnson et al. (1955). Genetic advance as percent of the mean (GAM) was calculated by dividing the expected genetic advance by the respective mean of the traits studied and multiplying by hundred.

Phenotypic and genotypic correlation coefficients were computed using GENRES statistical software (Pascal Intl Software Solutions, 1994) using the procedure suggested by Miller et al. (1958) from the corresponding variance and covariance components. The

Table 1: Description of the 49 bread wheat genotypes used in the study

S.N.	Genotype	Status	S.N.	Genotype	Status
1	'Alidoro'	Released	26	'ETBW 8514'	Elite line
2	'Biqa'	Released	27	'ETBW 8515'	Elite line
3	'Danda'a'	Released	28	'ETBW 8516'	Elite line
4	'Digelu'	Released	29	'ETBW 8517'	Elite line
5	'ETBW 6861'	Elite line	30	'ETBW 8518'	Elite line
6	'ETBW 6940'	Elite line	31	'ETBW 8519'	Elite line
7	'ETBW 7038'	Elite line	32	'Gassay'	Released
8	'ETBW 7058'	Elite line	33	'Hidasse'	Released
9	'ETBW 7101'	Elite line	34	'Hoggana'	Released
10	'ETBW 7120'	Elite line	35	'Honqolo'	Released
11	'ETBW 7147'	Elite line	36	'Hulluka'	Released
12	'ETBW 7194'	Elite line	37	'Jeferson'	Released
13	'ETBW 7213'	Elite line	38	'Kakaba'	Released
14	'ETBW 7364'	Elite line	39	'King Bird'	Registered
15	'ETBW 7368'	Elite line	40	'Mada-Wolabu'	Released
16	'ETBW 7871'	Elite line	41	'Mekelle-3'	Released
17	'ETBW 7872'	Elite line	42	'Mekelle-4'	Released
18	'ETBW 8506'	Elite line	43	'Ogolcho'	Released
19	'ETBW 8507'	Elite line	44	'Pavon-76'	Released
20	'ETBW 8508'	Elite line	45	'Shorima'	Released
21	'ETBW 8509'	Elite line	46	'Sofumar'	Released
22	'ETBW 8510'	Elite line	47	'TAY'	Released
23	'ETBW 8511'	Elite line	48	'UTQUE96/3/PYN/BAU//MILLAN'	Released
24	'ETBW 8512'	Elite line	49	'WORRAKATTA/PASTOR'	Released
25	'ETBW 8513'	Elite line			

significance of genotypic correlation coefficients were tested using the formula adopted by Robertson (1959). Path coefficient analysis was done following the method suggested by Dewey and Lu (1959).

3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF VARIANCE

As the relative efficiency of the simple lattice design was less than that of the randomized complete block design (RCBD) for most characters, and blocks within replication sum of squares were non-significant, the analysis of variance (ANOVA), therefore, was performed using RCBD model. The combined ANOVA for the two locations was run as the assumption for homogeneity of error variances was met.

The result of the combined analysis for different studied traits is shown in Table 2. Mean squares of genotypes for all characters studied were significant ($p < 0.05$), indicating the existence of genetic variability within genotypes to be exploited in breeding programs. The coefficient of determination (R^2) ranged from 0.77 for number of grains per spike to 0.95 to grain yield indicating that from 77 % to 95 % of the variation in the genotypes was explained by the traits considered. The location effect was significant ($p < 0.01$) for all traits, indicating the different climatic conditions in the two locations. Furthermore, location × genotype interaction effect was significant for all traits except number of spikelets per spike indicating different performance of bread wheat genotypes across the two locations (Table 2). The present investigation is in conformity with early findings (Tesfaye et al., 2014; Ferede and Worede, 2016; Mesele et al., 2016).

3.2 GENOTYPIC AND PHENOTYPIC COEFFICIENTS OF VARIATION

The genotypic coefficient of variation (GCV) ranged from 1.88 % for days to maturity to 8.66 % for spike length; and phenotypic coefficient of variation (PCV) ranged from 2.3 % for days to maturity to 13.3 % for number of productive tillers (Table 3). Maximum value of GCV was recorded for spike length (8.66 %), followed by number of productive tillers (8.4 %), number of grains per spike (6.4 %) and thousand-seed mass (6.15 %); whereas the highest value of PCV was recorded for productive tillers (13.3 %) followed by grain yield (11.35 %), spike length (10.3 %) and harvest index (9 %).

The magnitude of PCV was much higher than the corresponding GCV for number of productive tillers, grain yield, harvest index and biological yield indicating that the apparent variation for the characters was not only genotypic but also environmental. This result agrees with the findings of Mohammedi et al. (2011).

3.3 HERITABILITY IN THE BROAD SENSE

Heritability estimate for characters under study is

Table 2: Estimated values of mean squares, coefficient of variation (CV) and R^2 (%) for 11 traits of 49 bread wheat genotypes combined over two locations

Traits	L (df = 1)	G (df = 48)	G × L (df = 48)	Error (df = 96)	CV (%)	LSD (5%)	R^2 (%)
DH	65.15**	58.12**	10.5**	3.11	2.65	2.48	0.92
DM	650.3**	34.67**	11.70*	5.9	1.9	3.41	0.84
PH (cm)	4662**	117.4**	44.7**	25.5	6.45	7.09	0.84
NPTP	9.48**	0.163**	0.10**	0.06	16.3	0.35	0.81
SL (cm)	99.26**	2.40**	0.710*	0.46	8.97	0.94	0.86
NSPS	321.4**	3.50**	1.30ns	1.13	7.3	1.48	0.84
NGS	922.4**	40.5**	17.00*	11.27	8.9	4.71	0.77
TSM (g)	9839**	41.30**	16.15*	6.8	7.11	3.9	0.94
BY (kg m^{-2})	23.40**	0.140**	0.088**	0.045	10	0.299	0.89
HI (%)	1.070**	0.00311**	0.0021*	0.0014	9.66	0.043	0.94
GY (qt ha^{-1})	35311.8**	61.72**	43.75**	22.5	13.7	6.66	0.95

L = Location, G = genotype, G × L = Genotype-location interaction, df = degrees of freedom, DH = Days to heading, DM = Days to maturity, PH = plant height, NPTP = number of productive tillers per plant, SL = Spike length, NSPS = Number of spikelets per spike, NGS = Number of grains per spike, BY = Biological yield, HI = Harvest index, TSM = Thousand-seed mass and GY = Grain yield presented in Table 3. In the study, heritability in broad sense ranged from 29 % for grain yield to 82 % for days to heading. Heritability is categorized as low (0-30 %), moderate (30-60 %) and high (60 % and above) as given by Comstock and Robinson (1952).

Accordingly, high heritability was estimated for days to heading (82 %), days to maturity (66.2 %), spike length (70.4 %), plant height (63.6), number of spikelets per spike (62.5) and thousand-seed mass (61 %). Similar results were documented by Laghari et al. (2010). Moreover, Ali et al. (2008) reported high estimates of heritability for spike length and number of spikelets per spike in bred wheat. However, in contrast to the results of this study, Tesfaye et al. (2014) reported low estimates of heritability for those traits. The reasons for the disagreement in the findings may be due to differences in the type and number of genetic materials used, and differences in environmental conditions.

Moderate heritability was obtained for number of grains per spike, number of productive tillers, harvest index and biological yield, indicating that the characters were influenced by environment to some extent. Low heritability was obtained for yield per ha (29 %). Low heritability estimates for yield, ranging from 7.4 % to 25 %, were documented for grain yield (Mohammadi et al., 2011; Tesfaye et al., 2014; Mesele et al., 2016).

3.4 EXPECTED GENETIC ADVANCE

Genetic advance as percent of the mean (GAM) ranged from 3.15 % for days to maturity to 14.9 % for spike length (Table 3). Relatively high GAM values were recorded for spike length (14.9 %) followed by number of productive tillers per plant (10.6 %), number of grains per spike (10 %), thousand-seed mass (10 %), days to heading (9.7 %) and plant height (9.07 %), indicating good response to selection. The present study was in close agreement with the findings of Mohammadi et al. (2011), Mesele et al. (2016) and Rahman et al. (2016). The genetic advance for grain yield was 2.36 qt ha^{-1}. This indicates by selecting 5 % of the high yielding genotypes from the base population, mean yield of the new population would increase from 34.6 to 36.96 qt ha^{-1}.

High heritability accompanied with relatively high genetic advance in case of days to heading, plant height, spike length and thousands-seed mass indicates that the heritability is the most likely due to additive gene effects. In such cases early generation selection for these traits may be effective. In the present study, high heritability estimates along with low genetic advance, however, indicates that non additive type of gene action and environment play significant role in the expression of the traits as observed in days to maturity. The result agrees with the findings of Majumder et al. (2008).

In general, traits like spike length and thousand-seed mass showed high heritability along with high GAM, PCV and GCV; while number of grains per spike had moderate heritability along with high GAM, PCV and GCV in this study. Thus, direct selection could be practiced to improve bread wheat for these traits.

Table 3: Estimates of range, means, genotypic (σ^2g) and phenotypic (σ^2p) variances, heritability (h^2) and genetic advance (GA) for 11 traits of 49 bread wheat genotypes, combined across the locations

Traits	Range	Mean ± SE	δ^2_g	δ^2_p	GCV (%)	PCV (%)	h^2 (%)	GA	GAM
DH	61-79.5	66.6±0.04	11.90	14.53	5.20	5.72	82.0	6.45	9.70
DM	124-136	127.6±0.22	5.74	8.67	1.88	2.30	66.2	4.02	3.15
PH	68-93.75	78.3±0.084	18.7	29.35	5.50	6.90	63.6	7.10	9.07
NPTP	1.2-1.95	1.5±0.214	0.016	0.040	8.40	13.3	38.7	0.16	10.6
SL	6.4-10.9	7.5±0.104	0.422	0.600	8.66	10.3	70.4	1.12	14.9
NSPS	13 -17.4	14.6±0.78	0.550	0.880	5.10	6.44	63.0	1.21	8.30
NGS	29-45.7	37.8±0.11	5.850	10.12	6.40	8.42	57.8	3.80	10.0
TSM	34.8-48	40.8±0.10	6.29	10.32	6.15	7.86	61.0	4.04	10.0
BY	1.8-2.70	2.12±0.123	0.013	0.035	5.38	8.82	37.0	0.143	6.73
HI	0.26-0.36	0.31±0.16	0.00025	0.00078	5.10	9.00	32.0	0.018	5.95
GY	26.5-43.8	34.6±0.20	4.50	15.43	6.13	11.35	29.1	2.360	6.80

GCV and PCV = Genotypic and phenotypic coefficient of variation, GAM = Genetic advance as percent of the mean, DH = Days to heading, DM = Days to maturity, PH = Plant height, NPTP = Number of productive tillers per plant, SL = Spike length, NSPS = Number of spikelets per spike, NGS = Number of grains per spike, TSM = Thousand-seed mass, BY = Biological yield, HI = Harvest index, GY = Grain yield

3.5 CORRELATIONS ANALYSIS OF QUANTITATIVE TRAITS

Genotypic and phenotypic correlations of all possible combinations of the traits under study are presented in Table 4. In general, the magnitude of the genotypic correlation coefficients (r_g) was higher than the corresponding phenotypic correlation coefficients (r_p). This reveals the superiority of genetic variance in expression of the traits and that association among characters is under genetic control.

Days to maturity was significantly associated with days to heading (r_g = 0.946**) and biological yield per plot (r_g = -0.306*) at genotypic level. The negative association with biological yield connote that late maturing genotypes tend to have low biological yield. The correlation between plant height and grain yield per ha was positive and significant at both genotypic and phenotypic levels (r_g = 0.384**, r_p = 0.354*) which indicates an increase in plant height also leads to an increase in grain yield. Similar results in association with bread wheat have been reported by Moghaddam et al. (1997) and Gelalcha and Hanchinal (2013).

Thousand-seed mass had positive and significant association with grain yield per ha at genotypic and phenotypic levels (r_g = 0.395*, r_p = 0.365). This result is in agreement with the works of Laei et al. (2012) and Zafarnaderi et al. (2013). There were also significant genotypic correlations with plant height (0.377**) and harvest index (0.396**). Biological yield was in positive and significant relationship with grain yield at both phenotypic and genotypic levels (r_g = 0.617**, r_p = 0.624**). These results are supported by the findings of Chowdhry et al. (1991) and Laei et al. (2012).

Harvest index had positive and significant relationship at both genotypic and phenotypic levels with grain yield per ha (r_g = 0.731**, r_p = 0.625*). These results are supported by the findings of Chowdhry et al. (1991), Laei et al. (2012) and Zafarnaderi et al. (2013). It was negatively correlated with days to heading, days to maturity, spike length and thousand-seed mass at genotypic level. The result agreed with the findings of Moghaddam et al. (1997), but contradicted with the findings of Zafarnaderi et al. (2013).

The study of correlation among yield and yield attributing traits showed that plant height, number of productive tillers per plant, thousand-seed mass, harvest index and biological yield had positive and significant association with grain yield at genotypic level. Therefore, these traits could be utilized for indirect selection in breeding programs to improve bread wheat for yield. However, it is probably better to investigate

Table 4: Genotypic correlation coefficient (r_g; above diagonal) and phenotypic correlation coefficient (r_p; below diagonal) of 11 traits of 49 bread wheat genotypes

Traits	DH	DM	PH	NPTP	SL	NSPS	NGS	TSM	BY	HI	GY
DH		0.946**	-0.165	-0.386**	0.148	0.095	-0.092	0.207	-0.168	-0.122	-0.184
DM	0.767**		-0.064	-0.099	0.155	0.107	-0.017	0.096	-0.306*	-0.033	-0.252
PH	-0.113	-0.039		0.26	0.565**	0.575**	0.625**	0.377**	0.363*	0.232	0.384**
NPTP	-0.132	-0.033	0.18		-0.261	-0.381**	0.159	0.288*	0.268	0.248	0.366*
SL	0.087	0.107	0.474**	-0.132		0.743**	0.032	0.218	-0.047	0.06	-0.047
NSPS	0.038	0.033	0.38**	-0.039	0.662**		0.248	-0.012	0.046	-0.014	0.004
NGS	-0.09	0.005	0.39**	0.064	0.007	0.188		0.019	0.061	0.177	0.176
TSM	0.154	0.161	0.372**	0.214	0.213	0.03	0.012		0.109	0.396**	0.395**
BY	-0.123	-0.057	0.375**	0.169	0.038	0.046	0.118	0.194		-0.067	0.617**
HI	-0.031	0.031	0.161	0.15	0.021	-0.165	0.064	0.322*	-0.144		0.731**
GY	-0.102	-0.021	0.354*	0.226	-0.014	-0.124	0.136	0.365*	0.624**	0.625**	

X^2 = 0.288, 0.372; * and ** = significant at 5 % and 1% probability levels, respectively, DH = Days to heading, DM = Days to maturity, PH = Plant height, NPTP = Number of productive tillers per plant, SL = Spike length, NSPS = Number of spikelets per spike, NGS = Number of grains per spike, BY = Biological yield, HI = Harvest index, TSM = Thousand-seed mass, GY = Grain yield the direct and indirect effects of these traits on grain yield.

3.6 PATH COEFFICIENT ANALYSIS

As the number of interdependent characters affecting a dependent character increases, correlation alone becomes insufficient to explain relationships among characters (Ariyo et al., 1987). In such cases, path coefficient analysis, identification of direct and indirect causes of association becomes indispensable.

Estimates of path coefficients were presented in Table 5. Maximum positive direct effect on grain yield per ha was exerted by harvest index (0.753), followed by biomass yield (0.753). The high direct effects of these traits on grain yield could be considered as causes of the strong correlation; an increase in harvest index and biological yield directly contribute to an increase in grain yield. Chowdhry et al. (1991) also reported positive direct effects of harvest index (0.443) and biological yield (0.327) on grain yield per plant. Thousand-seed mass was the other trait with positive direct effect (0.161) on yield; it also had substantial effect on grain yield indirectly through harvest index (0.298*). On the other hand, negative direct effects were exerted on grain yield by plant height (-0.215) and number of productive tillers per plant (-0.078). However, the consequent counter balancing of the positive and substantial indirect effects of thousand-seed mass, harvest index and biological yield led to positive and significant correlation of these traits with grain yield. This justifies the importance of splitting genotypic correlation coefficients into direct and indirect effects by using path coefficient analysis.

On the basis of estimates of path coefficients, it could be suggested that harvest index followed by biological yield and thousand-seed mass are the direct contributors to grain yield in the present investigation. The result agrees with Gashaw et al. (2007) and Gelalcha and Hanchinal (2013). Biological yield, harvest index and thousand-seed mass, which had highly significant correlation with grain yield and positive direct effects, could be used as selection index in grain yield improvement of bread wheat.

To this end, the residual effect in the present study (0.126) shows that 87.4 % of the variability in grain yield was explained by the component traits, while 12.6 % is due to the interventions of unexplained factors (error and traits not included). The result is in conformity with the findings of Gashaw et al. (2007) and Gelalcha and Hanchinal (2013), who reported residual effects of 0.065 and 0.0083, respectively.

4 CONCLUSIONS

Overall variability within a crop is due to heritable and non-heritable components. In the present investigation, maximum GCV values of spike length (8.66 %) followed by number of productive tillers (8.4 %), number of grains per spike (6.4 %) and thousand-seed mass (6.15 %) shows the presence of sizable variability for these traits. Improvement of bread wheat could be based on direct selection for days to heading, plant height, spike length, number of grains per spike and thousand-seed mass as these traits had moderate to high values of heritability and genetic advance as percent of the mean. Significant positive correlation along with strong positive direct effects on grain yield were achieved by thousand-seed mass, harvest index and biological yield; consequently, these traits could be used as indirect selection criteria to improve bread wheat grain yield.

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Table 5: Estimate of direct (bold face and diagonal) and indirect (off diagonal) effects at genotypic level in five traits of 49 bread wheat genotypes

Traits	PH	NPTP	TSM	BY	HI	r_g
PH	-0.215	-0.020	0.061	0.273	0.174	0.384**
NPTP	-0.056	-0.078	0.046	0.202	0.187	0.366*
TSM	-0.081	-0.023	0.161	0.082	0.298*	0.395**
BY	-0.078	-0.021	0.018	0.753**	-0.050	0.617**
HI	-0.050	-0.019	0.064	-0.050	0.753**	0.731**

Residual effect = 0.126, * and ** significant at 0.05 and 0.01 probability levels, PH = Plant height, NPTP = Number of productive tillers per plant, TSM = Thousand-seed mass, BY = Biological yield, HI = Harvest index, r_g = Genotypic correlation

for availing wheat seeds, to research assistants of SARC for the help on the research field.

6 REFERENCES

Acquaah, G. (2007). *Principles of plant genetics and breeding*. Black well Publishing, USA.

Ali, Y., Atta, B.M., Akhter, J., Monneveux, P. and Lateef, Z. (2008). Genetic variability, association and diversity studies in wheat (*Triticum aesitum* L.) germplasm. *Pakistan Journal of Botany*, *40*(5), 2087-2097.

Ariyo, O.J., Aken'ova, M.E. and Fatokun, C.A. (1987). Plant character correlation and path analysis of pod yield in Okra (*Abelmoschus esculentus*). *Euphytica, 36*, 677-686. https://doi.org/10.1007/BF00041518

Burton, G.W. and DeVane, E.H. (1953). Estimating heritability in tall fescue (*Festuca arundinacea*) from replicated clonal material. *Agronomy Journal, 45*, 487-488. https://doi.org/10.2134/agronj1953.00021962004500100005x

Chowdhry, M.S., Alam, K. and Khaliq, I. (1991). Harvest index in bread wheat. *Pakistan Journal of Agricultural Sciences*, *28(2)*, 207- 210.

Comstock, R. R. and Robinson, H. F. (1952). Genetic parameters, their estimation and significance. *Proceedings of the* 6th International Grassland Congress (pp. 248-291). Washington, DC.

Central Statistical Agency (CSA). (2015). *Agricultural sample survey for 2014/2015: Area and production of major crops*. Volume I. Addis Ababa, Ethiopia.

Dewey, D.R. and Lu, K.H. (1959). A correlation and path coefficient analysis of components of crested wheat grass seed production. *Agronomy Journal*, *51*, 515-558. https://doi.org/10.2134/agronj1959.00021962005100090002x

Dwivedi, S.L., Sahrawat, K.L., Upadhyaya, H.D., Mengoni, A., Galardini, M., Bazzicalupo, M., Biondi, E.G., Hungria, M., Kaschuk, G., Blair, M.W., Ortiz, R. (2015). Advances in host plant and rhizobium genomics to enhance symbiotic nitrogen fixation in grain legumes. *Advances in Agronomy, 129*, 1-116. https://doi.org/10.1016/bs.agron.2014.09.001

Fehr, W.R. (1987). *Principles of cultivar development: Theory and technique*. Volume I. McGraw-Hill. New York.

Ferede, M. and Worede, F. (2016). Grain yield stability and phenotypic correlation analysis of bread wheat (*Triticum aestivum* L.) genotypes in north western Ethiopia. *Food Science and Quality Management, 48*, 51-59.

Gashaw, A., Mohammed, H. and Singh, H. (2007). Selection criterion for improved grain yields in Ethiopian durum wheat genotypes. *African Crop Science Journal*, *15(1)*, 25-31.https://doi.org/10.4314/acsj.v15i1.54407

Gelalcha, S., and Hanchinal, R. R. (2013). Correlation and path analysis in yield and yield components in spring bread wheat (*Triticum aestivum* L.) genotypes under irrigated condition in Southern India. *African Journal of Agricultural Research, 8(24)*, 3186-3192. https://doi.org/10.5897/AJAR2013.6965

Hartley, H.O. (1950). The maximum F-ratio as a short cut test for heterogeneity of variances. *Biometrika, 37*, 308-312. https://doi.org/10.2307/2332383

Haussmann, B.I.G., Parzies, H.K., Presterl, T., Susic, Z., and Miedaner, T. (2004). Plant genetic resources in crop improvement. *Plant Genetic Resources,* 2(1): 3-21. https://doi.org/10.1079/PGR200430

Johnson H.W., Robinson, H.F. and Comstock, R.E. (1955). Estimates of genetic and environmental variability in soyabeans. *Agronomy Journal*, *47*, 314-318. https://doi.org/10.2134/agronj1955.00021962004700070009x

Laei, G., Afshari, H., Kamali, M. R. J. and Hassanzadeh, A. (2012). Study yield and yield components comparison correlation some physiological characteristics, 20 genotypes of bread wheat. *Annals of Biological Research*, *3(9)*, 4343-4351.

Laghari, K. A., Sial, M. A., Arain, M. A., Dahot, M. U., Mangrio, M. S. and Pirzada, A. J. (2010). Comparative performance of wheat advance lines for yield and its associated traits. *World Applied Sciences, 8*, 34-37.

Majumder, D.A.N., Shamsuddin, A.K.M., Kabir, M.A. and Hassan, L. (2008). Genetic variability, correlated response and path analysis of yield and yield contributing traits of spring wheat. *Journal of the Bangladesh Agricultural University, 6(2)*, 227-234. https://doi.org/10.3329/jbau.v6i2.4815

Mesele, A., Mohammed, W. and Dessalegn, T. (2016). Estimation of heritability and genetic advance of yield and yield related traits in bread wheat (*Triticum aestivum* L.) genotypes at Ofla district, Northern Ethiopia. *International Journal of Plant Breeding and Genetics, 10*, 30-37. https://doi.org/10.3923/ijpbg.2016.31.37

Miller, P.A., Williams, J.C., Robinson, H.F. and Comstock, R.E. (1958). Estimates of genotypic and environmental variances and covariances in upland cotton and their implications in selection. *Agronomy Journal*, *50*, 126-131. https://doi.org/10.2134/agronj1958.00021962005000030004x

Moghaddam, M., Ehdaie, B. and Waines, J.G. (1997). Genetic variation and interrelationships of agronomic characters in landraces of bread wheat from southeastern Iran. *Euphytica, 95*, 361-369. https://doi.org/10.1023/A:1003045616631

Mohammadi, M., Karimizadeh, R., Shefazadeh, M.K. and Sadeghzad, B. (2011). Statistical analysis of durum wheat yield under semi-warm dryland condition. *Australian Journal of Crop Science*, *5(10)*, 1292-1297.

Rahman, M.A., Kabir, M.L., Hasanuzzaman, M., Rahman, M.A., Rumi, R.H. and Afrose, M.T. (2016). Study of variability in bread wheat (*Triticum aestivum* L.). *International Journal of Agronomy and Agricultural Research, 8(5),* 66-76.

Robertson, G.E. (1959). The sampling variance of the genetic correlation coefficient. *Biometrics, 15,* 469-485. https://doi.org/10.2307/2527750

Tesfaye, T., Genet, T. and Desalegn, T. (2014). Genetic variability, heritability and genetic diversity of bread wheat (*Triticum aestivum* L.) genotype in western Amhara region, Ethiopia. *Wudpecker Journal of Agricultural Research*, *3(1)*, 26-034.

Yao, J., Ma, H., Yang, X., Yoa, G.U. and Zhou, M. (2014). Inheritance of grain yield and its correlation with yield components in bread wheat (*Triticum aestivum* L.). *Af-*

rican Journal of Biotechnology, *13*, 1379-1385. https://doi.org/10.5897/AJB12.2169

Zafarnaderi, N., Aharizad, S. and Mohammadi, S.A. (2013). Relationship between grain yield and related agronomic traits in bread wheat recombinant inbred lines under water deficit condition. *Annals of Biological Research*, *4(4)*, 7-11.

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Style length and flower morphology of three eggplant (*Solanum melongena* L.) cultivars from Iran affected by fruit load

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Style length and flower morphology of three eggplant (*Solanum melongena* L.) cultivars from Iran affected by fruit load

Abstract: A common feature of eggplant is its heterostyly. Long-style flowers bear fruits whereas short style ones fail to do so. Heterostyly is influenced by some factors such as genotype, climatic conditions and fruit load. In this study three eggplant cultivars from Iran were cultivated under greenhouse condition. The influence of presence of fruit (two fruits and four fruits) or absence of that on style length and some other flower morphological was studied in three positions of single, basal and additional. The presence of fruit, specially four fruits reduced style length, stigma width as well as mass of flower, pistil and stigma compared to the control in all times during fruit growth, and after fruit harvest they increased again. Fruit load didn't affect the number of stamen and stamen length. These effects were observed in all three positons of single, basal and additional flowers of all three cultivars. Generally this study showed that fruit load has decreasing effect on style length and size of flowers forming after fruit setting, which reversed after fruit harvesting.

Key words: eggplant; floral morphology; heterostyly; presence of fruit; style length

Vpliv števila plodov na dolžino vrata pestiča in morfologijo cveta pri treh sortah jajčevca (*Solanum melongena* L.) v Iranu

Izvleček: Splošno poznana lastnost jajčevca je heterostilija. Cvetovi z dolgim vratom cvetiča imajo plodove, tisti s kratkim vratom pa ne. Na heterostilijo vplivajo nekateri dejavniki kot so genotip, podnebne razmere in obloženost s plodovi. V raziskavi so bile gojene tri sorte jajčevca v rastlinjaku. Preučevan je bil vpliv števila plodov (dva in štirje plodovi) in njihova odsotnost na dolžino vrata pestiča in nekatere druge morfološke lastnosti cvetov v odvisnosti od njihovega položaja in sicer posamezni, prvi v socvetju in naslednji. Prisotnost plodov, še posebej štirih, je zmanjšala dolžino vrata pestiča, širino brazde kot tudi maso cveta, vratu in brazde pestiča v primerjavi s kontrolo v celotnem obdobju rasti. Po obiranju plodov se je vrednost teh parametrov spet povečala. Obloženost s plodovi ni vplivala na število prašnikov in njihovo dolžino. Ti učinki so bili opaženi pri vseh treh položajih plodov (posamezni, prvi in naslednji), pri vseh treh sortah. Na splošno je raziskava pokazala, da je obloženost s plodovi vplivala na zmanjšanje dolžine vratov pestiča in velikosti cvetov, ki so nastali po zasnovi plodov, po njihovem obiranju pa so se vrednosti teh parametrov spet povečale.

Ključne besede: jajčevec; morfologija cvetov; heterostilija; prisotnost plodov; dolžina vratu pestiča

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1 INTRODUCTION

Eggplant (*Solanum melongena* L.) from the Solanaceae family, is belonging to tropical and subtropical regions (San José et al., 2016). India is primary centre of origin (Meyer et al., 2012); China and Japan are secondary centres of origin, and today this crop is cultivated worldwide from Mediterranean to Africa, Europe and America (Frary et al., 2007; Daunay, 2008). Its total world annual production reached over 55 million tons in 2019, which with an annual production of 670158 tons, Iran is the fifth leading eggplant producer after China, India, Egypt and Turkey (Faostat, 2019).

Flowering and fruit setting are the most important factors in determining the yield of eggplant that is influenced by the genotype, the environmental conditions, the flower's position on the plant and fruit load (Mohideen et al., 1977; Nothmann et al., 1983; Sun et al., 1990; Kowalska, 2003, 2006; Banik et al., 2018). Eggplant flowers are large and usually violet-colored. They consist of five united and persistent sepals, five united and cup-shaped petals, usually five stamens alternating with the corolla, united carpels, and superior ovaries arranged either singly or in inflorescence. The number of flower bud is different in inflorescences and it is between 2-7 flower buds (Rashid & Singh, 2000; Hazra et al., 2003; Jagatheeswari, 2014). Eggplant flowers reportedly exhibit a form of heterostyly. Based on this property, eggplant flowers are classified into the three groups of long style, medium style, and short style flowers based on the style length relative to that of the stamen (Prakash, 1968; Rylski et al., 1984; Handique & Sarma, 1995; Prasad & Sękara & Bieniasz, 2008). Style length plays an important role in the fruit set of eggplant since pollen liberated from pores at the apex of the anther cone thereby promoting the fertilization of long-styled pistils (Rylski et al., 1984; Passam & Bolmatis, 1997). Though short style flowers are not totally infertile, their fruit setting rate is much less than those of long and medium style flowers (Srinivas et al., 2016; Pohl et al., 2019). Since a considerable portion of eggplant flowers are short style ones, their failure to set fruit decreases their fruit-yielding potential significantly (Chadha & Saimbhi, 1977). Although heterostyly in eggplant flowers is a varietal characteristic (Rylski et al., 1984; Kowalska, 2006; Sękara & Bieniasz, 2008), it is affected by such factors as plant age, fruiting dynamics, and environmental conditions (Lenz, 1970; Sun et al., 1990). For instance, Nothmann et al. (1983) showed that low temperatures may have an adverse effect on fruit setting by reducing the length of style. Some researchers have also shown that treatment of seeds with gamma rays caused a change in heterostyly by increasing the long style flowers and decreasing the short style ones (Handique & Sarma, 1995). Moreover, exogenous application of some plant growth regulators such as auxin and kinetin was effective on changing heterostyly by changing the proportion of long and short style flowers (Lenz, 1970; Handique & Sarma, 1995; Passam et al., 2001). Moniruzzaman et al. (2015) and Hoque et al. (2018) also reported that using IAA, significantly increased the percentages of long and medium style flowers in eggplant.

Although the effect of fruit load on style length of some cultivars has previously been studied by some researchers (Khah et al., 2000; Passam et al., 2001), in the present paper we studied this effect on style length and some other flower morphological traits in all three positions of single, basal and additional flowers of three eggplant cultivars from Iran.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS

Three eggplant cultivars (TN74128, TN74243 and TN74239) obtained from the Gene Bank of the Agricultural Research Institute of Iran were used in this study. From each cultivar, 60 seeds were sown in the middle of March into boxes with a peat and perlite substrate at a ratio of 4:1; v/v. Six weeks after sowing, 18 uniform seedlings were selected from each genotype, and transplanted into an experimental field at spaces of 60 × 60 cm at Isfahan University of Technology, Isfahan, Iran (latitude 32° 42′ N; longitude 51° 28′ E; altitude 1624 m). The soil of the experimental site was sandy loam with neutral pH suitable for cultivation. Field was ploughed 2-3 times and organic manure 25 t ha^{-1} was applied. The fertilizer 20-20-20 including NPK 20-20-20+B+Cu+Fe+Mn+Mo+Zn was also applied once a month at a concentration of 1 mg l^{-1}. Plants were irrigated using the drip irrigation method, and weeding of the field was carried out several times manually. Pest management were conducted according to recommended standards during the growing season.

2.2 TREATMENTS AND OBSERVATIONS

In this trial three treatments applied including: 1) all flowers collected at anthesis (NF), 2) two fruits allowed setting, but all subsequent flowers collected at anthesis (2F), 3) four fruits allowed setting and all subsequent flowers collected at anthesis (4F). Each treatment comprises 9 plants that all of them were samples through the trial. In all three cultivars, single flower that were formed in the third week were kept on plants for turning into fruit (for treatments of 2 fruits and 4 fruits), and from the fifth week, studies relevant to the flowers forming after fruit setting were carried out. For this purpose, flowers were excised twice a week. They were classified according to whether they were single, basal (first flower in inflorescence) or additional (second flower in inflorescence) flower. Then they immediately transferred to the laboratory. It was assessed the number of stamen, the length of style and stamen, the width of stigma, and the mass of flower, pistil and stigma. The length of style and stamen, as well as the width of stigma were measured using a caliper on the millimeter scale (style and stamen length measurements were made from the point of attachment of the style to the ovary). Flower, pistil, and stigma mass were also measured using a sensitive digital scale and reported in milligram (flower and pistil), and microgram (stigma). The ninth week, fruits were harvested, and the observations lasted two weeks later, until the eleventh week.

2.3 DATA ANALYSIS

The results were statistically analysed by analysis of variance (ANOVA) using the SAS software, and in order to compare differences, least significant differences (LSD) test ($p < 0.05$) was applied for traits with significant F in ANOVA. Due to the lack of coordination of different treatments at the time of measuring the factors, the resulting data obtained from the first four weeks of the experiment (before fruit setting) was carried out with Split-plot according to randomized complete block design, included the position of flowers on the plant (single, basal or additional). For plants without any fruits, and in order to analyse the data from the fifth to eleventh week was used the split-plot factorial based on randomized complete block design. Treatments included the number of fruits per plant (0, 2 and 4 fruits) and flower position on the plant (single, basal or additional) in 3 replications and each replicate contains 3 plants.

3 RESULTS

3.1 STYLE LENGTH AND OTHER FLOWER MORPHOLOGICAL TRAITS AFFECTED BY FRUIT LOAD

As can be seen in Table 1, the presence of two and especially four fruits reduced the style length of the flowers that were later formed. The presence of fruit also significantly affected the size of the other female parts including width of stigma and mass of pistil and stigma, which reduced them in all three cultivars. The presence of four fruits per plant reduced the mass of the next flowers significantly in all three cultivars. The number and the length of stamen in any of the cultivars were not influenced by the number of fruits (data not shown).

3.2 STYLE LENGTH AND OTHER FLOWER MORPHOLOGICAL TRAITS BASED ON FLOWER POSITION

As expected, in Table 2, all traits measured included style length, number of stamens, stamen length, stigma width and the mass of flower, pistil and stigma were significantly less in additional flowers in all three cultivars compared to the basal and single flowers. Some of these traits are not significant between the basal and single flowers, and some of them in the basal flowers are a little more than single ones.

3.3 FLOWER MORPHOLOGICAL TRAITS AT DIFFERENT STAGES OF FRUIT GROWTH AFFECTED BY FRUIT LOAD

3.3.1 Style lenght

As shown in Fig. 1, in cultivar TN74128, the minimum and maximum (min and max) of style length in the basal flower were 13.41 and 14.31 mm, up to the 9th week in NF, while were 12.88 and 13.45 mm in 2F, and 12.03 and 12.33 mm in 4F, which increased to 13.30 mm in the 10th and 11th weeks after harvesting of fruits in 4F. This factor in the additional flower was 8.35 and 12.56 mm in control, 5.73 and 9.93 mm in 2F, and 4.1 and 9.36 mm in 4F, which increased after fruit harvest compared to the 9th week. In single flowers, this value was 13.06 and 13.58 mm in control, which did not differ significantly with 2F. While in 4F were 11.31 and 12.10 mm, which increased to 13.01 mm after harvest.

The min and max style length of basal flower in cultivar TN74243 were 11.40 and 12.59 mm until the 11th week. There was no significant difference in 2F with control. But in 4F it was 10.65 and 11.68 mm, which increased to 12.27 mm after the 9th week. In the additional flower, a significant decrease in the style length of all three treatments was observed from the 5th week to the 7th that began to increase after the 9th week. For single flower, this was 11.71 and 13.07 mm in control. In

Table 1: Effect of fruit load on style length and some flower morphological traits in three eggplant cultivars

Fruit load	Style length (mm)	Stigma width (mm)	Flower mass (mg)	Pistil mass (mg)	Stigma mass (µg)
Cultivar TN74128					
No fruit	12.72a	1.84a	0.88a	0.17a	2.70a
2 fruit	11.53b	1.67b	0.87a	0.16a	2.15b
4 fruit	10.34c	1.56c	0.73b	0.13b	1.80c
Cultivar TN74243					
No fruit	10.66a	1.74a	0.86a	0.15a	2.36a
2 fruit	9.88b	1.65ab	0.83a	0.14b	1.97b
4 fruit	8.94c	1.58b	0.78b	0.11c	1.80c
Cultivar TN74239					
No fruit	9.76a	1.52a	0.76a	0.12a	2.0a
2 fruit	9.20b	1.45b	0.71b	0.11b	1.67b
4 fruit	8.41c	1.35c	0.66c	0.10c	1.48c

The values with similar letters in each column for each cultivar have no significant difference using LSD test at 5 % probability.

Table 2: Differences between position of flower in the style length and other flower morphological traits of three eggplant cultivars

Flower position	Style length (mm)	Number of stamen	Stamen length (mm)	Stigma width (mm)	Flower mass (mg)	Pistil mass (mg)	Stigma mass (µg)
Cultivar TN74128							
Basal	13.29a	6.67a	14.14a	1.98a	1.03a	0.21a	3.08a
Additional	8.38c	6.33c	13.78b	1.21b	0.46b	0.04b	0.71c
Single flower	12.92b	6.55b	14.04a	1.89a	0.99a	0.21a	2.86b
Cultivar TN74243							
Basal	11.93a	6.32a	14.24a	1.89a	1.01a	0.19a	2.98a
Additional	5.63b	6.10b	13.81c	1.14b	0.46b	0.032b	0.35c
Single flower	11.92a	6.34a	14.00b	1.94a	1.01a	0.18a	2.80b
Cultivar TN74239							
Basal	11.51a	6.40a	14.18a	1.66a	0.88a	0.16a	2.53a
Additional	4.30b	5.92b	13.68b	1.08c	0.38b	0.02c	0.25c
Single flower	11.56a	6.33a	14.09a	1.58b	0.86a	0.15b	2.36b

The values with similar letters in each column for each cultivar have no significant difference using LSD test at 5 % probability

2F were 11.21 and 12.25 mm, which increased to 12.46 mm after harvest, and in 4F were 10.78 and 11.93 mm, which no difference was created after the 9th week.

The min and max style length of the basal flower in cultivar TN74239 was 11.19 and 11.84 mm until the 11th week. 2F did not show any significant difference with control, while in 4F, were 10.71 and 11.01 mm, which increased to 11.6 mm in the 11th week. In the additional flower, from the 5th week to the 8th and 9th weeks, there was a significant decrease in the style length of all three treatments that increased from the 10th to 11th weeks. The min and max style length of single flower was 11.10 and 12.49 mm in NF, and no significant difference was observed between 2F with control, while this amount in 4F was 10.15 and 11.0 mm, which increased to 12.45 mm after harvest.

3.3.2 Stigma width

According to Fig. 2 in cultivar TN74128, the min and max width of stigma in basal flower in control was 1.97 and 2.31 mm up to the 9th week, 1.75 and 2.35 mm in 2F, and 1.49 and 2.18 mm in 4F, which increased in two last treatments in the 10th week compared to the 9th week. There was also the similar trend in the additional flowers. In single flower, this factor was 1.95 and 2.14 mm in control, 1.71 and 2.02 mm in 2F, and 1.55 and 2.07 mm in 4F, which increased at the 10th and 11th weeks in both last treatments.

There was no significant difference between control and 2F in stigma width of basal flower in cultivar TN74243, while in 4F at all times, especially on the 9th week, the width of the stigma was less than the control and 2F, which increased after fruit harvest. The stigma width of the additional flowers had a sharp decrease in all three treatments from the 5th week to the 8th and 9th weeks, and then increased at the 10th and 11th weeks. In single flower, these were 1.89 and 2.26 mm in control, while was lower in 2F and 4F, which increased at the 10th and 11th weeks.

In cultivar TN74239, at all times, the stigma width of the basal flower was lower in 2F and 4F with a slight difference compared to the control, and after harvest increased. The additional flower showed a decrease trend from 5th week to 8th week in all three treatments and then began to increase. Min and max of stigma width in single flower were 1.52 and 1.68 mm in control until the 9th week. There was no significant difference in 2F, and in 4F were 1.18 and 1.7 mm, which increased after fruit harvest in both treatments.

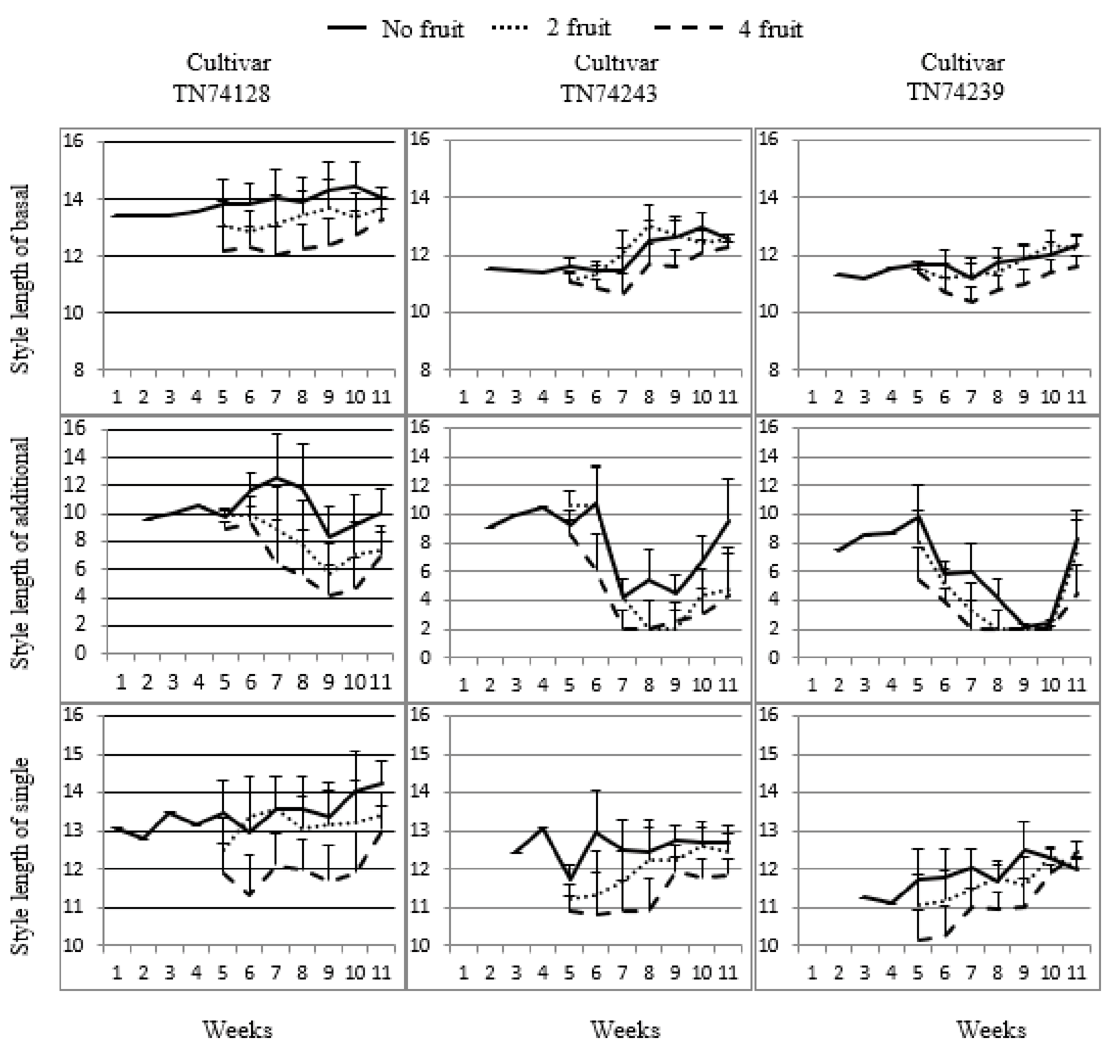

Figure 1: Style length of basal, additional and single flower in millimeter in three eggplant cultivars along the growing period affected by fruit load. Vertical bars represent standard deviation of the means

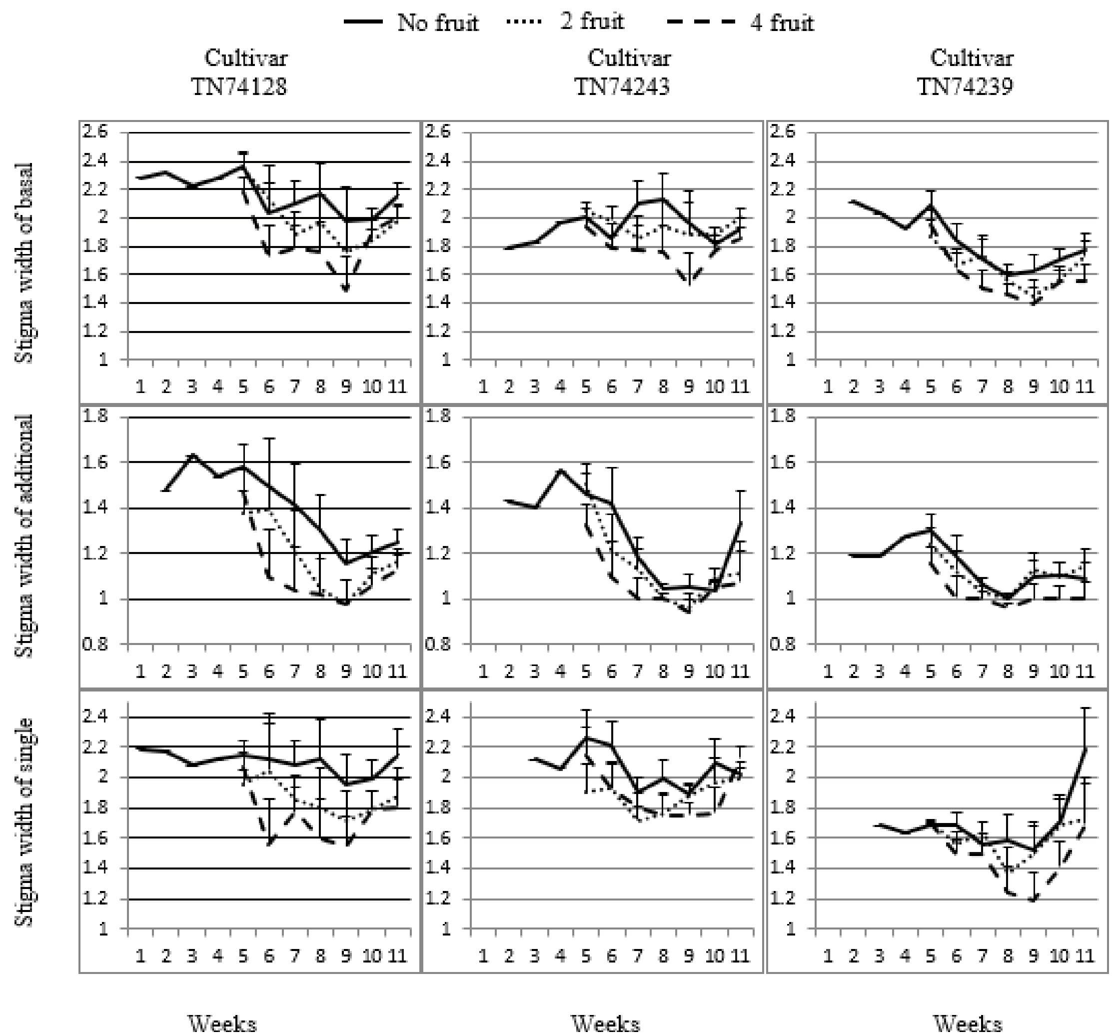

Figure 2: Stigma width of basal, additional and single flower in millimeter in three cultivars of eggplant along the growing period affected by fruit load. Vertical bars represent standard deviation of the means

3.3.3 Flower mass

No significant differences were found between 2F and 4F with control in basal and additional flowers of all cultivars. But in single flower the min and max flower mass of control in cultivars TN74128, TN74243 and TN74239 was 1.03 and 1.46, 0.96 and 1.33, and 0.88 and 1.20 mg, respectively, while in 4F, this factor was significantly lower (0.74 and 1.09, 0.80 and 1.20, and 0.71 and 0.89 mg, respectively), which increased slightly after harvest (Fig. 3).

3.3.4 Pistil mass

The min andmax pistil mass of basal flower in cultivar TN74128 until the 9th week was 0.16 and 0.33 mg in control. 2F did not show any significant difference with control, while in 4F was 0.13 and 0.22 mg, which showed a significant increase after harvesting compared to the last weeks. This factor in additional flower was 0.027 and 0.074 mg in control, 0.026 and 0.058 mg in 2F, and 0.012 and 0.044 mg in 4F, which after the harvest increased. In single flower, this amount was 0.22 and 0.34 in control, 0.15 and 0.28 in 2F and 0.15 and 0.2 mg in 4F, which after harvest increased compared to the 9th week.

The min and max pistil mass of basal flower in cultivar TN74243 in control was 0.15 and 0.26 mg. 2F didn't have significant difference with control, and in 4F, it was 0.14 and 0.22 mg. The pistil mass of additional flower was significantly reduced in all three treatments from the 5th week to the 8th week, and after the 10th week it increased slightly. In single flower, there was no significant difference between 2F with control, but in 4F was lower than the control at all times, which increased after the 9th week.

In cultivar TN74239, the pistil mass of basal flower was not significantly different in 2F and 4F with control, although this factor was less than the control at all times. In the additional flower, the pistil mass decreased significantly from the 5th to 7th weeks in all three treatments and increased slightly after the 10th week. In the single flower, 2F did not show any difference with the control, but in 4F at all times, the mass of the pistil was less than that of the control and after harvest increased compared to the previous one (Fig. 4).

3.3.5 Stigma mass

In cultivar TN74128, the min and max stigma mass of basal flower until the 9th week in control was 3.16 and 3.91 µg, in 2F, 2.53 and 3.73 µg, and in 4F, 1.99 and 3.04 µg, which increased after fruit harvest. Min and max of this factor in additional flower of control, 2F and 4F was 0.78 and 1.28, 0.58 and 0.75, and 0.37 and 0.46 µg, respectively, which increased slightly after the 9th week. In single flower, this factor was 2.22 and 4.22 µg in control, 2.36 and 2.85 µg in 2F, and 1.73 and 2.67 µg in 4F, which at the 10th and 11th weeks was more than to the previous weeks.

In cultivar TN74243, the min and max stigma mass of basal flower in control was 2.44 and 3.66 µg. This amount in 2F was 2.20 and 3.05 µg and in 4F was 2.10 and 2.75 µg, which increased in all three treatments after the 9th week. The stigma mass of the additional flower was 0.25 and 0.62 µg in the control. In 2F, 0.10 and 0.35 µg, and in 4F, without significant dif-

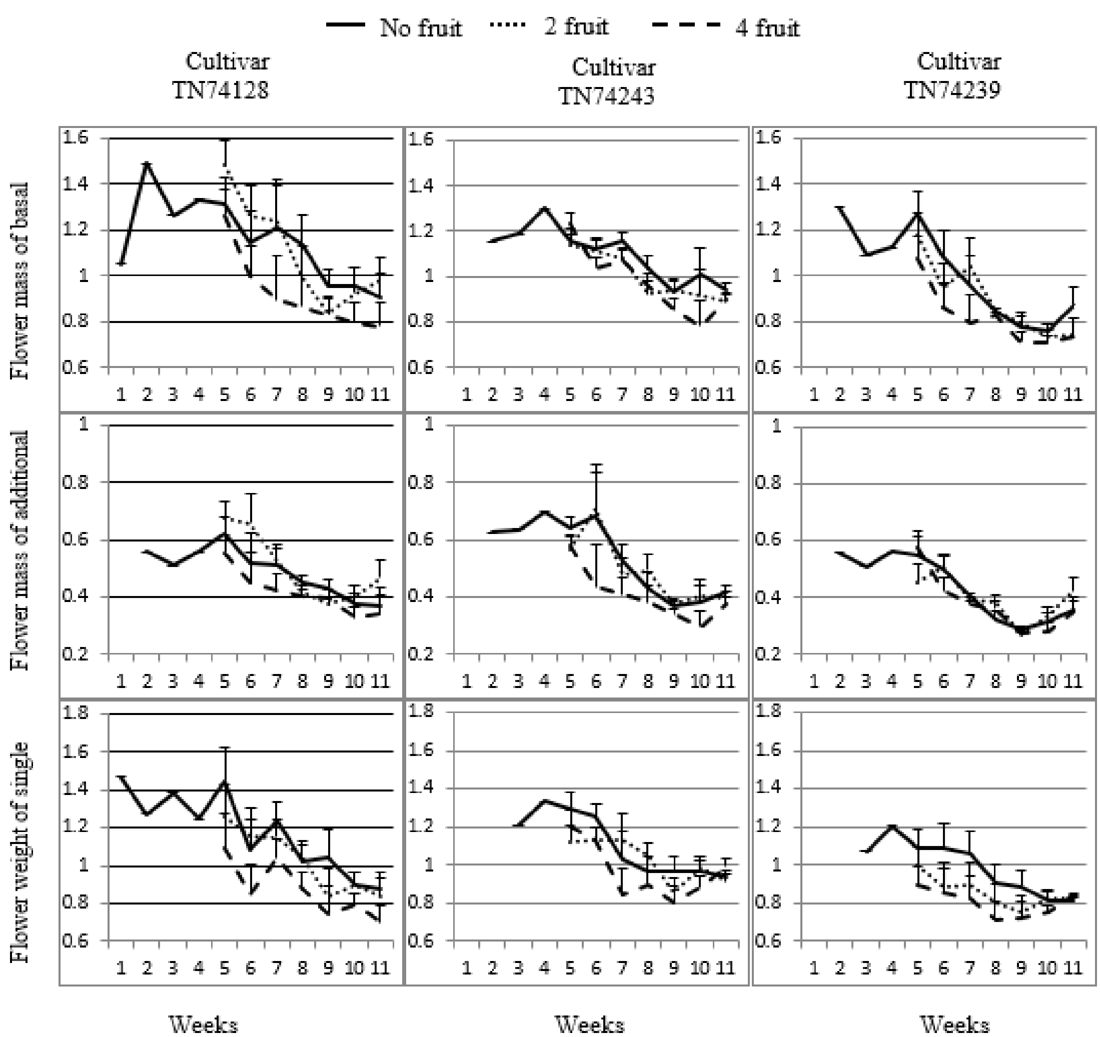

Figure 3: Flower mass of basal, additional and single flower in milligram in three cultivars of eggplant along the growing period affected by fruit load. Vertical bars represent standard deviation of the means

ferences with 2F was 0.10 and 0.32 µg, which increased after harvest. In single flower, this factor was 2.20 and 3.45 µg in control, in 2F, 2.06 and 2.90 µg, and in 4F, 1.66 and 2.85 µg that at the 9th and 10th week reached to their maximum.

In cultivar TN74239, the min and max stigma mass of basal flower in control was 2.12 and 3.57 µg, in 2F, 1.80 and 2.94 µg, and in 4F, 1.98 and 2.52 µg that increased after harvest than previous weeks. This factor showed a significant reduction in additional flowers in all three treatments from 5th to 8th and 9th weeks, while the graph of 2F and 4F were lower than the control, and then increased at the 11th week. The min and max stigma mass of single flower in control was 1.98 and 2.60 µg. This amount in 2F was 1.72 and 2.55 µg, and in 4F, 1.15 and 2.20 µg, which reached to the maximum value in all three treatments in the 10th and 11th weeks (Fig. 5).

4 DISCUSSION

The presence of two or four fruits in all three cultivars reduced the length of style, as well as the width of stigma in all three positions of the next basal, additional and single flowers. This difference was especially significant and remarkable in 4F. This process continued from the beginning until the time of fruit harvesting, and after harvesting began to increase again. The mass of basal and additional flower in three cultivars was not significantly affected by 2F and 4F, while mass of single flower was especially decreased in 4F as compared to the control, which increased again after harvesting. This factor generally decreased from the beginning of flowering to the end of the fruit growth period in all three cultivars. The mass of pistil also showed a similar trend to the mass of the flower during the growth

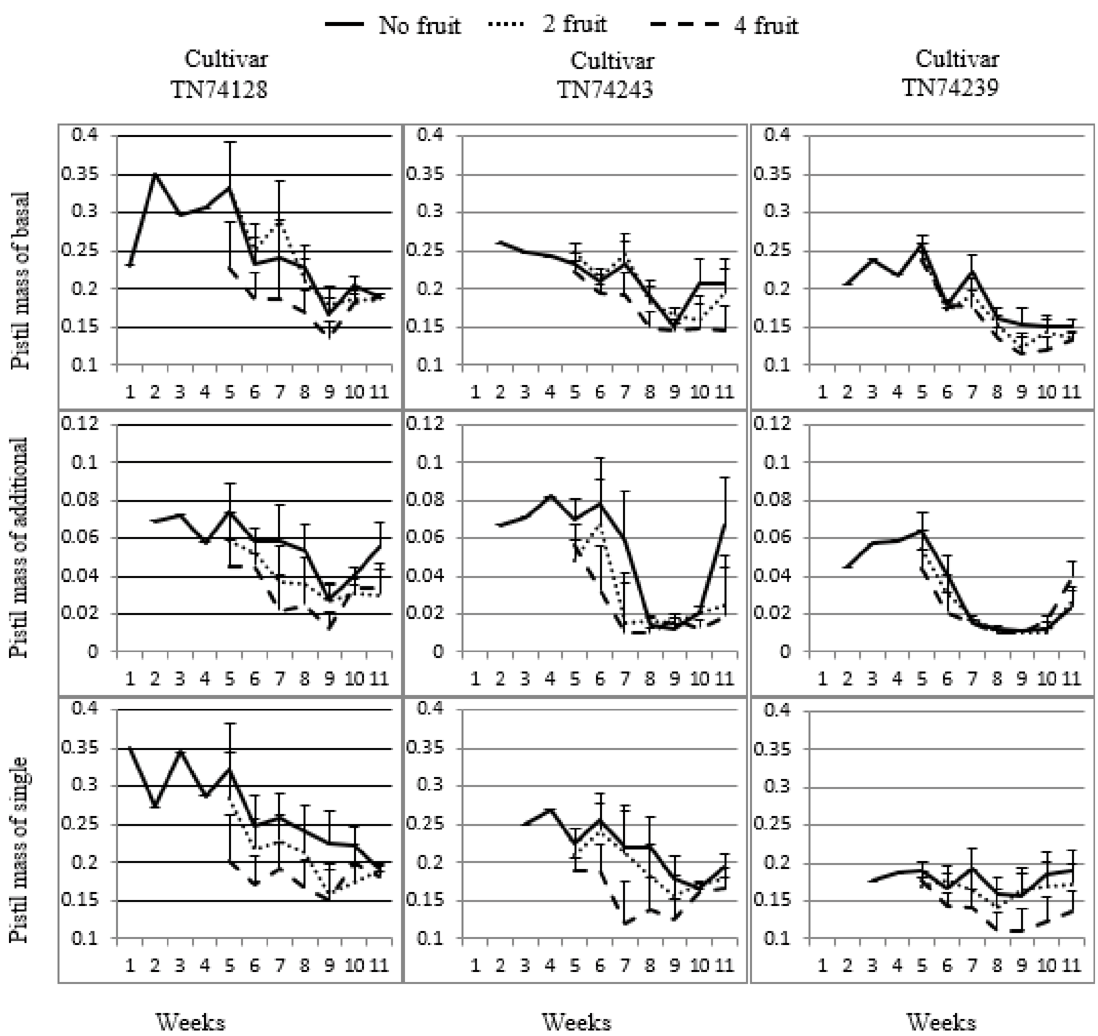

Figure 4: Pistil mass of basal, additional and single flower in milligram in three cultivars of eggplant along the growing period affected by fruit load. Vertical bars represent standard deviation of the means

period. In most cases, 2F did not affect the mass of pistil so much, while in 4F, this factor was more affected, so that it decreased until the fruit harvesting, and after that increased again. The mass of stigma was influenced by two and four fruits during the growth period, so that the presence of fruit per plant in all three cultivars reduced the mass of stigmata of flowers that formed after fruit setting, and after fruit harvest increased again. The treatment of two fruits and four fruits did not affect the number and the length of stamen in any of the cultivars during the growth period.

These results are in agreement with the study of Khah et al. (2000) on four eggplant varieties in the greenhouse conditions. They showed not only the length of style, but also the mass of flower, the mass of pistil and the number of flowers decreased by the number of fruits, and increased again after fruit harvest. Lenz (1970) also examined this issue in Hoagland's culture and expressed that the formation of the fruit affected style length of the next flowers. Moreover, Passam et al. (2001) reported similar results. Their experiments were conducted under favorable climatic conditions for fruit formation. According to them, the presence of fruit caused length of style was smaller during the period of fruit growth, which increased after the maturity of the fruit. The mass of the flower was also affected by the presence of fruits, and this factor also similar to our results showed a downward trend in the trial period. Pistil mass also had a pattern similar to flower mass, and no significant difference was observed in cultivars in stamen length between treatments.

Developing fruits reduce the growth of roots, stems and leaves in eggplants (Mochizuki, 1959), as occurs in other plant species (Leonard, 1962). Lenz (1970)

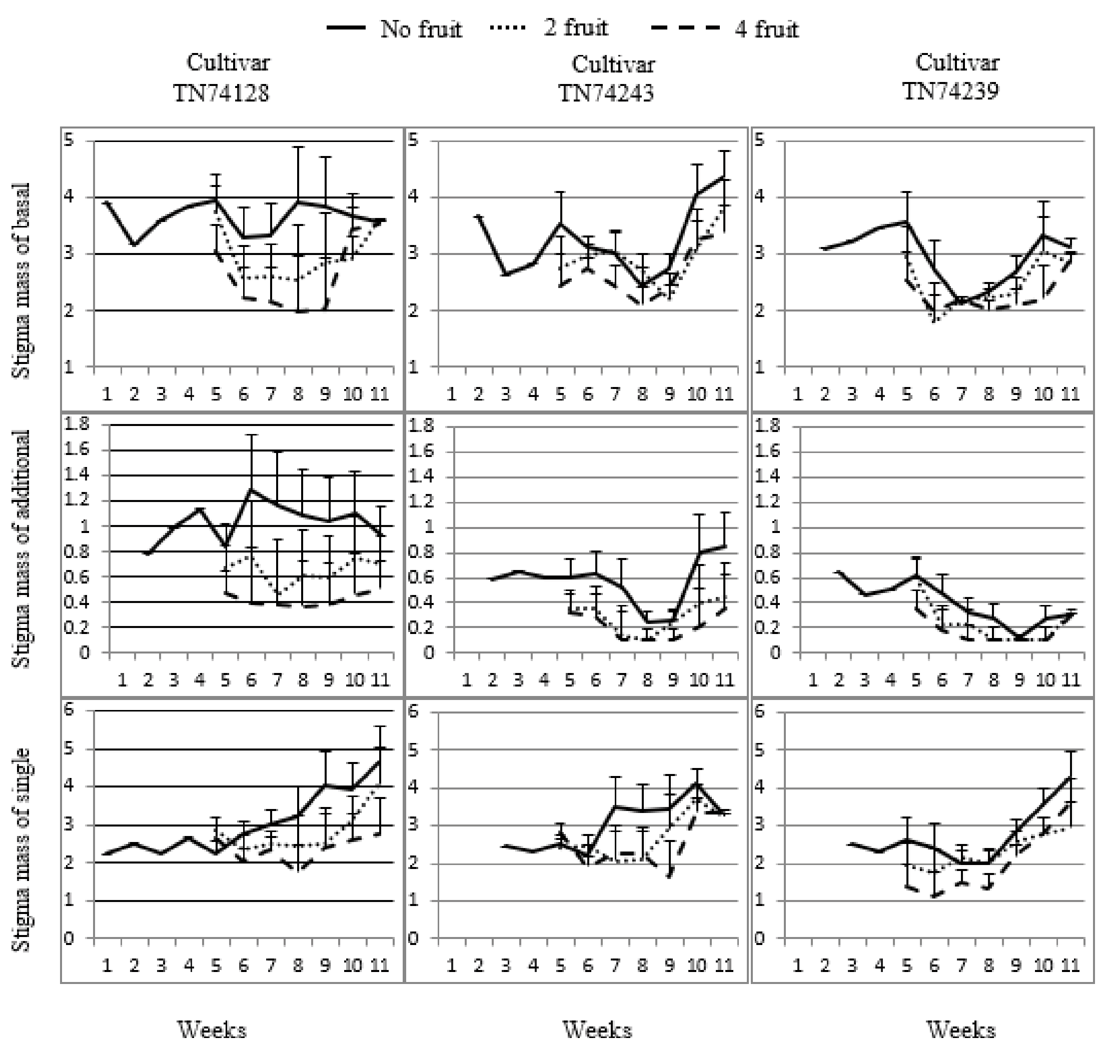

Figure 5: Stigma mass of basal, additional and single flower in microgram in three eggplant cultivars along the growing period affected by fruit load. Vertical bars represent standard deviation of the means

also showed this growth inhibition for flower style. This inhibitory growth is due to the competition of nutrients and assimilates, and may also result in hormones produced in the fruit. According to the Lenz (1970), both the length of style and stamen are influenced by auxin. Therefore, auxin existing in developing fruits can inhibit the growth of the style, which suggests that developing fruits control the sex expression of the eggplant. Claussen (1986) also states that since fruits are strong sink, the use of leaf carbohydrates reduces vegetative growth and decreases flower size. The issue of the dominance of the first fruit in plants from Cucurbitaceae family has also been proven. In these plants, the developing fruit can prevent the formation of the next fruit and the growth of new and young fruits. This inhibition can be due to competition for available assimilates, or due to the dominance of growth regulators created by the developing fruit. The pollen-dependent seed content seems to play a role in the dominance of the first fruit (Stephenson et al., 1988; Bangerth, 1989). However, Passam et al. (2001) demonstrated in a trial that the use of auxin in the absence of fruit did not affect the length of the style. Therefore, they stated that although the seeds of developing fruits through the synthesis of natural auxins or other growth regulators may have some effect on the length of the style, it seems that the main factor is the presence of fruit and the stage of fruit growth.

5 CONCLUSION

In summary, although fruit load did not affect the number and length of stamen of flowers which form later, reduced style length, stigma width, mass of flower, pistil and stigma of those flowers, which increase again after fruit harvesting. Therefore, the presence of fruit on plant reduces the chance of fruit formation from subsequent flowers that shows timely harvest of fruit can be effective in the formation of more long style flowers during plant growth period and so, more fruit setting.

6 REFERENCES

Bangerth, F. (1989). Dominance among fruits/sinks and the search for a correlative signal. *Physiologia Plantarum*, *76*(4), 608–614. https://doi.org/10.1111/j.1399-3054.1989.tb05487.x

Banik, S. C., Islam, S., Sarker, B., Chowdhury, D. D., & Uddin, M. N. (2018). Influence of flower types on fruit setting and yield dynamics of summer brinjal (*Solanum melongena* L.). *Asian Journal of Agricultural and Horticultural Research*, 1–9. https://doi.org/10.9734/AJAHR/2018/41185

Chadha, M. L., & Saimbhi, M. S. (1977). Varietal variation in flower types in brinjal (*Solanum melongena* L.). *Indian Journal of Horticulture*, *34*(4), 426–429.

Claussen, W. (1986). Influence of fruit load and environmental factors on nitrate reductase activity and on concentration of nitrate and carbohydrates in leaves of eggplant (*Solanum melongena*). *Physiologia Plantarum*, *67*(1), 73–80. https://doi.org/10.1111/j.1399-3054.1986.tb01265.x

Daunay, M.C. (2008). Eggplant. In J. Prohens-Tomas & F. Neuz (Eds.), *Vegetables II* (pp. 163–220). Springer. https://doi.org/10.1007/978-0-387-74110-9_5

Faostat, F. (2019). Agriculture Organization of the United Nations statistics division. *Production Available in http://Faostat3. FAO. Org/Browse/Q/QC/S. Accessed December 22, 2020.*

Frary, A., Doganlar, S., & Daunay, M. C. (2007). Eggplant. In C. Kole (Ed.), *Vegetables. Genome Mapping and Molecular Breeding in Plants* (pp. 287–313). Springer. https://doi.org/10.1007/978-3-540-34536-7_9

Handique, A. K., & Sarma, A. (1995). Alteration of heterostyly in *Solanum melongena* L. through gamma-radiation and hormonal treatment. *Journal of Nuclear Agriculture and Biology*, *24*(2), 121–126.

Hazra, P., Manda, J., & Mukhopadhyay, T. P. (2003). Pollination behaviour and natural hybridization in *Solanum melongena* L., and utilization of the functional male sterile line in hybrid seed production. *Capsicum and Eggplant Newsletter*, *22*, 143–146.

Hoque, A. A., Ahmed, Q. M., Rahman, M. M., & Islam, M. A. (2018). Effect of application frequency of naphthalene acetic acid on physiomorphological characters and yield of brinjal. *Research in Agriculture, Livestock and Fisheries, 5(2),* 151-155. https://doi.org/10.3329/ralf.v5i2.38051

Jagatheeswari, D. (2014). Morphological studies on flowering plants (Solanaceae). *International Letters of Natural Sciences*, *15,* 36-43. https://doi.org/10.18052/www.scipress.com/ILNS.15.36

Khah, E. M., Antonopoulos, A., & Passam, H. C. (2000). Floral behaviour and fruit set in four cultivars of aubergine. *Acta Horticulturae*, *579*, 259–264. https://doi.org/10.17660/ActaHortic.2002.579.43

Kowalska, G. (2003). The influence of heterostyly, pollination method and hormonization on eggplant's (*Solanum melongena* L.) flowering and fruiting. *Acta Agrobotanica*, *56*(1–2), 61–76. DOI: https://doi.org/10.5586/aa.2003.007

Kowalska, G. (2006). Eggplant (*Solanum melongena* L .) flowering and fruiting dynamics depending on pistil type as well as way of pollination and flower hormonization. *Folia Horticulturae*, *18*(1), 17–29.

Lenz, F. (1970). Effect of fruit on sex expression in eggplant (*Solanum melongena* L.). *Horticultural Research*, *10*, 81–82.

Leonard, E. R. (1962). Inter-relations of vegetative and reproductive growth, with special reference to indeterminate plants. *The Botanical Review*, *28*(3), 353–410. https://doi.org/10.1007/BF02868688

Meyer, R. S., Karol, K. G., Little, D. P., Nee, M. H., & Litt, A. (2012). Phylogeographic relationships among Asian eggplants and new perspectives on eggplant domestica-tion. *Molecular Phylogenetics and Evolution, 63,* 685–701. https://doi.org/10.1016/j.ympev.2012.02.006

Mochizuki, T. (1959). The carbon metabolism of eggplants as affected by bearing fruits. *Bulletin of the Faculty of Agriculture and Life Science, Hirosaki University*, *5*, 28–31.

Mohideen, M. K., Muthukrishnan, C. R., Rajagopal, A., & Metha, V. A. (1977). Studies on the rate of flowering, flower types and fruit set in relation to yielding potential of certain eggplant (*Solanum melongena* L.) varieties with reference to weather conditions. *South Indian Horticulture*, *25*, 56–61.

Moniruzzaman, M., Khatoon, R., Hossain, M. F. B., Jamil, M. K., & Islam, M. N. (2015). Effect of GA and NAA on physio-morphological characters, yield and yield components of Brinjal (*Solanum melongena* L.). *Bangladesh Journal of Agricultural Research, 39,* 397–405. https://doi.org/10.3329/bjar.v39i3.21983

Nothmann, J., Rylski, I., & Spigelman, M. (1983). Interactions between floral morphology, position in cluster and 2,4-D treatments in three eggplant cultivars. *Scientia Horticulturae*, *20*(1), 35–44. https://doi.org/10.1016/0304-4238(83)90109-7

Passam, H. C., Baltas, C., Boyiatzoglou, A., & Khah, E. M. (2001). Flower morphology and number of aubergine (*Solanum melongena* L.) in relation to fruit load and auxin application. *Scientia Horticulturae*, *89*(4), 309–316. https://doi.org/10.1016/S0304-4238(00)00242-9

Passam, H. C., & Bolmatis, A. (1997). The influence of style length on the fruit set, fruit size and seed content of aubergines cultivated under high ambient temperature. *Tropical Science*, *37*, 221–227.

Pohl, A., Grabowska, A., Kalisz, A., & Sękara, A. (2019). Biostimulant application enhances fruit setting in eggplant—An insight into the biology of flowering. *Agronomy, 9*, 482. https://doi.org/10.3390/agronomy9090482

Prasad, D. N., & Prakash, R. (1968). Floral biology of brinjal (*Solanum melongena* L). *Indian Journal of Agricultural Sciences*, *38*(6), 1053.

Rashid, M. A., & Singh, D. P. (2000). *A manual on vegetable seed production in Bangladesh.* AVRDC-USAID-Bangladesh Project, Horticulture Research Centre, Bangladesh Agricultural Research Institute.

Rylski, I., Nothmann, J., & Arcan, L. (1984). Differential fertility in short-styled eggplant flowers. *Scientia Horticulturae*, *22*(1–2), 39–46. https://doi.org/10.1016/0304-4238(84)90081-5

San José, R., Plazas, M., Sánchez-Mata, M. C., Cámara, M., & Prohens, J. (2016). Diversity in composition of scarlet (*S. aethiopicum*) and gboma (*S. macrocarpon*) eggplants and of interspecific hybrids between *S. aethiopicum* and common eggplant (*S. melongena*). *Journal of Food Composition and Analysis*, *45*, 130–140. DOI: 10.1016/j.jfca.2015.10.009

Sękara, A., & Bieniasz, M. (2008). Pollination, fertilization and fruit formation in eggplant (*Solanum melongena* L.). *Acta Agrobotanica*, *61*(1), 107. DOI: 10.5586/aa.2008.014

Srinivas, G., Jayappa, A. H., & Patel, A. I. (2016). Heterostyly: A threat to potential fruit yield in brinjal (*Solanum melongena* L.). *Advancements in Life Sciences, 5,* 1211–1215.

Stephenson, A. G., Devlin, B., & Horton, J. B. (1988). The effects of seed number and prior fruit dominance on the pattern of fruit production in *Cucurbita pepo* (zucchini squash). *Annals of Botany*, *62*(6), 653–661. https://doi.org/10.1093/oxfordjournals.aob.a087705

Sun, W., Wang, D., Wu, Z., & Zhi, J. (1990). Seasonal change of fruit setting in eggplants (*Solanum melongena* L.) caused by different climatic conditions. *Scientia Horticulturae*, *44*(1–2), 55–59. DOI : 10.1016/0304-4238(90)90016-8

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Effects of *Prosopis africana* (Guill. & Perr.) Taub. and *Ficus mucoso* Ficalho ethanolic leaves extract in the control of *Callosobruchus maculatus* (Fabricius, 1775) in stored cowpea

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Effects of *Prosopis africana* (Guill. & Perr.) Taub. and *Ficus mucoso* Ficalho ethanolic leaves extract in the control of *Callosobruchus maculatus* (Fabricius, 1775) in stored cowpea

Abstract: The study investigated the effectiveness of *Prosopis africana* and *Ficus mucoso* ethanolic leave extract in the control of *Callosobruchus maculatus* infesting cowpea. Treatments were applied at different concentrations (10 %, 30 % , 50 %, and 0 %) on cowpea. Five pairs of newly emerged adult *C. maculatus* were introduced into each treatment. The two botanicals were evaluated on the insecticidal effects it has on the insect and data were recorded on adult mortality, oviposition rate, larvae, pupae, and adult emergence, seed viability, and phytochemicals present in both botanicals. Results revealed that both treatments had insecticidal potentials, adversely reducing the number of eggs, larvae, and pupae of *C. maculatus* with *P. africana* having the highest mean mortality rate at 50 % concentration. Observations further indicated that the botanicals had no negative effect on seed viability. The phytochemical analysis revealed the presence of some bioactive compounds such as terpenoids, flavonoids, alkaloids, saponin, steroids, and tannin, *P. africana* mostly rich in them than *F. mucoso*. Though both extracts were effective, *P. africana* performed better in the control of the bruchid beetle indicating plausible usefulness in sustainable pest management by smallholder farmers and consumers of cowpea in environments where the plants are in abundance.

Key words: *Callosobruchus maculatus*; cowpea; botanicals; storage entomology; plant-based insecticide; coleoptera

Učinki etanolnih izvlečkov iz listov vrst *Prosopis africana* (Guill. & Perr.) Taub. in *Ficus mucoso* Ficalho na uravnavanje škodljivca *Callosobruchus maculatus* (Fabricius, 1775) v shranjenem zrnju kitajske vinje

Izvleček: V raziskavi so bili preučevani učinki etanolnih izvlečkov iz listov vrst *Prosopis africana* in *Ficus mucoso* na uravnavanje škodljivca *Callosobruchus maculatus* v semenu kitajske vinje. Obravnavanja so obsegala različne koncentracije izvlečka in sicer 10 %, 30 %, 50 %, in 0 %. Pet parov na novo izleglih odraslih osebkov škodljivca je bilo izpostavljenih izvlečkom v vseh obravnavanjih. Insekticidni učinki obeh rastlinskih vrst na hrošča so bili ovrednoteni glede na smrtnost odraslih osebkov, velikost ovipozicije, glede vplivov na ličinke in bube in izleganje odraslih osebkov. Ovrednoten je bil tudi učinek izvlečkov obeh vrst na vitalnost semen vinje in njihova kemična sestava. Izsledki so pokazali, da imata obe rastlinski vrsti insekticidni potencial, ker sta imeli negativni učinek na število jajčec, ličink in bub škodljivca, pri čemer je izvleček iz vrste *P. africana* povzročil največjo smrtnost pri 50 % koncentraciji. Nadalje so opazovanja pokazala, da izvlečka obeh vrst nista imela negativnega učinka na vitalnost shranjenih semen vinje. Fitokemična analiza izvlečkov je odkrila prisotnost nekaterih bioaktivnih snovi kot so terpenoidi, flavonoidi, alkaloidi, saponini, steroidi, in tanini, pri čemer je bila vrsta *P. africana* bogatejša na njih kot vrsta *F. mucoso*. Čeprav so sta bila oba izvlečka učinkovita, je izvleček iz vrste *P. africana* deloval bolje pri uravnavanju populacije hrošča, kar kaže na verjetno koristnost uporabe pri trajnostnem obravnavanju škodljivcev pri malih kmetih in potrošnikih kitajske vinje v okoljih, kjer sta obe vrsti rastlin v izobilju.

Ključne besede: *Callosobruchus maculatus*; kitajska vinja; rastlinski pripravki; entomologija shranjevanja pridelkov; insekticidi na osnovi rastlin; hrošči

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1 INTRODUCTION

Vigna unguiculata (L.) Walp (Cowpea), is an important legume crop in the tropics, and ensures the provision of plant-based protein for most people and also the fixation of nitrogen into the soils (Umeozor, 2005). Production of cowpea is limited by several abiotic and biotic factors, both in the field and in storage. Among the constraining biotic factors are insect pests (Swella & Mushobozy, 2007) mainly due to infestation by the cowpea bruchid, *Callosobruchus maculatus* (Fabricius, 1775) (*Coleoptera*: Bruchidae) which is a cosmopolitan field-to-store pest, and has been ranked as the principal post-harvest pest of cowpea especially in the tropics. It causes substantial qualitative and quantitative losses, manifested by seed perforation, and reductions in mass, market value, and germination ability of seeds. In storage conditions, 100 % infestation of cowpea, occurring within 3 to 5 months of storage, is not uncommon (Lale & Mustapha, 2000).

Toxic residual insecticides have been used routinely for many years to control insect pests in stored grain. These insecticides are primarily organophosphorous and pyrethroid compounds (Arthur, 1996). Several methods have also been employed over the years to protect cowpea from damages by the pest, using chemical insecticides which is the most prevalent (Abdullahi et al., 2011). However, the use of chemical insecticides is fast becoming less desirable because of the resistance in major insects, regulatory restrictions on the use of insecticides, awareness of environmental pollution, the increasing cost of insecticides, erratic supplies, worker's safety and, consumer desire for a pesticide-free product, which has led to pest management specialists reappraising natural products for potential usage (Haghtalab et al., 2009).

Chemical methods are also directly or indirectly dangerous to the user and non-target organisms, destruction of beneficial organisms, residual toxicity, widespread environmental hazards, development of resistance by insect species, etc. are always in association with its usage (Oni & Ileke, 2008; Oni, 2011). This necessitated the need for a safer alternative and sustainable control strategy. It is in this regard that extracts of plants have been thought of from different parts of the world. The method has been described as a cheaper eco-friendly and safer means of controlling insect pests of stored cowpea (Adedire et al., 2011).

Singh and Saratchandra (2005) reported that most plant species exhibit insect deterrent ability further indicative of the fact that some plant extracts can inhibit normal development in insects. Also, most of these plants can be acquired locally and freely and are easy to handle by smallholder farmers without any accruing adverse effects to man and the environment. In view of the increasing economic importance of cowpea and the intensity of damages due to insect pest infestation, an attempt was made to provide a safer method for the control of *C. maculatus* in stored grains using the plant extracts *Prosopis africana* and *Ficus mucoso.* Previously, there have been reports on the effectiveness of these plants genus in the control of pests and pathogens (Dangarembizi et al., 2012; Elaigwu et al., 2018; Zerihun & Ele, 2018; Shinkafi & Abdullahi, 2018) but without any scientific study targeted at investigating their potential effectiveness in curtailing the ruthless destruction by *C. maculatus* in stored seeds. The plants have promising potential to be considered as an alternative seed treatment to synthetic insecticides therefore the reason why this research work was initiated, to investigate the insecticidal activities of the two botanicals on the field-to-store insect pest. Furthermore, this research presents the first report on the use of *P. africana* and *F. mucoso* leave extracts in the control of the infamous cowpea weevil- *C. maculatus*.

2 MATERIALS AND METHODS

2.1 SOURCE AND TYPE OF SEED

The cowpea seeds used were the variety IT99K-573-1-1 which was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The seeds were white in colour and medium in size. The cowpea seeds were stored in the freezer compartment of a refrigerator at -17 °C for four days before the seeds were used for the experiment.

2.2 INSECT CULTURE

Callosobruchus maculatus used for this study was obtained from Nigeria Stored Product Research Institute (NSPRI), Ilorin Kwara State. The cowpea variety IT99K-573-1-1 was used to maintain insect culture by placing seeds in a plastic container of medium size to culture the insect at room temperature (22 °C). Plastic containers used for insect culture were covered with muslin clothe at the top held with a rubber band to allow for ventilation.

2.3 COLLECTION AND PREPARATION OF PLANT MATERIALS

Africana mesquites (*Prosopis africana* (Guill. & Perr.) Taub.) and Sand paper (*Ficus mucoso* Ficalho) leaves were plucked within University of Ilorin premises. Leaves were washed and air-dried for 14 days in the open field of the faculty of agronomy pavilion. The dried leaves were ground using mortar and pestle and then passed through 90-micron mesh sieve to obtain a uniform powder.

2.4 EXTRACTION OF PLANT MATERIALS

Extraction of each plant material was carried out in the laboratory by soaking 500 g of each of the plant powder in 2 l of ethanol for 72 hours with occasional stirring. The solution of each plant material was filtered using Whatman No. 1 filter paper. The extracts were concentrated using a rotary evaporator at a maximum temperature of 45 °C. The resulting crude extract was stored in a plastic container at room temperature until ready for use.

2.5 METHODS OF PHYTOCHEMICAL SCREENING

Chemical tests were carried out on the ethanolic extracts for the qualitative determination of phytochemical constituents as described by Harborne (1973), Trease and Evans (1989), and Sofowora (1993).

2.6 EXPERIMENTAL PROCEDURE

The ethanolic leaf extracts of *P. africana* and *F. mucoso* were dissolved in distilled water to prepare solutions of different concentrations (10, 30, and 50 % w/v). 100 g of cowpea seeds were treated with 0.5 ml of *Prosopis africana* and *Ficus mucoso* ethanolic extract in six replicates at different concentrations (10, 30, 50 % w/v) in transparent plastic containers (7 × 8 cm) also, untreated seeds were included as a control (0 %). The plant extract was applied to the seed samples with a micro-syringe and was thoroughly mixed with a wooden spatula. The seeds were air-dried in the laboratory for 5 minutes before introducing five pairs of freshly emerged adults of *Callosobruchus maculatus*. To prevent escape and allow for ventilation, muslin cloth fastened with a rubber band were used to cover containers after applying the various treatments.

Adult weevil mortality was observed daily for 4 days at 24, 48, 72, and 96 hours respectively and the number of dead weevils was counted and recorded. The insects were confirmed dead when there was no response to probing with a sharp entomological needle at the abdomen. Furthermore, to check for the emergence of new generation of the insect after applying the treatment on cowpea, a pointed forceps was used to pick five seeds at random from each replicate on the 4^{th}, and 7^{th} day after infestation, and selected seeds were examined for eggs after dissection with a sharp blade and the dissected beans were examined for larvae and pupae. The pointed forceps were used to prevent damage of the egg on the seeds. The emergence of the first filial (F_1) adult generation was also observed from 28 to 32 days after infestation.

Seed viability test was carried out on ten randomly selected seeds from each replication with the various treatments and concentration levels at the end of the experiment using floatation and germination techniques. The floatation method was adopted from the technique described by Ehiagbonare and Enabulele (2007). The percentage viability of seeds was calculated using the following formula: . Here, S = Number of treated seeds used per replicate. SF = Number of floating seeds per replicate.

The germination method was slightly modified from the technique described by Holly (2006). Cowpea seeds were placed on a petri dish already layered with dampened Whatman filter paper and left at room temperature (26-28 °C) for a period of 7 days during which time, samples were regularly checked for sprouting, and moisture was added to prevent complete dryness of the samples. The percentage viability of germinated cowpea seeds was recorded using the formula:

2.7 STATISTICAL ANALYSIS

Data were subjected to two-way analysis of variance (ANOVA). Where treatment means were significant, multiple comparisons of treatments was done using the Tukey's honestly significant difference test at 5% level of significance. All statistical analysis was done using the IBM SPSS version 26.

3 RESULTS

3.1 EFFECT OF BOTANICAL TYPES ON ADULT MORTALITY OF *Callosobruchus maculatus*

Table 1 shows the effectiveness of the botanical types at different concentrations on the mortality of adult *C. maculatus*. At 24, 48, 72, and 96 hours after treatment (HAT) both *P. africana* and *F. mucoso* had the most significantly highest rate of *C. maculatus* mortality at the concentration level of 50 % when compared to the control which had the least. Furthermore, the two botanical treatments at 30 % also had effective control of the insect pest population from 24 to 96 HAT as presented in Table 1. The mean number of insect mortality at the treatments concentration level of 10 % indicated a slower rate of control in comparison to the concentration levels of 50 and 30 %.

The overall effects of the different botanical treatment are also indicated in Table 1. At 24, 48 and 72 HAT, *P. africana* had a significantly ($p < 0.05$) higher mortality rate of the mean 5.650 ± *0.365, 12.623 ± 0.205 and 13.359 ± 0.025 respectively when compared to F. mucoso* which was lower at the mean numbers of 1.541 ± *0.234, 5.600 ± 0.300 and 10.863 ± 0.044 respectively. However, at 96 HAT, the leave extracts of P. africana* and *F. mucoso* showed no significant difference of adult mortality of *C. maculatus* thus having the same effective level of control on the insect pest.

3.2 EFFECT OF THE BOTANICAL TREATMENTS ON THE OVIPOSITION, LARVAE AND PUPAE EMERGENCE OF *C. maculatus*

Table 2 presents the activities of the botanical treatment in restricting the oviposition, larvae and pupae emergence of the insect pest. Post hoc analysis indicated the two different botanical treatments at 50 % concentration to possess the least mean number of eggs and also larvae and pupae population. This was followed by the concentration rate of 30 % only for seeds treated with *P. africana*. *F. mucoso* had no significant differences with the control at the concentration rate of 30 % and 10 % in all the various early life stages in Table 2.

In total, the control had the most numbers of eggs, larvae and pupae of the insect pest. There was no significant difference in the two botanical treatments in the number of eggs laid by the pest. Furthermore, seeds that were treated with *P. africana* had the least larvae and pupae emergence compared to *F. mucoso* and the control (Table 2).

Table 1: Effect of *P. africana* and *F. mucoso* concentrations on adult mortality of *C. maculatus*

		Adult mortality rate (HAT)			
Botanical type	Concentrations	24	48	72	96
P. africana	10 %	0.000 ± 0.000[c]	6.670 ± 1.620[c]	13.33 ± 0.590[b]	16.670 ± 0.033[b]
	30 %	10.00 ± 2.000[ab]	20.00 ± 3.452[ab]	20.00 ± 3.452[a]	16.670 ± 0.740[b]
	50 %	13.331 ± 0.612[a]	23.33 ± 4.213[a]	20.00 ± 3.562[a]	26.67 ± 1.111[a]
F. mucoso	10 %	0.000 ± 0.000[c]	6.670 ± 1.620[c]	6.670 ± 1.620[d]	16.671 ± 0.323[b]
	30 %	6.670 ± 1.020[b]	16.67 ± 0.321[ab]	16.671 ± 0.321[ab]	16.671 ± 0.033[b]
	50 %	13.330 ± 0.723[a]	20.000 ± 3.521[ab]	20.00 ± 3.510[a]	23.33 ± 2.271[ab]
Control	0.0	0.000 ± 000[c]	0.000 ± 0.000[d]	0.000 ± 0.000[e]	3.330 ± 1.730[c]
Total effect of the botanical treatments					
P. africana		5.650 ± 0.36[5a]	12.623 ± 0.205[a]	13.359 ± 0.025[a]	15.848 ± 0.022[a]
F. mucoso		1.541 ± 0.234[b]	5.600 ± 0.300[b]	10.863 ± 0.044[b]	15.467 ± 0.404[a]
Control		0.000 ± 0.000[c]	0.000 ± 0.000[c]	0.000 ± 0.000[c]	0.000 ± 0.000[b]

Values with the same letter (s) in the same column are not significantly different from each other at $p < 0.05$, HAT = Hour after treatment

Table 2: Effect of botanical types on the early life stages of *C. maculatus*

		Life stages		
Treatment	Conc. (%)	Oviposition	Larvae	Pupae
P. africana	10	4.333 ± 2.733^{abc}	2.000 ± 0.632^{bc}	2.667 ± 1.366^{ab}
	30	2.500 ± 0.547^{bcd}	2.000 ± 1.265^{bc}	1.833 ± 1.169^{ab}
	50	1.333 ± 0.516^{d}	0.833 ± 0.753^{c}	1.000 ± 0.894^{b}
F. mucoso	10	5.000 ± 1.673^{ab}	4.000 ± 1.265^{a}	3.500 ± 1.049^{a}
	30	3.167 ± 0.983^{abcd}	2.500 ± 1.517^{abc}	2.500 ± 1.643^{ab}
	50	1.833 ± 0.753^{cd}	1.667 ± 0.516^{bc}	2.167 ± 0.753^{ab}
Control	0	5.333 ± 1.751^{a}	4.333 ± 1.366^{a}	3.667 ± 1.211^{a}
Total effect of the botanical treatments				
P. africana		2.722 ± 1.994^{b}	1.611 ± 1.036^{c}	1.833 ± 1.294^{b}
F. mucoso		3.333 ± 1.749^{b}	2.722 ± 1.487^{b}	2.722 ± 1.274^{ab}
Control		5.500 ± 1.567^{a}	3.833 ± 1.403^{a}	3.583 ± 1.443^{a}

Values with the same letter (s) in the same column are not significantly different from each other at $p < 0.05$

Table 3: Effect of botanical treatment on emergence of *C. maculatus* adults

		Adult emergence (DAT)				
Treatment	Conc. (%)	28	29	30	31	32
P. africana	10	2.500 ± 2.168^{abc}	2.833 ± 2.317^{bc}	3.333 ± 2.582^{ab}	3.833 ± 0.983^{ab}	3.500 ± 2.258^{abc}
	30	1.667 ± 1.633^{bc}	2.333 ± 2.338^{c}	4.333 ± 1.751^{ab}	2.333 ± 1.033^{b}	1.500 ± 1.378^{c}
	50	0.333 ± 0.817^{c}	0.333 ± 0.516^{c}	1.667 ± 1.211^{b}	1.667 ± 0.817^{b}	1.333 ± 0.516^{c}
F. mucoso	10	4.167 ± 1.169^{ab}	5.833 ± 2.483^{ab}	4.000 ± 0.632^{ab}	4.500 ± 2.509^{ab}	2.833 ± 1.169^{abc}
	30	2.500 ± 2.509^{abc}	3.333 ± 2.422^{bc}	3.500 ± 1.517^{ab}	3.167 ± 1.941^{ab}	2.167 ± 1.472^{bc}
	50	1.667 ± 2.066^{bc}	2.333 ± 1.211^{c}	4.667 ± 1.211^{ab}	3.500 ± 1.049^{ab}	2.667 ± 1.506^{abc}
Control	0	5.167 ± 1.722^{a}	6.667 ± 1.366^{a}	6.333 ± 2.582^{a}	5.833 ± 2.041^{a}	4.833 ± 1.169^{a}
Total effect of the botanical treatments						
P. africana		1.500 ± 1.791^{b}	1.833 ± 2.121^{c}	3.111 ± 2.139^{b}	2.611 ± 1.289^{b}	2.111 ± 1.779^{b}
F. mucoso		2.778 ± 2.157^{b}	3.833 ± 2.503^{b}	4.056 ± 1.211^{b}	3.722 ± 1.904^{ab}	2.556 ± 1.338^{b}
Control		5.167 ± 1.801^{a}	6.333 ± 1.497^{a}	5.750 ± 2.179^{a}	4.750 ± 2.379^{a}	4.583 ± 1.311^{a}

Values with the same letter (s) in the same column are not significantly different from each other at $p < 0.05$, DAT = Days after treatment

3.3 EFFECT OF THE BOTANICAL TREATMENT ON THE EMERGENCE OF ADULT *C. maculatus* F_1 PROGENY

Results in Table 3 indicated both *P. africana* and *F. mucoso* leaves extract treatment at 50 % to have significantly ($p < 0.05$) reduced the population of newly emerging *C. maculatus* adults at 28 and 29 days after treatment (DAT) when compared to the control. Subsequent observations of the treatment effects on 30, 31 and 32 DAT revealed seeds treated with P. *africana* at 50 % concentration had the lowest number of adults that emerged than the rest of the treatments and the control as shown in Table 3.

The overall treatment effects of the two botanicals on the emergence of F1 progeny of *C. maculatus* adults is presented in Table 3. At 28, 30 and 32 DAT, there was no significant difference between *P. africana* and *F. mucoso* leaves extract treatment which had the least population of the insect pest compared to the control. At 29 and 31 DAT, it was observed that overall treatment effect of *P. africana* had the least F1 progeny emergence than the *F. mucoso* treated seeds and the control (Table 3).

3.4 EFFECT OF THE BOTANICAL TREATMENT ON COWPEA SEED VIABILITY

The results for floatation and germination test to check for cowpea seed viability after treatment with *P. africana* and *F. mucoso* leaves extract is presented in Table 4. Overall, there was no significant ($p > 0.05$) differences detected between the various treatment groups and their concentrations in both the floatation and germination test carried out.

On the other hand, the total effects of the treatments indicated a significant difference with the control (7.167 ± 1.115) having the least seed viability in the floatation test in comparison to both the *P. africana* (8.833 ± *1.505)* and *F. mucoso* (8.778 ± *1.263) treated seeds which had* no difference (Table 4). The germination test indicated no significant (P>0.05) difference in seed viability considering the total effect of the botanical treatments as inferred in Table 4.

Table 5: Qualitative analysis of phytochemical composition of ethanolic leave extracts of *Prosopis africana* and *Ficus mucoso*

Phytochemicals	*Prosopis africana*	*Ficus mucoso*
Saponins	+	+
Tannins	++	++
Flavonoids	++	-
Terpenoids	+++	-
Alkaloids	++	+
Steroids	+	++

Key: - = not present, + = present in very small concentration, ++ = present in moderately high concentration, +++ = present in very high concentration

Table 4: Effects of botanical treatment on seed viability

		Seed viability (%)	
Treatment	Conc. (%)	Floatation	Germination
P. africana	10	8.500 ± 1.378[ab]	6.333 ± 1.751[a]
	30	8.333 ± 2.066[ab]	6.000 ± 1.789[a]
	50	9.667 ± 0.516[a]	4.000 ± 1.414[a]
F. mucoso	10	8.000 ± 1.673[ab]	4.667 ± 2.160[a]
	30	9.167 ± 0.753[ab]	5.667 ± 2.422[a]
	50	9.16 7± 0.983[ab]	4.500 ± 1.378[a]
Control	0	7.500 ± 1.049[ab]	5.000 ± 2.168[a]
Total effect of the botanical treatments			
P. africana		8.833 ± 1.505[a]	5.444 ± 1.886[a]
F. mucoso		8.778 ± 1.263[a]	4.944 ± 1.984[a]
Control		7.167 ± 1.115[b]	6.250 ± 2.179[a]

Values with the same letter (s) in the same col

3.5 QUALITATIVE ANALYSIS OF PHYTOCHEMICAL COMPOSITION OF ETHANOLIC LEAVES EXTRACT OF BOTANICALS

Table 5 shows the qualitative analysis of phytochemical composition of ethanolic leaves extract of the two botanicals. The result of the phytochemical screening indicated that saponins were present in very small concentrations in *Prosopis africana* and *Ficus mucoso*. Tannin was found to be present in moderately high concentrations in *Prosopis africana* and *Ficus mucoso*. Flavonoids were also found to be present in moderately high concentration in *P. africana* and not present in *F. mucoso.* Terpenoid was found to be present in very high concentration in *P. africana* and not present in *F. mucoso*. Furthermore, alkaloids were present in moderately high concentration in *P. africana* while in very small concentration in *F. mucoso.* Lastly, steroid was indicated in very small concentration in *P. africana* and present in moderately high concentration in *F. mucoso* as shown in Table 5.

4 DISCUSSION

Several plant products have been studied to possess insecticidal properties against a wide range of insects, particularly agricultural pests (Abdullahi & Muhammad, 2004; Bishnu & Weisman, 2005; Swella & Mushobozy, 2007; Ajayi, 2007; Raja & William, 2008; Alan et al., 2009; Aly & Sahar, 2010). Extracts from such a plant have a natural tendency to break down rapidly and are environmentally safer as they produce no residue effect (Islam, 2006). Herein in this work, we presented the first evidence-based trials on the bioactive potential of two different plant ethanolic leave extracts namely *P. africana* and *F. mucoso* in the control of *C. maculatus* infesting stored cowpea. The results obtained revealed that the extract of the two botanicals caused a significant rate of mortality on the insect pest. However, the cowpea seeds treated with *P. africana* leaves extract recorded the highest mean mortality of adult *C. maculatus* compared to *F. mucoso*, especially at the highest treatment concentration. The insecticidal effect of the plants ethanolic extracts on *C. maculatus* in the treated cowpea seeds might be a result of direct contact. Most insects breathe by means of trachea which usually open at the surface of the body through spiracles, the extract mixed with the seeds might have blocked these spiracles thereby leading to suffocation and death of the insects (Adedire & Akinkurolele, 2005; Rahman & Talukder, 2006; Akinkurolele et al., 2006).

On the oviposition and emergence of a new generation of *C. maculatus* (larvae, pupae stage, and adult emergence) both *P. africana* and *F. mucoso* leave extracts were significantly effective in suppressing the population of the insect especially when the dosage of the extracts was increased (although, seed lots treated with *P africana* were observed to have had the least population of the insect pest). This means that the plants leave extracts had insecticidal properties that inhibited egg-laying, larvae, pupation, and the emergence of new adults of the insect, the repellant effects by the plant materials inactivated the insect pest (Lale & Ofuya, 2001; Adedire et al., 2011; Ileke & Olotuah, 2012). Similar work using other plant materials was also reported by Lale & Ofuya (2001) who stated that botanicals with toxic constituents are effective in the suppression of the various life stages of insect pests, though, he did not work on *P. africana* and *F. mucoso*, this work shows that the two botanicals could be applied in restricting the various early life stages of *C. maculatus*.

The phytochemical analysis of the two botanicals revealed that they contained bioactive compounds such as alkaloids, steroids, saponins, tannins, flavonoids, and terpenoids with *P. africana* containing the vast majority of the compounds in considerable amounts when compared to *F. mucoso*. The secondary plant metabolites may be responsible for the insecticidal properties of the leave extracts (Kabaru & Gichia, 2009). Iwuala et al. (1981) stated that aromatic compounds such as terpenoids, flavonoids, and saponins have ovicidal, toxic, and deterrent effects on coleopterous insect pests infesting stored products. The presence of terpenoids in *P. africana* indicated that the plant extract could act as an antifeedant, growth disruptor and possessed considerable toxicity toward insects' pests of stored seeds (Khalid et al., 1989). Okwu (2001) also stated that steroidal compounds which were more in *F. mucoso* play importance in pharmacy due to their relationship with such compounds as sex hormones and as such may be also responsible for disrupting the life cycle of the insect pest. The phytochemicals richly identified in *P. africana* leaves extract may be attributed to the reason it has the highest mean mortality rate and lowest oviposition and emergence of the insect pest when compared to *F. mucoso*.

Seed viability test indicated no negative effects of the two botanical treatments on cowpea even at increasing application rate of the treatment which did not differ from seeds that were not treated (the control) hence safe for usage on stored seeds before planting. It is conceivable to infer here that both *P. africana* and *F. mucoso* plant extracts offered a cheaper and sustainable alternative to synthetic insecticides (Mukanga et al., 2010) in the control of *C. maculatus* infesting stored cowpea seeds.

5 CONCLUSION

Many studies have addressed the insecticidal efficacy of some plant species. *Prosopis africana* and *Ficus mucoso* ethanolic leaves extract were evaluated for insecticidal activity in this study. This research indicated that the plant material may be suitable for developing plant-based insecticides which are biodegradable and ecologically friendly. This could be further adopted by smallholder farmers in the usage against major storage pests of cowpea. *P. africana* leaves extract had more insecticidal potential compared to *F. mucoso,* the latter could still be utilized especially in environments where they are growing in abundance if the former is lacking and as such could be integrated with other pest management approach in suppressing insect pests.

6 REFERENCES

Abdullahi, N., Majeed, Q. & Oyeyi, T. I. (2011). Studies on the efficacy of *Vittallaria paradoxa* seed oil on the oviposition, hatchability of eggs and emergence of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) on treated cowpea seed. *Journal of Entomology*, *8*, 391–397. https://doi.org/10.3923/je.2011.391.397

Abdullahi, Y.M. & Muhammad, S (2004). Effects of some plant powders on cowpea weevil *Callosobruchus maculatus* during storage. *African Journal of Biotechnology*, *3*(1), 67-70.

Adedire, C, Akinkurolere, R. O. & Ajayi, O. O. (2011). Susceptibility of some maize cultivars in Nigeria to infestation and damage by maize weevil, *Sitophilus zeamais* (Motsch) (Coleoptera: Curculionidae). *Nigerian Journal of Entomology*, *28*, 55-63.

Adedire, C. O. & Akinkurolele, R. O. (2005). Bioactivity of four plant extract on coleopterous pests of stored cereals and grain legumes in Nigeria. *Zoological Research*, *26*, 243-249.

Ajayi, F. A. (2007). Effect of grain breakage and application of edible oils to protect pearl millet, *Pennisetum glaucum* (L.) R. Br., against infestation by adult *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae) in Lafia, Nigeria. *Nigerian Journal of Entomology*, *24*, 107-113.

Ajayi, F. A., Wintola, H. U. (2006). Suppression of the cowpea bruchid (*Callosobruchus maculatus* (F.) infesting stored cowpea (*Vigna unguiculata* (L.) Walp.) seeds with some edible plant product powders. *Pakistani Journal of Biological Sciences*, *9*(8), 1454-1459. https://doi.org/10.3923/pjbs.2006.1454.1459

Akinkurolele, R. O., Adedire, C. O. & Odeyemi, O. O. (2006). Laboratory evaluation of the toxic properties forest *Anchomanes difformis* against pulse beetles *Callosobrunchus maculatus*. *Insect Science*, *13*, 25-29. https://doi.org/10.1111/j.1744-7917.2006.00064.x

Alan, C, Dobson, H, Grzywacz, D, Hodges, R, Orr, A. & Stevenson, P. (2009). *Review of pre- and post-harvest pest management for pulses with special reference to Eastern and Southern Africa*. Natural Resources Institute prepared for and funded by McKnight Foundation, Collaborative Crops Research Program.

Aly, S. D., Sahar, I. A. (2010). Efficacy of spearmint oil and powder as alternative of chemical control against *Callosobruchus maculatus* in Cowpea Seeds. *Egyptian Academic Journal of Biological Sciences*, *2*(1), 53-61. https://doi.org/10.21608/eajbsf.2010.17463

Arthur, F. (1996). Grain protectants: Current status and prospects for the future. *Journal of Stored Products Research*, *32*, 293–302. https://doi.org/10.1016/S0022-474X(96)00033-1

Bishnu, C. & Weisman, Z. (2005). Larvicidal effects of aqueous extracts of *Balanites aegyptiaca* (desert date) against the larvae of *Culex pipiens* mosquitoes. *African Journal of Biotechnology*, *4*(11), 1351-1354.

Dangarembizi, R., Erlwanger, K., Moyo, D. & Chivandi, E. (2012). Phytochemistry, pharmacology and ethnomedicinal uses of *Ficus Thonningii* (Blume Moraceae): A Review. *African journal of traditional, complementary, and alternative medicines: AJTCAM / African Networks on Ethnomedicines*, *10*. 203-212. 10.4314/ajtcam.v10i2.4. https://doi.org/10.4314/ajtcam.v10i2.4

Ehiagbonare, J. E. & Enabulele, S. A. (2007). Effect of storage regime, presorting treatments, light and dark on seed germination of *Chrysophylluum delevoyi* (De Wild). *Nigerian Journal of Applied Science, 25*, 151-156.

Elaigwu, M., Oluma, H.O.A. & Onekutu, A. (2018). Phytochemical and antifungal activity of leaf extracts of *Prosopis africana* and *Anacardium occidentale* against *Macrophomina* root rot of *Sesamum indicum* L. in Benue State, Central Nigeria. *Journal of Geoscience and Environment Protection, 6*, 66-76. https://doi.org/10.4236/gep.2018.67005

Haghtalab, N., Shayesteh, N. & Aramideh, S. (2009). Insecticidal efficacy of castor and hazelnut oils in stored cowpea against *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *Journal of Biological Sciences*, *9*, 175–179. https://doi.org/10.3923/jbs.2009.175.179

Harborne, J. B. (1973). Phytochemical methods: *A guide to modern techniques of plant analysis*. Chapman and Hall Ltd, London; Pp. 279.

Holly, S. K. (2006). *Seed Germination*. Library, Gardening in Western Washington. Presented by WSU Extension.

Ileke, K. D. & Olotuah, O. F. (2012). Bioactivity of *Anacardium occidentale* (L) and *Allium sativum* (L) powders and oils extracts against cowpea bruchid, *Callosobruchus maculatus* (Fab.) (Coleoptera: Chrysomelidae). *International Journal of Bio*logy, *4*(1), 23-28. https://doi.org/10.5539/ijb.v4n1p96

Islam, Shahidul (2006). Sweetpotato (*Ipomea batatas* L.) leaf: its potential effect on human health and nutrition. *Journal of Food Science*, *71*, 13-21. https://doi.org/10.1111/j.1365-2621.2006.tb08912.x

Iwuala, M. O. E., Osisiogwu, I. U. W. & Agbakwuru E. U. P. (1981). *Dennetia* oil, a potential new insecticide: Tests with adults and nymphs of *Periplanata america* and *Zonocerus variegatus. Journal of Economic Entomo*logy, *74*, 249-252. https://doi.org/10.1093/jee/74.3.249

Kabaru, M. & Gichia, L (2009). Insecticidal activity of extracts derived from different parts of the mangrove tree *Rhizophora mucronata* (Rhizophoraceae) Lam. against three arthropods. *African Journal of Science and Technology, 2*, 10. https://doi.org/10.4314/ajst.v2i2.44668

Khalid, Sami, Duddeck, Helmust & Gonzalez-Sierra, Manuel (1981). Isolation and characterization of an antimalarial agent of the neem tree *Azadirachta indica*. *Journal of natural products*, *52*, 922-6. https://doi.org/10.1021/np50065a002

Lale, N. E. S & Mustapha, A. (2000). Potential of combining neem (*Azadirachta indica* A. Juss.) seed oil with varietals resistance for the management of the cowpea bruchid, *Callosobruchus maculatus* (F.). *Journal of Stored Products Research*, *36*, 215–222. https://doi.org/10.1016/S0022-474X(99)00035-1

Lale, N.E.S., and Ofuya, T.I. (2001). Overview of pest problems and control in the tropical storage environment. In: Ofuya, T.I. and Lale, N.E. S. (eds.) *Pests of Stored Cereals and Pulses in Nigeria: Biology, Ecology and Control.* Dave Collins Publications, Akure, Nigeria. pp. 1-23.

Okwu, D. E (2001). Evaluation of chemical composition spices and flavoring agents. *Global Journal of pure and Applied Science*, *7*, 455-459. https://doi.org/10.4314/gjpas.v7i3.16293

Oni, M. O & Ileke, K. D. (2008). Fumigant toxicity of four botanical plant oils on survival, egg laying and progeny development of the dried yam beetle, *Dinoderus porcellus* (Coleoptera: Bostrichidae) Ibadan. *Journal of Agricultural Research*, *4*(2), 31-36.

Oni, M. O. (2011). Evaluation of seeds and fruit powder of *Capsicum annum* and *C. frutescens* for control of *Callosobruchus maculatus* (Fab.) in stored cowpea and *Sitophilus zeamais* in stored maize. *International Journal of Biology*, *3*(2), 185-188. https://doi.org/10.5539/ijb.v3n2p185

Rahman, A. & Talukder, F. A. (2006). Bio efficacy of some plant derivatives that protect grain against the pulse beetle, *Callosobruchus maculatus. Journal of Insect Science*, *6.* 3. https://doi.org/10.1673/1536-2442(2006)6[1:BOSPDT]2.0.CO;2

Raja, M. & William S. J. (2008). Impact of volatile oils of plant against cowpea beetle *Callosobruchus maculatus* (Fabr) (Coloeoptera: Bruchidae). *International Journal Integrative Biology*, *2*(1), 62-64.

Singh R. N. and Saratchandra B (2005). The development of botanical products with special reference to Seri-ecosystem. *Caspian Journal of Environmental Sciences*, *3*(1), 1-8.

Shinkafi, S. A & Abdullahi, H. (2018). Antifungal activity and phytochemical analysis of *Ficus sycomorus* leaf extract on *Malassezia glubosa*. *Advances in Plants and Agricultural Research,* 8(6), 432-436. https://doi.org/10.15406/apar.2018.08.00362

Sofowora, A. (1993) *Medicinal Plants and Traditional Medicine in Africa*. Spectrum Books Ltd., Ibadan, 191-289.

Swella, G. B. & Mushobozy, D. M. K. (2007). Evaluation of the efficacy of protectants against cowpea bruchids (*Callosobruchus maculatus* (F.)) on cowpea seeds (*Vigna unguiculata* (L.) Walp.). *Plant Protection Science*, *43*(2), 68-72. https://doi.org/10.17221/2256-PPS

Trease, G. E. & Evans, W. C. (1989). *Pharmacognosy*. Bailliere Tindall.

Umeozor, O. C. (2005). Effect of the infection of *Callosobruchus maculatus* (Fab.) on the weight loss of stored cowpea (*Vigna unguiculata* (L.) Walp). *Journal of Applied Sciences and Environmental Management*, *9*(1), 169-172.

Zerihun M, Ele E (2018). Insecticidal activities of leaf, seed and stem bark extracts of *Prosopis juliflora* against the cotton (*Aphis gossypii Glover*) aphid. *Academic Research Journal of Agricultural Science Research*, *6*(3), 202-221.

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Izhodišča pri izboru in načinu umeščanja vrtnic (*Rosa* spp.) na javne in poljavne mestne površine: primer četrtne skupnosti Bežigrad, Ljubljana

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Preferences in selection and planting types of roses (*Rosa* spp.) in urban public and semi-public areas: a case study of Bežigrad community, Ljubljana

Abstract: Roses have an indisputable leading role in private gardens. They also appear in public areas. They are very interesting plants for public urban areas because they represent aesthetic, ecological, tehnical and sociological potential. In public areas varietes are selected according to various criteria such as resistance to heat, low temperatures an drought, repetitive flowering and ease of maintenance. The aim of our study is to present preferences in the selection of groups of roses, their colors, types of plantings, the abudance of roses in planting and the height of individual roses on difrent subtypes of public ans semi-public green areas of the Bežigrad community, Ljubljana. The results of the study showed that the most comomn roses in urban public areas are floribundas. Dominated type of planting is a few plants together in a group. In neighborhoods and block settlements are dominated individual plants. The most common color of roses is red. In urban public areas are planted only roses up to 1 m height. In semi-public areas are also higher roses. The abudance of roses in semi-urban areas varies from 1 to over 30 roses in planting. In urban public areas are most common planting with 10 to 20 roses and those with more than 30 roses.

Key words: roses; urban public areas; urban semi-public areas; community of Bežigrad

Izhodišča pri izboru in načinu umeščanja vrtnic (*Rosa* spp.) na javne in poljavne mestne površine: primer četrtne skupnosti Bežigrad, Ljubljana

Izvleček: Vrtnice so na slovenskih zasebnih vrtovih zelo pogoste okrasne rastline, vedno bolj pa se sadijo tudi na javne površine. So zelo zanimive rastline za javne mestne površine, saj predstavljajo estetski, ekološki, tehnični in sociološki potencial. Za javni mestni prostor se izbira sorte po kriterijih, kot so: odpornost na vročino, nizke temperature, sušo, ponavljajoče cvetenje in enostavnost vzdrževanja. Namen naše raziskave je bil predstaviti preference pri izboru skupin vrtnic, njihove barve, vrste zasaditev, številčnost vrtnic v zasaditvi ter višina posameznih vrtnic na različnih podtipih javnih in poljavnih zelenih površinah četrtne skupnosti Bežigrad, Ljubljana. Rezultati raziskave so pokazali, da so na javni površini najpogostejše mnogocvetne vrtnice. Prevladujejo zasaditve po nekaj rastlin skupaj v gruči, medtem ko v soseskah in blokovskih naseljih prevladujejo posamezne rastline. Najpogostejša barva vrtnic je rdeča. Na javnih mestnih površinah so zasajene samo vrtnice, ki dosežejo višino 1 m, na poljavnih pa tudi višje. Številčnost vrtnic na poljavnih mestnih površinah je različna, vse od 1 do nad 30 vrtnic v zasaditvi. Na javnih mestnih površinah smo opazili predvsem zasaditve z 10 do 20 vrtnicami in pa take, kjer je bilo vrtnic nad 30.

Ključne besede: vrtnice; javne mestne površine; poljavne mestne površine; četrtna skupnost Bežigrad

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1 UVOD

Vrtnice so na slovenskih zasebnih vrtovih zelo pogoste okrasne rastline, vedno bolj pa se sadijo tudi na javne mestne površine, saj nekatere uspevajo v razmeroma neugodnih razmerah in jih odlikuje dolgo obdobje cvetenja. V javnem mestnem prostoru sorte vrtnic izbiramo po kriterijih, kot so odpornost na vročino, nizke temperature in sušo, ponavljajoče cvetenje, enostavnost vzdrževanja ter odpornost proti rastlinskim boleznim. Zaradi izjemne raznolikosti in sortne pestrosti vrtnice delimo v skupine, ki se razlikujejo po načinu rasti, morfologiji, zahtevnosti vzdrževanja in namenu sajenja. V javne mestne nasade se večinoma sadi manj zahtevne skupine vrtnic, ki jih v prostor najpogosteje umeščajo v obliki živih mej, obrob ali kot talne prekrovne rastline. Plezalke in vzpenjalke so v javnem mestnem prostoru manj zastopane, saj je njihovo vzdrževanje zahtevnejše (Zgonec, 1981; Cottini, 2003).

Ker se površine namenjene zelenju v mestih krčijo in so velikokrat umeščene zgolj v obcestni prostor, je smotrno izbrati rastline, ki zavzemajo večplastno vlogo. Vrtnice so na javnih površinah zato izjemno zanimive rastline, saj v stanovanjske soseske in širši mestni prostor vnašajo barvitost (estetski potencial), predstavljajo pašo za čebele in mesta za gnezdenje ptic (ekološki potencial), so primerne za sajenje na nagnjene površine zmernih nagibov (tehnični potencial) in priljubljene pri večini prebivalcev (sociološki potencial).

V raziskavi želimo predstaviti preference pri izboru skupin vrtnic (rožni grmi, pritlikave vrtnice, plezalke, prekrovne vrtnice, mnogocvetne, debelne vrtnice, retrovrtnice, vzpenjalke, poliante, atrijske, velecvetne vrtnice…), njihove barve, vrste nasadov, številčnost vrtnic v nasadu ter višina posameznih vrtnic na različnih podtipih javnih (javni parki, nasadi, drevoredi, zelenice, zelene površine ob javnih cestah lokalnih poteh in drugih javnih komunikacijah ter vodnih površinah, zelene površine ob spomeniških, zgodovinskih ter posameznih turističnih objektih v javni lasti ali če je njihovo urejanje v pristojnosti občine in zelene površine na pokopališčih) in poljavnih mestnih zelenih površinah (zelene površine pred gostinskimi objekti, lokali, televizijskimi objekti, pošto, sosesko, blokovskim naseljem, izobraževalnimi ustanovami in zdravstvenimi ustanovami) in poljavnih mestnih površin, na primeru četrtne skupnosti Bežigrad, Ljubljana. Uporabili smo izsledke na podlagi magistrskega dela (Kunc, 2019). Dobljene podatke raziskave želimo primerjati s preferencami izbora vrtnic glede na barvo, višino ter druge parametre, v državah po svetu.

2 MATERIALI IN METODE

Četrtna skupnost Bežigrad je ena izmed 17 četrtnih skupnosti v Ljubljani in po številu prebivalcev druga največja četrtna skupnost (Mestna občina Ljubljana, 2019). Na dan 31. 12. 2018 jih je imela 35.200. Zajema 724 ha površine in vključuje ljubljanske severne četrti: Bežigrad, Zupančičeva jama, Savsko naselje, Rapova jama, del Jarš, Tomačevo, BS 3, Študentsko naselje, Brinje in Stadion. Meje četrtne skupnosti so: na jugu Kurilniška ulica, Vilharjeva c. do Savske c. Na vzhodu Savska c., Žalska c. - mimo pokopališča, preko Groblja in Save. Na severu do levega brega Save in nato do krožišča Tomačevo. Območje zajema tudi del Jarš in Tomačevega. Proti zahodu poteka meja po obvoznici do podvoza pod kamniško progo. Na zahodu pa vzdolž kamniške proge mimo gorenjske železniške postaje do Kurilniške ulice, kjer se pentlja zaključi (Četrtna skupnost Bežigrad, 2019; Četrtna skupnost Bežigrad, 2015).

Kot glavno metodo našega raziskovalnega dela smo uporabili terensko raziskavo analiziranega območja. Analizo smo opravili v času polnega cvetenja, junija leta 2019. S pomočjo karte četrtne skupnosti Bežigrad smo ugotovili točne lokacije pojavljanja vrtnic. Poleg tega smo pridobili tudi informacije o tem, katere od analiziranih mestnih površin so javne ter katere so poljavne. Območje četrtne skupnosti Bežigrad smo sistematično terensko pregledovali. Zabeležili smo si lokacijo, skupino vrtnic, njihovo barvo, vrsto zasaditve, številčnost vrtnic v zasaditvi, višino rastlin ter podtip javne oziroma poljavne površine na kateri se vrtnice nahajajo. Stanje na terenu smo fotografirali.

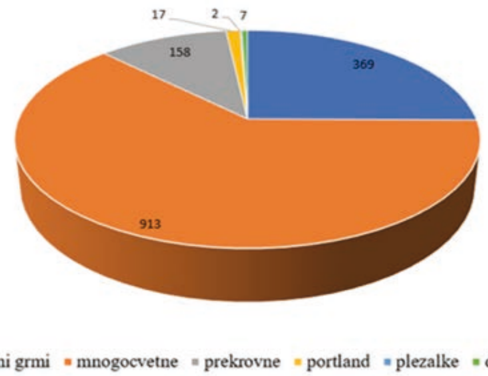
Na koncu smo vse dobljene podatke statistično obdelali in rezultate grafično prikazali ter rezultate primerjali z rezultati raziskav o vrtnicah na javnih mestnih površinah v drugih državah.

3 REZULTATI IN DISKUSIJA

Vrtnice se na javnih površinah nahajajo na Vojkovi cesti ob stavbi Upravne enote Ljubljana, izpostava Bežigrad ter pred pietetnim objektom (Žale) in v javnem parku (Park literatov). Na poljavnih površinah smo jih opazili pred gostinskima objektoma gostilna Pod kostanji in Vivo catering. Pred televizijskim objektom (POP TV), pred Pošto na Dunajski cesti. Največje število jih je bilo v soseskah in blokovskih naseljih. Lokacije, kjer smo jih opazili so: Peričeva, Ptujska, Linhartova ulica, Ulica Metoda Mikuža, Študentski dom na Vojkovi ulici, Črtomirova, Neubergerjeva, Topniška, Smoletova ulica, Ulica Pohorskega bataljona, Šarhova, Hubadova, Pegamova, Glavarjeva, Mašera-Spasića, BS3 balinarsko društvo, Turnerjeva ulica, Ulica Luize Pesjakove, Majaronova in Šerkova ulica. Poleg tega smo jih opazili še pri izobraževalnih ustanovah: Univerza v Ljubljani Fakulteta za socialno delo (Topniška ulica), Vrtec Mladi rod: Enota Mavrica (Črtomirova ulica), Vrtec Jekla (Glavarjeva ulica), Osnovna šola Bežigrad (Črtomirova ulica), Osnovna šola Franceta Bevka (Ulica Pohorskega bataljona), Univerzitetni rehabilitacijski Inštitut Republike Slovenije – Soča (Linhartova ulica). Manjši nasad smo opazili tudi pred Poslovno hišo Slovenčeva (PHS), na Slovenčevi ulici (Karta četrtne skupnosti…, 2019).

3.1 PRISOTNOST SKUPIN

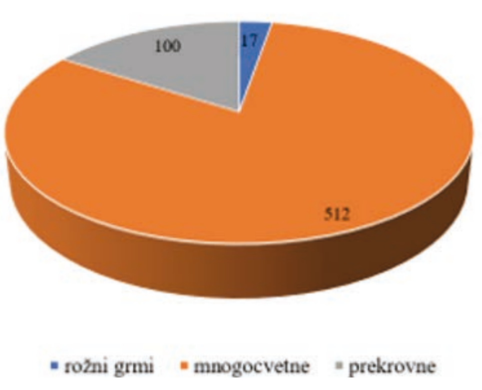
Na analiziranem območju smo opazili 1466 vrtnic. Od tega se jih je 873 (57,1 %) nahajalo na javnih ter 629 (42,9 %) na poljavnih mestnih površinah. Skupine vrtnic na posameznih tipih površin so prikazane v preglednici 1. Prevladujejo mnogocvetne vrtnice. Teh je na analiziranem območju 62,3 %. Sledijo rožni grmi, ki jih je 25,2 %. Prekrovnih vrtnic je 10,8 %, vrtnic ‚Portland' je 1,1 %, 0,5 % je debelnih in 0,2 % je plezalk (Slika 1).

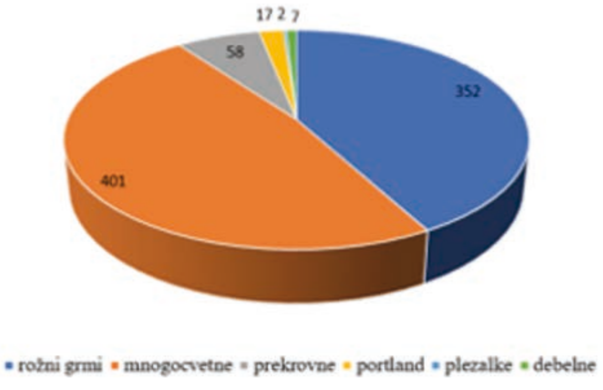


Slika 1: Analiza prisotnih skupin vrtnic na javnih in poljavnih mestnih površinah četrtne skupnosti Bežigrad

Na javnih mestnih površinah so močno prevladovale mnogocvetne vrtnice (81,4 %), v manjšem deležu so se pojavljale še prekrovne vrtnice (15,9 %) in rožni grmi (2,7 %) (Slika 2). Raznolikost skupin na poljavnih mestnih površinah je bolj pestra. V največjem deležu so zastopane mnogocvetne vrtnice (48 %), takoj za njimi sledijo rožni grmi (42,1 %). V manjših deležih pa smo opazili še prekrovne vrtnice (6,9 %), vrtnice portland (2 %), debelne vrtnice (0,8 %) in plezalki (0,2 %) (Slika 2).

Mnogocvetne vrtnice imenujemo tudi floribunde, klobčasto ali šopastocvetne vrtnice. Zaradi dobrega zdravstvenega stanja in odpornosti proti boleznim in škodljivcem so zelo primerne za sajenje na javne površine. Med prvim cvetenjem zrastejo do 70 cm visoko. Rožni grmi so višji od mnogocvetnih vrtnic in prav tako zelo primerni za sajenje na javnih površinah. Njihova povprečna končna višina znaša okrog 200 cm. Prekrovne vrtnice temeljito prerastejo tla. Lahko so polegle čisto po tleh, lahko so višine mnogocvetnih vrtnic, ali pa dosežejo višino rožnih grmov. Na javnih površinah nadomeščajo mnogocvetne vrtnice. Vrtnice ‚Portland' imajo pokončno rast in na javnih površinah niso pogoste. Plezalke zrastejo od 3 do 8 m visoko. Primerne so za plezanje po lokih, ograjah, senčnicah in stebrih. So zahtevne za gojenje, zato se na javnih površinah ne pojavljajo pogosto. Debelne vrtnice so pokončne ali povešave rasti z deblom visokim 75 do 150 cm in imajo obliko drevesa. Zaradi težavnega vzdrževanja se v javnem prostoru ne pojavljajo pogosto (Sojer, 2019; Cotini, 2003; Mastnak, 2008; Zgonec, 1981; Wilson Nurseries…, 2019; Garden Grower, 2010).





Slika 2: Analiza prisotnih skupin vrtnic na javnih (levo) in poljavnih (desno) mestnih površinah v četrtni skupnosti Bežigrad

Preglednica 1: Skupine vrtnic na posameznih tipih mestnih površin v četrtni skupnosti Bežigrad

Skupine vrtnic	Tipi površin
Mnogocvetne	Javne površine (pietetni objekt, park), poljavne površine (gostinski objekt, soseske in blokovska naselja, izobraževalne ustanove, poslovna stavba)
Rožni grmi	Javne površine (pietetni objekt), poljavne površine (gostinski objekt, Pošta, soseske in blokovska naselja)
Prekrovne	Javne površine (občinski objekt), poljavne površine (gostinski objekt, televizijski objekt, Pošta, soseske in blokovska naselja, izobraževalne ustanove)
Portland	Poljavne površine (soseske in blokovska naselja)
Plezalke	Poljavne površine (soseske in blokovska naselja)
Debelne	Poljavne površine (izobraževalne ustanove)

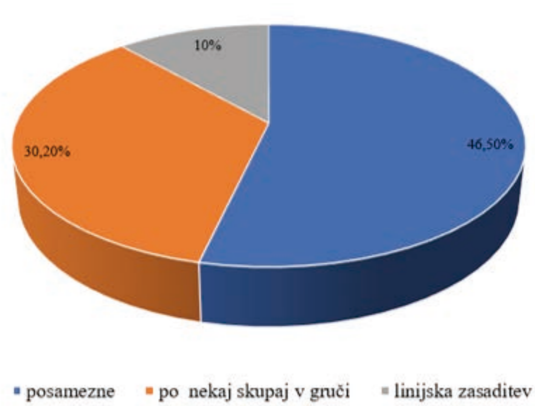
3.2 VRSTE NASADOV

Vrtnice se na analiziranem območju pojavljajo v treh tipih nasadov: kot posamezne rastline, po nekaj skupaj v gruči in kot linijski nasad. Na celotnem območju prevladujejo lokacije, kjer rastejo posamezne rastline (46,5 %), malo manj je nasadov v gruči (30,2 %), najmanj pa je linijskih nasadov (10 %) (Sliki 3 in 4).

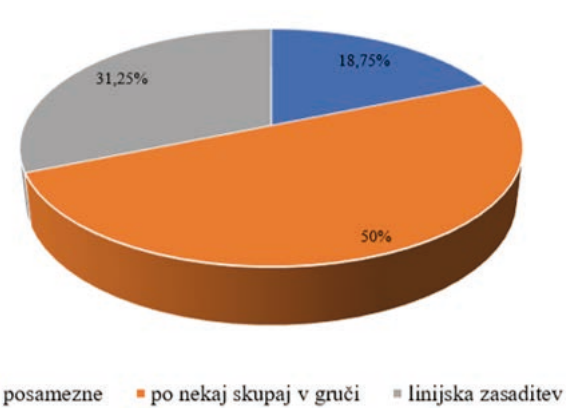
Na javnih mestnih površinah prevladujejo vrtnice, ki so posajene po nekaj skupaj v gruči (50 %) (slika 6), sledijo linijski nasadi (31,25 %), najmanj je posameznih rastlin (18,75 %) (slika 5).

V primerjavi z vrstami nasadov na lokacijah na javnih mestnih površinah je precej drugačno stanje v soseskah in blokovskih naseljih. S kar 63 % prevladujejo lokacije, kjer so vrtnice posajene posamezno, v kombinaciji z drugimi okrasnimi rastlinami (Slika 7). V enakih deležih (18,5 %) se pojavljajo v linijskih nasadih in v nasadih v gruči (Slika 8).

Slika 4: Primer linijskega nasada pred vhodom v Poslovno hišo Slovenčeva



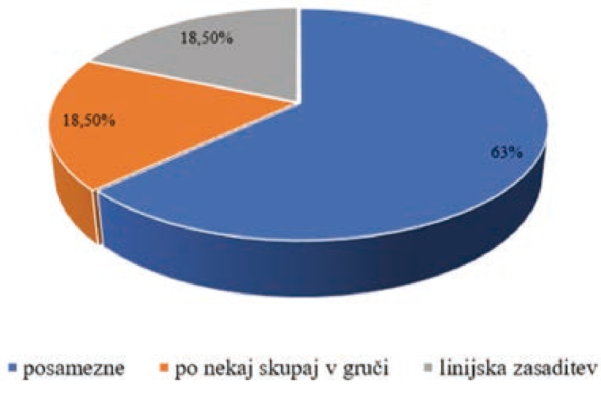
Slika 3: Analiza vrste nasadov na javnih in poljavnih mestnih površinah četrtne skupnosti Bežigrad



Slika 5: Analiza vrste nasadov na javnih mestnih površinah v četrtni skupnosti Bežigrad

Slika 6: Primer nasadov po nekaj rastlin skupaj v gruči na območju Žal

Slika 7: Primer posameznega nasada na območju sosesk in blokovskih naseljih v četrtni skupnosti Bežigrad



Slika 8: Analiza vrste nasada v soseskah in blokovskih naseljih v četrtni skupnosti Bežigrad

3.3 BARVA

Nasadi vrtnic so barvno usklajeni. Večji nasadi so roza, rdeče, oranžne ali pa bele barve.

Na celotnem analiziranem območju prevladujejo rdeče vrtnice, teh je 49,3 %, sledijo roza, ki jih je 31,4 %, belih je 8,8 %, oranžnih je 6,1 %, vijolično-rdečih je 2,4 %, roza-oranžnih je 1,5 % in 0,5 % je rumenih (Slika 9).

Na poljavnih površinah prevladujejo rdeče vrtnice, ki jih je 42,8 %, sledijo jim roza, ki jih je 41,7 %, 7,8 % je belih, 4,4 % je oranžnih , 2,4 % je roza-oranžnih in 0,9 % je rumenih (Slika 10). Podobna barvna sestava je tudi na javnih površinah. Prevladujejo rdeče (58 %), sledijo roza (17,6 %), belih je (10,2 %), oranžnih (8,5 %). Se pa na javnih površinah pojavljajo tudi vijolično-rdeče vrtnice. Teh je najmanj in sicer 5,7 % (Slika 11).

3.4 VIŠINA VRTNIC

Po višini smo vrtnice razdelili v 4 velikostne razrede: 0 – 1 m, 1 – 2 m, 2 – 3 m in 3 m in več (Preglednica 2). Opaziti je možno, da se na javne mestne površine sadi samo vrtnice, ki zrastejo do 1 m. Medtem, ko se za poljavne površine izbira vrtnice vseh štirih velikostnih skupin.

3.5 ŠTEVILČNOST VRTNIC V NASADU

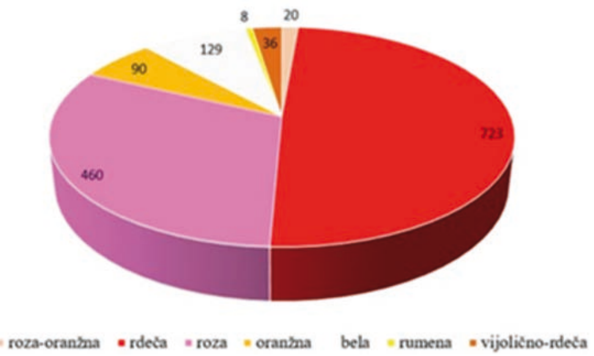
Glede na številčnost vrtnic v nasadu smo jih razdelili v 4 različne skupine: 0 – 10, 10 – 20, 20 – 30 in nad 30. Podatki so zbrani v Preglednici 3, iz katere je mogoče razbrati, da se v vseh 4 skupinah pojavljajo vrtnice na poljavnih površinah. Na javnih površinah pa se pojavljajo samo v skupini 10 – 20 in nad 30.

Za primerjavo smo pregledali preference pri izboru vrtnic za javne mestne površine drugod po svetu. Na sarajevskih (Avdić in sod., 2013) javnih mestnih površinah so prevladovale velecvetne vrtnice (48,94 %). Takoj za njimi so bile mnogocvetne vrtnice (46,81 %), v enakem majhnem deležu pa so se pojavljale še vzpenjalke in samonikli šipki. Glede barve, za razliko od bežigrajskih barvno usklajenih nasadov, sadijo skupaj popolnoma naključne barve. Na splošno pa v Sarajevu prevladujejo vrtnice bele in roza barve.

V Pekingu (Wang in sod., 2017) je najpogostejša rdeča barva vrtnic (57 %). V manjšem deležu se udi rumene, bele in večbarvne vrtnice. V Pekingu so nasadi precej večji kot na našem analiziranem območju. 40 % nasadov je z manj kot 100 vrtnicami, 24 % pa takih, kjer raste med 100 in 200 vrtnic. Tudi tukaj za javne površi ne izbirajo sorte, katerih višina ne presega 1 m.

Iranska raziskava preferenc (Rahnema in sod., 2018) okrasnih rastlin med obiskovalci njihovih mestnih parkov je pokazala, da so vrtnice s 25 %, takoj za tulipani, druge najbolj priljubljene okrasne rastline. Tudi prebivalci Irana imajo najraje rdečo barvo (52,1 %) cvetov, sledi vijolična in oranžna.

Vrtnice so priljubljene okrasne rastline na poljavnih in zasebnih površinah tudi v Turčiji (Gurkan Kaya in sod., 2018). V Antalyji so vrtnice zasedle četrto mesto med rastlinami, ki se najpogosteje pojavljajo na poljavnih iz zasebnih površinah. Pred njimi so le limonovci, pinije in pomarančevci.



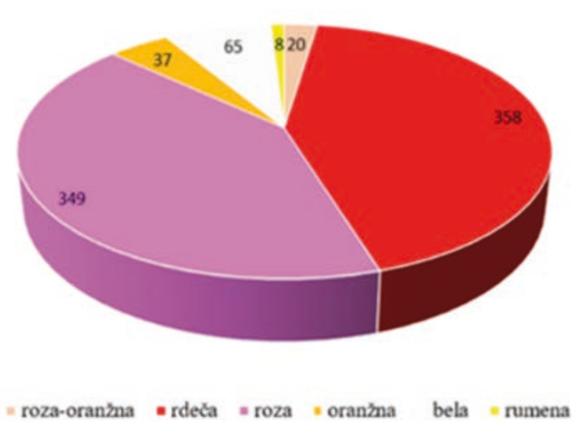
Slika 9: Analiza barve cvetov vrtnic na javnih in poljavnih površinah četrtne skupnosti Bežigrad

Preglednica 2: Višina vrtnic na različnih tipih mestnih površin v četrtni skupnosti Bežigrad

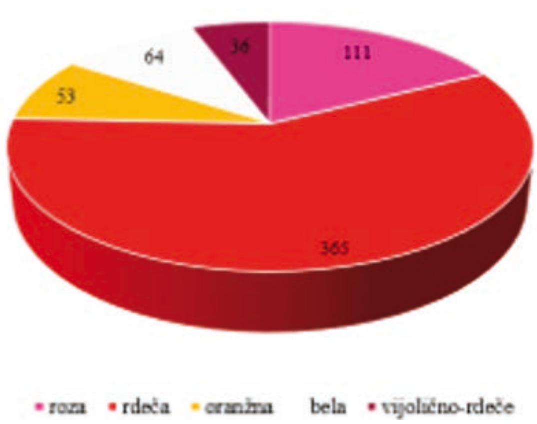
Višina vrtnic	Tipi površin
0 – 1 m	Javne površine (občinski objekt, pietetni objekt, park) in poljavne površine (gostinski objekt, soseske in blokovska naselja, izobraževalne ustanove, televizijski objekt, Pošta)
1 – 2 m	Poljavne površine (izobraževalne ustanove)
2 – 3 m	Poljavne površine (pietetni objekt, Pošta, gostinski objekt, soseske in blokovska naselja)
3 m in več	Poljavne površine (soseske in blokovska naselja)

Preglednica 3: Številčnost vrtnic v nasadu na različnih tipih mestnih površin v četrtni skupnosti Bežigrad

Številčnost vrtnic v nasadu	Tipi površin
0 – 10	Poljavne površine (gostinska objekta, televizijski objekt, Pošta, soseske in blokovska naselja, izobraževalne ustanove)
10 – 20	Javne površine (pietetni objekt), poljavne površine (soseske in blokovska naselja, poslovna stavba)
20 – 30	Poljavne površine (soseske in blokovska naselja, izobraževalne ustanove)
Nad 30	Javne površine (občinski objekt, pietetni objekt, park) in poljavne površine (soseske in blokovska naselja)



Slika 10: Analiza barve cvetov na poljavnih mestnih površinah četrtne skupnosti Bežigrad



Slika 11: Analiza barve cvetov vrtnic na javnih mestnih površinah četrtne skupnosti Bežigrad

4 SKLEPI

Iz ugotovitev lahko zaključimo, da so tako na javnih kot tudi na poljavnih površinah najbolj zastopane mnogocvetne vrtnice. Na analiziranem območju smo opazili 1466 vrtnic, od tega se jih je 57,1 % nahajalo na javnih, ter 42,9 % na poljavnih površinah. Prevladujejo mnogocvetne vrnice, sledijo rožni grmi, prekrovne vrtnice in vrtnice portland. Najmanj pa je debelnih vrnic in plezavk. Vzrok za tako redko pojavnost omenjenih skupin je v tem, da so težje za gojenje ter vzdrževanje. Na javnih površinah so najpogostejši nasadi po nekaj vrtnic skupaj v gruči, medtem ko v soseskah in blokovskih naseljih močno prevladujejo posamezne rastline. Barvna sestava vrtnic ni pestra. Močno prevladuje rdeča, v manjših deležih pa se pojavljajo tudi roza, oranžne, bele, rumene, roza-oranžne in vijolično-rdeče. Za poljavne površine se izbira vrtnice vseh velikostnih skupin, medtem ko za javne površine samo vrtnice višine do 1 m. Glede številčnosti rastlin v nasadu lahko sklepamo, da se v vseh 4 skupinah pojavljajo vrtnice na poljavnih površinah. Na javnih površinah pa se pojavljajo samo v skupini 10 – 20 in nad 30. Ugotovili smo, da se vrtnice v soseskah in blokovskih naseljih pojavljajo v kombinaciji z drugimi okrasnimi rastlinami. Za razliko od omenjenih območij pa se na ostalih analiziranih lokacijah vrtnice pojavljajo kot samostojne rastline oziroma v nasadih vrtnic.

5 VIRI

Avdić J., Bečić B., Sarajlič N., Arar K. (2013). Roses (*Rosa* spp.) in public green spaces of Sarajevo. *Works of the Faculty of agriculture and food sciences*, University of Sarajevo, 61, 66/1: 209-212

Cottini P. (2003). Vrtnice, sorte in način gojenja (izbira, sajenje, nega, obrezovanje). Ljubljana, Rože in vrt, *Delo Revije*: 34 str.

Četrtna skupnost Bežigrad. (2015). Wikipedija. https://sl.wikipedia.org/wiki/Četrtna_skupnost_Bežigrad

Četrtna skupnost Bežigrad. Mestna občina Ljubljana. (2019). https://www.ljubljana.si/sl/moja-ljubljana/cetrtne-skupnosti-v-ljubljani/cetrtne-skupnosti-v-ljubljani-2/cetrtna-skupnost-bezigrad/

Gurkan Kaya L., Kaynakci-Elinc Z., Yucedag C., Cetin M. (2018). Enviromental outdoor plant preferences: A practical approach for choosing outdoor plants in urban or suburban residental areas in Antalya, Turkey. *Fresenius Enviromental Bulletin*, *27*(12), 7945-7952

Garden Grower. (2010). http://www.garden-grower.com/flowers/pruning-roses.shtml (21. 2. 2019)

Rahnema S., Sedaghathoor S., Sadegh Allahyari M., Damalas C. A., El Bailali H. (2018). Preferences and emotion perceptions of ornamental plant species for green space designing among urban park users in Iran. *Urban Forestry & Urban Greening*, *30,* 1-11.

Karta četrtne skupnosti Bežigrad, (2019). Naš Bežigrad. *Glasilo četrtne skupnosti Bežigrad Mestne občine Ljubljana*, *8*(1), 12-13.

Kunc N. (2019). Izbor in pojavnost vrtnic (*Rosa* spp.) na javnih površinah v Mestni občini Ljubljana, četrtna skupnost Bežigrad. *Magistrsko delo*. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 44 str.

Mastnak M. (2008). *Vrtnice.* Ljubljana, Kmečki glas: 184 str.

Mestna občina Ljubljana. (2019a).Wikipedija. https://sl.wikipedia.org/wiki/Mestna_občina_Ljubljana

Sojer E. (2019). Rast in razvoj vrtnic (*Rosa* spp.) v prvem letu po presajanju. *Diplomsko delo (VS)*. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 35 str.

Wang H., Yang Y., Li M., Liu J.,, Jin W. (2017). Residents‘ preferences for roses. Features of rose plantings and the relations between them in built-up areas of Beijing, China. *Urban Foestry & Urban Greening*, *27*, 1-8. https://doi.org/10.1016/j.ufug.2017.06.011

Wilson Nurseries & Landscape Supply. (2019). *Basic Rose Care*: 2 str. https://www.wilsonnurseries.com/wp-content/uploads/2015/01/Basic-Rose-Care.pdf (2. 3. 2019)

Zgonec S. (1981). *Vrtnice*. Ljubljana, ČZP Kmečki glas: 191 str

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Phosphate fertilization regulates arbuscular mycorrhizal symbiosis in roots of soybean (*Glycine max* L.) cultivars in a humid tropical soil

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Phosphate fertilization regulates arbuscular mycorrhizal symbiosis in roots of soybean (*Glycine max* L.) cultivars in a humid tropical soil

Abstract: The effect of phosphate fertilization on arbuscular mycorhizal symbiosis and grain yields of soybean cultivars was investigated on P deficient soil. A two-year field study (2017-2018) consisting of two soybean cultivars (TGx 1448-2E and TGx 1440-1E) and three phosphate rates [0, 20 and 40 kg P_2O_5 ha^{-1}) was laid out in a randomized complete block design with three replications. The results showed that P fertilization significantly ($p < 0.001$) reduced AMF root colonization of both cultivars in the two cropping years. The arbuscular, vesicular, internal hyphae and total colonization in the root cortex of the soybean cultivars were significantly ($p < 0.001$) reduced with high P (40 kg) application. However, moderate P (20 kg) promote AMF symbiosis in roots of 'TG x 1448-2E'. Dry mass (root and shoot), P uptake and grain yield of the soybean cultivars were significantly ($p < 0.001$) increased with increasing P ferilization. There was a strong linear relationships between root colonization and total dry matter mass (r = 0.81), P uptake (r = 0.81) and grain yield (r = 0.85). Thus, it could be concluded that moderate P fertilizer application is needed to promote mycorrhizal symbiosis in soybean and sustainable crop production in humid tropical soil.

Key words: arbuscules; biomass; internal hyphae; soybean; phosphorus uptake; vesicles; Nigeria

Gnojenje s fosfatom uravnava arbuskularno mikorizno simbiozo v koreninah sort soje (*Glycine max* L.) v vlažnih tropskih tleh

Izvleček: Učinek gnojenja s fosfatom na arbuskularno mikorizno simbiozo (AMF) in pridelek zrnja je bil preučevan pri sortah soje na tleh s pomanjkanjem fosforja. Dvoletni poljski poskus (2017-2018), ki je vključeval dve sorti soje ('TGx 1448-2E' in 'TGx 1440-1E') in tri gnojenja s fosfatom [0, 20 in 40 kg P_2O_5 ha^{-1}) je bil izveden kot popolni naključni bločni poskus s tremi ponovitvami. Rezultati so pokazali, da je gnojenje s fosforjem značilno ($p < 0{,}001$) zmanjšalo mikorizacijo obeh sort soje v dveh letih poskusa. Število arbuskulov, veziklov in interkortikalnih hif se je pri obeh sortah soje značilno zmanjšalo ($p < 0{,}001$) pri uporabi velikih količin P (40 kg). Zmerno gnojenje s P (20 kg) pa je pospešilo AMF simbiozo v koreninah 'TG x 1448-2E'. Suha masa (korenin in poganjkov), privzem P in pridelek zrnja so se pri obeh sortah soje značilno povečali ($p < 0{,}001$) s povečevanjem gnojenja s fosforjem. Obstajala je močna linearna, pozitivna povezava med kolonizacijo korenin in celokupno suho maso (r = 0,81), privzemom fosforja (r = 0,81) in pridelkom zrnja (r = 0,85). Iz vsega tega lahko zaključimo, da je potrebno zmerno gnojenje s fosforjem za pospeševanje mikorizne simbioze soje za njeno trajnostno pridelavo v vlažnih tropskih tleh.

Ključne besede: arbuskuli; biomasa; interkortikalne hife; soja; privzem fosforja; vezikli; Nigerija

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1 INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) belonging to the phylum Glomeromycota form symbiotic relationships with roots of most terrestrial plants, inculding most agricultural crops (Smith and Read, 2008). AMF symbiosis is the most common type and very frequent in most soils, especially phosphorus (P) defiecient soils of tropical and sub-tropical regions (Brundrett, 2009). AMF is of great relevance in most agroecosystems because of their contributions in improving uptake of water and nutrients, especially P and to a lesser extent nitrogen in exchange for plant derived carbon ((Jiang et al., 2017). The mediated improvement in nutrients uptake by AMF helps to increased growth and development of plants, and confer resistance to abiotic and biotic stress (Smith and Read, 2008; Gianinazzi et al., 2010). Moreover, AMF improve soil structure and helps to mitigate drought and salinity stress, in plants (Smith et al., 2011). Thus, AMF symbiosis may be critical to increasing crop yields and ecosystem sustainability in a low-input manner (Castillo et al., 2016).

Despite the long history of AMF coevolution with most plants in various ecosystems, resulting in their adaptation to specific areas (Gosling et al., 2013), majority of research on AMF symbiosis involves controlled experiments such as laboratory or pots and ignore native AMF taxa that could alter plant responses (Adeyemi et al., 2019). In addition, there are limited reports on the influence of various farming practices such as fertilizer application, tillage practices on AMF symbiosis in humid tropical soils of Nigeria. It is evident that important gaps in understanding the regulation of AMF symbiosis still remain. Previuos studies have shown that intensive application of chemical fertilizers, particularly phosphate fertilizers reduced development of AMF symbiosis in roots of plants (Johnson et al., 2015). Conversely, in soils with inherent soil availability, the benefit of the symbiosis could be enhanced with moderate P fertilization (Chalk et al., 2006). Interestingly, the use of native AMF as biological fertilizers has been recommended because of their better adaptation to local conditions. (Berruti et al., 2016).

Soybean (*Glycine max* L.) is an economically important crop grown in various parts of the world because of its high quality protein and oil content for human and livestock consumption. Root colonization of oil-seed crops including soybean by native AMF taxa has been reported earlier (Adeyemi et al., 2019). Despite this, in Nigeria, little attention has been paid to development of AMF symbiosis of native taxa under different phospahte fertilization (Sakariyawo et al., 2016; Adeyemi et al., 2017, 2019, 2020). Thus, little is known on how P fertilization regulates AMF symbiosis in this agroecosystem. Thus, assessing the regulation of AMF symbiosis might serve to establish a link in the use of native AMF as biolfertilizers for improving crop productivity and sustainable agriculture.

To the best of our knowledge, this present work would be an encompassing assessment of phosphate fertilization effects on AMF symbiosis in the humid tropical soil of southwest Nigeria. The objective of the present was to investigate how phosphate fertilization regulates arbuscular mycorhizal symbiosis, dry matter accumulation, P uptake as well as grain yield of soybean cultivars on P deficient soil in a humid tropical soil of southwest Nigeria. Thus, this study hypothesized that: 1) phosphate fertilization would influence the development of AMF symbiosis in roots of the soybean cultivars, 2) AMF symbiosis in soybean would be regulated by varietal difference between the soyean cultivars, and 3) assuming the soybean cultivars differ in terms of dry matter accumulation and P uptake, root colonization of the cultivars will be also different.

2 MATERIALS AND METHODS

2.1 STUDY AREA

The study was carried out at the Teaching and Research Farms of the Federal University of Agriculture, Abeokuta, Ogun State, Nigeria (Latitude 7° 15’ N, Longitude 3° 28’ E.), during the 2017 and 2018 cropping seasons. The soil was sandy loam and classified as kandic paleustalf in the alfisol order of the United States Department of Agriculture (USDA) soil taxonomy. The area is tropical derived savanna and is characterized by bimodal rainfall pattern; it has two distinctive seasons (dry and wet). Mean annual temperature is about 27.5 °C, and the total annual rainfall was 839.7 mm and 1403.3 mm in 2017 and 2018 respectively with maximum rainfall in the period between May to September. The pre-planting soil analysis was done by collecting soil samples from the study field at a depth of 0-20 cm. The collected soil samples were air dried, bulked and sieved in the laboratory through a 2 mm mesh to determine the basic physical and chemical soil properties of the study area. The particle size distribution (clay, silt, sand) was determined using the hydrometer method and soil pH (1:1 soil: water ratio) using the electrometric method (Page, Miller and Keeney, 1982). The organic carbon using Walkley–Black wet oxidation method (Nelson and Sommers, 1982), available phosphorus using the Bray No. 1 method (Bray and Kurtz, 1945), total nitrogen using Kjeldahl distillation method

(Bremner and Mulvaney, 1982) and exchangeable basic cations using ammonium acetate method (Moss, 1961). The modified wet sieving and sucrose techniques of Giovannetti and Mosse (1980) were used to determine the initial mycorrhizal spore density in the soil (48 spores/100 g of soil). The experimental site had no previous known history of inoculation with AMF. The native AMF spore density in the soil was determined using modified wet sieving and sucrose techniques of Giovanetti and Mosse (1980) in 100 g of soil. The initial soil properties are presented in Table 1.

2.2 EXPERIMENTAL TREATMENTS AND DESIGN

The study consisted of two soybean cultivars (TGx 1448-2E and TGx 1440-1E) and three phosphate rates [0 (P0), 20 (P20) and 40 (P40) kg P_2O_5 ha^{-1}) laid out in a randomized complete block design with three replications. The seed of the soybean cultivars were obtained from the Institute of Agricultural Research and Training, Ibadan, Nigeria.

The land was manually cleared and the experimental blocks were marked out using pegs, measuring tape and ropes, and then labeled after double plowing. Each block size was 4 m × 4 m (16 m^2) and net plot size of 3 m × 3 m (9 m^2). The distance between blocks was 1 m and 2 m between replicates. The soybean seeds were sown manually in a row at a depth of 2 cm–5 cm and spacing of 50 cm (inter-row) × 10 cm (intra-row).The P fertilizer was supplied using single superphosphate (SSP, 20 % P_2O_5) immediately after sowing. Weeds were controlled manually using hoes and cutlasses.

Table 1: Selected basic soil properties of the study site

Soil Property	Value
pH (1:1 H_2O)	5.7
Sand (%)	70.8
Silt (%)	12.7
Clay (%)	16.5
Textural Class	Sandy loam
Total N (%)	0.09
Organic matter (%)	1.77
Available P (mg kg^{-1})	6.13
K (cmol kg^{-1})	0.61
Ca (cmol kg^{-1})	6.68
Mg (cmol kg^{-1})	1.47
Na (cmol kg^{-1})	0.29
Initial spore density	125 spores/100 g of soil

2.3 DATA COLLECTION

2.3.1 Root colonization

Fine roots of soybean were collected from 10 randomly selected plants in each plot and washed in tap water before storage in 50 % ethanol to preserve the roots. The roots were rinsed thoroughly to remove the ethanol, cut into 1 cm segments before they were cleared in hot KOH solution (10 % w/v, at 90 °C) for 1 hour and stained with trypan blue lacto-glycerol (1:1:1:0.5 g) at 90 *°C for 30 minutes (Phillips and Hayman*, 1970). The percentage root length colonized by AMF (% RLC) was measured on 25 root segments under a stereo microscope at 100 × magnification scoring the presence or absence of arbuscules, vesicles and hyphae (Giovannetti and Mosse, 1980). The % RLC was calculated using the equation 1 according to Adeyemi et al. (2021).

$$\% \text{ RLC} = \frac{\text{Number of colonized root segments}}{\text{Total number of root segments}} \times 100 \qquad (1)$$

2.3.2 Soybean biomass and grain yield

Ten plants were harvested from each plot, separated into roots and shoots, and then oven-dried at 70 °C to a constant. The roots and shoots were added to determine the total dry mass. At harvest maturity, the pods from each plot were manually threshed, air-dried to determine the grain yield in kg per hectare.

2.3.3 Phosphorus uptake

The oven-dried soybean samples were ground into fine particles. Sub-samples (1 g) were taken and analyzed colorimetrically by the molybdate blue method (Murphy and Riley, 1962) after digesting in concentrated H_2SO_4 to determine P concentration. The P uptake was determined by multiplying the P concentration by the dry matter.

2.4 STATISTICAL ANALYSIS

Data collected were analyzed by two-way analysis of variance using Genstat Release 12.1, (Copyright

2009, VSN International Ltd). Root colonization (%) was subjected to arcsine transformation for normalization of the data. Fishers protected LSD was used to separate means at significance level of $p < 0.05$. Microsoft excel 2016 was used to generate the figures.

3 RESULTS AND DISCUSSION

The effect of P rates, varieties and their interaction on root colonization, dry biomass, P uptake and grain yield are summarized in Table 2. The total root colonization of the soybean cultivars ranged from 18.3-60.8 % in 2017 and 18.3-59.2 % in 2018 cropping season in this study (Fig. 1). This result suggest a moderate capacity of the infection of the native AMF in the study area. This confirms the presence of native or indegenous AMF and the symbiotic association with soybean roots in the derived savannah of Nigeria. Previous study in this agroecosystem have reported the presence of indiegenous AMF and root colonization of agrcultural crops such as soybean, maize, sunflower and sesame (Adeyemi et al., 2019, 2020; Sakariyawo et al., 2019), which showed similar root colonization to those obtained in the present study. 'TGx 1448-2E' was highly colonized by AMF compared to 'TGx 1440-1E' on average in both seasons. The difference observed in root colonization between the two cultivars can be explained by the nutrients demand, particularly P and carbon supplied from the cultivars, allowing both symbiont partners to adjust the symbiosis accordingly (Kiers et al., 2011). This is in agreement with reports of Hetrick et al. (1996), who reported that mycorrhizal dependency was positively correlated with P uptake. The ability of the cultivars to uptake P depends on the morphological and physiological characteristics of their roots (Schachtman et al., 1998; Rao et al., 1999). In addition, plant species with high phosphatase exudation in the

Table 2: Factorial ANOVA of treatment effects on root colonization, growth and P uptake of soybean in 2017 and 2018 cropping seasons

	p-values		
	Cultivars	P rates	Cultivar × P rates
	2017		
Root colonization			
Arbuscules	0.710	< 0.001	0.303
Hyphae	0.002	< 0.001	0.051
Vesicles	0.152	< 0.001	0.418
Total	< 0.001	< 0.001	0.033
Dry mass			
Shoot	0.240	< 0.001	0.487
Root	0.286	< 0.001	0.978
Total	0.431	< 0.001	0.591
P uptake	0.045	0.025	0.820
Grain yield	< 0.001	0.001	0.460
	2018		
Root colonization			
Arbuscules	0.31	< 0.001	0.667
Hyphae	0.023	< 0.001	0.221
Vesicles	0.069	< 0.001	0.540
Total	0.051	< 0.001	0.313
Dry mass			
Shoot	0.240	< 0.001	0.487
Root	0.373	< 0.001	< 0.988
Total	0.398	< 0.001	0.547
P uptake	< 0.001	< 0.001	0.593
Grain yield	< 0.001	< 0.001	0.186

roots may not depend on AMF colonization. Furthermore, Adeyemi et al. (2021a) reported that mycorrhizal colonization of soybean varies greatly within cultivars. Werner and Kiers (2015) have also reported spatial precision among plant species or cultivars in selecting symbiotic partners.

Our results showed that phosphate fertilization significantly ($p < 0.001$) affected the root colonization (arbuscules, internal hyphae, vesicles and total) of the soybean cultivars by AMF in both cropping seasons (Fig.1). The root colonization of plants by AMF has been reported to be influenced by several factors including crop management practices such as plant species or cultivars selection, soil P availability, P fertilization (Fernández et al., 2011; Verbruggen et al., 2013; Adeyemi et al., 2019). Application of high phosphate, 40 kg P_2O_5 ha^{-1} (P40) significantly ($p < 0.001$) suppressed percentage root colonization in both soybean cultivars in terms of arbuscular, hyphae, vesicular and total root colonization of the soybean roots. This conforms to the reports of previous studies (Ortas, 2012; Wang et al., 2016; Thioub et al., 2019), who observed that high P fertilization disrupt mycorrhizal symbiosis. The increased root colonization in control plots in this study can be explained by the inherent low soil P availability of the study area (Tab. 1). However, the enhanced root colonization of 'TGx 1448-2E' with moderate phosphate rate (P20) in the present study confirms the result of Chalk et al. (2006), who reported that increased root colonization in soil with low available P when fertilized with moderate P than unfertilized soil. Among the AMF structures examined for the soybean root colonization, internal hyphae colonization was significantly ($p < 0.051$) dominant in both cultivars and under all P rates in this study. This can be explained by its function in transfer of nutrients and water to the plants (Smith and Read, 2008). The reduced arbuscular colonization can be explained by the early and premature degradation of arbuscules in the root cortex (Breuillin et al., 2010). The significant reduction of the presence of the AMF structures in the root cortex under high P fertilization could decrease P uptake via the mycorrhizal pathway and increase P uptake via plant direct pathway, thus suppressing development of AMF symbiosis (Smith et al., 2011). Additionally, the significant decrease in root colonization with high P fertilization can be linked with inhibition of plant symbiotic genes and symbiotic related P transporters (Breuillin et al., 2010). Berruti et al. (2014) reported that high fertilizer levels in soil drastically alters the interaction between plants and soil microbes. In addition, the report of Fernández et al.

A.

B.

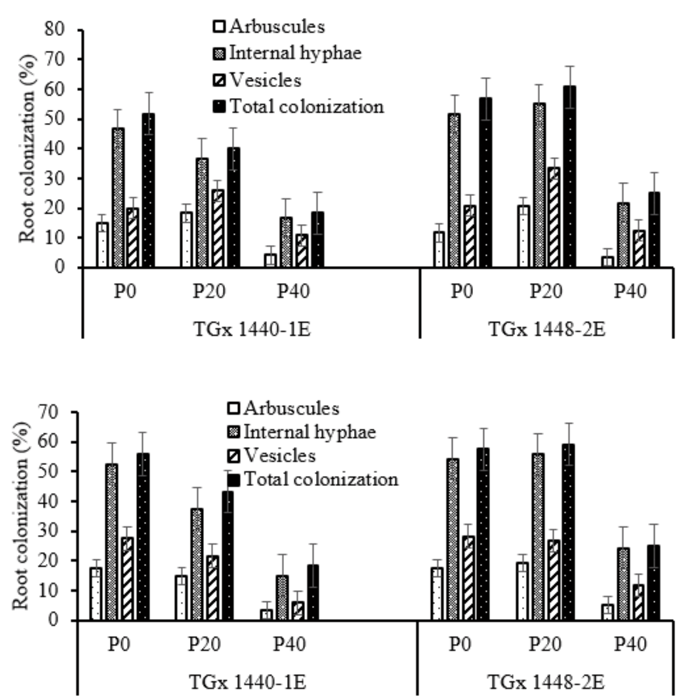
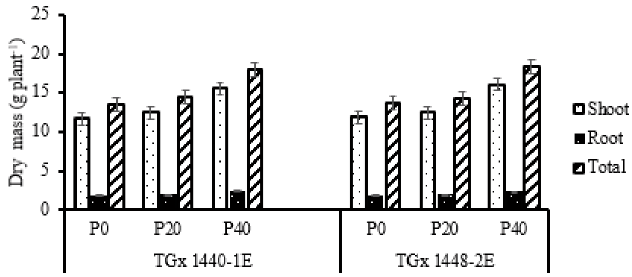


Fig. 1: Percentage of root colonized (arbuscules, internal hyphae, vesicles, and total colonization) in soybean cultivars as influenced by phosphate rates during 2017 (A) and 2018 (B) cropping seasons. P0, P20 and P40 indicate 0, 20 and 40 kg P_2O_5 ha^{-1} respectively. Bars indicate mean values ± standard errors of differences (n = 3) at $p < 0.05$

A.



B.

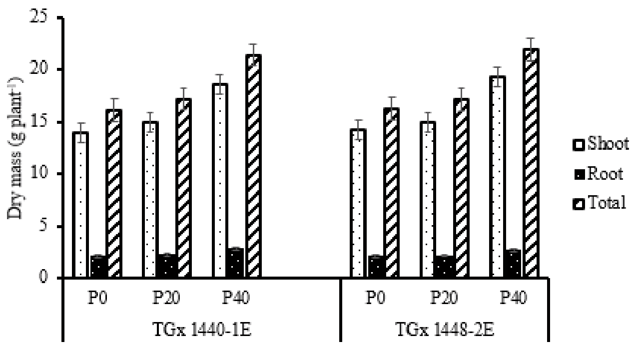


Fig. 2: Dry mass (shoots, roots and total) of soybean cultivars as influenced by phosphate rates during 2017 (A) and 2018 (B) cropping seasons. P0, P20 and P40 indicate 0, 20 and 40 kg P_2O_5 ha^{-1} respectively. Bars indicate mean values ± standard errors of differences (n = 3) at $p < 0.05$

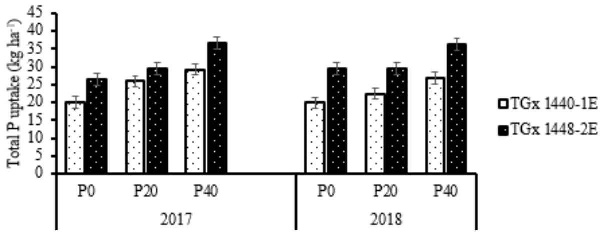


Fig. 3: Total phosphorus uptake in soybean cultivars as influenced by phosphate rates during 2017 and 2018 cropping seasons. P0, P20 and P40 indicate 0, 20 and 40 kg P_2O_5 ha^{-1} respectively. Bars indicate mean values ± standard errors of differences (n = 3) at $p < 0.05$.

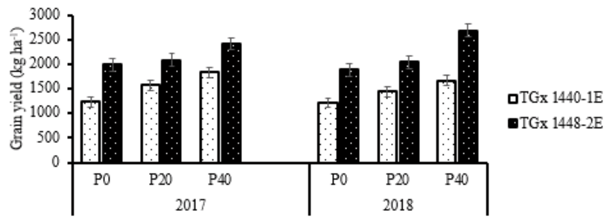


Fig. 4: Grain yield of soybean cultivars as influenced by phosphate rates during 2017 and 2018 cropping seasons. P0, P20 and P40 indicate 0, 20 and 40 kg P_2O_5 ha^{-1} respectively. Bars indicate mean values ± standard errors of differences (n = 3) at $p < 0.05$.

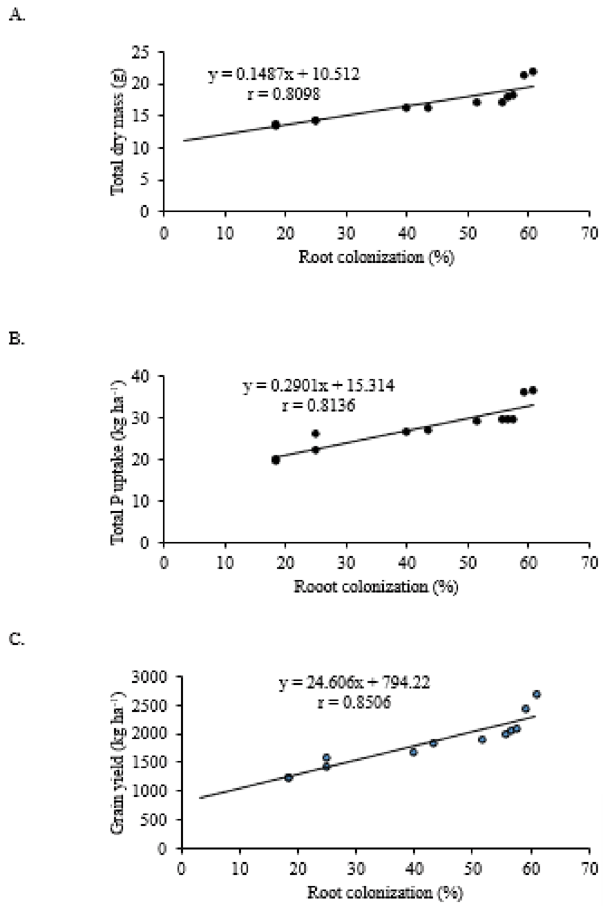


Fig. 5: Relationships between root colonization and total dry mass (A), P uptake (B) and grain yield (C) of soybean

(2011) showed that AMF root colonization in soybean was negatively related to soil available phosphorus.

The present study also revealed the positive effect of phosphate fertilization on plant biomass, P uptake and grain yield of the soybean cultivars. In both cultivars, the dry biomass (Fig. 2), P uptake (Fig. 3) and grain yield (Fig. 4) significantly ($p < 0.001$) increased with increasing P rates compared to the control in both seasons. Promotion of plant growth including soybean has been reported in several other studies. The increased growth of the soybean cultivars with increasing P rate could be attributed to the role of P in major metabolic processes in plants such as photosynthesis, energy transfer, biosynthesis of macromolecules, respiration and signal transduction involve phosphorus (Khan et al., 2010).

The results of this study confirmed very strong linear relationships between the root colonization by AMF and total dry matter (r = 0.81), P uptake (r = 0.81) and grain yield (r = 0.85). This indicates that increase in root colonization increased dry matter, P uptake and grain yield and vice versa (Fig. 5). Several studies have reported that root colonization by AMF has a positive effect on plant growth (Thioub et al., 2019; Adeyemi et al., 2020; 2021b). This is often attributed to increased water and nutrients uptake, particularly P (Cozzolino et al., 2013; Williams et al., 2013; Adeyemi et al., 2021c).

4 CONCLUSION

The results of the present study show that AMF were present and associated with roots of soybean in transitory rainforest of southwest Nigeria. Phosphate fertilization regulates AMF symbiosis in roots of soybean cultivars. High phosphate fertilization suppressed percentages of root colonization of the soybean cultivars in both cropping seasons. Moderate phosphate fertilization (P20) promote AMF symbiosis in roots of 'TGx 1448-2E' in terms of root colonization (arbuscules, internal hyphae, vesicles and total colonization). The biomass (shoot and root), P uptake and grain yield of both soybean cultivars were promoted with increasing phosphate fertilization, with the highest observed under P40 rate. The present study also conclude that the mycorrhizal symbiosis, P uptake and grain yield can be enhanced in soybean with moderate P fertilizer application. Thus, it is necessary to develop sustainable farming practices with reduce P fertilizer application to maximize the benefits of the AMF symbiosis in agricultural soils. However, further research is needed need to gain a better understanding of how AMF taxa functional roles differ in diverse ecosystems in Nigeria in response to different agronomic practices including complementary studies on AMF nutrient demands, host effects and feedbacks mechanisms.

5 REFERENCES

Adeyemi, N., Sakariyawo, O. and Atayese, M. (2017). Yield and yield attributes responses of soybean (glycine max l. merrill) to elevated CO2 and arbuscular mycorrhizal fungi inoculation in the humid transitory rainforest. *Notulae Scientia Biologicae, 9*(2), 233–41. https://doi.org/10.15835/nsb9210002

Adeyemi, N. O., Atayese, M. O., Olubode, A. A. (2019). Identification and relative abundance of native arbuscular mycorrhizal fungi associated with oil-seed crops and maize (*Zea mays* L.) in derived savannah of Nigeria. *Acta Fytotechnica et Zootechnica, 22*(3), 84–89. https://doi.org/10.15414/afz.2019.22.03.84-89.

Adeyemi, N. O., Atayese, M. O., Olubode, A. A. and Akan, M. E. (2020). Effect of commercial arbuscular mycorrhizal fungi inoculant on growth and yield of soybean under controlled and natural field conditions. *Journal of Plant Nutrition, 43*(4), 487–99. https://doi.org/10.1080/01904167.2019.1685101

Adeyemi, N. O., Atayese, M. O.,, Sakariyawo, O. S., Azeez, J. O., Olubode, A. A., Ridwan, M., Adebiyi, A., Oni, A., and Ibrahim, I. (2021a): Influence of different arbuscular mycorrhizal fungi isolates in enhancing growth, phosphorus uptake and grain yield of soybean in a phosphorus deficient soil under field conditions, *Communications in Soil Science and Plant Analysis*, https://doi.org/10.1080/00103624.2021.1879117

Adeyemi, N. O., Atayese, M. O., Sakariyawo, O. S., Azeez, J. O., and Ridwan, M. (2021b). Arbuscular mycorrhizal fungi species differentially regulate plant growth, phosphorus uptake and stress tolerance of soybean in lead contaminated soil. *Journal of Plant Nutrition*, *44*(11), 1633–1648. https://doi.org/10.1080/01904167.2021.1871748

Adeyemi, N. O., Atayese, M. O., Sakariyawo, O. S., Azeez, J. O., Sobowale, S. P. A., Olubode, A., Mudathir, R., Adebayo, R., and Adeoye, S. (2021c). Alleviation of heavy metal stress by arbuscular mycorrhizal symbiosis in *Glycine max* (L.) grown in copper, lead and zinc contaminated soils. *Rhizosphere*, *18*, 100325. https://doi.org/10.1016/j.rhisph.2021.100325

Berruti, A., R. Borriello, A. Orgiazzi, A. C. Barbera, E. Lumini, and V. Bianciotto. (2014). Arbuscular mycorrhizal fungi and their value for ecosystem management. *Biodiversity - the Dynamic Balance of the Planet,* 159–191. https://doi.org/10.5772/58231

Berruti, A., Lumini, E., Balestrini, R., (2016). Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. *Frontier in Microbiol*ogy, *6*, 1. https://doi.org/10.3389/fmicb.2015.01559

Bray, R. H., and L. T. Kurtz. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Science,* 591), 39–46. https://doi.org/10.1097/00010694-194501000-00006

Bremner, J. M., and C. S. Mulvaney (1982). Nitrogen – Total. In *Methods of soil analysis. American society of agronomy. Soil science of America,* ed. A. L. Page, R. H. Miller, and D. R. Keeney, 595–624, Madison, Wisconsin, USA: American Society of Agronomy. https://doi.org/10.2134/agronmonogr9.2.2ed.c31

Breuillin, F., J. Schramm, M. Hajirezaei, A. Ahkami, P. Favre, U. Druege, B. Hause, M. Bucher, T. Kretzschmar, E. Bossolini, C. Kuhlemeier, E. Martinoia, P. Franken, U. Scholz and D. Reinhardt. (2010). Phosphate systemically inhibits development of arbuscular mycorrhiza in petunia hybrida and represses genes involved in mycorrhizal functioning. *The Plant Journal, 64*, 1002–17. https://doi.org/10.1111/j.1365-313X.2010.04385.x

Brundrett M.C. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant and Soil, 320*, 37–77. https://doi.org/10.1007/s11104-008-9877-9

Castillo, C., Borie, F., Oehl, F., et al. (2016). Arbuscular mycorrhizal fungi biodiversity: prospecting in southern-central zone of Chile. A review. *Journal of Soil Science and Plant Nutr*ition, *16*, 400–422. https://doi.org/10.4067/S0718-95162016005000036

Chalk, P.M., Souza, R.D.F., Urquiaga, S., et al. (2006). The role of arbuscular mycorrhiza in legume symbiotic performance. *Soil Biology and Biochemistry*, *38*, 2944–2951. https://doi.org/10.1016/j.soilbio.2006.05.005

Cozzolino, V., Di Meo, V., and Piccolo, A., (2013). Impact of arbuscular mycorrhizal fungi applications on maize production and soil phosphorus availability. *Journal of Geochemical Explor*ation, *129*, 40–44. https://doi.org/10.1016/j.gexplo.2013.02.006

Fernández M.C., Boem F.H.G., and Gerardo R.G. (2011). Effect of indigenous mycorrhizal colonization on phosphorus-acquisition efficiency in soybean and sunflower. *Journal of Plant Nutrition and Soil Sci*ence, *174*, 673–677. https://doi.org/10.1002/jpln.201000109

Gianinazzi, S., Gollotte, A., Binet, M.N., et al. (2010). Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza, 20*(8), 519–530. https://doi.org/10.1007/s00572-010-0333-3

Giovanneti, M. and Mosse, B. (1980). An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist*, *84*(3), 489–500. https://doi.org/10.1111/j.1469-8137.1980.tb04556.x

Gosling, P., Mead, A., Proctor, M., et al. (2013). Contrasting arbuscular mycorrhizal communities colonizing different host plants show a similar response to a soil phosphorus concentration gradient. *New Phytologist, 198*(2), 546–556. https://doi.org/10.1111/nph.12169

Jiang, Y.N., Wang, W.X., Xie, Q.J., et al. (2017). Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. *Science*, *356*, 1172–1175. https://doi.org/10.1126/science.aam9970

Johnson, N.C., Wilson, G.W.T., Wilson, J.A., et al. (2015). Mycorrhizal phenotypes and the law of the minimum. *New Phytologist, 205*, 1473–1484. https://doi.org/10.1111/nph.13172

Khan, M. S., Zaidi, A., Ahemad, M., et al. (2010). Plant growth promotion by phosphate solubilizing fungi – current perspective. *Archives of Agronomy and Soil Science, 56*(1), 73–98. https://doi.org/10.1080/03650340902806469

Kiers Et, Duchamel M, Beesetty Y, et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science, 333*, 880–882. https://doi.org/10.1126/science.1208473

Murphy, J., and Riley, J. P. A. (1962). Modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5

Nelson D.W. and L.E. Sommers. (1982). Total carbon, organic carbon, and organic matter. In *Methods of Soil Analysis*. A. L. Page, R.H. Miller, D.R. Keeney (Eds.). Part II, 2nd ed., 539-580, American Society of Agronomy, Madison, USA. https://doi.org/10.2134/agronmonogr9.2.2ed.c29

Ortas, I. (2012). The effect of mycorrhizal fungal inoculation on plant yield, nutrient uptake and inoculation effectiveness under long-term field conditions. *Field Crop Research, 125*, 35–48. https://doi.org/10.1016/j.fcr.2011.08.005

Page, A. L., R. H. Miller, and D. R. Keeney. (1982). *Method of soil analysis, part 2 Agronomy monograph 9, part 2 agr.* Ed. Wisconsin, Madison: American Society of Agronomy

Phillips, J., and Hayman, D. (1970). Improved producers for clearing roots and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society, 55*, 158–160. https://doi.org/10.1016/S0007-1536(70)80110-3

Rao, 1.M., Friesen, D.K., and Osaki, M. (1999): Plant adaptation to phosphorus limited tropical soil. In *Handbook of Plant and Crop Stress.* Ed. M Pessarakli, p. 61-95, Marcel Dekker, Inc. New York. https://doi.org/10.1201/9780824746728.ch4

Sakariyawo O.S., Adeyemi, N.O., Atayese, M.O., et al. (2016) Growth, assimilate partitioning and grain yield response of soybean (*Glycine max* L. Merrrill) varieties to carbon dioxide enrichment and arbuscular mycorrhizal fungi in the humid rainforest. *Agro-science, 15*, 29-40. https://doi.org/10.4314/as.v15i2.5

Schachtman, D.P., Reid, R.J., and Ayling, S.M. (1998): Phosphorus uptake by plants: From soil to cell. *Plant Physiology*, *116*, 447-453. https://doi.org/10.1104/pp.116.2.447

Smith S.E, Jakobsen L, Grønlund M, et al. (2011). Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiology, 156*, 1050–1057. https://doi.org/10.1104/pp.111.174581

Smith, S.E., and Read, D.J. (2008): *Mycorrhizal symbiosis*, 3rded. Academic Press, London, UK.

Thioub, M., Ewusi-Mensah, N., Sarkodie, J., et al. (2019). Arbuscular mycorrhizal fungi inoculation enhances phosphorus use efficiency and soybean productivity on a haplic acrisol. *Soil & Tillage Research, 192*, 174–186. https://doi.org/10.1016/j.still.2019.05.001

Verbruggen, E., Van Der Heijden, M. G. A., Rillig, M. C., et al. (2013). Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success. *New Phytologist, 197*, 1104–1109. https://doi.org/10.1111/j.1469-8137.2012.04348.x

Wang, X., Zhao, S., and Bücking, H., (2016). Arbuscular mycorrhizal growth responses are fungal specific but do not differ between soybean genotypes with different phosphate efficiency. *Annals of Botany, 118*, 11–21. https://doi.org/10.1093/aob/mcw074

Werner, G. and Kiers, E. T. (2015). Partner selection in the mycorrhizal mutualism. *New Phytologist. https://doi.org/10.1111/nph.13113*

Williams, A., Ridgway, H.J., and Norton, D.A., (2013). Different arbuscular mycorrhizae and competition with an exotic grass affect the growth of *Podocarpus cunninghamii* Colenso cuttings. *New Forest, 44*, 183–195. https://doi.org/10.1007/s11056-012-9309-9

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Insekticidni proteini in njihova uporaba za zatiranje koloradskega hrošča (*Leptinotarsa decemlineata* [Say, 1824])

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Insecticidal proteins and their potential use for Colorado potato beetle (*Leptinotarsa decemlineata* [Say, 1824]) control

Abstract: Plants respond to pest attack, among other mechanisms, by producing specific proteins with insecticidal properties. Proteins with toxic effects on insects have also been discovered in many other organisms, especially fungi and bacteria. Due to their biological function, insecticidal proteins represent an important potential in the development of more environmentally friendly plant protection methods. Increasing knowledge about the mode of action of insecticidal proteins and the identification of genes encoding their synthesis enable the breeding of transgenic plants resistant to insect pests and the development of new bioinsecticidal agents. The Colorado potato beetle (*Leptinotarsa decemlineata*) is one of the most important pests of potato, so the study of such control methods is crucial for the development of sustainable integrated pest management strategies of potato. This review highlights the properties of some groups of insecticidal proteins and their modes of action, and summarizes examples of studies of their use for the control of Colorado potato beetle.

Key words: entomotoxic proteins; bioinsecticide; plant toxins; potato; insecticide resistance

Insekticidni proteini in njihova uporaba za zatiranje koloradskega hrošča (*Leptinotarsa decemlineata* [Say, 1824])

Izvleček: Rastline se na napad škodljivcev odzovejo med drugim tudi s tvorbo specifičnih proteinov z insekticidnim delovanjem. Proteine s toksičnim delovanjem na žuželke so odkrili tudi v številnih drugih organizmih, predvsem glivah in bakterijah. Zaradi omenjene biološke funkcije insekticidni proteini predstavljajo pomemben potencial v razvoju okolju prijaznih metod varstva rastlin. Poznavanje mehanizmov delovanja insekticidnih proteinov in identifikacija genov za njihovo sintezo omogočata žlahtnjenje transgenih sort rastlin odpornih na škodljive žuželke ter razvoj bioinsekticidnih učinkovin. Koloradski hrošč je pomemben škodljivec krompirja, zato je preučevanje tovrstnih načinov varstva rastlin ključno za razvoj trajnostnih strategij integriranega varstva krompirja. V prispevku povzemamo lastnosti nekaterih skupin insekticidno delujočih proteinov in njihovih mehanizmov delovanja ter primerov preučevanja njihove uporabe za zatiranje koloradskega hrošča.

Ključne besede: entomotoksični proteini; bioinsekticidi; rastlinski toksini; odpornost; krompir

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1 UVOD

Rastline so med evolucijo razvile različne mehanizme za obrambo pred rastlinojedimi organizmi. Odziv rastlin na mehanske poškodbe zaradi napada grizočih žuželk vključuje morfološke prilagoditve in sintezo različnih kemičnih snovi, od preprostejših spojin do kompleksnejših proteinskih struktur, ki zavirajo rast, razvoj ter razmnoževanje škodljivih organizmov (Tripathi in Mishra, 2016). Pomemben del induciranega obrambnega odziva rastlin na napad škodljivcev je tvorba insekticidno delujočih proteinov. Najbolj znani proteini s tovrstnim odzivom na škodljivce so lektini in inhibitorji različnih prebavnih encimov, kot so α-amilaze in proteaze ter nekateri drugi (Dang in Van Damme, 2015).

Insekticidne proteine poleg rastlin proizvajajo tudi številni drugi organizmi. Do danes so bili identificirani različni proteini rastlinskega, živalskega in mikrobiološkega izvora s toksičnim delovanjem na vrsto žuželk. Sodobne biotehnološke metode so v zadnjih desetletjih omogočile velik napredek tudi pri identifikaciji genov, odgovornih za sintezo omenjenih snovi in transformacijo rastlinskih tkiv za namene povečanja odpornosti proti rastlinskim škodljivcem (Carlini in Grossi-De-Sá, 2002; Tripathi in Mishra, 2016).

V kmetijstvu se za omejevanje izpada pridelka zaradi škodljivih organizmov tradicionalno poslužujemo uporabe kemičnih insekticidov, kar pa zaradi številnih negativnih okoljskih učinkov in naraščajoče problematike odpornosti proti aktivnim snovem povečuje potrebo po iskanju alternativnih pristopov varstva rastlin (Trdan, 2013, 2016). Uporaba transgenih rastlin predstavlja pomemben potencial za zmanjševanje odvisnosti kmetijske pridelave od uporabe sintetičnih insekticidov (Tripathi in Mishra, 2016).

V tem smislu imajo vse večji pomen kot metoda nekemičnega varstva rastlin tudi biopesticidi. Biopesticidi so snovi na podlagi mikroorganizmov ali naravnih produktov in v tem smislu predstavljajo okoljsko sprejemljivejši način zatiranja škodljivih organizmov. Po nekaterih definicijah se med biopesticide uvrščajo tudi snovi, ki jih tvorijo rastline z dodanim genetskim materialom z uporabo metod genskega inženiringa (Plant-Incorporated-Protectants ali PIPs) (United States Environmental Protection Agency, 2017). Uporaba insekticidnih proteinov bakterijskega izvora predstavlja enega pomembnejših mehanizmov delovanja biopesticidov (Paul in Das, 2020).

Insekticidni proteini se razlikujejo glede na izvor in način delovanja na ciljne organizme. Koloradski hrošč je najpomembnejši škodljivec krompirja, problematika njegovega zatiranja pa je zaradi pojava odpornih populacij proti večini kemičnih insekticidov in splošne okoljske problematike rabe kemičnih sredstev za varstvo rastlin predmet številnih raziskav alternativnih, okoljsko sprejemljivejših pristopov za njegovo zatiranje (Bohinc in sod., 2019; Laznik in sod., 2010), ki vključujejo tudi uporabo insekticidnih proteinov (Alyokhin in sod., 2008; Cingel in sod., 2017).

2 SKUPINE INSEKTICIDNIH PROTEINOV

2.1 RASTLINSKI LEKTINI

Lektini so proteini neimunskega izvora, ki imajo mesta za specifično vezavo ogljikovih hidratov. Lektini so v naravi splošno razširjene spojine, večina jih je rastlinskega izvora, najdemo pa jih tudi v različnih vrstah živali, gliv, bakterij in virusov (Peumans in Van Damme, 1995). V rastlinah so koncentracije lektinov navadno največje v semenih in drugih založnih organih, kjer predstavljajo zalogo skladiščih proteinov, sodelujejo pri komunikaciji gostiteljskih rastlin s simbiotskimi mikroorganizmi, kot so bakterije iz rodu *Rhizobium* in mikorizne glive. Vsebnost lektinov se v rastlinskih tkivih poveča tudi ob napadu škodljivih organizmov, na podlagi česar so ugotovili njihovo vlogo pri induciranem odzivu rastlin na napad škodljivih organizmov, predvsem žuželk. Povečana vsebnosti lektinov v rastlinskih celicah je pogojena s tvorbo rastlinskih hormonov, ki sprožijo ekspresijo genov za njihovo sintezo ob napadu herbivorov. Lektini, ki jih proizvajajo rastline, se ob zaužitju vežejo s specifičnimi ogljikovimi hidrati, vezanimi v glikoproteinske in glikolipidne strukture, na peritrofični membrani žuželčjega epitela (Vandenborre in sod., 2011). Zaradi specifičnosti vezave posameznih lektinov je njihov toksični učinek, ki se odraža v omejevanju prehranjevanja, rasti in razvoja ciljnega organizma na posamezne vrste žuželk, nepredvidljiv. Do danes so bili med drugim izolirani številni lektini iz več rastlinskih vrst z dokazanim toksičnim delovanjem na grizoče žuželke iz reda metuljev (Lepidoptera), polkrilcev (Hemiptera) in hroščev (Coleoptera) (Carlini in Grossi-De-Sá, 2002; Vandenborre in sod., 2011). Prepoznani insekticidno delujoči lektini so bili preizkušeni v številnih laboratorijskih raziskavah na podlagi lektin-vsebujočih diet in poskusih s transgenimi rastlinami z vnosi genov, ki kodirajo zapise za njihovo sintezo. Po vsebnosti lektinov so bogata predvsem semena nekaterih vrst stročnic, iz katerih izhaja največ potencialno učinkovitih lektinov, ki so bili preučeni v raziskavah. Prvi so o učinkovitosti lektina PHA, ki so ga izolirali iz semen fižola (*Phaseolus vulgaris* L.), poročali Gatehouse in sod. (1984) in dokazali njegovo toksično delovanje na ličinke hrošča *Callosobruchus maculatus* (Fabricius, 1775). Poleg stročnic so pozneje proti omenjenemu škodljivcu ugotovili delovanje lektinov iz številnih rastlinskih vrst (Zhu in sod., 1996). Za zatiranje ličink koloradskega hrošča (*Leptinotarsa decemlineata* [Say, 1824]) so Wang in sod. (2003) v laboratorijskih poskusih pri nanosu na liste dokazali učinkovitost lektina imenovanega gleheda, ki so ga izolirali iz listov bršljanaste grenkuljice (*Glechoma hederacea* L.). V tej raziskavi nobena od ličink, ki so se prehranjevale na listih krompirja (*Solanum tuberosum* L.) tretiranih z lektinom, ni dokončala razvojnega kroga. Obstoj rastlinskih lektinov je znan že stoletja, njihov pomen v fiziologiji rastlin pa še ni povsem razjasnjen. Dokazano učinkovitost lektinov, izoliranih iz različnih rastlinskih vrst, je pri genski transformaciji in vnosu v ciljne rastline težko predvideti; njihova učinkovitost je lahko vprašljiva tudi zaradi številnih neznank o načinih delovanja proti žuželkam na molekularni ravni (Dang in Van Damme, 2015), slabše pa so preučeni tudi njihovi vplivi na neciljne organizme (Vandenborre in sod., 2011, Dang in Van Damme, 2015).

Glede na strukturo razvrščamo lektine v več različnih skupin, mednje pa uvrščamo tudi t.i. ribosom-inaktivirajoče proteine (RIP), ki s specifično N-glukozidazno aktivnostjo povzročijo deaktivacijo ribosomov (Vandenborre in sod., 2011). Najbolj znan in preučen RIP je ricin, ki so ga najprej izolirali iz kloščevca (*Ricinus communis* L.) in pozneje iz drugih vrst iz rodu *Ricinus*. Njegovo toskično delovanje so med drugim ugotovili tudi na hroščih, in sicer na vrstah *Callosobruchus maculatus* in *Anthonomus grandis* Boheman, 1843 (Gatehouse in sod., 1984). Do danes so tudi na podlagi analize genomov ugotovili prisotnost drugih RIP sekvenc v številnih drugih rastlinah (Dang in Van Damme, 2015). RIP se sicer za namene žlahtnjenja odpornih sort rastlin redkeje uporabljajo, predvsem zaradi nespecifične toksičnosti za sesalce (Carlini in Grossi-De-Sá, 2002). Nasprotno pa so gene za sintezo nekaterih lektinov iz nerastlinskih virov že vnesli v rastline, kot na primer gene za sintezo lektinov iz jajčnih beljakov za zatiranje koloradskega hrošča, ki so jih preizkušali v poskusih s transgenimi rastlinami krompirja (Cooper in sod., 2009).

2.2 INHIBITORJI PREBAVNIH ENCIMOV

Tako kot ostali heterotrofni organizmi, tudi rastlinojede žuželke za zadovoljevanje svojih energetskih potreb in prebavo rastlinskih tkiv izločajo vrsto prebavnih encimov. Pomembnejši med njimi so proteaze in glikohidrolaze, ki omogočajo razgradnjo molekul beljakovin in sladkorjev v rastlinskem soku. Encimske lastnosti posameznih žuželk so prilagojene njihovemu načinu prehranjevanja ter biokemijskim lastnostim gostiteljskih rastlin.

Inhibitorji prebavnih encimov so snovi, ki predstavljajo pomemben del imunskega odziva rastlin na napad škodljivih organizmov. Nahajajo se v rastlinskem tkivu, pretežno v semenih in gomoljih, njihova sinteza pa poteka kot del induciranega odziva rastlin na grizenje, bodenje ali sesanje (Ryan, 1990, Fürstenberg-Hägg in sod., 2013).

Različne skupine proteolitičnih encimov ali proteaz v prebavilih žuželk sodelujejo pri presnovi proteinov in zagotavljajo vir aminokislin. Regulacija proteolitske aktivnosti je zaradi vitalne funkcije tega procesa v organizmih izrednega pomena in poteka tudi na podlagi inhibitorjev, ki z vezavo na specifične katalitične domene proteaz tvorijo komplekse ter onemogočajo njihovo aktivnost (Ryan, 1990). Vloga inhibitorskih proteinov v organizmih je večplastna, različni organizmi, med njimi tudi rastline, jih med drugim tvorijo tudi v obrambne namene. Največ inhibitorjev proteaz tvorijo rastline iz družin metuljnic (Fabaceae), razhudnikovk (Solanaceae) in trav (Poaceae). Iz njihovih tkiv so izolirali številne proteine za inhibicijo specifičnih proteaz, ki jih izločajo žuželke in na podlagi molekularnih tehnik določili gene za njihovo sintezo ter jih vnesli v druge gostiteljske vrste (Dang in Van Damme, 2015; Singh in sod., 2020).

V prebavilih koloradskega hrošča prevladujejo cisteinske proteaze in aspartatne proteaze, ki jih inhibirajo obrambni aspartatni in cisteinski inhibitorji (cistatini) (Wolfson in Murdock, 1987; Srp in sod., 2016). Na podlagi ugotovitev o akumulaciji inhibitorjev proteaz v krompirju in paradižniku (*Solanum lycopersicum* L.) zaradi napada ličink koloradskega hrošča, sta o njihovi vlogi pri razvoju odpornosti sklepala Green in Ryan (1972). Eni izmed prvih cisteinskih inhibitorjev, ki so jih s pomočjo rekombinantne bakterijske RNA uspešno izolirali iz rastlin in preizkusili za namene zatiranja hroščev, so bili cistatini iz riža (*Oryza sativa* L.) - orizacistatini (Chen in sod., 1992). Leto pozneje so Michaud in sod. (1993) dokazali specifičnost njihove vezave na cisteine, ki so jih izolirali iz prebavil koloradskega hrošča. Na podlagi vnosa orizacistatinov v krompir so te preizkusili za namene zatiranja koloradskega hrošča in ugotovili vpliv na povečanje smrtnosti ličink (Lecardonnel in sod., 1999; Cingel in sod., 2017). Podobne učinke na zmanjšanje rasti ličink so ugotovili tudi pri vnosu inhibitorjev cistatinov iz papaje (*Carica papaya* L.), soje (*Glycine max* [L.] Merr.) in ječmena (*Hordeum vulgare* L.) (Visal in sod., 1998; Lalitha in sod., 2005; Álvarez-Alfageme in sod., 2007). Ashouri in sod. (2017) so inhibitorje cisteinov koloradskega hrošča določili tudi v semenih sončnic (*Helianthus annuus* L.).

Iz prebavil koloradskega hrošča so izolirali tudi aspartatne proteaze, njihove inhibitorje pa so odkrili v paradižniku. Po uspešnem vnosu v rastline krompirja so preizkušali njihovo potencialno učinkovitost in ugotovili številne fiziološke posledice za ličinke L3 stopnje koloradskega hrošča, ki so se odražale v manjši ješčnosti, masi in prirastu, medtem ko pri starejših ličinkah (stopnja L4) tega učinka niso zaznali (Brunelle in sod., 2004). O podobnem fenomenu prilagoditve koloradskega hrošča so poročali že Cloutier in sod. (2000), ki pri proučevanju orizacistatinov niso ugotovili predhodno dokazano učinkovitega delovanja na odrasle hrošče.

Znano je namreč, da naštete načine encimske inhibicije v rastlinah žuželke zaradi izdatnejših potreb po aminokislinah in posledičnega selekcijske pritiska, s prilagoditvami encimske aktivnost hitro zaobidejo. Žuželke se na nove prehrambene razmere prilagodijo na različne načine, kot so hiperprodukcija proteaz in povečano prehranjevanje z gostiteljskim tkivom, sintezo novih odpornih proteaz in proteolitsko deaktivacijo rastlinskih inhibitorjev (Ryan, 1990; Cingel in sod., 2016). Podrobneje so mehanizme prilagoditve koloradskega hrošča na proteolitsko inhibicijo, ki se odraža v spremembah sinteze cisteinov, preučevali Gruden in sod. (2004).

Naprednejši biotehnološki pristopi vključujejo tehnike t.i. piramidenja genov, kjer z vnosom več sekvenc omogočajo izražanje različnih tipov inhibitorjev in s tem manjšo možnost razvoja odpornosti škodljivih organizmov (Schlüter in sod., 2010; Martinez in sod., 2016). Učinkovitost takšnega pristopa so na podlagi koekspresije genov za sintezo dveh različnih cistantinov v krompirju, dokazali tudi Cingel in sod. (2017) pri zatiranju koloradskega hrošča.

Inhibitorje proteaz, ki sodelujejo v metabolizmu koloradskih hroščev, pa so v preteklih raziskavah izolirali tudi iz drugih nerastlinskih virov. Gruden in sod. (1998) so ugotovili toksično delovanje cistatinov iz rdeče morske vetrnice (*Actinia equina* (L., 1758).

Encimi imajo odločilno vlogo tudi pri metabolizmu ogljikovih hidratov. Alfa amilaze (α amilaze) so hidrolitični encimi, ki so zastopani v živalih, rastlinah in mikroorganizmih. Ti encimi katalizirajo hidrolizo glikozidnih vezi polisaharidov in imajo pomembno vlogo pri razgradnji kompleksnih sladkorjev, kot sta škrob in glikogen. Predvsem za žuželke, ki se pretežno prehranjujejo s semeni, torej rastlinskimi organi z veliko vsebnostjo škroba, je aktivnost α amilaze odločilna za njihovo preživetje (Franco in sod., 2002). Rastline se tudi na žuželčje α amilaze odzivajo s sintezo specifičnih inhibitorjev z različnimi mehanizmi inhibicije, ki se odraža v zmanjšanju metabolizma ogljikovih hidratov kot pomembnega vira energije. Večja vsebnost inhibitorjev je značilna predvsem za semena žit in stročnic (Carlini in Grossi-De-Sá, 2002; Fürstenberg-Hägg in sod., 2013). V več raziskavah so ugotovili učinkovitost inhibitorjev iz pšenice (*Triticum aestivum* L.), ječmena, riža, koruze (*Zea mays* L.) in več rastlinskih vrst iz družine ščirovk (Amaranthaceae) za zaviranje aktivnosti α amilaz različnih skladiščnih škodljivcev, kot so mokarji (*Tribolium* spp.), žitni žužki iz rodu *Sitophilus* ter žitniki iz rodu *Oryzaephilus* (Fürstenberg-Hägg in sod., 2013; Rane in sod., 2020). Natančneje so bili preučeni tudi različni inhibitorji α amilaz iz fižola za zatiranje nekaterih lepencev iz poddružine Bruchinae, ki povzročajo škodo na semenih stročnic (Carlini in Grossi-De-Sá, 2002; Rane in sod., 2020). Ashouri in sod. (2017) so iz rdečega fižola izolirali tudi inhibitorje, ki so v prehranjevalnih testih vplivali na zmanjšano aktivnost α amilaz ličink koloradskega hrošča (Ashouri in Farshbaf Pourabad, 2021).

2.3 BAKTERIJSKI INSEKTICIDNI PROTEINI

Bakterijski toksini so najpogosteje uporabljene mikrobne učinkovine pri zatiranju rastlinskih škodljivcev. Do sedaj je bilo odkritih več kot sto različnih vrst bakterij, ki izkazujejo entomopatogeno delovanje, med njimi predvsem bakterije iz družin Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae in Micrococcaceae (Kalha in sod., 2014). Izmed bakterijskih insekticidih proteinov so najbolje preučeni in v najširši uporabi δ toksini, ki jih proizvaja gram pozitivna bakterija *Bacillus thuringiensis* Berliner, 1915, Bt. Pomembnejši med njimi so Cry toksini, ki izkazujejo toksično delovanje na vrsto žuželk, med drugim tudi na hrošče. Bt proizvaja Cry toksine v času sporulacije in jih kopiči v obliki kristalnih struktur (Crickmore in sod., 1998). Cry toksine uvrščamo v skupino t. i. porotvornih toksinov (PFT), za katere je značilna afiniteta vezave na biološke membrane in tvorba transmembranskih por. Po zaužitju kristalnih struktur se namreč pod vplivom nizkega pH prebavnih sokov in proteazne aktivnosti v črevesju gostitelja iz njih sprostijo toksini, ki z vezavo na specifične proteinske receptorje in povzročanjem poškodb na membranah povzročijo celično smrt ter s tem zmanjšano prehranjevalno sposobnost žuželk, ki posledično odmrejo (Bravo in sod., 2007). Zanje je značilno visoko specifična insekticidna učinkovitost oz. delovanje zgolj na določene vrste oziroma skupine žuželk, ki je pogojena z biokemično strukturo in metabolno aktivnostjo ciljnega organizma. Bt toksini niso strupeni za vretenčarje in rastline ter so popolnoma biorazgradljivi, zaradi česar so še posebno zanimivi za uporabo v varstvu rastlin (Palma in sod., 2014). Trenutna Bt nomenklatura obsega več sto sekvenc za sintezo različnih proteinov, med njimi je največja skupina Cry3, iz katerih izhaja največ bakterijskih toksinov, ki so toksični za žuželke (Berry in Crickmore, 2017). Izmed proteinov iz te skupine izhaja največ toksinov, ki izkazujejo insekticidno delovanje na koloradskega hrošča, predvsem Cry3A in Cry3B, ki so bili največkrat vneseni v transformirane rastline krompirja. Prav transgena sorta krompirja ‚NewLeaf' (Monsanto Corp.), v katero so vnesli Cry3A, z namenom povečevanja odpornosti proti koloradskemu hrošču, velja za prvo komercialno gensko spremenjeno sorto rastline, ki je bila v Združenih državah Amerike registrirana leta 1995 za pridelavo v prehrambne namene. Reed in sod. (2001) so v dveletnem poljskem poskusu dokazali primerljivo učinkovitost uporabe sorte ‚NewLeaf' kot načina varstva pred koloradskim hroščem z manj negativnimi vplivi na neciljne organizme v primerjavi z rabo insekticidov. Kljub vsemu je bila omenjena sorta pozneje zaradi splošno negativnega javnega mnenja glede uporabe gensko spremenjenih rastlin umaknjena s tržišča (Grafius in Douches, 2008). Razvoj in pridelava sort na podlagi Cry3A se predvsem zaradi naraščajoče problematike razvoja odpornosti koloradskega hrošča proti insekticidom in trendov zmanjševanja negativnih vplivov kmetijske pridelave na okolje nadaljuje tudi drugod po svetu (Kamionskaya in sod., 2012; Mi in sod., 2015; K. Wang in sod., 2019). Do danes so v številnih raziskavah potrdili insekticidno delovanje na koloradskega hrošča tudi pri toksinih iz drugih skupin Cry proteinov, kot so Cry1, Cry2, Cry3, Cry5, Cry7 in Cry8 (K. Wang in sod., 2019; Balaško in sod., 2020; Domínguez-Arrizabalaga in sod., 2020).

Bt, poleg endogenih Cry toksinov, proizvajajo tudi druge proteine z insekticidnim delovanjem na hrošče, ki jih v medcelični prostor izločajo v fazi vegetativne oblike. Vip1 in Vip2 toksini se podobno kot Cry vežejo na prebavni epitel žuželk in ga poškodujejo (Chakroun in sod., 2016). Znani so tudi Sip toksini, ki se uvrščajo v skupino t. i. sekrecijskih proteinov, in jih bakterije izločajo v svojo okolico. Izmed slednjih toksin tipa Sip1A, ki ga izloča Bt, dokazano učinkuje tudi proti ličinkam koloradskega hrošča (Donovan in sod., 2006).

Poleg Bt so vir proteinov z insekticidnim delovanjem na koloradskega hrošča našli še v nekaterih drugih vrstah bakterij, kot na primer v *Chromobacterium* sp. (Martin in sod., 2006), *Photorhabdus luminescens, Photorhabdus luminescens* (Thomas and Poinar, 1979) Boemare et al., 1993 emend. Fischer-Le Saux et al., 1999 (Blackburn in sod., 2005) in *Leclercia adecarboxylata* (Leclerc 1962) (Muratoglu in sod., 2011). Toksini iz drugih vrst bakterij lahko predstavljajo pomemben potencial za uporabo pri genski transformaciji, tudi kot nadomestilo za Bt proteine v smislu preprečevanja razvoja odpornosti koloradskega hrošča proti Cry toksinom (Wang in sod., 2019). Žuželke namreč pod vplivom selekcijskega pritiska s fiziološkimi prilagoditvami, ki omogočajo razgradnjo ali deaktivacijo specifičnih proteinskih toksinov ali mutacijami receptorjev na prebavnem epitelu, spontano razvijajo odpornost na Cry toksine (Bravo in sod., 2011). Iz preteklosti so znani nekateri primeri populacij koloradskega hrošča, odpornih proti Cry3A proteinom (Whalon in sod., 1993). V primeru širše uporabe Bt sort krompirja pa je moč pričakovati, da bi ta problematika lahko postala pereč problem (Domínguez-Arrizabalaga in sod., 2020).

Varstvo pred rastlinskimi škodljivci na podlagi bakterijskih proteinov poleg razvoja transgenih rastlin temelji tudi na njihovi uporabi v obliki mikrobnih bioinsekticidov, namenjenih nanosu s pršenjem. Prav pripravki na podlagi Bt toksinov so glavno gonilo razvoja bioinsekticidov, njihov tržni odstotek na globalnem trgu tovrstnih proizvodov pa naj bi dosegal skoraj 90 % (Jallouli in sod., 2020). Večina Bt insekticidov vsebuje Cry3 kristalne strukture, ki se jih v vodni raztopini na rastline nanaša foliarno. Za zatiranje ličink koloradskega hrošča je znana uporaba Cry3A, ki ga proizvaja *Bt* subsp. *tenebrionis* in je na trgu dostopen v obliki komercialnega proizvoda Novodor (Domínguez-Arrizabalaga in sod., 2020). Takšen način uporabe bakterijskih proteinov je v praksi bolj uveljavljen, saj je uvajanje pridelave gensko spremenjenih rastlin predvsem v Evropi še vedno zelo striktno regulirano in večinoma prepovedano (Direktiva EU 2001/18/ES). Kljub učinkovitemu delovanju Bt pripravkov je slabost foliarnega nanosa Cry proteinov, v primerjavi z izražanjem v transformiranih rastlinah, njihova omejena učinkovitost, ki je posledica vpliva okoljskih dejavnikov, predvsem podvrženosti proteinov svetlobni razgradnji zaradi UV svetlobe (Bravo in sod., 2011).

2.4 PROTEINI IZ GOB

Višje glive (Eumycota) iz dveh različnih debel, in sicer prostotrosnice (Basidiomycota) ter zaprtotrosnice (Ascomycota), na nadzemskem delu tvorijo reprodukcijske organe - trosišča, ki jim pravimo gobe. Z njimi se v naravi hranijo številni fungivori, med njimi tudi žuželke. Glive so med evolucijo razvile različne obrambne mehanizme, ki gobe varujejo pred fungivori, saj je s tem pogojena njihova zmožnost razmnoževanja. Večina obrambnih procesov temelji na tvorbi proteinov, med katerimi so številni toksični tudi za žuželke (Wang in sod., 2002).

Za glive je značilna velika vsebnost lektinov, ki predstavljajo pomemben vir založnih beljakovin, imajo pomembno vlogo v simbiotskih in parazitskih odnosih ter sodelujejo pri obrambnih odzivih na napad škodljivih organizmov. Glede na strukturo lektine iz gob razvrščamo v šest različnih skupin; pri nekaterih so ugotovili njihove toksične učinke na nekatere vrste žuželk (Varrot in sod., 2013). Lektin, ki so ga izolirali iz glive *Rhizoctonia solani* J.G. Kühn, spada v skupino hololektinov. Le-ti imajo podobne strukturne in sekvenčne lastnosti kot rastlinski lektin ricin. Walski in sod. (2014) so v poskusih ugotovili njegov vpliv na zmanjšano rast in upočasnjen razvoj ličink riževega mokarja (*Tribolium castaneum* [Herbst, 1797]). Pohleven in sod. (2011) so preučevali insekticidni učinek lektinov iz poprhnjene livke (*Clitocybe nebularis* [Batsch] P. Kumm., CNL), pri čemer so ugotovili, da je uporaba lektina CNL vplivala na zmanjšanje prehranjevanja ličink koloradskega hrošča. Vse omenjene raziskave toksičnih lektinov iz gliv na žuželke so bile opravljene laboratorijsko na podlagi prehranskih testov; poskusi s transgenimi rastlinami še niso bili opravljeni.

Gobe so bogat vir proteinov in proteaznih inhibitorjev. Zlasti iz gliv iz debla prostotrosnic so izolirali številne inhibitorje proteolize, ki so jim natančneje določili pomembne biokemične in strukturne lastnosti, po katerih se ločijo od inhibitorjev proteaz iz drugih virov (Erjavec in sod., 2012; Sabotič in Kos, 2012). Pri tem gre pretežno za serinske (mikospini) in cisteinske (mikocipini) inhibitorje, ki izkazujejo tudi insekticidne lastnosti (Sabotič in sod., 2016). Makrocipini iz skupine mikocipinskih inhibitorjev žuželčjih cisteinov, ki so jih izolirali iz orjaškega dežnika (*Macrolepiota procera* [Scop.] Singer), so v prehranjevalnih testih povzročili zmanjšanje rasti ličink koloradskega hrošča, njihov insekticidni učinek pa je bil dokazan tudi v transgenih rastlinah krompirja (Šmid in sod., 2013). Šmid in sod. (2015) so v prehranjevalnih testih ugotovili tudi toksični vpliv klitocipina iz poprhnjene livke, ki je povzročil večjo smrtnost ličink, vendar je v transgenem krompirju zaradi manjšega izražanja genov izkazoval manjšo insekticidno učinkovitost v primerjavi z makrocipinom.

2.5 EGEROLIZINI

Proteini iz družine egerolizinov sodijo v skupino porotvornih proteinov, ki jih proizvajajo različni evkariontski organizmi ter bakterije. Njihova biološka vloga še ni povsem razjasnjena, znana pa je njihova velika afiniteta vezave na membranske lipide in sposobnost tvorbe transmembranskih por (Berne in sod., 2009; Butala in sod., 2017). Bakterijski egerolizini različnih virov se znotraj večkomponentnih kompleksov specifično vežejo v prebavilih žuželk in nanje delujejo toksično. Glede na strukturo med egerolizine uvrščamo tudi nekatere Cry proteine, ki jih tvori Bt. Za binarni kompleks Cry34Ab1 / Cry35Ab1 je značilna specifična vezava v prebavnem epitelu koruznega hrošča (*Diabrotica virgifera virgifera* LeConte, 1868) in tvorba transmembranskih por. Omenjeni kompleks so na podlagi transformacije vnesli v hibrid koruze, ki je na voljo v komercialni pridelavi. Večina študij se sicer osredotoča predvsem na preučevanje egerolizinov iz gob, med katerimi imajo številni tudi insekticidne lastnosti. Za egerolizine, ki jih tvorijo glive iz rodu ostrigarjev (*Pleurotus* sp.), je značilna specifična vezava z lipidom, in sicer s sfingolipidom ceramid fosfoetanolaminom, ki je prisoten v membranah nevretenčarjev, ni pa ga v vretenčarjih. Omenjena lastnost pogojuje perspektivnost nadaljnjih raziskav njihove uporabe za proizvodnjo selektivnih insekticidov. Insekticidno delovanje egerolizinov iz ostrigarjev omogoča vezava v proteinske komplekse s partnerskimi proteini, ki vsebujejo specifične MACPF (angl. membrane attack complex/perforin) domene, ki tvorijo transmembranske pore (Butala in sod., 2017; Panevska in sod., 2020). Panevska in sod. (2019) so v raziskavi med drugim preučevali tudi toksičnost egerolizinskih kompleksov iz ostrigarjev proti ličinkam koloradskega hrošča. Določili so tri egerolizinske proteine: ostreolizin A6 (OlyA6), pleurotolizin A2 (PlyA2) in erilizin A (EryA). Ti skupaj s partnerskim proteinom pleurotolizinom B (PlyB) tvorijo transmembranske pore v črevesnih celicah, kar se odraža v povečani smrtnosti ličink zaradi stradanja. Rezultati nakazujejo nadaljnjo možnost uporabe egerolizinskih kompleksov iz ostrigarjev kot bioinsekticidnih komponent ali virov odpornosti proti koloradskemu hrošču pri žlahtnjenju rastlin.

3 SKLEPI

Insekticidno delujoči proteini, ki jih tvorijo različne vrste organizmov, predstavljajo pomemben potencial v varstvu rastlin pred škodljivci. V primerjavi s sintetičnimi insekticidi je njihova prednost bolj specifično delovanje, hitra razgradnja in manjša toksičnost za neciljne organizme. Razvoj odpornih sort rastlin je eden glavnih žlahtniteljskih ciljev, ki ga lažje dosegajo z uporabo sodobnih biotehnoloških postopkov in proizvodnje gensko spremenjenih rastlin.

Škodljivci se tekom generacij relativno hitro prilagajajo na spremenjene okoljske razmere in oblikujejo nove obrambne mehanizme. Kljub temu so za preprečevanje razvoja odpornosti pomembna preučevanja in raziskave drugih vrst insekticidih proteinov iz različnih virov. Sodobni biotehnološki pristopi omogočajo hkraten vnos dveh ali več genov iz različnih virov za sintezo proteinov z različnimi načini delovanja (piramidenje genov) ali fuzijo gena, ki sočasno kodira dva proteina (fuzijski proteini). Takšen način proizvodnje transgenih rastlin omogoča zanesljivejšo in dolgotrajnejšo učinkovitost proti kateri škodljivi organizmi težje razvijejo odpornost.

Izmed proteinov, ki izkazujejo insekticidno delovanje, so najbolj preučeni bakterijski proteini, med njimi predvsem Cry proteini, ki jih proizvaja Bt. Geni za njihovo sintezo so največkrat uporabljeni pri transformaciji v rastlinska tkiva za namene razvoja odpornih sort rastlin. Pridelava transgenih sort krompirja odpornih na koloradskega hrošča trenutno v praksi ni razširjena, kljub temu pa potekajo številne raziskave vnosa insekticidno delujočih proteinov iz različnih virov, ki v laboratorijskih razmerah izkazujejo učinkovitost pri zatiranju tega škodljivca.

Zaradi splošnih zadržkov javnosti in zakonodaje proti uveljavljanju gensko spremenjenih rastlin v kmetijstvu, je pomemben razvoj drugih možnosti uporabe insekticidnih proteinov, predvsem v obliki bioinsekticidov. Tudi tukaj prednjači uporaba Bt formulacij, njihova uporaba pa je v zadnjih 30 letih v porastu tudi zaradi tendence po zmanjševanju uporabe kemičnih insekticidov. Uporaba bioinsekticidov predstavlja pomembno alternativo kemičnim insekticidom tudi pri zatiranju koloradskega hrošča. Proti njegovim ličinkam delujejo toksini, ki jih izloča Bt subsp. *tenebrionis*, pomembno vlogo pri razvoju novih insekticidnih učinkovin pa imajo tudi proteini iz drugih virov. Predvsem višje glive predstavljajo pomemben vir lektinov, encimskih inhibitorjev in egerolizinov, ki delujejo toksično na ličinke koloradskega hrošča in številne druge žuželke.

4 VIRI

Álvarez-Alfageme, F., Martínez, M., Pascual-Ruiz, S., Castañera, P., Diaz, I., Ortego, F. (2007). Effects of potato plants expressing a barley cystatin on the predatory bug *Podisus maculiventris* via herbivorous prey feeding on the plant. *Transgenic Research*, *16*(1), 1–13. https://doi.org/10.1007/s11248-006-9022-6

Alyokhin, A., Baker, M., Mota-Sanchez, D., Dively, G., Grafius, E. (2008). Colorado potato beetle resistance to insecticides. *American Journal of Potato Research, 85*(6), 395–413. https://doi.org/10.1007/s12230-008-9052-0

Ashouri, S., Farshbaf Pourabad, R. (2021). Regulation of gene expression encoding the digestive α-amylase in the larvae of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) in response to plant protein extracts. *Gene*, *766*, 145159. https://doi.org/10.1016/j.gene.2020.145159

Ashouri, S., Farshbaf Pourabad, R., Kocadağ Kocazorbaz, E., Zihnioglu, F. (2017). Influence of red kidney bean seed proteins on development, digestive α-amylase activity and gut protein pattern of *Leptinotarsa decemlineata* (Say). *Journal of the Entomological Research Society*, *19*(3), 69–83.

Balaško, M. K., Mikac, K. M., Bažok, R., Lemic, D. (2020). Modern techniques in colorado potato beetle (*Leptinotarsa decemlineata* Say) control and resistance management: History review and future perspectives. *Insects*, *11*(9), 1–17. https://doi.org/10.3390/insects11090581

Berne, S., Lah, L., Sepčić, K. (2009). Aegerolysins: Structure, function, and putative biological role. *Protein Science, 18(*4), 694–706. https://doi.org/10.1002/pro.85

Berry, C., Crickmore, N. (2017). Structural classification of insecticidal proteins – Towards an in silico characterisation of novel toxins. *Journal of Invertebrate Pathology*, *142*, 16–22. https://doi.org/10.1016/j.jip.2016.07.015

Blackburn, M. B., Domek, J. M., Gelman, D. B., Hu, J. S. (2005). The broadly insecticidal Photorhabdus luminescens toxin complex a (Tca): Activity against the Colorado potato beetle, *Leptinotarsa decemlineata*, and sweet potato whitefly, *Bemisia tabaci*. *Journal of Insect Science*, *5*. https://doi.org/10.1093/jis/5.1.32

Bohinc, T., Vučajnk, F., Trdan, S. (2019). The efficacy of environmentally acceptable products for the control of major potato pests and diseases. *Zemdirbyste-Agriculture*, *106*(2), 135–142. https://doi.org/10.13080/z-a.2019.106.018

Bravo, A., Gill, S. S., Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon : official journal of the International Society on Toxinology*, *49*(4), 423—435. https://doi.org/10.1016/j.toxicon.2006.11.022

Bravo, A., Likitvivatanavong, S., Gill, S. S., Soberón, M. (2011). *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology, 41(*7), 423–431. https://doi.org/10.1016/j.ibmb.2011.02.006

Brunelle, F., Cloutier, C., Michaud, D. (2004). Colorado potato beetles compensate for tomato cathepsin D inhibitor expressed in transgenic potato. *Archives of Insect Biochemistry and Physiology*, *55*(3), 103–113. https://doi.org/10.1002/arch.10135

Butala, M., Novak, M., Kraševec, N., Skočaj, M., Veranič, P., Maček, P., Sepčić, K. (2017). Aegerolysins: Lipid-binding proteins with versatile functions. *Seminars in Cell and Developmental Biology, 72*, 142–151). https://doi.org/10.1016/j.semcdb.2017.05.002

Carlini, C. R., Grossi-De-Sá, M. F. (2002). Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon, 40*(11), 1515–1539. https://doi.org/10.1016/S0041-0101(02)00240-4

Chakroun, M., Banyuls, N., Bel, Y., Escriche, B., Ferré, J. (2016). Bacterial Vegetative Insecticidal Proteins (Vip) from Entomopathogenic Bacteria. *Microbiology and Molecular Biology Reviews*, *80*(2), 329 LP – 350. https://doi.org/10.1128/MMBR.00060-15

Chen, M. S., Johnson, B., Wen, L., Muthukrishnan, S., Kramer, K. J., Morgan, T. D., Reeck, G. R. (1992). Rice cystatin: Bacterial expression, purification, cysteine proteinase inhibitory activity, and insect growth suppressing activity of a truncated form of the protein. *Protein Expression and Purification*, *3*(1), 41–49. https://doi.org/10.1016/1046-5928(92)90054-Z

Cingel, A., Savić, J., Lazarević, J., Ćosić, T., Raspor, M., Smigocki, A., Ninković, S. (2016). Extraordinary adaptive plasticity of colorado potato beetle: "Ten-striped Spearman" in the era of biotechnologicalwarfare. *International Journal of Molecular Sciences, 17*(9). MDPI AG. https://doi.org/10.3390/ijms17091538

Cingel, A., Savić, J., Lazarević, J., Ćosić, T., Raspor, M., Smigocki, A., Ninković, S. (2017). Co-expression of the proteinase inhibitors oryzacystatin I and oryzacystatin II in transgenic potato alters Colorado potato beetle larval development. *Insect Science*, *24*(5), 768–780. https://doi.org/https://doi.org/10.1111/1744-7917.12364

Cloutier, C., Jean, C., Fournier, M., Yelle, S., Michaud, D. (2000). Adult Colorado potato beetles, *Leptinotarsa decemlineata* compensate for nutritional stress on oryzacystatin I-transgenic potato plants by hypertrophic behavior and over-production of insensitive proteases. *Archives of Insect Biochemistry and Physiology*, *44*(2), 69–81. https://doi.org/10.1002/1520-6327(200006)44:2<69::AID-ARCH2>3.0.CO;2-6

Cooper, S. G., Douches, D. S., Grafius, E. J. (2009). Combining engineered resistance, avidin, and natural resistance derived from & lt; I & gt; *Solanum chacoense* & lt;/I & gt; bitter to control Colorado potato beetle (Coleoptera: Chrysomelidae). *Journal of Economic Entomology*, *102*(3), 1270–1280. https://doi.org/10.1603/029.102.0354

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D. H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews*, *62*(3), 807–813. https://doi.org/10.1128/mmbr.62.3.807-813.1998

Dang, L., Van Damme, E. J. M. (2015). Toxic proteins in plants. *Phytochemistry, 117*(1), 51–64). https://doi.org/10.1016/j.phytochem.2015.05.020

Domínguez-Arrizabalaga, M., Villanueva, M., Escriche, B., Ancín-Azpilicueta, C., Caballero, P. (2020). Insecticidal activity of *Bacillus thuringiensis* proteins againstcoleopteran Pests. *Toxins*, *12*(7), 430. https://doi.org/10.3390/toxins12070430

Donovan, W. P., Engleman, J. T., Donovan, J. C., Baum, J. A., Bunkers, G. J., Chi, D. J., Clinton, W. P., English, L., Heck, G. R., Ilagan, O. M., Krasomil-Osterfeld, K. C., Pitkin, J. W., Roberts, J. K., Walters, M. R. (2006). Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. *Applied Microbiology and Biotechnology*, *72*(4), 713–719. https://doi.org/10.1007/s00253-006-0332-7

Erjavec, J., Kos, J., Ravnikar, M., Dreo, T., Sabotič, J. (2012). Proteins of higher fungi - from forest to application. *Trends in Biotechnology, 30*(5), 259–273. https://doi.org/10.1016/j.tibtech.2012.01.004

Franco, O. L., Rigden, D. J., Melo, F. R., Grossi-de-Sá, M. F. (2002). Plant α-amylase inhibitors and their interaction with insect α-amylases. *European Journal of Biochemistry*, *269*(2), 397–412. https://doi.org/https://doi.org/10.1046/j.0014-2956.2001.02656.x

Fürstenberg-Hägg, J., Zagrobelny, M., Bak, S. (2013). Plant defense against insect herbivores. *International Journal of Molecular Sciences*, *14*(5), 10242–10297. https://doi.org/10.3390/ijms140510242

Gatehouse, A. M. R., Dewey, F. M., Dove, J., Fenton, K. A., Pusztai, A. (1984). Effect of seed lectins from Phaseolus vulgaris on the development of larvae of *Callosobruchus maculatus*; mechanism of toxicity. *Journal of the Science of Food and Agriculture*, *35*(4), 373–380. https://doi.org/https://doi.org/10.1002/jsfa.2740350402

Grafius, E. J., Douches, D. S. (2008). The present and future role of insect-resistant genetically modified potato cultivars in IPM. V *Integration of Insect-Resistant Genetically Modified Crops within IPM Programs* (str. 195–221). Springer Netherlands. https://doi.org/10.1007/978-1-4020-8373-0_7

Green, T. R., Ryan, C. A. (1972). Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. *Science*, *175*(4023), 776–777. https://doi.org/10.1126/science.175.4023.776

Gruden, K., Kuipers, A. G. J., Gunčar, G., Slapar, N., Štrukelj, B., Jongsma, M. A. (2004). Molecular basis of Colorado potato beetle adaptation to potato plant defence at the level of digestive cysteine proteinases. *Insect Biochemistry and Molecular Biology*, *34*(4), 365–375. https://doi.org/10.1016/j.ibmb.2004.01.003

Gruden, K., Štrukelj, B., Popovič, T., Lenarčič, B., Bevec, T., Brzin, J., Kregar, I., Herzog-Velikonja, J., Stiekema, W. J., Bosch, D., Jongsma, M. A. (1998). The cysteine protease activity of Colorado potato beetle (*Leptinotarsa decemlineata* Say) guts, which is insensitive to potato protease inhibitors, is inhibited by thyroglobulin type-1 domain inhibitors. *Insect Biochemistry and Molecular Biology*, *28*(8), 549–560. https://doi.org/10.1016/S0965-1748(98)00051-4

Jallouli, W., Driss, F., Fillaudeau, L., Rouis, S. (2020). Review on biopesticide production by *Bacillus thuringiensis* subsp. *kurstaki* since 1990: Focus on bioprocess parameters. V *Process Biochemistry* (Let. 98, str. 224–232). Elsevier Ltd. https://doi.org/10.1016/j.procbio.2020.07.023

Kalha, C. S., Singh, P. P., Kang, S. S., Hunjan, M. S., Gupta, V., Sharma, R. (2014). Entomopathogenic viruses and bacteria for insect-pest control. V *Integrated Pest Management: Current Concepts and Ecological Perspective* (str. 225–244). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398529-3.00013-0

Kamionskaya, A. M., Kuznetsov, B. B., Ismailov, V. Y., Nadikta, V. D., Skryabin, K. G. (2012). Genetically transforming Russian potato cultivars for resistance to Colorado beetle. *Clon Transgen*, *1*, 101. https://doi.org/10.4172/2168-9849.1000101

Lalitha, S., Shade, R. E., Murdock, L. L., Bressan, R. A., Hasegawa, P. M., Nielsen, S. S. (2005). Effectiveness of recombinant soybean cysteine proteinase inhibitors against selected crop pests. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *140*(2), 227–235. https://doi.org/10.1016/j.cca.2005.02.007

Laznik, Ž., Tóth, T., Lakatos, T., Vidrih, M., Trdan, S. (2010). Control of the Colorado potato beetle (*Leptinotarsa de-*

cemlineata [Say]) on potato under field conditions: a comparison of the efficacy of foliar application of two strains of *Steinernema feltiae* (Filipjev) and spraying with thiametoxam. *Journal of Plant Diseases and Protection*, *117*(3), 129–135. https://doi.org/10.1007/BF03356348

Lecardonnel, A., Chauvin, L., Jouanin, L., Beaujean, A., Prévost, G., Sangwan-Norreel, B. (1999). Effects of rice cystatin I expression in transgenic potato on Colorado potato beetle larvae. *Plant Science*, *140*(1), 71–79. https://doi.org/10.1016/S0168-9452(98)00197-6

Martin, P. A. W., Blackburn, M., Shropshire, A. D. S. (2006). Two new bacterial pathogens of Colorado potato beetle (Coleoptera: Chrysomelidae). *Journal of Economic Entomology*, *97*(3), 774–780. https://doi.org/10.1603/0022-0493(2004)097[0774:tnbpoc]2.0.co;2

Martinez, M., Santamaria, M. E., Diaz-Mendoza, M., Arnaiz, A., Carrillo, L., Ortego, F. (2016). Phytocystatins: Defense proteins against phytophagous insects and acari. *International Journal of Molecular Sciences, 17*(10). MDPI AG. https://doi.org/10.3390/ijms17101747

Mi, X., Ji, X., Yang, J., Liang, L., Si, H., Wu, J., Zhang, N., Wang, D. (2015). Transgenic potato plants expressing cry3A gene confer resistance to Colorado potato beetle. *Comptes Rendus - Biologies*, *338*(7), 443–450. https://doi.org/10.1016/j.crvi.2015.04.005

Michaud, D., Nguyen-Quoc, B., Yelle, S. (1993). Selective inhibition of Colorado potato beetle cathepsin H by oryzacystatins I and II. *FEBS Letters*, *331*(1–2), 173–176. https://doi.org/10.1016/0014-5793(93)80320-T

Michiels, K., Van Damme, E. J. M., Smagghe, G. (2010). Plant-insect interactions: what can we learn from plant lectins? *Archives of Insect Biochemistry and Physiology*, *73*(4), 193–212. https://doi.org/https://doi.org/10.1002/arch.20351

Muratoglu, H., Demirbag, Z., Sezen, K. (2011). The first investigation of the diversity of bacteria associated with *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Biologia*, *66*(2), 288–293. https://doi.org/10.2478/s11756-011-0021-6

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., Caballero, P. (2014). Bacillus thuringiensis toxins: An overview of their biocidal activity. *Toxins, 6*(12), 3296–3325). MDPI AG. https://doi.org/10.3390/toxins6123296

Panevska, A., Hodnik, V., Skočaj, M., Novak, M., Modic, Š., Pavlic, I., Podržaj, S., Zarić, M., Resnik, N., Maček, P., Veranič, P., Razinger, J., Sepčić, K. (2019). Pore-forming protein complexes from *Pleurotus* mushrooms kill western corn rootworm and Colorado potato beetle through targeting membrane ceramide phosphoethanolamine. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-41450-4

Panevska, A., Skočaj, M., Modic, Š., Razinger, J., Sepčić, K. (2020). Aegerolysins from the fungal genus *Pleurotus* – Bioinsecticidal proteins with multiple potential applications. *Journal of Invertebrate Pathology*, 107474. https://doi.org/10.1016/j.jip.2020.107474

Paul, S., Das, S. (2020). Natural insecticidal proteins, the promising bio-control compounds for future crop protection. *The Nucleus*. https://doi.org/10.1007/s13237-020-00316-1

Peumans, W. J., Van Damme, E. J. (1995). Lectins as plant defense proteins. *Plant Physiology*, *109*(2), 347–352. https://doi.org/10.1104/pp.109.2.347

Pohleven, J., Brzin, J., Vrabec, L., Leonardi, A., Čokl, A., Štrukelj, B., Kos, J., Sabotič, J. (2011). Basidiomycete *Clitocybe nebularis* is rich in lectins with insecticidal activities. *Applied Microbiology and Biotechnology*, *91*(4), 1141–1148. https://doi.org/10.1007/s00253-011-3236-0

Rane, A. S., Venkatesh, V., Joshi, R. S., Giri, A. P. (2020). Molecular investigation of Coleopteran specific α-amylase inhibitors from Amaranthaceae members. *International Journal of Biological Macromolecules*, *163*, 1444–1450. https://doi.org/10.1016/j.ijbiomac.2020.07.219

Reed, G. L., Jensen, A. S., Riebe, J., Head, G., Duan, J. J. (2001). Transgenic Bt potato and conventional insecticides for Colorado potato beetle management: comparative efficacy and non-target impacts. *Entomologia Experimentalis et Applicata*, *100*(1), 89–100. https://doi.org/10.1046/j.1570-7458.2001.00851.x

Ryan, C. A. (1990). Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology*, *28*(1), 425–449. https://doi.org/10.1146/annurev.py.28.090190.002233

Sabotič, J., Kos, J. (2012). Microbial and fungal protease inhibitors - Current and potential applications. *Applied Microbiology and Biotechnology, 93*(4), 1351–1375). https://doi.org/10.1007/s00253-011-3834-x

Sabotič, J., Ohm, R. A., Künzler, M. (2016). Entomotoxic and nematotoxic lectins and protease inhibitors from fungal fruiting bodies. *Applied Microbiology and Biotechnology, 100*(1), 91–111). https://doi.org/10.1007/s00253-015-7075-2

Schlüter, U., Benchabane, M., Munger, A., Kiggundu, A., Vorster, J., Goulet, M. C., Cloutier, C., Michaud, D. (2010). Recombinant protease inhibitors for herbivore pest control: A multitrophic perspective. *Journal of Experimental Botany*, *61*(15), 4169–4183. https://doi.org/10.1093/jxb/erq166

Singh, S., Singh, A., Kumar, S., Mittal, P., Singh, I. K. (2020). Protease inhibitors: recent advancement in its usage as a potential biocontrol agent for insect pest management. *Insect Science, 27*(2), 186–201. https://doi.org/10.1111/1744-7917.12641

Šmid, I., Gruden, K., Buh Gašparič, M., Koruza, K., Petek, M., Pohleven, J., Brzin, J., Kos, J., Žel, J., Sabotič, J. (2013). Inhibition of the growth of Colorado potato beetle larvae by macrocypins, protease inhibitors from the parasol mushroom. *Journal of Agricultural and Food Chemistry*, *61*(51), 12499–12509. https://doi.org/10.1021/jf403615f

Šmid, I., Rotter, A., Gruden, K., Brzin, J., Buh Gašparič, M., Kos, J., Žel, J., Sabotič, J. (2015). Clitocypin, a fungal cysteine protease inhibitor, exerts its insecticidal effect on Colorado potato beetle larvae by inhibiting their digestive cysteine proteases. *Pesticide Biochemistry and Physiology*, *122*, 59–66. https://doi.org/10.1016/j.pestbp.2014.12.022

Srp, J., Nussbaumerová, M., Horn, M., Mareš, M. (2016). Digestive proteolysis in the Colorado potato beetle, *Leptinotarsa decemlineata:* Activity-based profiling and imaging of a multipeptidase network. *Insect Biochemistry and Molecular Biology*, *78*, 1–11. https://doi.org/10.1016/j.ibmb.2016.08.004

Trdan, S. (2013). Insecticides - Development of safer and more effective technologies. V *Insecticides - Development of Safer and More Effective Technologies*. InTech. https://doi.org/10.5772/3356

Trdan, S. (2016). Insecticides Resistance. V *Insecticides Resistance*. InTech. https://doi.org/10.5772/60478

Tripathi, A. K., Mishra, S. (2016). Biotechnological Approaches. V *Ecofriendly Pest Management for Food Security* (str. 685–701). Elsevier Inc. https://doi.org/10.1016/B978-0-12-803265-7.00022-1

USEPA. (2010). *BIOPESTICIDES REGISTRATION ACTION DOCUMENT. Bacillus thuringiensis Cry3Bb1 Protein and the Genetic Material Necessary for Its Production in MON 863 and MON 88017 Corns*. http://www.epa.gov/pesticides/biopesticides/pips/cry3bb1-brad.pdf

Vandenborre, G., Smagghe, G., Van Damme, E. J. M. (2011). Plant lectins as defense proteins against phytophagous insects. *Phytochemistry, 72*(13), 1538–1550). https://doi.org/10.1016/j.phytochem.2011.02.024

Varrot, A., Basheer, S. M., Imberty, A. (2013). Fungal lectins: Structure, function and potential applications. *Current Opinion in Structural Biology, 23*(5), 678–685). https://doi.org/10.1016/j.sbi.2013.07.007

Visal, S., Taylor, M. A. J., Michaud, D. (1998). The proregion of papaya proteinase IV inhibits Colorado potato beetle digestive cysteine proteinases. *FEBS Letters*, *434*(3), 401–405. https://doi.org/10.1016/S0014-5793(98)01018-7

Walski, T., Van Damme, E. J. M., Smagghe, G. (2014). Penetration through the peritrophic matrix is a key to lectin toxicity against *Tribolium castaneum*. *Journal of Insect Physiology*, *70*, 94–101. https://doi.org/10.1016/j.jinsphys.2014.09.004

Wang, K., Shu, C., Zhang, J. (2019). Effective bacterial insecticidal proteins against coleopteran pests: A review. *Archives of Insect Biochemistry and Physiology*, *102*(3), e21558. https://doi.org/https://doi.org/10.1002/arch.21558

Wang, M., Triguéros, V., Paquereau, L., Chavant, L., Fournier, D. (2002). Proteins as active compounds involved in insecticidal activity of mushroom fruitbodies. *Journal of economic entomology*, *95*(3), 603–607. https://doi.org/10.1603/0022-0493-95.3.603

Wang, W., Hause, B., Peumans, W. J., Smagghe, G., Mackie, A., Fraser, R., Van Damme, E. J. M. (2003). The Tn antigen-specific lectin from ground ivy is an insecticidal protein with an unusual physiology. *Plant Physiology*, *132*(3), 1322–1334. https://doi.org/10.1104/pp.103.023853

Whalon, M. E., Miller, D. L., Hollingworth, R. M., Grafius, E. J., Miller, J. R. (1993). Selection of a Colorado potato beetle (Coleoptera: Chrysomelidae) strain resistant to *Bacillus thuringiensis*. *Journal of Economic Entomology*, *86*(2), 226–233. https://doi.org/10.1093/jee/86.2.226

Wolfson, J. L., Murdock, L. L. (1987). Suppression of larval Colorado potato beetle growth and development by digestive proteinase inhibitors. *Entomologia Experimentalis et Applicata*, *44*(3), 235–240. https://doi.org/10.1111/j.1570-7458.1987.tb00550.x

Zhu, K., Huesing, J. E., Shade, R. E., Bressan, R. A., Hasegawa, P. M., Murdock, L. L. (1996). An insecticidal N-acetylglucosamine-specific lectin gene from *Griffonia simplicifolia* (Leguminosae). *Plant Physiology*, *110*(1), 195 LP – 202. https://doi.org/10.1104/pp.110.1.195