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CONTENTS

SILVA M.G., ALVES L.S., SOARES T.M., GHEYI H.R., BIONE M.A.A.
Growth, production and water use efficiency of chicory (*Cichorium endivia* L.) in hydroponic systems using brackish waters 243

AL-ASHOUSH A.M., SHIBLI R.A., TAHTAMOUNI R.W., AL-QUDAH T.S., ABU-IRARMAILEH B.B.
Enhancement of Pentacyclic Triterpenoids (Betulinic and Oleanolic acids) production from callus cultures of *Lantana camara* L. 255

BONYANPOUR A.R., JAMALI B.
Seasonal enzymatic and non-enzymatic antioxidant responses in seven Iranian pomegranate cultivars 265

ZEYNALOVA A., NOVRUZOV E., BARTOLINI P., BRUNETTI C., MASERTI B.
Phenolic fingerprint in wild growing pomegranate fruits from Azerbaijan 277

RAHIMI-DVIN S., GHARAGHANI A., POURKHALOEE A.
Genetic diversity, population structure, and relationships among wild and domesticated almond (*Prunus* spp.) germplasms revealed by ISSR markers 287

HERÊNIO GONÇALVES JÚNIOR D., DE MELO RODRIGUES L., ALVES DE SANTANA FILHO J., NASCIMENTO LIMA W., FERREIRA ALVES A.
Genetic parameters, correlations and path analysis in cowpea genotypes for yield and agronomic traits growin in *Cerrado*/Amazon Rainforest ecotone 301

OTIENO P.C., NYALALA S., WOLUKAU J.
Optimization of biosolids as a substrate for tomato transplant production 313

JADIDI E., TATARI M., GHASEMNEZHAD M., SALEMI H.R.
The salinity tolerance of pomegranate cultivars: Effects of salt stress on root and leaf mineral content 325

VIERA NASCIMENTO M., RIBEIRO ÁVILA M.C., FIORI DE ABREU-TARAZI M., OLIVEIA NOGUEIRA A.P., CARDOSO CAMPOS L.F., DOS REIS NASCIMIENTO A.
Identification of promising tomato breeding lines with determinate growth by selection index 337

SHORT NOTE

BENJELLOUN J., TAOUFYQ A., EL ABIDINE TRIQUI Z., ALAMI Q.L., LAYACHI R., SMOUNI A., BOUZROUD S., GUEDIRA A.
Improvement of *in vitro* germination of *Cycas revoluta* zygotic embryos using gelrite as gelling agent 349

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Advances in Horticultural Science

Growth, production and water use efficiency of chicory (*Cichorium endivia* L.) in hydroponic systems using brackish waters

M.G. Silva [1 (*)], L.S. Alves [2], T.M. Soares [1], H.R. Gheyi [1], M.A.A. Bione [1]

[1] *Soil and Water Engineering Nucleus, Federal University of Recôncavo of Bahia, Cruz das Almas, Bahia, Brazil.*

[2] *Department of Technology, State University of Feira de Santana, Feira de Santana, Bahia, Brazil.*

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(*) Corresponding author:
mairtong@hotmail.com

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Abstract: Plants response to the same level of salinity can be increased in hydroponic cultivation compared to under soil conditions. The study aimed at evaluating the chicory growth in DFT (Deep Flow Technique) hydroponic system using brackish water, comparing the results with those obtained in NFT (Nutrient Film Technique) system. The experiment was carried out in a randomized block design with eight replicates. Each plot (replicate) was represented by a hydroponic channel with 15 plants. Four treatments were used, consisting of plants grown in the DFT system submitted to three levels of electrical conductivity of nutrient solution - ECsol (2.57, 3.43 and 4.75 dS/m) and in the NFT system under ECsol of 2.57 dS/m. Plant height, number of leaves, fresh and dry matter of shoot, water consumption, water use efficiency and water content in shoot at 20 and 25 days after transplanting (DAT) were evaluated. In each harvest, a mean value was obtained per plot through of the harvest collection of five plants. At 25 DAT, the largest reductions in production and water use efficiency of chicory were observed under higher salinity (ECsol 4.75 dS/m). In the DFT system no symptoms of toxicity that could be attributed to salinity were observed.

1. Introduction

Under natural conditions, plants are frequently exposed to complex interactions which involve numerous environmental factors (Rejeb *et al.*, 2014; Zribi *et al.*, 2017; Prisa, 2019), such as salinity, water deficit, temperature and others (Ramakrishna and Ravishankar, 2011; Szareski *et al.*, 2018).

In arid and semi-arid regions of different parts of the world, such as the Brazilian Northeast (Rocha Neto *et al.*, 2017), among the various abiotic stresses, saline stress, which expresses the concentration of soluble salts in the soil or water (Breś *et al.*, 2016), has been pointed out as the

main cause for the decrease of yield in most agricultural crops (Younis *et al*., 2013; Boughalleb *et al*., 2017; Rezaei *et al*., 2017).

Soil salinization can be of primary origin (natural) and/or secondary origin, caused by anthropic activity, such as the use of saline water in irrigation (Shahrayini *et al*., 2018; Sienkiewicz-Cholewa *et al*., 2018). Secondary salinization is a consequence of inadequate irrigation management because natural drainage in the semi-arid regions is limited due to the low rainfall (Endo *et al*., 2011; Suassuna *et al*., 2017), which is not sufficient to leach the salts from the root zone to deeper soil layers and thus maintain compatible levels of salts in the root zone of crops (Ünlükara *et al*., 2008; Shrivastava and Kumar, 2015).

Plant species respond differently to salt stress (Tabatabaei and Ehsanzadeh, 2016). Some are able to produce satisfactory yields under saline conditions, while others are not (Zrig *et al*., 2016; Qrunfleh *et al*., 2017). The responses of plants are variable among the different organs, species/cultivars, development stages and duration of exposure to the salts (Parvaiz and Satyawati, 2008; Abbas *et al*., 2015), which usually lead to reductions in phytomass production, yield or survival rates (Munns and Tester, 2008; Xu *et al*., 2018).

Salt stress can limit the exploitation of most agricultural crops, making the agricultural activity economically unviable. To mitigate the problems of salinity, hydroponic cultivation (soilless cultivation) has been pointed out as a technique suitable for the use of saline water because the response of plants to salinity is better than in soil, when irrigated with the same water (Silva *et al*., 2018 a). In this system, there is higher and immediate availability of water and nutrients to plants because there is no matric potential, which is one of the main causes of reduction in the free energy of water in the soil.

Studies on the tolerance of several species to salinity in hydroponic systems have demonstrated that, through adequate management of water and cultivation practices, it is possible to produce commercially using brackish waters (Dias *et al*., 2011), especially leafy vegetables such as lettuce (Soares *et al*., 2015; Cova *et al*., 2017; Silva *et al*., 2018 b). In this context, other crops of economic potential, such as chicory, have been investigated using brackish waters under hydroponic conditions (Tzortzakis, 2009, 2010; Atzori *et al*., 2016, 2019 a). Atzori *et al*. (2016) reported that chicory crop proved to have a considerable higher tolerance to salinity compared to lettuce in hydroponics. According to Cecílio Filho *et al*. (2015), on average chicory is a more profitable crop than lettuce, and its requirements in terms of management in hydroponic systems are similar.

In Brazil and in several parts of the world, the NFT (Nutrient Film Technique) hydroponic system is the most used commercially. NFT is an active system which requires pumping to recirculate the nutrient solution, usually at 15 min intervals (Zanella *et al*., 2008). Thus, the use of NFT system may be limited in places where there are frequent interruptions in supply of electricity (Santos Júnior *et al*., 2015; Silva *et al*., 2016). To overcome this problem, some researchers adopted the DFT (Deep Flow Technique) system in PVC pipes (Santos Júnior *et al*., 2015; Silva *et al*., 2016; Cova *et al*., 2017; Campos Júnior *et al*., 2018 a; Gondim Filho *et al*., 2018; Martins *et al*., 2019 a, b; Santos *et al*., 2019; Silva Júnior *et al*., 2019), in which plant roots remain continually immersed in the nutrient solution which is recirculated but not as frequently as in NFT. Thus, in case of short interruptions in electricity supply plants do not undergo water restriction (Silva *et al*., 2018 a).

Therefore, this study aimed to evaluate the growth, production, water consumption, water use efficiency and quality of plants of the chicory (*Cichorium endivia* L.) using nutrient solutions prepared in brackish waters in DFT system, comparing the results with those obtained using solution prepared in fresh water in NFT system.

2. Materials and Methods

Experiment location

The study was carried out in a greenhouse (East-West orientation), from June to August (Fall-Winter) of 2016. The greenhouse was 7.0 m wide and 24 m long, with ceiling height of 2.8 m, protected on the sides by black shade (50% luminosity) screen and covered with low density polyethylene film, with anti-ultraviolet additive and with a thickness of 150 microns. The study site was in the Experimental Area of the Graduate Program in Agricultural Engineering of the Federal University of Recôncavo of Bahia, located in the municipality of Cruz das Almas, Bahia, Brazil (12° 40’ 19” S, 39° 06’ 23” W, and at an elevation of 220 m a.s.l).

Treatments, experimental design and structure

The experiment was carried out in a randomized block design, with four treatments and eight replicates. Chicory plants were submitted to three levels

of electrical conductivity of nutrient solution - ECsol (2.57, 3.43 and 4.75 dS/m) in hydroponics DFT and under ECsol of 2.57 dS/m in hydroponics NFT, being cultivated 15 plants in each cultivation channel in the central part. Nutrient solutions were prepared by adding adequate amounts of fertilizer salts according to recommendation of Furlani *et al*. (1999). The nutrient solution of 2.57 dS/m was obtained by addition of only fertilizer salts to public-supply water (0.34 dS/m). For ECsol levels of 3.43 and 4.75 dS/m, the nutrient solutions were prepared in waters with EC of 1.5 and 3.0 dS/m obtained by addition of NaCl to public-supply water, which were also used to replace the volume consumed by the plants in the respective treatments during the cultivation period.

In both systems, channels made of PVC pipes (6-m length and 0.075 m in diameter) were used, with circular holes of 0.05 m in diameter, spaced 0.25 m apart. Benches with trestles made of PVC pipes of 0.05 m in diameter were used to support the hydroponic channels. Three hydroponic channels were used per bench, with horizontal spacing of 0.30 m. One corridor (0.5-m width) was left between the benches to facilitate transit and operability.

In the DFT hydroponic system, caps were attached at both ends of each hydroponic channel installed with zero slope, and a drain was installed in the caps to maintain a mean level of the nutrient solution of 0.02 m, conducting the excess solution through a hose back to the solution tank. In the NFT system the hydroponic channels were installed with a 4% slope.

Each experimental unit consisted of an independent hydroponic channel, containing a plastic tank (60-l capacity) to store the nutrient solution, and an electric pump to inject the nutrient solution into the channel. The tank had a ballcock valve to maintain a volume of 50 l of the solution and connected to water reservoir, built with PVC pipes of 0.15 m in diameter. A transparent hose with a tape ruler was installed vertically on the outside of the reservoir to verify the water level. The reservoir and supply tank were connected by a hose; the water level was manually controlled through a ball valve which was opened daily at prefixed hours to maintain the water level in the reservoir and quantify water consumption.

Crop conduction and nutrient solution management

Seeds of broad-leaved chicory cv. 'Dafne' were sown on phenolic foam (2 x 2 x 2 cm), by planting one seed per cell. After germination, seedlings were daily irrigated with public-supply water until 10 days after sowing (DAS). After this period, the seedlings were transferred to a nursery (NFT system), where they received nutrient solution (Furlani *et al*., 1999) at 50% concentration for 15 days. Irrigations in the nursery were controlled by an analog timer at intermittent intervals of 15 min, from 06:00 to 18:00 h. During the period from 18:00 to 06:00 h, the nutrient solution was recirculated once every 2 h, with duration of 15 min. The seedlings were transplanted to the definitive cultivation system with mean height of 0.133 m and four true leaves.

The programming to control circulation of the nutrient solution in the cultivation channels in both systems (NFT and DFT) was similar to that used during the initial stage i.e. at intervals of 15 min, from 06:00 to 18:00 h. During the period from 18:00 to 06:00 h, the nutrient solution was recirculated once every 2 h, with duration of 15 min.

During the experiment, ECsol and pH of the solution were monitored in the central position of each hydroponic channel, using portable conductivity and pH meter. At the end of the experiment, the ECsol means values were 1.88 and 2.03 dS/m for the treatment under ECsol de 2.57 dS/m, respectively in the NFT e DFT systems, and of 3.24 and 4.88 dS/m for the treatments under ECsol de 3.43 e 4.75 dS/m in the DFT system, with no replacement of nutrient to the solution during the cycle. When the pH values were outside the ideal range (between 5.5 and 6.5) for hydroponic cultivation, corrections were made by addition of calcium hydroxide.

Variables evaluated

Harvests were performed at 20 and 25 days after transplanting (DAT). The strategy of performing two harvests along the experiment was to identify the best period for plant harvest, and also to evaluate the absolute growth rate of plants under different treatments before harvest and to assess possibility of early harvest. In each harvest, a mean value was obtained per plot (hydroponic channel) through of the harvest of five plants for the determination of plant height, number of leaves and shoot fresh matter. Immediately after weighing the plants, the fresh material was placed in paper bags and dried in an air circulation oven at temperature of 65°C until constant weight, to quantify shoot dry matter.

The volume evapotranspired per plant was determined daily by dividing the volume of nutrient solution consumed in the plot by the number of plants under cultivation at that moment in the plot, according to equation described by Lira *et al*. (2018). Cumulative water consumption was calculated for

the periods of 1 to 20 and 1 to 25 DAT. Water use efficiency (WUE) was based on the relationship between shoot fresh (SFM) or dry matter (SDM) production and the cumulative water consumption per plant, according to equation 1:

$$WUE (g/l) = (SFM \text{ or } SDM)/WC \quad (1)$$

where SFM is shoot fresh matter, in g; SDM is shoot dry matter, in g; WC is cumulative water consumption during the period, in l/plant.

The water content in shoot (WCS) was calculated according to equation 2:

$$WCS (\%) = [(SFM - SDM)/SFM] \times 100 \quad (2)$$

The absolute growth rate (AGR) of SFM was calculated according to equation 3:

$$AGR (g/day) = (SFM2 - SFM1)/(T2 - T1) \quad (3)$$

where SFM_1 and SFM_2 are shoot fresh matter at times T_1 (20 DAT) and T_2 (25 DAT), in g.

Statistical analysis

The results were subjected to analysis of variance by F test and the means were compared by Tukey test (P = 0.05). The standard deviations of means were also calculated.

3. Results

Visual symptoms of the chicory plants

Under salt stress no symptoms of toxicity by the ions Na^+ and/or Cl^- were observed which could compromise the visual quality of chicory plants (Fig. 1 A). Only in the NFT system without salt stress (ECsol of 2.57 dS/m), after 20 DAT, chicory leaves exhibited necrosis on the edges (Fig. 1 B), an abnormality known as tipburn. Although tipburn symptoms were observed in all plots of this system, but only in some plants.

Growth and production of the chicory

The F-test of the analysis of variance showed a significant effect of the treatments on the number of leaves, plant height, shoot dry matter and water consumption, only at 25 DAT. For shoot fresh matter the treatments had significant effect at 20 and 25 DAT, and only at 20 DAT on the water content in shoot, water use efficiency and the absolute growth rate (20-25 DAT) based on shoot fresh matter (Tables 1 and 2).

At 20 DAT, the overall mean for the number of leaves was 8.2, regardless of the hydroponic systems

Fig. 1 - Visual aspect of chicory (*Cichorium endivia* L. cv. Dafne) plants in the NFT (without salt stress) and DFT (with and without salt stress) hydroponic systems, at 25 days after transplanting (A) and plants with tipburn in NFT system without salt stress (B).

Table 1 - Summary of the F-test for number of leaves (NL), plant height (PH), shoot fresh matter (SFM), shoot dry matter (SDM) and absolute growth rate of SFM (AGR-SFM) of chicory cultivated under different treatments in hydroponic systems, at 20 and 25 days after transplanting (DAT)

SV	df	Days after transplanting (DAT)								
		NL		PH		SFM		SDM		AGR-SFM
		20	25	20	25	20	25	20	25	20-25
Blocks	7	NS	NS	*	**	*	*	NS	**	*
Treatment	3	NS	**	NS	*	**	**	NS	**	**
Error	21	-	-	-	-	-	-	-	-	-
CV (%)		9.40	9.47	3.30	3.01	9.38	10.04	17.00	15.09	15.81

SV= Source of variation; df= degrees of freedom; CV= coefficient of variation; *, ** significant respectively at P<0.05 and P<0.01; NS= not significant.

Table 2 - Summary of the F-test for water content in shoot, water consumption (WC), and water use efficiency based on shoot fresh matter - SFM (WUE-SFM) and shoot dry matter - SDM (WUE-SDM) of chicory cultivated under different treatments in hydroponic systems, at 20 and 25 days after transplanting (DAT)

SV	df	Days after transplanting (DAT)							
		Water content		WC		WUE-SFM		WUE-SDM	
		20	25	20	25	20	25	20	25
Blocks	7	NS	NS	**	**	**	NS	NS	NS
Treatments	3	**	NS	NS	*	*	NS	NS	NS
Error	21	-	-	-	-	-	-	-	-
CV (%)		0.77	0.63	15.25	14.22	16.20	15.27	19.92	19.87

SV= Source of variation; df= degrees of freedom; CV= coefficient of variation; *, ** significant respectively at P<0.05 and P<0.01; NS= not significant.

and water salinity levels. At 25 DAT, the lowest number of leaves (9.4) was observed in plants under the highest salinity (ECsol of 4.75 dS/m) compared with the other treatments (12.3, 11.9 and 11.1 leaves) (Fig. 2 A). Within a 5-day interval (20 to 25 DAT), under the highest salinity (ECsol of 4.75 dS/m) in the DFT system the increase in the number of leaves did not exceed by 2.0 leaves, while in the NFT system without salt stress (ECsol of 2.57 dS/m) the increase reached approximately 4.0 leaves.

In general, plant height was little influenced by the treatments. On average, plant height at 20 DAT was approximately 30.0 cm. Within a 5-day interval (20 to 25 DAT), the maximum increment of height occurred in the NFT system (2.7 cm), whereas in the DFT system (without and with salt stress) the increments did not exceed 1.0 cm. At ECsol of 2.57 dS/m and regardless of the hydroponic system, the means did not differ statistically, as well as there was no significant difference among the means of the DFT system under different salinity levels (Fig. 2 B).

Differently from the growth variables (Fig. 2 A and 2 B), at 20 DAT, the shoot fresh matter in the DFT system was 26.4% higher than in the NFT system under cultivation conditions without salt stress (ECsol of 2.57 dS/m). In the DFT system even under salt stress conditions (ECsol of 3.43 and 4.75 dS/m), the means of 70.16 and 65.03 g/plant did not differ statistically from that obtained in the NFT system without salt stress (Fig. 2 C). Since the means for shoot dry matter at 20 DAT did not differ statistically (Fig. 2 E), the superiority in the production of shoot fresh matter obtained in the DFT system can be explained by the higher water content in the tissues (Fig. 3 A), since plants responded similarly in terms of number of leaves and height.

In a marked manner, within the interval of only 5-days (from 20 to 25 DAT), the accumulation of fresh matter in plants grown in the NFT system was superior to that recorded during the first 20 days, with growth rate of 16.20 g/day (Fig. 2 D), totaling 144.63 g/plant at 25 DAT (Fig. 2 C). This value was statistical-

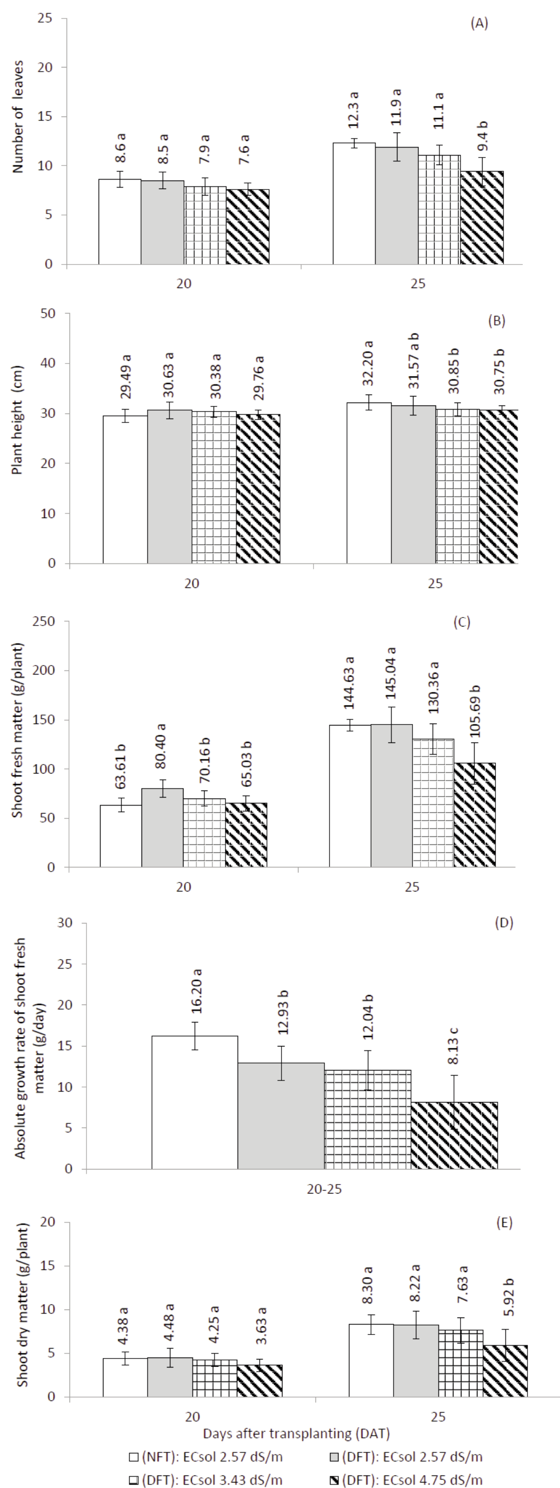

Fig. 2 - Mean number of leaves (A), plant height (B), shoot fresh matter (C), and shoot dry matter (E) of chicory (*Cichorium endivia* L. cv. Dafne) plants in the NFT (without salt stress) and DFT (with and without salt stress) hydroponic systems, at 20 and 25 days after transplanting (DAT) and absolute growth rate of shoot fresh matter during the period 20-25 DAT (D). Means followed by different letters indicate significant differences at 0.05 probability level (Tukey-test). Bars indicate the standard deviations of the means of the eight replicates.

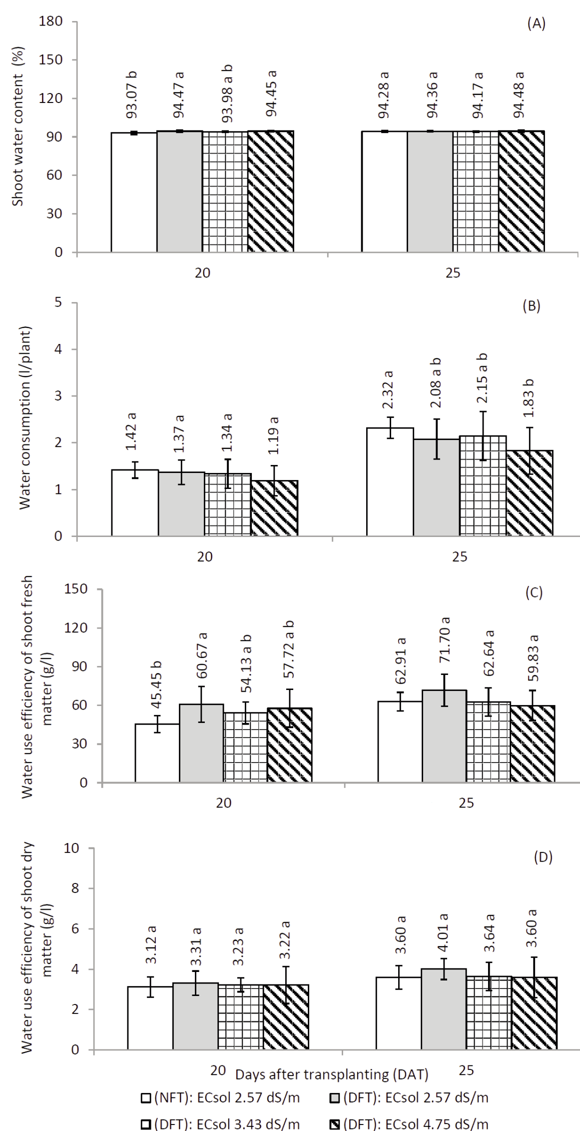

Fig. 3 - Mean water content in shoot (A), water consumption (B) and water use efficiency of shoot fresh matter (C) and shoot dry matter (D) of chicory (*Cichorium endivia* L. cv. Dafne) plants in the NFT (without salt stress) and DFT (with and without salt stress) hydroponic systems, at 20 and 25 days after transplanting (DAT). Means followed by different letters indicate significant differences at 0.05 probability level (Tukey-test). Bars indicate the standard deviations of the means of the eight replicates.

ly similar to the means obtained in the DFT system at ECsol of 2.57 and 3.43 dS/m (145.04 and 130.36 g/plant), with the respective growth rates of 12.93 and 12.04 g/day. At the highest salinity level (ECsol of 4.75 dS/m), the mean of 105.69 g/plant was statistically inferior to those of the other treatments. Based on the results, plants should be harvested at 25 days after transplanting in the hydroponic system, totaling a 50-days cycle from sowing. For the studied cultivar of chicory, on average, the cycle ranges from 45 to 55 days.

At 25 DAT, a similar behavior to that of fresh matter was observed for shoot dry matter (Fig. 2 E). The reduction in shoot dry matter was due to the decrease in the number of leaves and plant height, since the mean water content in the shoots of plants under different treatments did not differ statistically (Fig. 3 A).

Consumption, water use efficiency and water content of the chicory

The F-test of the analysis of variance showed a significant effect of the treatments on the water content in shoot and water use efficiency based on shoot fresh matter for the first evaluation period (20 DAT). For the second evaluation period (25 DAT), there was a significant effect only on the water consumption (Table 2).

For the cumulative water consumption in the period of 20 days, the mean consumption was 1.38 l/plant (Fig. 3 B) and it was not affected by studied treatments. Thus, higher water use efficiency (60.67 g/l) in the DFT system compared to the NFT system (45.45 g/l) (Fig. 3 C) is due to the greater accumulation of fresh matter (Fig. 2 C). In the DFT system, at ECsol levels of 3.43 and 4.75 dS/m, the means of water use efficiency (54.13 and 57.72 g/l) did not differ from those in the condition without salt stress (ECsol of 2.57 dS/m).

At 25 DAT, the water consumption of chicory plants in the DFT system (2.08 l/plant) was similar to that of the NFT system (2.32 l/plant) for the condition without salt stress (ECsol of 2.57 dS/m), with significant reduction only at the highest salinity level (ECsol of 4.75 dS/m) (Fig. 3 B). Within a 5-day interval (20 to 25 DAT), plants increased water use efficiency based on shoot fresh matter, i.e., within five days the consumed water volume was converted to greater biomass accumulation, with overall mean of 64.27 g/l, regardless of the hydroponic systems and salinity levels of nutrient solution (Fig. 3 C). The means of water use efficiency based on shoot dry matter were of 3.22 and 3.71 g/l at 20 and 25 DAT, regardless of hydroponic systems and salinity levels of nutrient solution (Fig. 3 D).

4. Discussion and Conclusions

In the present study (Fig. 1 B), the occurrence of tipburn observed in the NFT system using nutrient solution (ECsol of 2.57 dS/m) prepared in fresh water is due to the higher absolute growth rate significantly higher (16.20 g/day) in comparison to other treatments in the period between 20 and 25 DAT (Fig. 2 D), increasing the demand for calcium. Tipburn symptoms have been reported in other studies with chicory under different conditions of cultivation (Feltrim *et al*., 2008; Sá and Reghin, 2008; Kowalczyk *et al*., 2016 a).

Regarding the visual aspect of chicory plants in the DFT system, with the level of ECsol of 4.75 dS/m there were no symptoms of toxicity due to salinity (Fig. 1 A). In some regions of the world, waters with high salt concentrations are the only source of water available for irrigation of the crops (Cova *et al*., 2020), causing serious problems of toxicity when the concentrations of Na^+ and/or Cl^- inside the plant are sufficiently high (Talhouni *et al*., 2019), resulting in necrosis of older leaves (Parvaiz and Satyawati, 2008; Tavakkoli *et al*., 2010). This occurs because plants virtually lose only water by transpiration, thus leading to accumulation of these ions in the leaves (Acosta-Motos *et al*., 2017; Ismail and Horie, 2017). The time for the damage by toxicity to be manifested depends on the Na^+ and/or Cl^- content in the leaves and on the effectiveness in the compartmentalization of these ions in leaf tissues and cells (Parvaiz and Satyawati, 2008; Giuffrida *et al*., 2013).

The data shown in figure 2 C demonstrate that the production of shoot fresh matter was not affected until 20 DAT, regardless of hydroponic systems and water salinity levels. In the DFT system, plants were supplied with water and nutrients all the time and this favored plants at young age (with smaller size) to produce more fresh matter than those in the NFT system until 20 DAT. After this period, as the volume of roots increased, the oxygen dissolved was depleted more rapidly, thus decreasing the growth rate (Fig. 2 D). With the increase in the volume of roots, there is greater demand for oxygen, according to Kläring and Zude (2009) and Mobini *et al*. (2015); therefore, reductions in oxygen concentrations are expected to occur in the adult stage of the plants (Kiferle *et al*., 2012; Niñirola *et al*., 2014).

The results of present study show that it is possible to produce chicory using brackish waters with reduction of about 27 and 28% in fresh or dry matter, with plants harvested at 25 days after transplanting (Fig. 2 C and 2 E). Such reduction in the yield under salt stress can be compensated by cultivating plants in the system for a longer period, because plants continue to accumulate biomass. Another strategy to compensate the reduction of yield may be by reducing spacing between plants, because under condi-

tions of stress plants occupy a smaller area allowing cultivating more plants per meter length of hydroponic channel as shown by Silva *et al.* (2019) in case of basil. Yet another possibility is to cultivate more than one plant per hole maintaining the spacing of 0.25 m to reach the ideal weight of the bunch for marketing.

In other studies under hydroponic conditions (NFT system), the cultivation of chicory with brackish waters was viable. There was no significant effect on the fresh matter and number of leaves at concentrations of 100 mM (Tzortzakis, 2009) and 40 mM of NaCl (Tzortzakis, 2010) in the nutrient solution, compared to the condition without stress (0 mM of NaCl). The concentration of 30 mM of NaCl did not cause significant effect on the fresh matter and/or number of leaves (Kowalczyk *et al*., 2012; Kowalczyk *et al*., 2016 b).

Chicory plants positively responded to the cultivation in the DFT system adapted in PVC pipes, with a constant 0.02-m depth of nutrient solution (approximately 6.0 l). With this volume of solution in each cultivation channel and assuming mean daily consumption of 0.144 l/plant, if there are interruptions in electricity supply, the system will be able to maintain 15 plants without water restriction for about three days. These results complement other studies which have shown feasibility for the cultivation of different plant species in the DFT system in tubes, such as lettuce (Cova *et al*., 2017), rocket (Campos Júnior *et al*., 2018 b), coriander (Silva *et al*., 2018 a), parsley (Martins *et al*., 2019 a) and chives (Silva Júnior *et al*., 2019). In studies with basil, there was no significant difference in the growth and production variables when plants were cultivated in the DFT and NFT in tubes using nutrient solution prepared in wastewater (Alves *et al*., 2019) and using nutrient solution prepared in fresh water (Santos *et al*., 2019).

The lack of significant effect on water use efficiency based on shoot dry matter, regardless of hydroponic systems and water salinity, demonstrates that the significant differences in shoot fresh matter were due to storage of water in plant tissues (Fig. 3 A). Under salt stress conditions, plants use stomatal closure as a strategy, reducing transpiration due to lower absorption of water (Aroca *et al*., 2012; Moosavi, 2012), which results in increased water use efficiency (Acosta-Motos *et al*., 2017; Morais *et al*., 2018; Soares *et al*., 2018), as reported in various studies under salt stress (Soares *et al*., 2010; Diniz *et al*., 2013; Santos Júnior *et al*., 2013; Soares *et al*., 2015; Lima *et al*., 2017; Coelho *et al*., 2018; Atzori *et al*., 2019 a).

The low water volume used to produce one chicory plant demonstrates high water use efficiency in the hydroponic cultivation, which corroborates with Atzori *et al*. (2019 b) who reported an increase in water use efficiency of chicory in hydroponics compared to conventional soil cultivation. Still in the present study, the quantification of water consumption along the crop cycle can contribute to better planning and use of water resources at places with low water availability because it is possible to estimate in advance the water volume required to produce a certain number of plants within a given period of time. Potentially, in hydroponic cultivation there is greater possibility of using water more efficiently which is not possible in conventional planting.

In conclusion, the data show that the variables of growth, production, water consumption and water use efficiency of chicory under conditions without salt stress (ECsol of 2.57 dS/m) were not significantly affected by the NFT and DFT hydroponic systems. Nutrient solution with salinity of up to 4.75 dS/m prepared in brackish water (NaCl) can be used in chicory cultivation in DFT system, despite small reductions in growth and production, but without any negative effects on the commercial quality of the product.

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Enhancement of Pentacyclic Triterpenoids (Betulinic and Oleanolic acids) production from callus cultures of *Lantana camara* L.

A.M. AL-Ashoush [1], R.A. Shibli [1 (*)], R.W. Tahtamouni [2], T.S. Al-Qudah [3], B.B. Abu-Irarmaileh [3]

[1] *Department of Horticulture and Agronomy, Faculty of Agriculture, University of Jordan, Amman, Jordan.*
[2] *Department of Biotechnology, Faculty of Agricultural Technology, Al-Balqa Applied University, Amman, Jordan.*
[3] *Hamdi Mango Center for Scientific Research (HMCSR), University of Jordan, Amman, Jordan.*

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(*) **Corresponding author:**
r.shibli@ju.edu.jo

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All relevant data are within the paper and its Supporting Information files.

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Abstract: *Lantana camara* L. is an ornamental plant with high medicinal value. This study aimed to investigate the possibility of enhancing the production of betulinic and oleanolic acids in *Lantana* callus by adding different types and levels of chemical elicitors (NaCl, sugars, growth regulators and heavy metals) to Murashige and Skoog (MS) medium. Data revealed that, adding NaCl to the culture medium affected callus fresh weight and color negatively, but it increased the extracted amounts of oleanolic and betulinic acids significantly to reach maximum levels of 0.702 and 0.051 mg/g DW at 120 mM NaCl compared to 0.659 mg/g DW and 0.014 mg/g DW obtained in the control. Meanwhile, increasing glucose level to 36.02 g/l in the medium maximized oleanolic acid accumulation to 0.829 mg/g DW, while betulinic acid accumulation reached 0.038 mg/g DW at 54.03 g/l glucose. In growth regulators experiment, highest callus fresh weight was observed in the control medium, while it declined to the minimum at 0.50 mg/l of Thiadiazuron (TDZ). Maximum values of both acids (0.685 and 0.033 mg/g DW) were recorded in MS medium plus 1.0 mg/l TDZ. Callus fresh weight decreased significantly in response to heavy metals addition, while adding chromium at 0.08 mg/l improved production of oleanolic acid to reach the maximum of 0.676 mg/g DW. Meanwhile betulinic acid was maximized at 0.057 mg/g DW in callus cultures exposed to 0.08 mg/l cobalt.

1. Introduction

Lantana camara L. is a popular flowering ornamental plant (Charitha and Ranwala, 2018), belonging to the family Verbenaceae (Mishra, 2015). It is native to tropical and sub-tropical areas of America (Singh and Saxena, 2016), West Africa (Waoo *et al.,* 2015 a) and tropical Asia

(Ghisalberti, 2000). This plant is routinely used as an evergreen aromatic ornamental or hedge shrub (Bhakta and Ganjewala, 2009; Zoubiri and Baaliouamer, 2012). It's commonly named as Lantana, red sage, Surinam tea plant, Spanish flag and West Indian lantana (Kalita *et al*., 2012).

Lantana camara L. is also listed as one of the most important medicinal plants, as it possesses many distinguished medicinal properties (Waoo *et al.,* 2014). The interest in *Lantana camara* L. has recently, increased because it is an excellent source of many chemical compounds of medicinal potential (Srivastava *et al.,* 2011; Saxena *et al.*, 2012; Waoo *et al.,* 2014) like pentacyclic triterpenoids which includes many active compounds such as betulinic and oleanolic acids (Kensa, 2011; Venkatachalam *et al.,* 2011; Kazmi *et al.,* 2012; Mariajancyrani *et al*., 2014).

Betulinic acid has recently gained increased attention as it possesses a remarkable variety of biological and medicinal properties (Moghaddam *et al.,* 2012; Pandey *et al.,* 2015). Oleanolic acid was also reported to possess many important biological activities (Ghosh *et al.,* 2010; Xia *et al.,* 2011; Singh *et al.,* 2012). So producing such valuable compounds in commercial amounts is of great importance.

In vitro culture was utilized as an efficient approach for production of secondary metabolites. Currently, *in vitro* culture techniques are used to facilitate the possibility of making quantitative and qualitative elicitation of the production of plant secondary metabolites by changing the culturing media composition (Affonso *et al.,* 2007; Alenizi *et al.,* 2020). According to Taiz and Zeiger (2002), adding elevated levels of growth regulators, sugars and heavy metals was found to drive the plant cell machinery to produce more secondary metabolites rather than cell division. For example, Pasqua *et al.* (2005) reported successful enhancement of anthocyanin production in *Camptotheca acuminata* cell cultures by adding different types and levels of growth regulators and sugars to the growth media. In addition, Lee *et al.* (2011) were able to produce more rutin from callus and adventitious roots of white mulberry tree by adding auxins, cytokinins, and nitrogen to the growth medium.

Heavy metals were also found to increase the production of bioactive compounds in many plants (Verpoorte *et al.,* 2002). So, this study was carried out to enhance the possibility of production of betulinic and oleanolic acids in *Lantana* callus cultures by modifying the culture medium using different types and levels of growth regulators, sugars, NaCl, and heavy metals in callus culture media.

2. Materials and Methods

Mother stock culture establishment and maintenance

In vitro grown callus cultures of *Lantana camara* L. were implemented by the Plant Tissue Culture and Microbiology Laboratories at Hamdi Mango Center for Scientific Research (HMCSR). The cultures were subcultured on callus maintenance semi solid medium consisting of 4.4 g/l of Murashige and Skoog (Murashige and Skoog, 1962) MS premix (Duchefa Biochemie) plus 34.2 g/l sucrose in addition to 1.0 mg/l Kinetin and 2.0 mg/l 2,4-D. Cultures were maintained in the growth room under dark conditions at 24± 1°C. Subculturing of callus was performed every 3-4 weeks by subdividing callus under sterile conditions.

Enhancement of betulinic and oleanolic acids production

To study the possibility of enhancing production of betulinic and oleanolic acids in *Lantana camara* L. callus cultures, different types and levels of chemical elicitors (NaCl, sugars, growth regulators, heavy metals) were added to the culture medium.

In NaCl experiment, callus clumps of 1.0 g were subcultured onto fresh MS callus maintenance medium described earlier and supplemented with NaCl at different concentrations (0, 40, 80, 120 and 160 mM). Meanwhile, the effect of sugars on betulinic and oleanolic acids was experimented by transferring 1.0 g of callus clumps onto maintenance medium supplemented with different types and levels of sugars: sucrose (34.2, 68.4 and 102.6 g/l); glucose or fructose (18.01, 36.02 and 54.03 g/l).

In heavy metals experiment, lead (Pb), cobalt (Co) or chromium (Cr) were prepared from their salts; lead nitrate Pb $(NO_3)_2$, potassium chromate (K_2CrO_4), and cobalt (II) chloride ($CoCl_2.6H_2O$) respectively. A stock solution of 100 mg/l was prepared from each salt. After that the requested amount of each heavy metal (0.08, 0.16 and 0.24 mg/l) was added to the callus medium prepared as described earlier.

To test the effect of plant growth regulators thidiazuron (TDZ) and kinetin on betulinic and oleanolic acids production, the explants were transferred onto hormone free MS medium for one week to remove any carry over effect of callus maintenance media. Next, callus clumps of 1.0 g were subcultured onto full strength MS solid medium supplemented with different levels (0.5, 1.0 and 1.5 mg/l) of either TDZ

or Kinetin in combination with 2.0 mg/l 2,4-D.

All cultures were maintained in the growth room under dark conditions at 24±1°C, and data were collected after 6 weeks for callus fresh weight and color before oven drying.

Extraction

Calluses from each treatment were oven dried at 50°C for 2 days. Then, the dried callus was ground using liquid nitrogen. Next, 100 mg were taken from the dried matter of each treatment and soaked in 10 ml of methanol and water in a ratio of 1:1. The samples were then placed on a shaker at slow speed for at least 72 hours at room temperature (Hussain *et al*., 2012). The plant extract was filtered and centrifuged at 5000 g for 10 min. The resulting supernatant was dried in a rotary evaporator at 40°C. Next, the dried extract was resuspended in the mobile phase for HPLC analysis.

Preparation of betulinic and oleanolic acids stock solutions and working standards

Betulinic and oleanolic acid stock solutions at concentration of 1000 mg/l were prepared by weighing 5 mg of each in 5 ml volumetric flask, dissolved and brought to volume by methanol HPLC grade. Both stock solutions were stored at 4°C in dark. Working solutions were prepared by serially diluting stock solutions using the mobile phase at concentrations of 800, 400, 200, 100, 50, 25 and 12.5 mg/l. Fresh working standards were prepared daily. Betulinic and oleanolic acids were eluted at 15.8 and 19 min, respectively. Calibration curves were constructed for betulinic and oleanolic acids before starting chemical analysis (r^2= 0.9999).

HPLC instrumentation and conditions

HPLC analysis was performed using a Shimadzu-LC system (Shimadzu Corporation, Kyoto, Japan) equipped with a CBM-20A controller, LC-20AT pump, and SPD-20AV UV/VIS detector Chromatographic separations were achieved using an Agilent Eclipse Plus C18 column and were carried out using an isocratic flow rate of 1 ml/min, a column temperature of 25° C, a mobile phase of acetonitrile: methanol: acidified water (70:20:10 v/v), with pH adjusted to 2.8 using 85% phosphoric acid. The ultraviolet (UV) detection was set at 210 nm. The injection volume was 20 µl of sample solution. Total run time was 19 min for each injection. Data were acquired and processed with LC-Solution software (Shimadzu Corporation, Kyoto, Japan).

Statistical analysis

Each treatment was arranged in a complete randomized design (CRD) and replicated ten times with two callus clumps/replicate. HPLC analysis was conducted on three replicates for each treatment with two samples/replicate. The collected data were statistically analyzed using SPSS analysis system. The analysis of variance (ANOVA) was used and mean separation was done according to the Tukey's HSD at probability level of 0.05.

3. Results

Effect of NaCl

After 6 weeks of incubation under different levels of NaCl, the obtained results indicated that, callus fresh weight and color (callus quality) declined in response to NaCl and the effects became more severe as NaCl level increased (Table 1). The maximum fresh weight 8.6 g was recorded in the control, while brownish yellow callus with minimal fresh weight value of (2.6 g) was obtained on the medium supplemented with 160 mM NaCl (Table 1).

On the other hand, adding NaCl to the growth medium enhanced the production of oleanolic acid in the callus cultures in response to NaCl level reaching the maximum level of 0.702 mg/g DW at 120 mM

Table 1 - Effect of NaCl on callus fresh weight, color and levels of oleanolic and betulinic acids in callus culture of *Lantana camara*

NaCl (mM)	Weight (g)	Color	Oleanolic acid (mg/g DW)	Betulinic acid (mg/g DW)
Control	8.60 ± 0.36 a *	White	0.659 ± 0.011 d	0.014 ± 0.0003 e
40	5.90 ± 0.35 b	White	0.668± 0.0028 c	0.021± 0.0531 d
80	4.70 ± 0.59 bc	Yellow	0.674± 0.0124 b	0.043 ± 0.0081 b
120	4.11 ± 0.32 c	Yellow	0.702 ± 0.0088 a	0.051 ± 0.0083 a
160	2.62 ± 0.53 d	Yellow to brown	0.656± 0.0096 d	0.035± 0.0057 c

* Values represent means ± standard error. Control: represents callus maintenance medium consisting of solid MS medium + 34.2 g/l sucrose+ 2.0 mg/l 2, 4-D + 1.0 mg/l kinetin. Means with different letters are significantly different according to Tukey HSD range test at P≤0.05.

NaCl compared to 0.659 mg/g DW extracted from the control treatment (Table 1). Also, the production of betulinic acid was enhanced by adding NaCl. The maximum value 0.051 mg/g DW was obtained at 120 mM NaCl compared to 0.014 mg/g DW extracted from the control (Table 1).

Effect of sugars

Sucrose. Our results revealed that, increasing sucrose concentration in the MS media led to a dramatic decline in fresh weight and quality of *Lantana camara* L. callus cultures (Table 2). Fresh weight of the white callus decreased significantly from 8.6 g in control treatment to reach the minimum (3.1 g) at 102.6 g/l sucrose where it turned dark brown (Table 2). Meanwhile, in response to level of sucrose, we observed significant increases in oleanolic and betulinic acids production. The maximum amounts of oleanolic acid (0.97 mg/g DW) and betulinic acid (0.035 mg/g DW) were recorded in media with 102.6 g/l of sucrose. This level of sucrose enhanced the production of betulinic and oleanolic acids in callus cultures to levels that exceeded values of those extracted from naturally growing plants (Table 2). On the other hand, callus was not healthy and the growth was very limited in all treatments compared to the control (Table 2).

Glucose. Adding glucose resulted in a significant decline in callus growth and quality as shown in Table 2. Instead increasing glucose level in the medium resulted in increasing level of oleanolic acid to reach the maximum at 36.02 g/l glucose (0.829 mg/g DW) compared to control (0.659 mg/g DW). Meanwhile, betulinic acid accumulation increased to 2.5 times when 54.03 g/l glucose was added to the media compared to the control (0.014 mg/g DW) as shown in Table 2.

Fructose. The obtained data indicated that, fructose was not a good choice for *Lantana camara* L. callus cultures in terms of growth, color and secondary metabolite production. Callus fresh weight decreased gradually with increasing fructose levels in the medium and minimum fresh weight of the brown callus (1.55 g) was obtained on 54.03 g/l fructose (Table 2). Meanwhile, adding fructose to the culture medium at levels higher than 18.01 g/l negatively affected oleanolic accumulation compared to the control, and the same for betulinic acid (Table 2).

Effect of plant growth regulators

Effect of Kinetin. Data obtained revealed a significant increase in fresh weight (8.6 g) at Kinetin concentration of 1.0 mg/l (Table 3). Meanwhile maximum amounts of oleanolic acid and betulinic acids (0.67 and 0.021 mg/g DW) were extracted from callus cultures grown on media supplemented with low rate of kinetin (0.5 mg/l), while the amount of fresh weight and both acids declined significantly as kinetin level increased in the media (Table 3).

Table 2 - Effect of sucrose; glucose and fructose on callus fresh weight, color and production of betulinic acid and oleanolic acid in callus culture of *Lantana camara*

Carbohydrate (g/l)	Weight (g)	Color	Oleanolic acid (mg/g DW)	Betulinic acid (mg/g DW)
Sucrose				
Control (34.2)	8.60 ± 0.36 a*	White	0.659± 0.011 c	0. 014 ± 0.0003 c
68.4	3.21 ± 0.16 b	Yellow	0.824 ± 0.0082 b	0.027± 0.013 b
102.6	3.11 ± 0.32 b	dark brown	0.970± 0.0081 a	0.035± 0.0291 a
Glucose				
Control	8.60 ± 0.36 a	White	0.659± 0.011 c	0.014 ± 0.0003 c
18.01	4.49 ± 0.197 b	Yellow	0.765± 0.0093 b	0.025 ± 0.0084 b
36.02	3.80 ± 0.56 bc	Yellow start to be brown	0.829± 0.0089 a	0.036± 0.0189 a
54.03	3.00 ± 0.46 c	Yellow start to be brown	0.764± 0.0077 b	0. 038 ± 0.0095 a
Fructose				
Control	8.60± 0.36 a	White	0.659 ± 0.011 a	0.014 ± 0.0003 a
18.01	4.40 ± 0.22 b	Yellow	0.661 ± 0.0035 a	0.012 ± 0.0066 ab
36.02	2.34 ± 0.47 c	Yellow	0.514 ± 0.0092 b	0.010 ± 0.0090 b
54.03	1.55 ± 0.13 d	Brown	0.380 ± 0.0060 c	0.010± 0.0089 b

* Values represent means ± standard error. Control: represents callus maintenance medium consisting of solid MS medium + 34.2 g/l sucrose+ 2.0 mg/l 2, 4-D + 1.0 mg/l kinetin. Means with different letters are significantly different according to Tukey HSD range test at P≤0.05.

Table 3 - Effect of different growth regulators in combination with 2.0 mg/l of 2, 4-D on callus fresh weight, color and production of betulinic and oleanolic acids in callus culture of *Lantana camara*

Growth regulator (mg/l)	Weight (g)	Color	Oleanolic acid (mg/g DW)	Betulinic acid (mg/g DW)
Kinetin				
0.5	4.63 ± 0.55 b*	White	0.672 ± 0.0091 a	0.021 ± 0.0067 a
1.0 (control)	8.60 ± 0.36 a	White	0. 659 ± 0.011 b	0.014 ± 0.0003 b
1.5	4.72 ± 0.44 b	Yellow	0. 621 ± 0.0001 c	0.014 ± 0.0010 b
TDZ				
Control	8.60 ± 0.36 a	White	0.659 ± 0.011 c	0.014 ± 0.0003 c
0.5	1.40 ± 0.14 c	Yellow	0. 651 ± 0.0088 d	0.025±0.023 b
1.0	3.44 ± 0.63 b	White	0.685 ± 0.0082 a	0.033±0.009 a
1.5	3.68 ± 0.23 b	White	0.668±0.0045 b	0.012± 0.0072 c

* Values represent means ± standard error. Control: represents callus maintenance medium consisting of solid MS medium + 34.2 g/l sucrose+ 2.0 mg/l 2, 4-D + 1.0 mg/l kinetin. Means with different letters are significantly different according to Tukey HSD range test at P≤0.05.

Effect of TDZ. The highest fresh weight of callus (8.6 g) was obtained in the control treatment followed by that obtained on 1.5 mg/l TDZ in combination with 2.0 mg/l 2,4-D (Table 3). However, the resulted callus was of good quality as it remained white in all treatments levels (Table 3). Moreover, a significant increase in oleanolic acid and betulinic acids was obtained in response to TDZ, and maximum values of both acids 0.685 and 0.033 mg/g DW were recorded in callus cultures grown in MS medium plus 1.0 mg/l TDZ, while adding higher levels of TDZ negatively impacted on oleanolic and betulinic acids (Table 3).

Effect of heavy metals

Effect of cobalt. Callus fresh weight and color were negatively affected by adding cobalt to the media at levels higher than 0.08 mg/l. Minimum weight of 5.32 g was recorded at Co level of 0.24 mg/l (Table 4). On the other hand, callus remained healthy white despite of Co addition at all levels (Table 4, Fig. 1). Meanwhile, amounts of oleanolic acid were negatively affected by adding Co to the media at all levels compared to the quantity extracted from the control (Table 4). On the other hand, production of betulinic acid was significantly improved by adding Co to the medium. At 0.24 mg/l

Table 4 - Effect of heavy metals on callus fresh weight, color and production of betulinic and oleanolic acids in callus culture of *Lantana camara*

Heavy metal (mg/l)	Weight (g)	Color	Oleanolic acid (mg/g DW)	Betulinic acid (mg/g DW)
Cobalt				
Control	8.60 ±0.36 a *	White	0.659 ± 0.011 a	0.014 ± 0.0003 d
0.08	6.87 ± 0.59 ab	White	0.501 ± 0.0066 b	0.020 ± 0.0003 c
0.16	5.41 ± 0.71 b	White	0.472 ± 0.0046 c	0.033± 0.0072 b
0.24	5.32 ± 0.75 b	White	0.215 ± 0.0082 d	0.057± 0.0102 a
Lead				
Control	8.60± 0.36 a	White	0.659 ±0.011 a	0.014 ± 0.0003 b
0.08	3.80 ± 0.76 b	White with red spots	0.344 ± 0.001b	0.020 ± 0.009 a
0.16	3.42 ± 0.90 b	Yellow with red spots	0.343 ± 0.006 b	0.021 ± .0001 a
0.24	1.57 ± 0.10 b	Yellow with red spots	0.117± 0.0085 c	0. 016 ± 0.008 b
Chromium				
Control	8.6 ±0.36 a	White	0.659 ± 0.011 c	0.014 ± 0.0003 a
0.08	3.60 ± 0.54 b	Yellow with brown spots	0.676 ± 0.001 a	0.013 ± 0.0003 a
0.16	2.81 ± 0.66 b	Yellow with brown spots	0.665 ± 0.0083 b	0.010 ± 0.0085 b
0.24	0.99 ± 0.13 c	Yellow with brown spots	0.435 ± 0.0071 d	0.010± .0069 b

* Values represent means ± standard error. Control: represents callus maintenance medium consisting of solid MS medium + 34.2 g/l sucrose+ 2.0 mg/l 2, 4-D + 1.0 mg/l kinetin. Means with different letters are significantly different according to Tukey HSD range test at P≤0.05.

it was four times higher than the control as shown in Table 4.

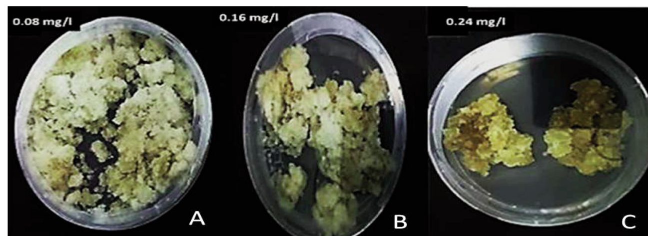

Fig. 1 - Effect of cobalt on callus fresh weight and color in callus culture of *Lantana camara* L. after 6 weeks of incubation in A medium containing 0.08; B medium containing 0.16 and C medium containing 0.24 mg/l cobalt.

Effect of lead. Data revealed that, callus growth, color and oleanolic acid production decreased dramatically with increasing Pb level in the media (Table 4, Fig. 2). The minimal value for fresh weight (1.57 g) was recorded in cultures grown in media supplemented with 0.24 mg/l Pb. Furthermore, adding Pb to the medium determined the appearance of red spots on the white callus at all Pb levels (Table 4, Fig. 2). Meanwhile, production of betulinic acid was improved significantly by adding either 0.08 or 0.16 mg/l Pb while it tended to decline at 0.24 mg/l Pb (Table 4).

Effect of chromium. The results obtained showed that callus growth was negatively affected by adding Cr in the medium (Table 4). Furthermore, adding Cr to the medium resulted in development of brown spots on the white callus at all levels (Table 4). Moreover, chromium had improved the production of oleanolic acid to reach the maximum (0.676 mg/g DW) at 0.08 mg/l, while it tended to decline at higher Cr levels (Table 4). Meanwhile, amounts of betulinic acid decreased in response to Cr to reach minimum level of (0.010 mg/g DW) at either 0.16 or 0.24 mg/l, respectively (Table 4).

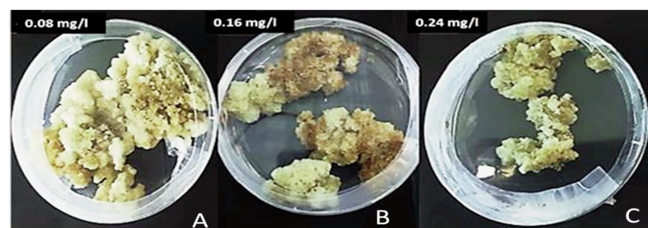

Fig. 2 - Effect of lead on callus fresh weight and color in callus culture of *Lantana camara* L. after 6 weeks of incubation in A medium containing 0.08; B medium containing 0.16 and C medium containing 0.24 mg/l lead.

4. Discussion and Conclusions

Effect of NaCl

Adding NaCl to the culture medium resulted in reduction in most growth parameters in callus cultures of *Lantana camara* which agrees with Nikman, *et al.* (2004) and Nikman *et al.* (2006) studies on *Nicotiana* seedlings and on calli and seedlings of two *Trigonella* species on culture media supplemented with elevated levels of NaCl, where growth and quality of the cultures declined sharply at levels high levels. Generally, quality parameters including color, declined in tissue cultured plant material of many plant species when exposed to osmotic stress due to addition of salts or sugars to the media (Taiz and Zeiger, 2002). This could be due to the continuous accumulation of osmotica, dehydration and phenolic compounds accumulation that results in a shift in color toward brown which might determine, with time, cell death.

Meanwhile, enhanced accumulation of oleanolic and betulinic acids in *Lantana camara* callus cultures was obtained at high NaCl levels agrees with Parida and Das (2005) who reported that salt stress might induce the production of secondary metabolites to maintain cell turgidity and to reduce dehydration. Also, Wang *et al.* (2015) confirmed in their study on cotton that salinity stress resulted from adding NaCl to the growth medium induced the production of some secondary metabolites such as gossypol, flavonoids and tannin.

Effect of sugars

It was clear from our data, that increasing sugar concentration in the medium inversely affected callus fresh weight and color. Elevated levels of sugars would make them act as osmotic agents instead of energy sources, and this would hinder growth rate of the explants (Tahtamouni *et al.,* 2016). Consequently cell volume would decrease as a result of low turgor pressure which would be translated in a form of growth reduction (Taiz and Zeiger, 2002).

Meanwhile, according to our results increasing sucrose level in the medium led to significant increase in production of oleolinic acid and betulinic acid. Generally, high levels of sucrose were reported to increase the secondary metabolite production in plant tissue cultures (Bandhakavi and Kamarapu, 2016). Similar results were also recorded in different species such as, grape berries (Dai *et al.,* 2014), *Ginkgo biloba* (Park *et al.*, 2004), and *Clematis pitcher* (Kawa-Miszczak *et al.,* 2009).

Enhancement of secondary metabolites production in response to glucose was also obtained by Verma *et al.* (2012) in their study about improving alkaloid content in callus culture of *Catharanthus roseus.*

Effect of plant growth regulators

Callus fresh weight was influenced by the level of Kinetin in the medium. This was in full agreement with Singh and Saxena findings (2016) where maximum callus growth was obtained when 2, 4-D was used in combination with Kinetin to induce callusing in yellow variety of *Lantana camara* L.

Effect of heavy metals

Our results showed that, increasing levels of the experimented heavy metals in the culture medium led to a gradual decline in callus fresh weight and color. A decline in cultures growth in response to addition of chromium to the medium was reported by Waoo *et al*. (2015 a) who observed that increasing chromium in the medium decreased shoot length and percentage of survival in *Lantana camara* L. Similar response was also reported in Waoo *et al*. (2015 b) study about the toxic effects of different lead concentrations on *in-vitro* grown shoot cultures of *Lantana camara*.

Our results had also indicated that production of oleanolic and betulinic acids varied with type and level of the added heavy metal. Chemical elicitation was commonly combined with osmotic stress that usually reduces growth (Taiz and Zeiger, 2002). In many studies, increasing biosynthesis of secondary metabolites by stressing the plant using chemical and/ or physical elicitation agents was possible. But adding such elicitors usually resulted in decreasing biomass of the plant, and consequently the levels of secondary metabolites was unchanged or declined, although the concentration is strongly enhanced at tissue or cell level (Gershenzon, 1984; Selmar and Kleinwächter, 2013; Paulsen and Selmar, 2016). Therefore, although biosynthesis of these compounds increased significantly in plant material like callus cultures or microshoots (Paulsen and Selmar, 2016) due to chemical elicitation, the total biomass sharply decline because of the negative effects of the elicitors. So, the total biosynthesis of secondary metabolites is dramatically decreased.

It can be concluded from our study that enhancement of oleanolic and betulinic acids production in *Lantana camara* callus cultures is possible by adding some types of chemical elicitors to the culture medium. Meanwhile, obtaining enough plant material is crucial to guarantee sustainable production of secondary metabolites in commercial amounts. This would open the gate for conducting research to improve our methodology to maintains proper amounts of plant material with enhanced levels of both acids.

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Seasonal enzymatic and non-enzymatic antioxidant responses in seven Iranian pomegranate cultivars

A.R. Bonyanpour [(*)], **B. Jamali**
Crop and Horticultural Science Research Department, Fars Agricultural and Natural Resources Research and Education Center (AREEO), Shiraz, Iran.

Key words: abiotic stress tolerance, antioxidant, *Punica granatum.*

Abbreviation: APX= Ascorbic peroxidase; CAT= Catalase; GSH= Glutathione; MDA= Malondialdehyde; MDG= Malas Daneh Ghermez; MMS= Malas Momtaz Yazd; SK= Shishe Kab; POD= Peroxidase; ZA= Zagh Aghda; SOD= Superoxide dismutase; SSF= Shirin Shahvar Fars; ZAA= Zard Anar Arsenjan; NB= Naderi Badrood.

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[(*)] **Corresponding author:**
arbonyanpour@yahoo.com

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Abstract: The present study was carried out as bifactorial in a completely randomized block design to compare seasonal changes of antioxidant response (enzymatic and non-enzymatic) in seven Iranian pomegranate cultivars ('Malas Mommtaz Saveh', 'Shishe Kab', 'Zagh Aghda', 'Naderi Badroud', 'Malas Daneh Ghermez', 'Shirin Shahvar Fars' and 'Zard Anar Arsenjan') for a deeper understanding of their physiological differences and selecting probable more tolerant and adaptable cultivars to environmental conditions. Uniform and healthy rooted (one-year) plants of seven Iranian commercial pomegranate cultivars were purchased from a commercial nursery and planted in an orchard site in Arsenjan region (one of the main hubs of pomegranate growing), Fars province, Iran. After full establishment of the trees, three rounds of sampling (fresh leaves) were conducted in spring, summer and fall. Results indicated that in summer, activity of enzymatic antioxidants and leaf content of non-enzymatic antioxidants (glutathione, α-tocopherol and total polyphenols) rose in comparison to the contents measured in the other seasons in all studied cultivars. Significant differences were observed among pomegranate cultivars for these parameters and also content of leaf pigments. 'Shishe Kab' was evaluated as a potential tolerant cultivar with high accumulation to changing environmental conditions, since this cultivar demonstrated the highest leaf content of non-enzymatic antioxidants, chlorophyll a/b ratio and lowest level of lipid peroxidation in warmest days of summer. Similarly, 'Zard Anar Arsenjan', 'Shirin Shahvar Fars' and 'Malas Daneh Ghermez' were evaluated as adaptable cultivars to regional conditions.

1. Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit-bearing decidu-

ous shrub or small tree native of Iran (Ebtedaie and Shekafandeh, 2016). Historical evidence reveals that the primary origin of pomegranate is Iran and that it has been spread from this region to other areas. A large number of pomegranate varieties can be found in Iran, more than 760 original, wild and decorative cultivars (Mousavinejad *et al*., 2009). With a production of 700,000 tons/year, Iran is the world's leading producer (Sarkhosh *et al*., 2009). Because of its high acclimation, pomegranate tree grows well in wide range of climates and soil conditions (Galindo *et al*., 2014).

Iran, with an annual precipitation of 200 mm, is considered as a dry country and due to global warming and climate change, abiotic stresses such as drought and salinity are predicted to intensify in near future (Ebtedaie and Shekafandeh, 2016). Planting fruit trees which are low water consumers such as pomegranate can be a suitable strategy for cultivating arid and semiarid regions (Greenwood *et al*., 2010; Jiménez *et al*., 2010).

One of the main steps in orchard establishment is selection of suitable cultivars. Previous investigations indicate varied levels of acclimation potential and tolerance to abiotic stress conditions among different pomegranate cultivars (Tabatabaei and Sarkhosh, 2006; Okhovatian-Ardakani *et al*., 2010; Ibrahim, 2016). That would be attributed to their varied enzymatic and non-enzymatic antioxidant potential in response to seasonal changes in environmental conditions (Jamali *et al*., 2016). Maintenance of a high antioxidant capacity to scavenge the toxic reactive oxygen species (ROSs) has been linked to increased acclimation of plants to environmental stresses (Sharma *et al*., 2012). ROSs are produced in plants as byproducts during many physiological and biochemical processes such as photosynthesis and respiration. Generation of ROSs causes rapid cell damage by triggering a chain reaction (Ahmad *et al*., 2010). Naturally occurring antioxidants in plant cells include: enzymatic and peptide defense mechanisms, non-enzymatic mechanisms, phenolic defense compounds, nitrogen compounds, carotenoids and chlorophyll derivatives. Both the enzymatic and non-enzymatic antioxidants play an important role as natural antioxidants (El-Missiry, 2012)

Previous literatures focused on comparison between differences in enzymatic and non-enzymatic antioxidants in various Iranian pomegranate cultivars are limited and more investigations seem necessary. The goal of present study was to compare seasonal changes in enzymatic and non-enzymatic antioxidant responses of seven Iranian commercial pomegranate cultivars for a deeper understanding of their acclimation to environmental and regional conditions.

2. Materials and Methods

Uniform and healthy rooted plants of seven Iranian cultivars of *Punica granatum* L. were purchased from a commercial nursery and planted in a completely randomized block design with 3 replications (each replication had 3 plants) with 3-meter distance in rows and 5-meter distance between rows in an orchard site in Arsenjan region (hub of pomegranate growing and production in Fars), Fars province, southern Iran. Average annual climate parameters in the experimental region were: precipitation (200 mm), relative humidity (Max: 55%, Min: 23%), temperature (Max: 42°C, Min: 4°C). All the cultivars were growing in same soil conditions and were irrigated in a similar way (Drip irrigation). Routine cultural practices suitable for commercial fruit production were carried out during the experimental period.

Cultivars included: Malas Momtaz Saveh (MMS), Naderi Badroud (NB), Malas Daneh Ghermez (MDG), Shirin Shahvar Fars (SSF), Zagh Aghda (ZA), Shishe Kab (SK) and Zard Anar Arsenjan (ZAA). After 4 years and full establishment of the plants, samples (fresh leaves) were taken from the trees. Leaf samples were taken from different orientations of the trees (north, south, west and east); 25 fully expanded mature leaves from each side of all trees (100 leaves per tree as bulk samples) were samples in liquid N_2 and transported to laboratory. Leaves were taken from shoots without terminal fruit. Leaves with abnormal symptoms such as chlorosis and mechanical lesions caused by pests or diseases were avoided. Leaf samples were taken at 3 times (June 2nd, August 10th and October 10th) during the growing season; temperature and humidity of experimental region at the time of leaf sampling are given in Table 1.

Table 1 - Average day and night temperature and relative humidity in the experiment region at the time of sampling

Date of sampling	Average day/night temperature (°C)	Relative humidity (%)
June 2nd	27/14	45
August 10th	38/25	23
October 10th	27/15	27

The following parameters were measured in studied cultivars for two consecutive years and an average was reported.

Enzymes extraction

For enzymes extraction, leaves (0.5 g) were ground to fine powder in liquid nitrogen with mortar and pestle and then homogenized in 2 mL extraction buffer (50 mM potassium-phosphate buffer, pH 8.0), 10% (w/v) polyvinylpyrrolidone (PVP), 0.1 mm ethylenediaminetetra acetic acid (EDTA), 1 mM dithiothreitol (DTT). The homogenate was centrifuged (15000 × *g*) at 4°C for 30 min. Then, the supernatants were collected.

Superoxide dismutase activity

The activity of SOD was determined by adding 0.1 mL of the enzymatic extract to a tube containing 13 mM l-methionine, 25 mM nitro-blue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM sodium carbonate and 2 mM riboflavin in a 50 mM phosphate buffer in pH 7.8 (Dhindsa *et al.,* 1980). Tube was placed under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as control. Reaction was stopped by switching off the lights and keeping the tubes in dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme that reduced the absorbance reading to 50% in comparison with tubes lacking enzyme. SOD activity was expressed as units per milligram of protein per minute.

Catalase activity

Catalase (CAT, EC 1.11.1.6) activity was measured spectrophotometrically according to the method of Chance and Maehly (1955), by monitoring the decline in absorbance at 240 nm due to H_2O_2 consumption. One milliliter of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was initiated by adding 50 µL of crude extract to this solution. CAT activity was expressed as units (µmol of H_2O_2 consumed per minute) per milligram of protein.

Peroxidase activity

The activity of guaiacol peroxidase (POX) was determined by adding 50 µl of the crude enzyme preparation to 2 ml of a solution containing 50 mM potassium phosphate buffer (pH 7.0)., 13 mM guaiacol and 5 mM H_2O_2.

Increase in absorbance due to oxidation of guaiacol (extinction coefficient: 26.6 $mM^{-1} \cdot cm^{-1}$) was monitored at 470 nm for a minute. Peroxidase activity was expressed as units (µmol guaiacol oxidised per minute) per milligram of protein.

Ascorbate peroxidase activity

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured spectrophotometrically according to Nakano and Asada (1981) by following the decline in absorbance at 290 nm due to ascorbate oxidation. The oxidation rate of ascorbate was estimated between 1 and 60s after starting the reaction with the addition of H_2O_2. One milliliter of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.15 mM H_2O_2, 0.1 mM EDTA and 50 µL of enzyme extract. APX activity was expressed as units (µmol of ascorbate oxidized per minute) per milligram of protein.

Protein content

Protein content was determined according to Bradford (1976) by using bovine serum albumin as a standard. For preparation of Bradford reagent, 100 mg of Coomassie Brilliant Blue G-250 weight and then dissolved in 50 mL 0f 95% ethanol. Then, 100 ml of 85% orthophosphoric acid (H_3P0_4) added to aforesaid solution and volume reached into1000 mL volume with distilled water. Bradford reagent filtered using Whatman paper to remove precipitates before use. for assessment the protein content, 5 mL of Bradford reagent and 100 µL protein extraction added in the test tube and shaken vigorously for a few seconds. Reaction mixture remained at ambient temperature for 5 min and absorbance was read using spectrophotometer at 595 nm. Bovine serum albumin (BSA) was used to elaborate a standard curve. The protein content was calculated according to the obtained equation.

Proline content

Proline was extracted and its concentration determined by the method of Bates *et al*. (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged (3000 × *g*) for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for one hour and then absorbance at 520 nm was determined. Contents of proline are expressed as $\mu mol \cdot g^{-1}$ fresh weight.

Leaf chlorophyll and carotenoids content

Leaf discs (0.5 g) were extracted in 5 mL of acetone (80%), then centrifuged (8000 × g) for 10 minutes. The supernatant was used to make a final volume of 100 mL of the leaf extract. Extraction of leaf tissue with the buffer continued until decoloration.

Absorbance of the extract was read at 470, 645 and 663 nm with a spectrophotometer and 80% acetone was used as a blank. Finally, chlorophyll (a and b) and carotenoids contents were calculated according to the following equations (Lichtenthaler, 1987):

$$\text{Chl a (mg. g}^{-1}\text{ fresh weight): }[(12.25A_{663} - 2.79A_{645}) \times v / 1000 \times W]$$
$$\text{Chl b (mg. g}^{-1}\text{ fresh weight): }[(21.50A_{645} - 5.10A_{663}) \times v / 1000 \times W]$$
$$\text{Chla + Chlb (mg. g}^{-1}\text{ f.w.): }[(7.15A_{663} + 18.71A_{645}) \times v /1000 \times W]$$
$$\text{Carotenoids (mg. g}^{-1}\text{ f.w.): }1000A_{470}\text{-1.82Chla- 85.02Chlb / 198}$$

where Chla = chlorophyll a; Chlb = chlorophyll b; Chla+Chlb = total chlorophyll; A = absorbance at ƛ (nm).

Leaf anthocyanins content

Total leaf anthocyanins were measured spectrophotometrically by pH differential method (Lee *et al*., 2005) with two buffer systems: potassium chloride buffer (pH 1.0,0.025 M) and sodium acetate buffer (pH 4.5, 0.4 M). Leaf samples (0.5 g) were extracted with 2 mL methanol: water: concentrated HCl solution (80:20:1 v/v/v). 0.4 mL of leaf extract was mixed with 3.6 mL of corresponding buffers and read against water as blank at 510 and 700 nm. Absorbance (A) was calculated as

$$A = (A_{510} - A_{700}) \text{ pH 1.0} - (A_{510} - A_{700}) \text{ pH 4.5}$$

Then total anthocyanins content was calculated using the equation:

$$\text{Anthocyanin (µg. g}^{-1}\text{ fresh weight)} = (A \times \text{Mw} \times \text{DF} \times 1000) / \varepsilon$$

Where *A* is the absorbance of the diluted sample and DF is the dilution factor (10), Mw is molecular weight of cyanidin-3-glucoside (449.2) and ε= 26,900 l/mol.cm, the molar extinction coefficient of cyanidin-3-glucoside.

Glutathione content

Glutathione (GSH) was estimated by the method of Moron *et al*. (1979). Two hundred mg of leaf tissue was homogenized in 2 mL of ice-cold 5% trichloroacetic acid. The homogenate was then centrifuged at 4°C at 17000 × *g* for 30 min. A volume of 75 µl of the clear supernatant was added to a cuvette containing 300 µl of phosphate buffer (0.2 M, pH 8.0) and 750 µL of 0.6 mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid) in phosphate buffer. The absorbance at 412 nm was read and glutathione content was derived against a standard curve prepared with known amounts of GSH in 5% trichloroacetic acid.

Malondialdehyde content

Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction as described by Ali *et al*. (2005), with slight modifications. Two hundred mg leaf samples were homogenized with 2 mL of 0.1% trichloroacetic acid and centrifuged at 10000 × *g* for 15 min. One mL of the supernatant was mixed with 2.5 mL 0.5% thiobarbituric acid in 20% trichloroacetic acid and incubated in hot water (95°C) for 30 min. Thereafter, it was cooled immediately on ice to stop the reaction and centrifuged at 10000 × *g* for 30 min. Absorbance at 532 and 600 nm was determined, and MDA concentration was estimated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm, using an absorbance coefficient of extinction (155 $mM^{-1} \cdot cm^{-1}$).

Leaf total polyphenols content

Leaf polyphenols content was determined with Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound. In brief, 1 g of lyophilized leaf samples were placed in an Eppendorf tube, with 1 mL of methanol (80%), grinded at 4°C and centrifuged at 10000 × *g* for 15 min. The extract was mixed with 0.5 mL of Folin-Ciocalteu reagent (diluted 1:1 with water) then 1 mL of a 5% sodium carbonate solution was also added. After 30 min, absorbance was measured at 725 nm and expressed as mg · g^{-1} fresh weight.

Leaf α-tocopherol content

α-Tocopherol was extracted according to Chong *et al*. (2004). Two hundred mg lyophilized sample was homogenized in 1 mL acetone with a prechilled mortar and pestle at 4°C. Following the addition of 0.5 mL hexane, the homogenate was first vortexed for 30 s, then centrifuged at 1000 × *g* for 10 min. The upper hexane layer was removed while the acetone layer containing vitamin E remained in the vial. A second aliquot of 0.5 mL hexane was added, and the extraction process was repeated at least twice. α-Tocopherol was estimated by the method of Kanno and Yamauchi (1997). A 0.4-ml aliquot of 0.1% (w/v) 3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4-triazine was added to 0.2 mL of pooled extract. The volume was made up to 3 mL with absolute ethanol, 0.4 mL 0.1% (w/v) ferric chloride ($FeCl_3 \cdot 6H_2O$) was added, and the content was gently mixed under dim light in a dark room to avoid photochemical reduction. After a 4 minutes reaction at room temperature, 0.2 mL 0.2 M orthophosphoric acid was added and the mixture left for another 30 min. Absorbance was determined at 554 nm spectrophotometrically and reported as µg. g^{-1} fresh weight. The blank was prepared in the same manner except that absolute ethanol was used instead of the sample. α-Tocopherol (Sigma Chemical) was used as a standard.

Leaf ascorbic acid content

Ascorbic acid was estimated by the method of Omaye *et al*. (1979). Briefly, to 1 g of lyophilized leaf sample, 10% ice-cold trichloroacetic acid was added and centrifuged for 20 min at 3500 × g in room temperature. One mL of the supernatant was mixed with 0.2 mL of DTC reagent and incubated for 3 hours at 37°C. Then 1.5 mL of ice-cold 65% H_2SO_4 was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 minutes. The color developed was read at 520 nm spectrophotometrically and Leaf ascorbic acid content reported as µg. g^{-1} fresh weight.

Statistical analysis

The experiment was carried out as a 7×3 bifactorial in a completely randomized block design (seven pomegranate cultivars as first factor and three sampling dates as second factor). Data were analyzed by SAS and means were compared using Duncan's multiple range tests at 5% probability level.

3. Results

The activity of SOD, CAT, POD and APX in the studied pomegranate cultivars is reported in Table 2. SK and MMS showed the highest SOD activity. No significant difference was observed among NB, SSF and ZAA. The maximum POD activity (50.41 units mg^{-1} protein) was found in NB which was significantly higher than other cultivars. Activity of this enzymatic antioxidant was not different in MMS, SK and MDG. The highest CAT activity was detected in SSF (27.18 units mg^{-1} protein), however ZAA, NB and MMS were not statistically different. Activity of CAT was not significantly different in SK and ZA compared to MDG. APX activity in ZAA was higher in comparison to the other studied cultivars. Activity of APX was not statistically different in SK and MDG.

Leaf contents of some non-enzymatic antioxidants in studied pomegranate cultivars are presented in Table 3. The highest leaf glutathione content (158.57

Table 2 - SOD, CAT, APX and POD activity in the leaves of different pomegranate cultivars

Cultivars	SOD (mg^{-1} protein min^{-1})	CAT (mg^{-1} protein)	APX (mg^{-1} protein)	POD (mg^{-1} protein)
MMS	76.72± 6.08 a	23.37± 2.54 ab	7.02± 0.66 d	38.56± 6.74b
NB	67.25± 8.25 bc	26.15± 1.34 ab	7.25± 0.66 d	50.41± 1.52a
MDG	63.02± 7.96 cd	19.07± 1 c	11.29± 1.20 b	37.83± 5.78b
SK	76.17± 3.48 a	22.76± 2.02 bc	11.19± 1.45 b	36.60±2.02 b
SSF	68.55± 6.56 bc	27.18± 2.90 a	9.50± 1.45 c	28.38± 4.58d
ZA	58.82± 8.76 d	19.98± 2.72 c	7.28± 0.66 d	24.30± 2.72e
ZAA	70.21 ± 7.02 ab	24.04± 2.40 ab	13.90± 0.88a	32.58± 1.15 c

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated mean ± standard error (n = 3).

Table 3 - Leaf concentration of some non-enzymatic antioxidants and Malondialdehyde (MDA) in seven Iranian cultivars

Cultivars	Glutathione (µg g^{-1} f.w.)	Proline (µg g^{-1} f.w.)	Ascorbic acid (µg g^{-1} f.w.)	α-tocopherol (µg g^{-1} f.w.)	Polyphenol (mg g^{-1}f.w.)	MDA (µg g^{-1} f.w.)
MMS	142.2±2.8 c	6.04±0.5 b	3.07±0.84 b	210.66±14.8 d	26.84±0.5 ab	32.99±1.15 a
NB	147.5±4.61 b	3.26±0.1 d	2.55±0.2 c	199.3±8.3 f	18.77±0.77 e	29.01±1.63 b
MDG	138.74±10.1 d	4±0.1 cd	2.48±0.13 c	172.6±19.47 g	22.42±1.0 d	26.01±0.87 c
SK	158.57±4.0 a	7.35±0.3 a	3.58±6.26 a	269.66±7.8 a	27.98±0.66 a	23.23±0.82 d
SSF	140.19±11.5 cd	5.92±0.08 b	2.48±0.13 c	172.6±19.47 g	23.55±1.11 cd	23.99±0.72 cd
ZA	128.36±5.7 f	4.62±0.3 c	3.01±0.2 b	204±10.12 e	18.77±0.77 e	30.32±0.54 ab
ZAA	134.28±4.5 e	6.98±0.32 ab	2.92±0.3 b	229.33±12.34 b	25.31±0.5 bc	24.42±2.08 cd

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated mean ± standard error (n = 3).

µg g^{-1} fresh weight) was observed in SK which was significantly higher than other cultivars. No significant difference was detected between MDG and SSF. Leaf total polyphenols in SK was 32% higher compared to NB, MMS was not significantly different in comparison to SK. Also, ZA, SSF and MDG were not different. SK demonstrated the highest leaf proline content (7.35 µmol g^{-1} fresh weight), however ZAA was not statistically different in comparison to this cultivar. Leaf ascorbic acid content was significantly higher in SK compared to other studied cultivars. This parameter was not statistically different in MMS, ZA, SSF and ZAA. Leaf α-tocopherol content in SK (269.66 µg g^{-1} fresh weight) was the highest among the studied cultivars. The highest MDA content was found in MMS (32.99 µmol g^{-1} fresh weight), however, ZA was not statistically different.

Seasonal changes of SOD, CAT, POD and APX in studied pomegranate cultivars are presented in figure 1 (a-d). SOD activity rose in summer in MMS, ZA, NB, SSF and ZAA. In fall, activity of this antioxidant enzyme decreased significantly in ZAA, SSF and ZA compared to summer. Higher activity of POD was observed in MMS, SK, ZA, NB, MDG and ZAA in summer in comparison to spring. This characteristic was not statistically different in summer compared to spring in SSF. A significant decline in POD activity in fall compared to summer was detected in MMS, SK, ZA and NB. CAT activity did not change significantly during spring, summer and fall in MMS, ZA, NB, MDG, SSF and ZAA. Activity of this enzyme decreased significantly in SK (about 37%) in summer. Rise in activity of APX was observed in MMS, SK and ZAA in summer. This parameter was not different in MMS, ZA, NB, MDG, SSF and ZAA in fall compared to summer.

Changes in content of some non-enzymatic antioxidants in studied pomegranate cultivars in spring, summer and fall are demonstrated in Fig. 2 (a-e). In all studied pomegranate cultivars leaf content of glutathione rose significantly in summer, and then declined in fall. The highest content of this non-enzymatic antioxidant (182.21 µg g^{-1} fresh weight) was detected in SK in summer. Leaf total polyphenols increased significantly in all studied cultivars in summer, whereas the content decreased in fall in MMS, SK, ZA, MDG, SSF and ZAA this characteristic decreased in fall. Proline in MMS, SK and ZA was higher in summer compared to spring and fall in NB, MDG, SSF and ZAA did not change significantly during spring, summer and fall. Ascorbic acid content increased (about 56%) in MDG in summer compared to spring then decreased significantly in fall. In contrary in MMS ascorbate content declined (34%) in summer then rose in fall. Other cultivars showed no significantly changes during these seasons. In all cultivars leaf α-tocopherol increased in summer then

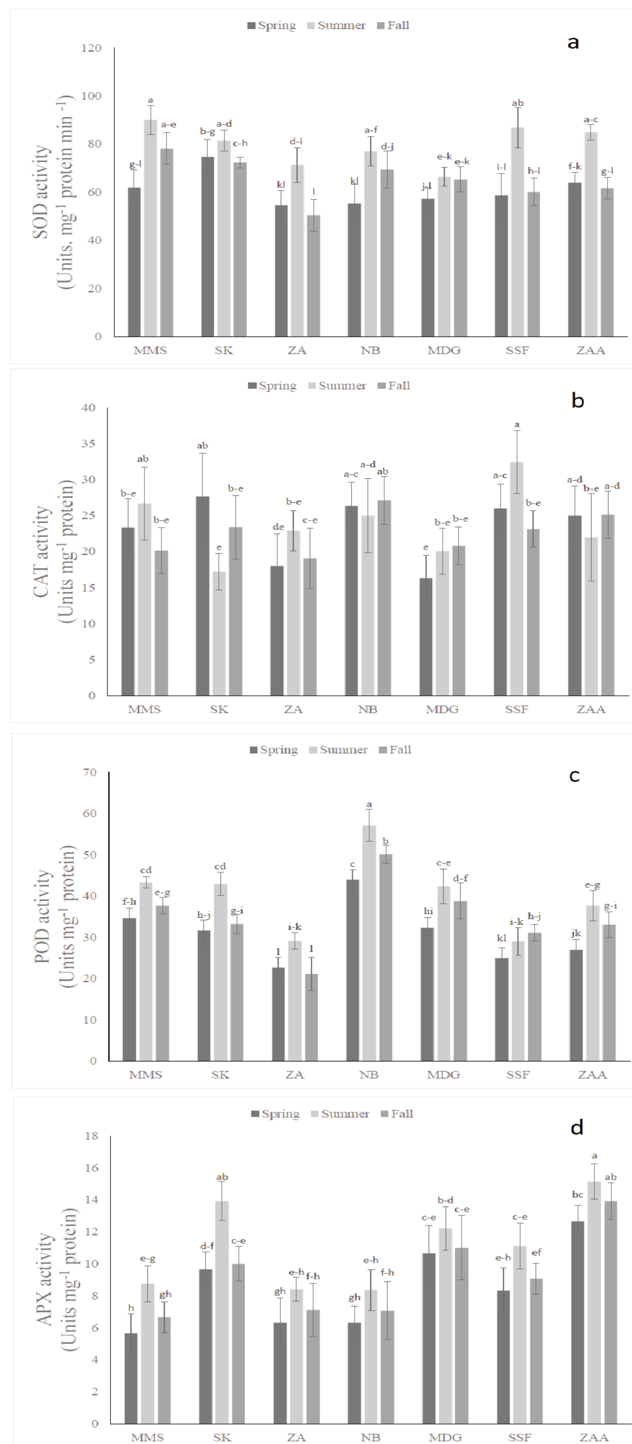

Fig. 1 - Seasonal activity changes of SOD (a), CAT (b), POD (c) and APX (d) in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

decreased in fall. This characteristic in MMS, ZA, NB, MDG and SSF was higher in fall in comparison to spring. ZAA and SK leaf α-tocopherol content was not statistically different in spring and fall.

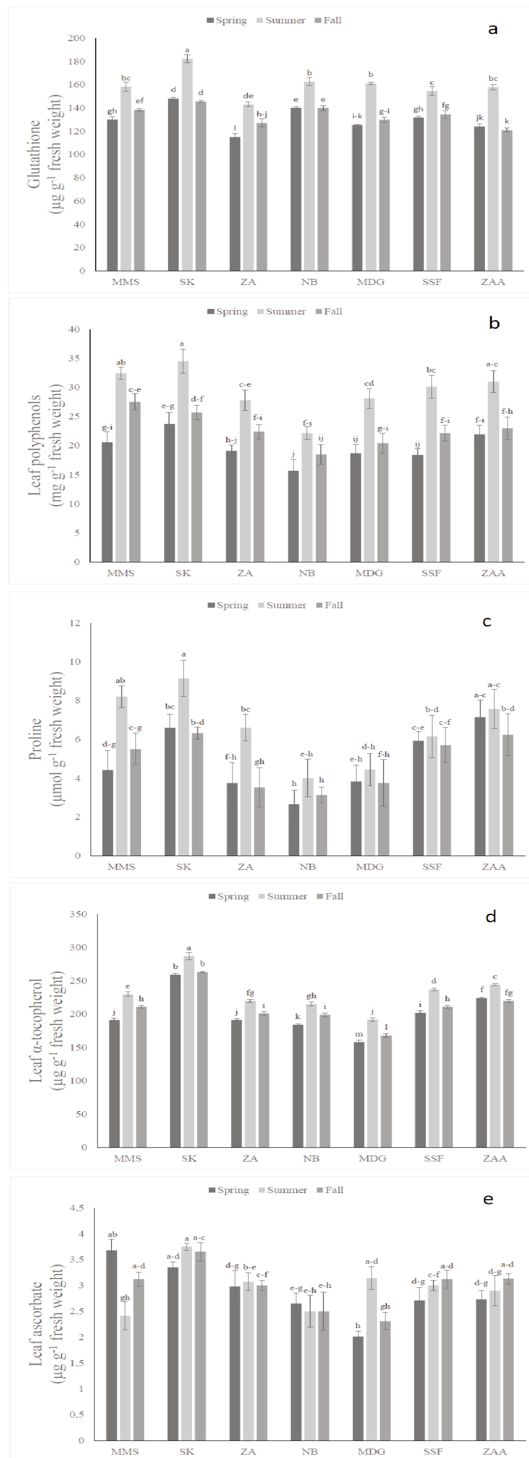

Fig. 2 - Seasonal changes in content of glutathione (a), polyphenols (b), proline (c), α-tocopherol (d) and ascorbic acid (e) in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

The highest MDA content was detected in summer, this parameter was higher in fall compared to spring. Leaf total proteins decreased significantly in summer (Fig. 3). Leaf MDA content increased in summer in all studied cultivars, and the rise was more pronounced in ZA, NB and MMS compared to SK, SSF and ZAA. In the former three cultivars MDA was higher than spring (Fig. 3).

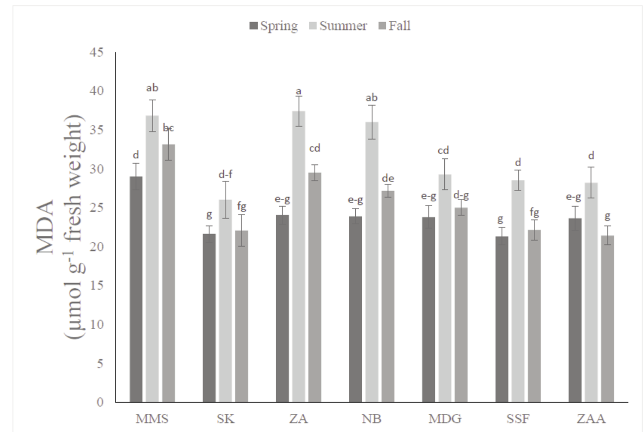

Fig. 3 - Seasonal changes in MDA content in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

Leaf pigments content in studied cultivars are indicated in Table 4. The highest total chlorophyll content was observed in SSF, but SK was not statistically different. Also no significant difference was observed between MDG and ZAA. The highest leaf anthocyanin (0.31 µg g^{-1} fresh weight) and carotenoid (0.34 mg g^{-1} fresh weight) were obtained from SK. No significant difference was observed between ZAA and SSF for these two parameters. Chlorophyll a/b ratio was significantly higher in SK and ZAA compared to other cultivars. NB and ZAA had significantly higher total leaf proteins in comparison to other cultivars. No significant difference was observed between SK, ZA, MDG and SSF.

Leaf total chlorophyll content was higher in spring in comparison to summer and fall. In summer the highest levels of anthocyanin and carotenoid contents were detected. Chlorophyll a/b ratio was significantly higher in summer or fall compared to spring (Fig. 4 a-d).

Changes in leaf pigments in spring, summer and fall in studied cultivars are shown in Fig. 4 (a-d). Leaf total chlorophyll content decreased in summer then rose in fall in all cultivars. In SK, ZA, NB, MDG, SSF and ZAA Leaf total chlorophyll was not statistically different in fall compared to spring. Leaf anthocyanin

and carotenoid content rose in all studied cultivars in summer. In fall leaf anthocyanin decreased in SK and ZA, similar decline in leaf carotenoid was observed in MMS, SK, ZA, NB and ZAA. Chlorophyll a/b ratio increased in MM, ZA and NB, decreased in ZAA and did not change in SK, MDG and SSF in summer. This parameter was not different in fall compared to spring in all cultivars.

4. Discussion and Conclusions

In present study mean activity of SOD, POD and APX rose significantly in summer similar to the concentration of non-enzymatic antioxidants. During summer months solar irradiance intensity and regional mean day temperature rise. Drought, salinity, high temperatures and UV-B radiation lead to enhanced generation of ROSs in plants due to disruption of cellular homeostasis (Mittler, 2002). Scavenging or detoxification of excess ROSs is achieved by an efficient antioxidative system comprising of the non-enzymatic as well as enzymatic antioxidants (Noctor and Foyer, 1998). Rise in activity of SOD, POD and APX as well as increase in concentration of non-enzymatic antioxidants in summer were a part of protective responses of studied plants. However, significant differences were observed

Table 4 - Leaf pigments and protein concentration in seven Iranian cultivars

Cultivars	Anthocyanin (µg g^{-1} f.w.)	Chlorophyll (mg g^{-1} f.w.)	Chlorophyll a/b ratio	Carotenoid (mg g^{-1} f.w.)	Proteins (mg g^{-1} f.w.)
MMS	0.29±0.01 b	1.17±0.59 c	1±0.02 d	0.24±0.006 d	19.62±0.95 c
NB	0.26±0.007 c	1.09±0.07 d	1.10±0.03 c	0.23±0.007 de	21.35±0.25 a
MDG	0.26±0.002 c	1.21±0.17 bc	1.17±0.01 b	0.27±0.009 c	20.32±1.25 b
SK	0.31± 0.002 a	1.34±0.06 a	1.23±0.01 a	0.34±0.008 a	20.48±0.98 b
SSF	0.29±0.01 b	1.36±0.04 a	1.15±0.008 b	0.31±0.004 b	20.62±1.59 b
ZA	0.25±0.006 c	1.03±0.06 e	1.07±0.03 c	0.22±0.005 e	20.48±0.40 b
ZAA	0.29±0.01 b	1.27±0.1 b	1.22±0.038 a	0.30±0.006 b	21.27±0.68 a

Means followed by the same letters within columns are not different at 5% probability using Duncan's test. All data indicated mean ± standard error (n = 3).

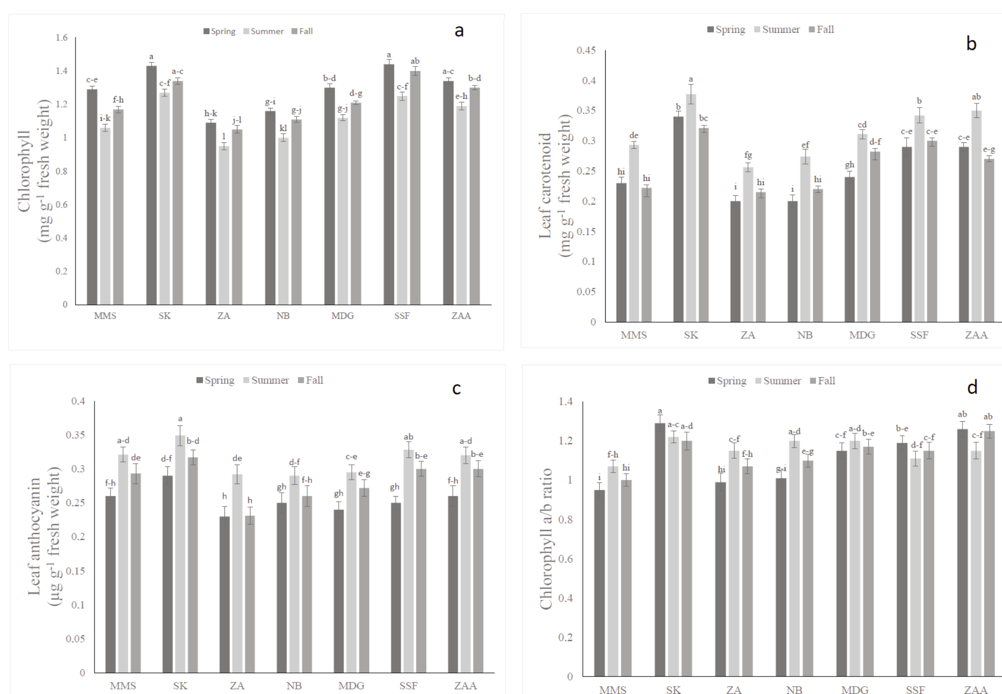

Fig. 4 - Seasonal changes in content of chlorophyll (a), carotenoid (b), anthocyanin (c), chlorophyll a/b ratio (d) in studied cultivars. Columns with different letters represent significant differences at 5% probability using Duncan's multiple range test, those are valid for all columns.

between cultivars.

Increased activity of SOD is often correlated with increased adaptability of the plants to environmental conditions (Zaefyzadeh *et al*., 2009; Sharma *et al*., 2012). High temperatures or drought cause either enhancement or depletion of CAT activity depending on the plant species (Sharma and Dubey, 2005; Han *et al*., 2009). CAT activity decreased in SK and did not change significantly in other cultivars in summer; similar results have been reported by Gholami *et al*. (2012). APX scavenges the H_2O_2 produced by SOD using ascorbate as the electron donor (Noctor and Foyer, 1998). Decline in ascorbate level in MMS in summer can be contributed to this process. However, this parameter did not change in SK in summer which can be due to higher tolerance of this cultivar to high temperature. Overexpression of a cytosolic APX-gene derived from pea in transgenic tomato plants ameliorated oxidative injury induced by chilling and salt stress (Wang *et al*., 2005). Similarly, over expression of the tApx gene in either tobacco or in *Arabidopsis* increased tolerance to oxidative stress (Yabuta, 2002).

Most of non-enzymatic antioxidants such as polyphenols, glutathione, proline and α-tocopherol rose in summer in our study concomitant with increase in mean day temperature and solar irradiance. This was in agreement with previous works. (Ghorbanali *et al*., 2012; Sivaci and Duman, 2014)

Glutathione is one of the most important cellular antioxidants (Sharma *et al*., 2012). It also plays an indirect role in protecting membranes by maintaining α-tocopherol and zeaxanthin in the reduced state (Hasanuzzaman *et al*., 2013). Glutathione accumulates to high concentrations, especially under stress conditions. Increase in glutathione concentrations during stress offsets stress initiated oxidation of glutathione and causes changes in gene expression directly or through interaction with regulatory proteins and/or transcription factors. This increase is equally important in signal transduction and defense against ROSs (Hasanuzzaman *et al*., 2013). Glutathione is a potential scavenger of $^1O_2$, H_2O_2 and $^{\bullet}OH$ (Gholami *et al*., 2012). Additionally, this tripeptide plays a key role in the antioxidative defense system by regenerating another potential water-soluble antioxidant like ascorbic acid (Foyer and Halliwell, 1976). Endogenous glutathione concentration has been reported to be associated with salt stress tolerance (Sumithra *et al*., 2006). Kattab (2007) reported that *Brassica napus* seed priming with glutathione improved seedling resistance probably by enhancing the activities of antioxidant enzymes.

Polyphenols have strong antioxidant properties and their presence at an elevated level is associated with increased abiotic stress tolerance. Increase in concentration of polyphenolic compounds following abiotic stress conditions such as high temperature has been reported by various authors in different species (Jamali *et al*., 2016). Bautista *et al*. (2016) compared the levels of total phenolic compounds and antioxidant flavonoids in a relatively large number of plant species from different families growing under varied environmental conditions. Their data strongly support a general and relevant role of these compounds in mechanisms of acclimation to environmental and regional conditions.

Proline accumulation can serve as a selection criterion for the tolerance and adaptability of most species (Parida and Das, 2005; Ashraf and Foolad, 2007; Ahmad *et al*., 2009). In addition to its role as a compatible osmolyte and osmoprotectant, several studies have attributed an antioxidant feature to this amino acid, suggesting ROSs scavenging activity and proline acting as a $^1O_2$ quencher (Smirnoff and Cumbes, 1989; Matysik *et al*. 2002). It has been reported that proline protects higher plants against osmotic stresses not only by adjusting osmotic pressure but also by stabilizing many functional units such as complex II electron transport, membranes, and proteins and enzymes such as rubisco (Hamilton and Heckathorn, 2001).

Several lines of evidence indicate that α-tocopherol plays a major role in plant stress tolerance, keeping an adequate redox state in chloroplasts (Munne-Bosch, 2005). Deficiency of this antioxidant leads to a slightly increased susceptibility to photo oxidative stress (Kanwischer *et al*., 2005). Other mechanisms such as increases in abscisic acid concentration under stress conditions are also known to enhance α-tocopherol (Singh *et al*., 2011).

In our study, ascorbic acid did not change significantly during spring, summer and fall. The increase of glutathione pool during summer could be necessary to regulate the levels of ascorbate (Foyer and Theodoulou, 2001).

Significant difference in concentration of leaf pigments was observed between cultivars and seasons in the present study. A decrease in the chlorophyll concentration in summer is a typical symptom of oxidative stress (Egert and Tevini, 2002). In addition to harvesting solar energy, carotenoids play protection roles keeping integrity of photosynthesis apparatus against photo oxidative damages by scavenging

free radicals (Andrade-Souza *et al*., 2011). Carotenoids are precursor of ABA which is an important phyto-hormone regulating plant responses to stresses. Presence of higher carotenoid concentration leads to lower photo-oxidative damage and higher potential for regulating plant growth under stress conditions (Han *et al*., 2008). Our results were in accordance with previous works (Christie *et al*., 1994; Chalker-Scott, 1999; Parida and Das, 2005). Plant tissues with a higher content of anthocyanins usually have a higher resistance to drought. The purple cultivar of pepper is more tolerant to water stress than the green cultivar (Bahler *et al*., 1991). The higher ratio of chlorophyll a/b which was observed in some of cultivars was considered to be the result of a decreased emphasis on light collection in relation to the rates of PSII photochemistry (Demmig-Adams and Adams, 1996).

It was suggested that SOD can be used as an indirect selection criterion for screening drought-resistant plant materials (Zaefyzadeh *et al*., 2009). Significant differences were observed for the measured parameters between studied pomegranate cultivars. SK cultivar is well adapted to changing environmental conditions since this cultivar had higher activity of SOD and elevated levels of non-enzymatic antioxidants compared to other cultivars. The presence of these compounds at higher concentration probably mitigated overproduction of ROSs in summer. This was in accordance with previous studies. Khayyat *et al*. (2014) compared salt tolerance in MMS and SK. They evaluated SK as more salt tolerant cultivar.

In various studies the chlorophyll concentration was used as a sensitive indicator of the cellular metabolic state (Chutipaijit *et al*., 2011). Higher chlorophyll concentration is related to elevated tolerance against abiotic stresses such as drought and salinity (Hasanuzzamn *et al*., 2013). Chlorophyll a/b ratio did not change in SK in summer which could be explained as no decrease of peripheral light-harvesting complexes and a higher stress tolerance in this cultivar (Liu *et al*., 2011). MDA concentration which is an index for lipid peroxidation was the lowest in SK. ZAA, SSF and MDG could be considered as cultivars with high tolerance capacity to abiotic stresses as well, and MYS, ZA and NB as cultivars with intermediate abiotic stress tolerance. Other important characteristics such as macro and micronutrients absorption (data not shown), endogenous plant growth regulators such as auxins, cytokinins and ABA (data not shown) and trees productivity and fruits quality (data not shown) were considered for this evaluation. However, as it was mentioned before, pomegranate cultivars have high compatibility capacity and they grow well in wide range of climates and soil conditions. This is the reason why Ghasemi Soloklui *et al*. (2012) did not find remarkable differences in freezing tolerance between seven pomegranate cultivars in midwinter.

Significant differences were observed between studied pomegranate cultivars for activity of enzymatic antioxidants, leaf content of non-enzymatic antioxidants and leaf pigment contents. SK was possibly evaluated as a cultivar that can acclimate against environmental condition changes extremely. ZAA, SSF and MDG cultivars were ranked following the SK, those may still responses against abiotic stress in a high level. However, MYS, ZA and NB were known as cultivars show stress tolerance moderately.

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Phenolic fingerprint in wild growing pomegranate fruits from Azerbaijan

A. Zeynalova [1], E. Novruzov [1], P. Bartolini [2], C. Brunetti [2 (*)], B. Maserti [2]

[1] *Institute of Botany, Azerbaijan National Academy of Sciences, Badamdar 40, Baku, AZ1004, Azerbaijan.*

[2] *Institute for Sustainable Plant Protection (IPSP), CNR, Via Madonna del Piano, 50019 Sesto Fiorentino (FI), Italy.*

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(*) **Corresponding author:**
cecilia.brunetti@ipsp.cnr.it

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All relevant data are within the paper and its Supporting Information files.

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Abstract: The demand for pomegranate (*Punica granatum* L.) juices is increasing worldwide due to its documented health-promoting effects which likely derive from phenolic compounds. This study reports the phenolic composition of the juices obtained from eight wild-growing pomegranate accessions collected in eight areas of Azerbaijan, characterized by different climate and soil composition. The anthocyanins found in all the accessions were cyaniding derivatives and pelargonidin derivatives, while only two accessions contained also delphinidin-3,5-O-diglucoside. The main hydrolysable tannins contained in the juices were punicalagin and ellagic acid derivatives. These bio-active metabolites found in the juices varied qualitatively and quantitatively among the eight accessions, thus constituting specific traits for selecting promising accessions that can be used as a nutritious food source. The different phenolic profiles might be determined both by genotype and the growing environmental conditions, or by their interaction. Our results suggest that some of the studied wild-growing pomegranate accessions might have a commercial value because of their richness in bioactive metabolites and might constitute a suitable source of genes for breeding programs.

1. Introduction

Punica granatum L. (pomegranate) originated in the region extending from Iran to northern India has been used since ancient times as fruit and for medical purpose. Recently an increasing of pomegranate cultivation and consumption has been reported because of its beneficial effects on human health (Kalaycıoğlu and Erim, 2017). In particular, the juice of pomegranate shows potent antioxidant (Lee *et al*., 2012; Aloqbi *et al.,* 2016), anti-atherosclerotic (Aviram *et al*., 2000) and anticancer effects (Derakhshan *et al*., 2018), due to its high concentration of punicalagin derivatives, ellagic acid derivatives (both in its free and conjugated forms), gallotannins and anthocyanins. The high concentration of polyphenols in pomegranate extracts has been also related to a strong anti-inflammatory, hepatoprotective and antigenotoxic properties has

been recently investigated (Sartippour *et al.,* 2008; Santhini *et al.,* 2011). Moreover, pomegranate extract has been reported to be beneficial to enhance fruit post-harvest stability due to its antimicrobial and antifungal effects, mainly correlate to the content of punicalagin derivatives (Rongai *et al*., 2018, 2019).

Azerbaijan is one of the richest floristic regions and a major focus in the South-West Asian and it is the origin center of many cultivated plants including pomegranate (Zeynalova and Novruzov, 2017). Pomegranate carries a deep cultural and historical heritage in Azerbaijan and occupies a special place in cuisine and traditional medicine (Karasharli, 1979). In several regions of Azerbaijan, wild pomegranate accessions grow spontaneously in self-maintaining populations in natural or semi-natural ecosystems independently of direct human action. In particular, wild pomegranate accessions can be found at the foothills and in lower mountain zones of the Greater and Lesser Caucasus, in the Kur-Araz and Samur-Divichinsk lowlands and in the mountain belt of the Talysh Plateau, on dry slopes up to 700 m above sea level (Asadov and Asadov, 2001).

Wild plant accessions of cultivated fruits gained attention to scientific community, since they have developed a high adaptability to climatic pressures and resistance to diseases and pests, accumulating high amount of secondary metabolites respect to cultivated varieties (Howard *et al*., 2003; Kassim *et al*., 2009). The genetic background of each accessions plays a key role in the responses to environmental conditions, resulting in accessions more or less sensitive to different environmental pressures (Schwartz *et al*., 2009), however, in most cases, climate effects on arils' composition is substantial since phenol metabolism, both synthesis and oxidation, is induced by stressful conditions, especially thermal stress (Di Stefano *et al*., 2019). At this regards, it has been shown that seasonal warming increases the content of diglucosylated anthocyanins (Borochov-Neori *et al*., 2011).

In the framework of a systematic study on the functional properties of wild pomegranate plants in Azerbaijan, this work reports the phenolic fingerprinting of the juices obtained from wild growing pomegranate accessions collected in eight different areas of Azerbaijan (Zeynalova *et al.*, 2019), as a starting point to select wild-growing suitable genotypes that can be used in breeding programs for the production of fruits and juices with a high content of bioactive metabolites.

2. Materials and Methods

Plant material collection

Fresh ripe pomegranate (*Punica granatum* L.) fruits were collected from seven districts of Azerbaijan. Geographical coordinates and altitude corresponding to each surveyed area are shown in (Fig. 1 and Table S1). The soil of the different areas was analyzed. Pomegranate fruits were collected at the same maturity stage from of 25th September and October 2nd 2018 during a field campaign extensively described in a companion paper (Zeynalova *et al*., 2019). Each accession sample (Pg) was the pool of three fruits from three different trees collected in each sampling station. Details on the physio-chemical and organoleptic characteristics of the fruits are reported in Zeynalova *et al*. (2019).

Juice preparation

After collection, fruit juices were prepared as described by Zeynalova *et al.* (2019). Briefly each fruit was weighed individually, then the arils, peel and calyx membranes were manually divided and juice was extracted by mechanical press, directly from arils to prevent the contamination of phenolic components from the skin or peels membrane into the juice, and then stored at -80°C until HPLC analysis.

Chemicals

All reagents and solvents were of HPLC grade (Sigma Aldrich, Italy). The following standards were used for identification and quantification purposes with HPLC-DAD: cyaniding 3-glucoside, delphinidin 3-

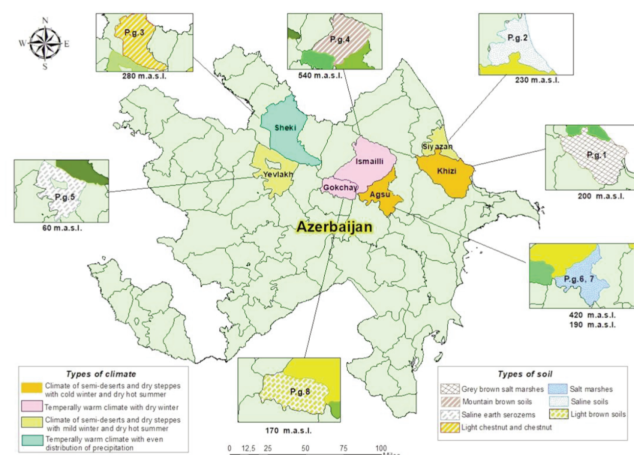

Fig. 1 - Selected sampling stations in Azerbaijan districts. The type climate and of soil of each district is reported. The pomegranate accession harvested in each area are also indicated on the map.

glucoside, pelargonidin 3-glucoside, cyanidin 3-rutinoside (Extrasynthese, Lyon, France); gallic acid (Fluka, Buchs, Switzerland); ellagic acid (Extrasynthese, Lyon, France); punicalagin A+B mixture (Sigma Aldrich, Italy).

Quantification of phenolic compounds by HPLC analysis

The juices obtained from the fruits of the eight pomegranate accessions were directly injected in the HPLC system. HPLC system consisted of Perkin Elmer Flexar HPLC equipped with a quaternary 200Q/410 pump and a LC 200 photodiode array detector. Anthocyanins and non-anthocyanin phenolics were separated and quantified using two different gradient elution conditions, as previously described in Fischer *et al*. (2011). The separation was carried out injecting 10 µL of the juices with analytical Zorbax SB-C18 (5 µm, 250 × 4.6 mm) column (Agilent Technologies, Italy), operating at 30°C and at a flow rate of 0,6 L min^{-1}. Individual phenols were identified comparing UV-spectral characteristics with those of authentic standards and on the basis of the identification reported in Fischer *et al.* (2011). The diode array detector was set at an acquisition range between 200 and 600 nm. Quantification was performed at 520 nm for anthocyanins and at 280 nm for tannins, utilizing calibration curves of corresponding standards.

Statistical analysis

Data corresponds to the juice extracted from a pool of three fruits for each pomegranate accession. Three technical replicates of each sample were analyzed. A one-way ANOVA with the "accession" as factor followed by Tukey post hoc test ($p<0.05$) was used to compare the compound in the different accessions. Heat map and PCA biplot analysis have been performed with ClustVis software (Metsalu and Vilo, 2015).

3. Results

Plant material

The climate was different in the seven districts, ranging from temperate warm climate (Gokchay, Ismailly) to semidesert dry steppes with hot summer (Agsu, Khizi). The sampling area were located at different altitude, ranging from 60 m (Yevlak) to 540 m (Ismailli). In the Agsu district two accessions were collected, one accession in the Agsu mountaın pass, whereas the other accession at 190 m a.s.l. (Fig. 1). Four areas presented salinized soil (Slyazan, Yevlakh and the two sampling points in the district of Agsu). Two areas have a typical mountain humus rich soil (Sheki, Ismailli).

Morphological traits

The shape and dimension of the fruits were very similar, except for Pg7 which showed a higher fruit length. The colors of the extracted juice ranged from a strong red (Pg 2) to light pink (Pg8) (Fig. 2 A, B).

Anthocyanins content in pomegranate juice

Six different anthocyanins were present in the pomegranate juice, namely delphinidin-3,5-O-diglucoside (Del-3,5-diglc), cyanidin-3,5-O-diglucoside (Cya-3,5-digl), cyanidin-3-O-glucoside(Cya-3-glc), cyanidin-3-O-pentoside (Cya-3-pent), pelargonidin-3,5-O-diglucoside (Pel-3,5-digl), pelargonidin-3-O-glucoside (Pel-3-glc) (Table 1). The anthocyanin profiles were significantly different qualitatively and qualitatively among the accessions. The accessions Pg3-8 contained only cyanidin derivatives, namely Cya-3,5-digl, Cya-3-glc and Cya-3-pent.The highest content of these compounds was detected in Pg 5,6,7 (Table 1). The accessions Pg 1 and Pg 2 showed a different anthocyanin composition, characterized by the presence ofDel-3,5-diglc, Cya-3,5-digl, Pel-3,5-digl and Pel-3-glc.The highest concentration Cya-3,5-digl of was determined in Pg1 (152.6±2.21mg L^{-1}) and Pg2 (102.3±1.39mg L^{-1}), whereas the lower level was measured in Pg 4 (5.450±0.63 mg L^{-1}) (Table 1). The compounds Cya-3-pent and Pel-3-glc are present in low concentration in the juices of all the accessions.

Content of hydrolyzable tannins and their derivates in pomegranate juice

Among the hydrolysable tannins, punicalagin α and β isomers, punicalagin derivatives,ellagic acid, ellagic acid glucoside, galloyl-glucose and hexahydroxydiphenoyl (HHDP)-hex-derivative were found in the studied accessions.All the pomegranate accession contained the punicalagin isomer α and β. Punicalagin isomer α concentration ranged from ~8mg L^{-1}inPg2 and Pg8 to 37.52 mg L^{-1}inPg1. The highest content of punicalagin isomer βwas found in Pg1 (61.65 mg L^{-1}), whereas the lowest was in Pg 2 and Pg 8 (~14mg L^{-1}) (Fig. 3).

In the juices obtained from the different pomegranate accessions were found also other punicalagin derivatives (Table 2). These compounds were identified because of their UV/VIS absorption spectrum which showed the maxima at 378 and 258

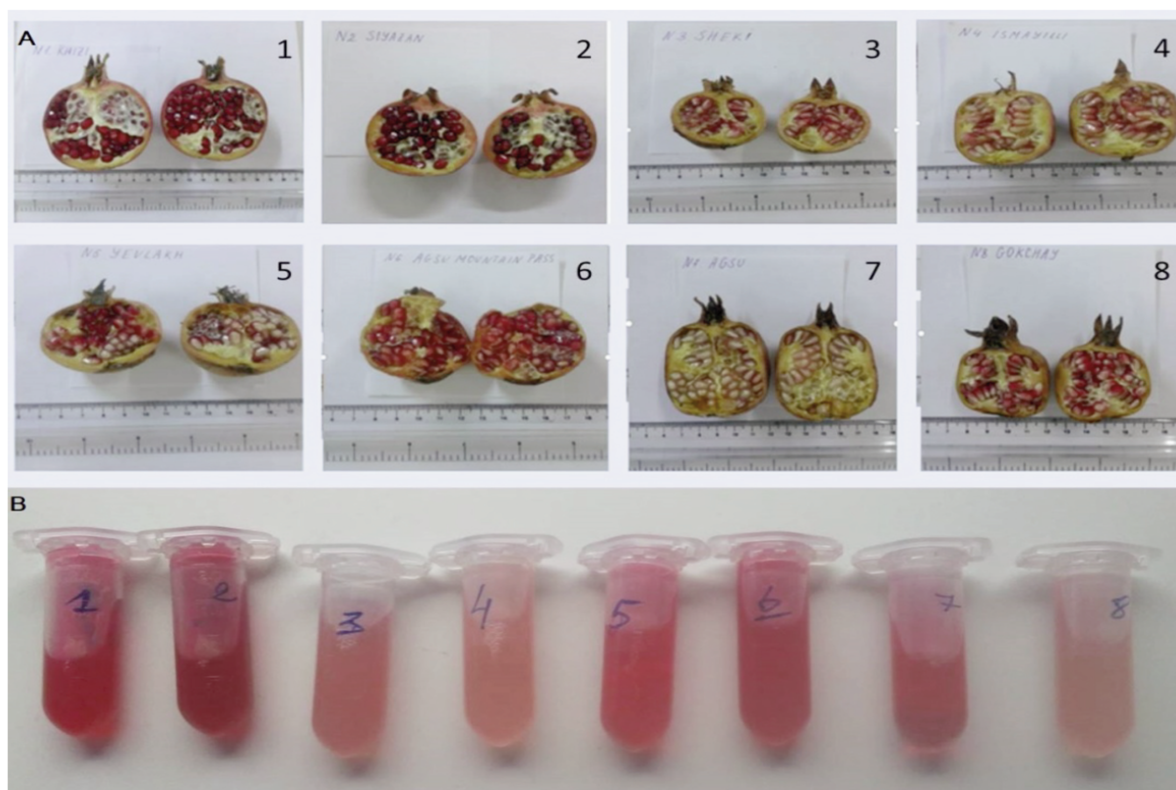

Fig. 2 - A typical fruit harvested in each sampling area (A) and an aliquot of the corresponding extracted juice (B).

Table 1 - Content of anthocyanin derivatives in the juices obtained from pomegranate fruits harvested in each sampling area

Pomegranate accessions	Del-3,5-diglc (mgL^{-1})	Cya-3,5-digl (mgL^{-1})	Pel-3,5-digl (mgL^{-1})	Cya-3-glc (mgL^{-1})	Pel-3-glc (mgL^{-1})	Cya-3-pent (mgL^{-1})
Pg1	75.58 ± 0.46 b	152.6 ± 2.21 a	0.102 ± 0.001 b	-	1.67 ± 0.165 a	-
Pg2	114.7 ± 1.85 a	102.3 ± 1.39 b	80.54 ± 1.20 a	-	0.65 ± 0.03 b	-
Pg3	-	7.910 ± 0.30 e	-	10.20 ± 0.04 d	-	0.551 ± 0.06 e
Pg4	-	5.450 ± 0.63 f	-	5.051 ± 0.08 f	-	0.061 ± 0.003 f
Pg5	-	26.94 ± 2.49 d	-	30.34± 2.85 c	-	2.451 ± 0.26 c
Pg6	-	49.75 ± 17.8 c	-	41.32 ± 5.04 a	-	6.581 ± 0.28 a
Pg7	-	41.12 ± 1.46 c	-	33.53 ± 1.09 b	-	4.663 ± 0.15 b
Pg8	-	7.680 ± 0.61 e		9.52 ± 0.48 e		0.532 ± 0.002 e

Values are reported as mean ± SD (n=3).
Numbers followed by different letters in the same column are statistically different according to Tukey's test ($p \leq 0.05$).
Del-3,5-diglc=delphinidin-3,5-O-diglucoside, Cya-3,5-digl=cyanidin-3,5-O-diglucoside, Cya-3-glc=cyanidin-3-O-glucoside, Cya-3-pent=cyanidin-3-O-pentoside, Pel-3,5-digl=pelargonidin-3,5-O-diglucoside, Pel-3-glc=pelargonidin-3-O-glucoside.

nm, as also observed for punicalagin isomer α and β and previously reported in other studies on pomegrante composition (Gil *et al*., 2000). Punicalagin derivatives 1 and 2 were more abundant in Pg 1, whereas punicalagin derivatives 2 was higher in Pg 6 and Pg 8. Punicalagin derivative 4 was maximum in Pg3 and Pg4, reaching concentration more than 19 mg L^{-1}), whereas the minimum content was found in Pg5 (4.54±0.49mg L^{-1}) (Table 2). Overall, considering the sum of all punicalagin derivatives, the highest content was found in Pg 1.

A significant variation in the content of ellagic acid derivatives was found among the accessions. In particular, the concentration of ellagic acid ranged between 2.01 to 18.81mg $L^{-1}$and increasing from Pg2< Pg1< Pg6 = Pg5< Pg8< Pg7< Pg3=Pg 4. A similar trend was also observed for the elleagic glucoside, whichranged from 2.92 to 27.36 mg L^{-1}, showing the highest values in Pg3 and Pg4 and the lowest in Pg2 (Fig. 4).

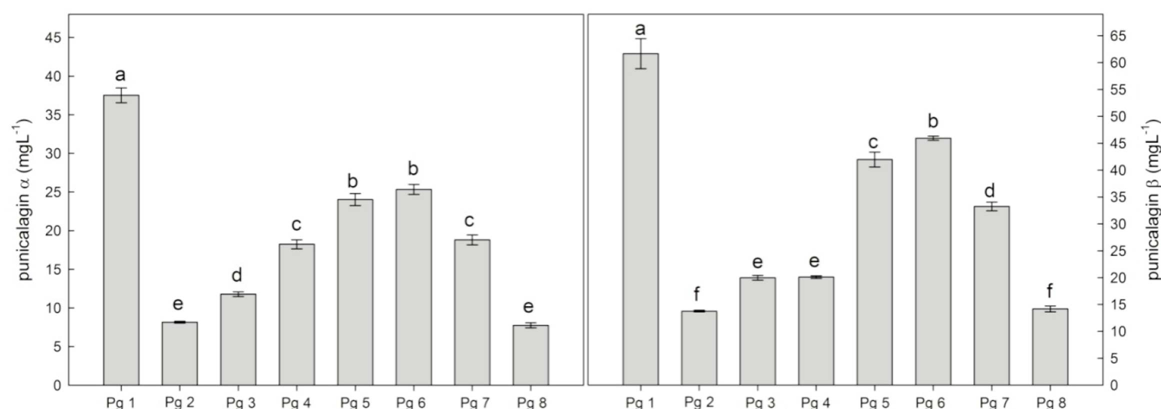

Fig. 3 - Concentration of punicalagin α and β in the juices obtained from pomegranate fruits harvested in each sampling area. The bars indicate the standard deviation (n=3). Different lowercase letters indicate significant differences according to Tukey's test ($p \leq 0.05$).

Table 2 - Content of four punicalagin derivatives in the juices obtained from pomegranate fruits harvested in each sampling area

Pomegranate accessions	Punicalagin derivative_ 1 (mgL^{-1})	Punicalagin derivative_2 (mgL^{-1})	Punicalagin derivative_3 (mgL^{-1})	Punicalagin derivative_4 (mgL^{-1})	Sum of all punicalagin derivatives (mgL^{-1})
Pg1	14.1 ± 0.73 a	21.7 ± 0.18 a	7.94 ± 0.19 b	8.44 ± 0.17 c	151.3 ± 4.66 a
Pg2	9.09 ± 0.03 c	8.17 ± 0.10 c	2.20 ± 0.01 e	6.15 ± 0.13 d	47.54 ± 0.38 f
Pg3	4.12 ± 0.06 d	10.3 ± 0.33 b	8.36 ± 0.18 b	19.7 ± 0.43 a	74.28 ± 1.41 e
Pg4	4.15 ± 0.05 d	10.4 ± 0.10 b	7.49 ± 0.18 b	19.7 ± 0.02 a	80.13 ± 1.13 d
Pg5	1.95 ± 0.09 f	8.72 ± 0.38 c	4.54 ± 0.49 d	4.54 ± 0.49 f	88.93 ± 3.29 b
Pg6	11.6 ± 0.48 b	-	9.66 ± 0.44 a	-	92.51 ± 0.88 b
Pg7	3.07 ± 0.02 e	11.0 ± 0.32 b	6.15 ± 0.18 c	11.3 ± 0.10 b	83.60 ± 1.29 c
Pg8	4.10 ± 0.15 d	6.53 ± 0.25 d	9.27 ± 0.40 a	5.71 ± 0.23 e	47.54 ± 1.90 f

The sum of all punicalagin derivatives include also punicalagin α and β.
Values are the mean ± SD (n =3). Values followed by the different letter in the same column are statistically different according to Tukey's test ($p \leq 0.05$).

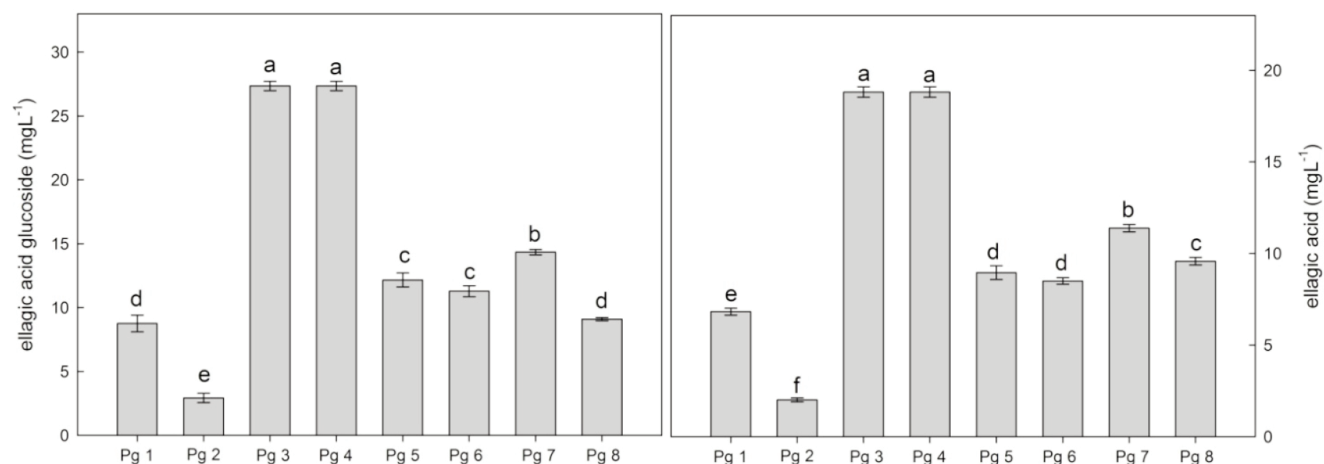

Fig. 4 - Concentration of ellagic acid glucoside and ellagic acid in the juices obtained from pomegranate fruits of the different accessions. The bars indicate the standard deviation (n=3). Different lowercase letters indicate significant differences according to Tukey's test ($p \leq 0.05$).

Other compounds were putatively identified, on the basis of the retention time and their UV/VIS absorption spectrum (Fischer *et al*., 2011), as galloyl-glucose and hexahydroxydiphenoyl-(HHDP)-hex-derivatives (following named HHDP-hex-derivatives). Indeed, the peak of galloyl-glucose showed a typical UV spectra similar to that of gallic acid (λmax = 269 nm), whereas the HHDP-hex-derivatives have similar retention time and λmax to pedunculag in derivatives (λmax = 258 and 375nm) reported in Fischer *et al*. 2011. Galloyl-glucose concentration increased from the minimum in Pg2 (~6 mg L^{-1})to the maximum in Pg7 (~18 mg L^{-1}) (Fig. 5). The concentration of HHDP-hex-derivatives was highest in Pg 1, Pg 3 and Pg 4. In particular, the HHDP-hex-derivative 1 was found in similar content in Pg 3 and Pg 4 (~50mg L^{-1})while HHDP-hex-derivative 2 ranged from 8.44 mg L^{-1}in Pg6 and Pg8 to 20.07 mg L^{-1}in Pg1 (Fig. 5).

4. Discussion and Conclusions

The current study reported interesting differences in thejuice phenolic fingerprinting of eight wild-growing pomegranate accessions harvested in eight areas of Azerbaijan characterized by different climate conditions, soil composition and altitude.

Specific anthocyanin composition of the eight pomegranate accessions

Anthocyanins are natural plant pigments that have beneficial effects for the plantsas well as for humans and animals. The presence of six anthocyanins, 3-*O*-glucosides and 3,5-*O*-diglucoside of delphinidin, cyanidin and pelargonidin, is in agreement with the anthocyanin composition determined in registered Turkish varieties (Türkyilmaz, 2013).The compound Cya-3,5-digl was present in all samples (Ben-Simhon *et al.,* 2015), however the higher value found in Pg1 and Pg2, and moderately in Pg6 and Pg7,accounted for the promininent red color showed by the four accessions respect to the other ones (Fig. 2B and Table 1). These outcomes are in agreement with those reported by Hasnaoui et al. (2011), who found that Cy-3,5-digl was the main anthocyanin in Tunisian pomegranate juice as well as in the Turskish “Izmir 1513” variety (170 mg L^{-1}),which were characterized by a strong red color of the juice (Türkyilmaz, 2013). In addition, in the accessions Pg 1 and Pg 2, the low concentration of Pel-3-glc, a pigment responsible for the orange

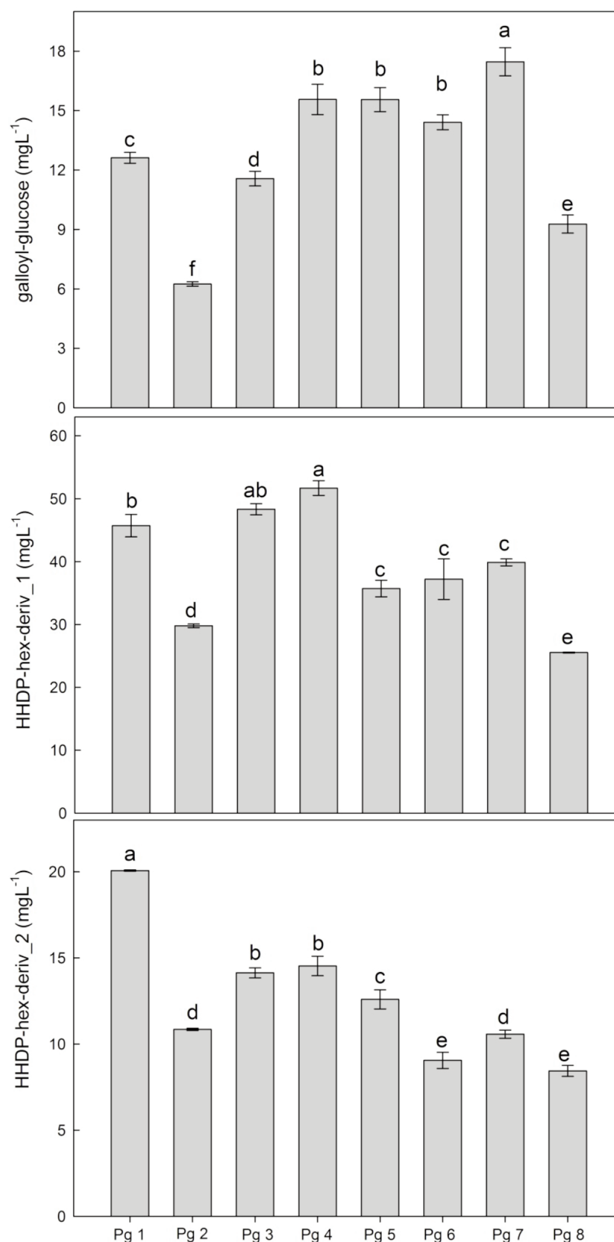

Fig. 5 - Concentration of galloyl-glucose and two HHDP-hex-derivatives (HHDP-hex-deriv_1 and HHDP-hex-deriv_2) in the juices obtained from pomegranate fruits of different accessions. The bars indicate the standard deviation (n=3). Different lowercase letters indicate significant differences according to Tukey’s test ($p \leq 0.05$).

pigmentation,is in agreement with those reported by Ben-Simhon *et al.* (2015), who found similar values for Pel-3-glc content in the pomegranate cultivar “Wonderful” (Table 1).

The quantitative and qualitative anthocyanin variations among the accessions might be related to the environmental conditions during the ripening period, as well as the to genotype adaptation

mechanisms to the growing area (Fig. 1). Borochov-Neori *et al*. (2011, 2014) reported an increment in diglucosylated anthocyanins after seasonal warming, and our data may concurr to suggest a correlation between high level of the anthocynain diglucosides and hot summer. Indeed, Pg 1 and Pg 2, which can be found in dry regions characterized by hot summers, contain the highest levels of delphinidin-3,5-O-diglucoside and cyanidin-3,5-O-glucoside, presumably due to a higher stability of 3,5-diglucosides compared to 3-glucosides (Garcı́a-Viguera and Bridle, 1999; Hernandez *et al*., 1999). However, the high levels of anthocyanins might be also the consequence of adaptation to saline soil or marshes as high amount of anthocyanin leads to increased salt tolerance in Arabidopsis (Oh *et al.,* 2011). The hypothesis that Pg1, Pg2, Pg6, Pg7 can be more tolerant to saline soil should be further explored in view of their possible use as sources of resistance genes in breeding programs. Additionally, the high and peculiar concentration of anthocyanins in Pg1 and Pg2 makes the two accessions of particular interest for utilization in human health, given the activity of these compounds as cellular antioxidant and against inflammation status (Blesso, 2019).

Different content of hydrolyzable tanninsin the eight pomegranate accessions

In addition to anthocyanins, the studied pomegranate juiceswere also rich of hydrolyzable tannins which are synthesized from galloyl glucose, with pentagalloyl glucose serving as the precursor for both higher-molecular-weight gallotannins and ellagitannins. Ellagitannins, which contain two or more neighboring galloyl groups that are oxidatively coupled to form rigid groups such as hexahydroxydiphenoyl (HHDP) units, are particularly abundant in pomegranate fruits and can be extracted in significant levels into the juice (Fig. 3, 4, 5) (Seeram *et al*., 2005). The high levels of ellagitannins, in particular punicalagin derivatives, found in our juices are in line with the those reported in literature (Fig. 3) (Mena *et al.,* 2012; Akhavan *et al*., 2015; Kalaycıoğlu and Erim, 2017; Balli *et al*., 2020). Even if these compounds can determine an unpleasant taste in juices, due to their interactions with the salivary proteins, they have been identified as the active antiatherosclerotic compounds in pomegranate juices responsible for the ability of this juice to protect human low-density lipoprotein cholesterol from oxidation *in vivo* (Aviram *et al*., 2000). In addition, ellagitannins show also strong radical scavenging and antioxidant ability as well as interesting antimutagenic and anticancer properties (Vattem and Shetty, 2005). Other than the well-known punicalagin application in human health (Scalbert and Williamson, 2000; Zarfeshany *et al*., 2014), it is noteworthy the noticeable antifungal activities of punicalagins due to the ability, similar to that of amphotericin B to form pore-like aggregates in the cellular membranes of fungi (Rongai *et al*., 2018). These recent findings suggest further potential utilization of punicalagins extracted from pomegranate fruits as environmentally-friendly natural formulations to control fungus and bacteria in crop cultivation.

Besides their multiple applications for human health and agriculture, ellagitannins have also important functions for plant physiology, indeed they can offer protection against biotic stresses (Furlan *et al*., 2011). Lastly, the concentration of ellagic acid derivatives seems to increase under salinity stress (Borochov-Neori *et al*., 2014), probably because of the ability of these compounds to alleviate osmotic stress as previously reported in other species (El-Souda *et al*., 2013).

Selection of pomegranate accessions through the phenolic fingerprint

The phenolic fingerprint of the juices obtained from the accessions collected in eight different areas of Azerbaijan allowed to select promising wild pomegranate accession particularly rich both in anthocyanins and ellagitannins. Among the investigated accessions, Pg 1 resulted the richest both in anthocyanins and hydrolysable tannins, particularly in punicalagin derivatives (Table 1-2 and Fig. S1). In this accession, collected in the region of Khizi, characterized by hot summer and cold winter but partially mitigated by its proximity to the Caspian Sea, the interaction between environmental conditions and the genotype induces the accumulation of both more stable anthocyanins, such as del-3,5-diglc and cya-3,5-digl, and different punicalagin derivatives. Similar anthocyanin composition was also found in Pg 2, which however showed an unbalalanced composotion because of the very low amount of tannins (Table 1-2 and Fig. S1). Indeed, the accumulation of both classes of polyphenols can be stimulated under warming conditions, but also somehow drastically reduced under prolonged and severe drought conditions (Mena *et al*., 2013). A PCA biplot was applied to highlight the correlations between the contents of phenolic compounds of various accession fruits (Fig. S2). The two PCs explained almost the 70% of the variance of the data.

The closer the accessions lie on the plot, the more similar they are in the composition of phenolic compounds. By contrast, distant accessions have significantly different compositional characteristics. Indeed, PC1 discriminates very well Pg 1 and Pg 2 from the other accessions, since the samples on the left of the biplot are more abundant in anthocyanins. PC2 allows to discriminate accessions on the basis of their tannin content, and it clearly shows the presence of three different groups: the richest accession (Pg1), accessions with similar tannin content (Pg 3,4,5,6,7) and the lowest accessions (Pg 2 and Pg 8). The accessions Pg 3,4,5,6,7 have a more balanced content of anthocyanins and tannins (Fig. S1). However, also among these accessions, environmental influences can be osbserved considering the accessions Pg 5, 6, 7, which have been collected in areas with hot summer conditions and salinity soils and result to have higher anthocyanin and punicalagin contents compared to Pg 3 and Pg 4 (Table 1, 2 and S1). By contrast, these latest accessions, collected in regions with a temperate climate and dark loamy soils, are characterized by a higher content of ellagic acid and its glycosylated derivative (Fig. 4 and Table S1). The results of this study, considering the recently work on the pomegranate genome (Qin *et al*., 2017), provides a valuable resource for selecting pomegranate accessions with interesting metabolic traits that could be used in breeding efforts to increase the production of this bioactive compounds.

The results reported in this study contribute to provide significant information about phenolic fingerprinting of juices extracted from different wild growing pomegranate accessions in the territory of Azerbaijan Republic. Data showed that those accessions are rich in bioactive metabolites, which are useful for human health and might be correlated to adaptation to specific environmental conditions. The results of our study suggest that Azerbaijan wild-growing accessions are promising sources of bioactive metabolites and they might be exploited in pomegranate breeding programs as donors of valuable traits for creating pomegranate varieties with high nutritional and medical properties and/or to ameliorate the resistance of commercial varieties to harsh conditions.

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Genetic diversity, population structure, and relationships among wild and domesticated almond (*Prunus* spp.) germplasms revealed by ISSR markers

S. Rahimi-Dvin [1], **A. Gharaghani** [1, 2 (*)], **A. Pourkhaloee** [3]

[1] *Department of Horticultural Science, College of Agriculture, Shiraz University, Shiraz, Iran.*

[2] *Drought Research Center, College of Agriculture, Shiraz University, Shiraz, Iran.*

[3] *Department of Horticultural Science, College of Agriculture, Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran.*

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(*) **Corresponding author:**
agharghani@shirazu.ac.ir

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: The use of diverse almond genetic resources to expand the genetic bases of commercial cultivars is important for almond breeders. Iran is within the center of origin for almond and enjoys a huge diversity of wild species and local cultivars of this important nut crop. Despite some reports, there is still a critical need to collect comprehensive information on the genetic diversity of almond germplasm in Iran. This study was conducted to evaluate the genetic diversity, structure, and relationships among a total of 75 individuals from 10 populations of 4 wild and cultivated almond species by using 12 inter-simple sequence repeat (ISSR) primer pairs. A total number of 353 DNA fragments were obtained of which 352 were polymorphic (99.69%). The average of polymorphism information content (PIC), marker index (MI), and resolving power (Rp) were 0.932, 27.211, and 7.882, respectively which indicated high discriminatory power of markers. Gene flow between wild and cultivated gene pools is shown to be moderate to high (Nm = 2.7607), which verifies the hypothesis of low genetic differentiation among populations. Cluster analysis based on unweighted pair-group, classified individuals into 7 major gene pools which showed the entire provenances were divided into 7 main groups. Overall high levels of genetic diversity were confirmed and useful information obtained on the differentiation and genetic structure of the studied almond germplasms. Future evaluation on morphological and physiological aspects, is necessary to identify the most promising individuals to be used directly in afforestation, landscape development as well as nut and oil production or indirectly in future almond and stone fruits breeding programs.

1. Introduction

Almond [*Prunus dulcis* (L.) Batsch] belongs to the Rosaceae family and

is one of the most important nut crops in the world which is known for its high nutritional value. Almond domestication occurred nearly 5000 years ago in the Fertile Crescent (Velasco *et al*., 2016). United States of America, Spain, Iran, Italy, Turkey, Tunisia, Morocco, Syria, Greece and Australia are the ten major producers of almond (Ardjmand *et al.*, 2014). Iran is the fifth world producer of almond (Gharaghani *et al.*, 2017) which produced approximately 2.99% of the total world production of cultivated almonds (Sorkheh *et al.*, 2016).

Due to the narrow genetic background of commercial cultivars, breeding programs of almond face many challenges. In modern plant breeding, native plants are considered as valuable gene pools for crossing programs which can be used to introduce new traits into commercial relatives. Wild almond species are found in the mountains and deserts of Central Asia from western China to Iran and Turkey (Rahemi *et al.*, 2012). Wild almond species could be valuable gene pools for breeding purposes due to late bloom, early maturity, adaption to drought and salinity, resistance to winter lower temperatures, reduced insect infestation and fungal attacks (Gharaghani *et al.*, 2017). Thus, knowledge about genetic diversity of wild genetic resources of almond is an essential prerequisite for involvement of native germplasm in almond breeding programs. On the other hand, assessment of genetic diversity and population structure is necessary to evaluate the existing levels of genetic variability and its patterns of distribution among the local populations, which is considered as a guarantee for conservation management of natural populations (Cohen *et al.*, 1991; Sreekanth *et al.*, 2012).

Iran is a center for genetic diversity of almond and nearly twenty wild species of almond have been reported from arid and semi-arid regions of this country (Sorkheh *et al.*, 2009). Different regions of Iran have variable environmental conditions including subtropical climate in the south, temperate in the north, and extended deserts in the middle which helps the distribution of wild species such as almonds. Wild almond germplasm forms the main part of distributed plant species in the mountainous and plain sub-regions of ecological zones in the Zagros of Iran where the annual precipitation rate is more than100 mm (Sabeti, 1994). Almond stands of the Irano-Turanian region have been observed in Badamak, Mohammadabad Maskun and Badameshk forests in Fars, Kerman and South Khorasan province of Iran, respectively (Talebi *et al*., 2013). Fars and Charmahal-o-Bakhtiari provinces cover parts of the central and southern Zagros where *Prunus scoparia* (Spach) C.K. Schneid*., P. elaeagnifolia* Spach., and *P. eburnean* Spach. are widely distributed (Gharaghani *et al.*, 2017).

Owing to some traits such as leaf shedding during hot seasons, and the remarkable capability of roots in water absorption, some of the wild almond species can resist draught (Madam *et al*., 2011). *P. scoparia* is a potentially multi-purpose wild almond species in Iran which has the potential to become the crop of choice for soil stabilization and landscape in arid and semi-arid areas (Mozaffarian, 2005). It has been used as a dwarfing rootstock for almond for centuries (Gharaghani and Eshghi, 2015). *P. scoparia* is a potential source of vegetable oil for human nutrition and health with relatively higher oxidative stability, higher unsaturated to saturated fatty acids ratio, calculated oxidisability value, total tocopherols and phenolics contents, and unsaponifiable matter contents, than those of olive oil (Farhoosh and Tavakoli, 2008). Zedu gum is exuded from the bark of *P. scoparia*, and its kernel oil are used in Iranian traditional medicine (Zargari, 1997). Zedu gum is also being used as emulsifier in cosmetic and textile industries (Rahimi *et al*., 2013). These species lie among the rare trees which naturally grow in barren soils (Ali *et al*., 2015). *P. elaeagnifolia* has been used as a rootstock for plum (Gholami *et al.,* 2010) in drought conditions in Iran and some other species have also been used as rootstocks for almond and peach by ancient Iranians in arid lands (Denisov, 1988). Grafting nectarine on wild almond trees as rootstock has also been reported by Alberghina in 1978. Wild almond species have also been used to afforest barren lands and to protect vegetative cover (Mardani, 2006).

DNA-based molecular markers are important tools to study genetic variation in population genetics. Various molecular markers including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), Inter-simple sequence repeat (ISSR), and single nucleotide polymorphism (SNP) have been previously used to describe genetic diversity and structure in the genus *Prunus* (Martins *et al.*, 2003; Shiran *et al.*, 2007; Sorkheh *et al.*, 2007; Wu *et al.*, 2008; Bouhadida *et al.*, 2009; Rahemi *et al.*, 2012). Among different DNA markers, ISSRs have greater reliability and reproducibility in comparison with RAPD system, as well as the lower cost of the analyses than AFLP

and SSR (Rodrigues *et al.*, 2013). Moreover, ISSR markers seem to be especially useful to study closely related individuals which show low levels of polymorphism (Zietkiewicz *et al.*, 1994).

Local wild species make up an excellent source of genetic diversity which can be used for crop improvement and breeding programs (Khadivi-Khub and Anjam, 2014). Despite some reports, there is still a critical need to collect more information on the genetic diversity of almond germplasm in Iran. Because of high value of *P. scoparia*, it is important that the necessary steps be taken to comprehensively evaluate, utilize and ensure the conservation of this unique wild species. The purpose of our study was to study the genetic diversity and population structure of a collection of wild and cultivated almond populations in Iran using ISSR markers. The emphasis of this study is on the populations of *P. scoparia* collected from different sites in central and southern Zagros regions, experiencing less natural precipitation and higher temperature comparing to other natural habitats of this species in Iran. These special climatic condition made these wild populations a promising source of genes evolved for drought and high temperature tolerance, which will be very valuable in facing harsh effects of climate change. We also sought to compare the diversity and illustrate the relationships of these populations with some populations of three other almond species including *P. elaeagnifolia*, *P. eburnea* and *P. dulcis* (common almond) from the same geographical region, to put more shed on the possible gene flow among them as well as to detect the footprint of these species in the genetic background of cultivated almond. The results of this study are useful for conservation of these wild stands as well as for decision making on direct or indirect utilization of them for afforestation, nut and oil production, landscape purpose and through breeding programs.

2. Materials and Methods

Field sampling

To detect higher genetic diversity (resulting from cross-pollination in natural habitat of the plant materials used herein) as well as the feasibility of plant materials collection (seeds instead of leaf samples) we chose to use raised seedling populations instead of natural populations in this study. In total, seeds of 72 wild almond trees were sampled from southern and central regions of Zagros Mountain in Iran during late spring to early summer of 2014. These regions are placed in Fars and Chaharmahal-and-Bakhtiari provinces. The studied genotypes belong to *P. scoparia* and *P. eburnean* in section Spartioides Spach. as well as *P. elaeagnifolia* and *P. dulcis* in section Euamygdalus Spach (Kester and Gradziel, 1996). In addition, seeds of 5 almond cultivars were sampled. Characteristics of the populations (collection sites, latitude, longitude, altitude, etc.) are listed in Table 1.

Plant materials and DNA extraction

The seeds of all species were mechanically scarified and then soaked in water for 24 h. They were mixed with perlite and stratified at 4±1°C for 45 days. After stratification, nuts were directly sown in 5 kg pots filled with a mixture of fine sand, soil and leaf mold. The pots were then transferred to the greenhouse with an average temperature of 26±3°C under daylight illumination conditions i.e. 800 $\mu mol\, m^{-2}\, s^{-1}$ about 10 hours. In December 2017 a total of 75 seedlings (each seedling represents an individual tree in natural habitat) comprised 10 populations (3 to 11 individuals per population) were selected. Stem pieces (200 mg) for each individual were collected into aluminum foil, immediately snap-frozen in liquid nitrogen and stored at -80°C until the DNA was extracted. Total genomic DNA was isolated following the cetyltrimethylammonium bromide (CTAB) protocol with minor modifications (Doyle and Doyle, 1987). DNA quantity and quality were determined by spectrophotometry and visual comparison of DNA electrophoresed on 1% agarose gel.

ISSR genotyping

In total, 12 ISSR primer pairs were selected based on literature review (Carvalho *et al.*, 2002; Martins *et al.*, 2003; Dje *et al.*, 2006; Zhao *et al.*, 2007; Oliveira *et al.*, 2010; Moulin *et al.*, 2012; Ahmed *et al.*, 2013; Muraseva *et al.*, 2018) and synthesized (by Metabion, Germany). Polymorphism of markers was first tested in a subset of samples and then polymerase chain reaction (PCR) conditions were optimized. The list of primers and their information are presented in Table 2.

The PCR mix contained 10 ng template DNA, 10 pmol of primer in a final 20 µl reaction volume. Conditions of the PCR amplification were as follows: 94°C (3 min), then 35 cycles at 94°C (45 s) / 38-61°C (varied for each primer according to Table 2) (45 s) / 72°C (1 min) and final extension at 72°C for 7 min. The amplified products were separated by 1% (w/v) agarose gel electrophoresis in 1× TBE buffer at constant voltage (100) for 45 min, stained with

Table 1 - List of the studied genotypes with indication of their regions and geographical coordinates of the collection sites

Species	No.	Population	E	N	Altitude (m)
Prunus scoparia	1	Shiraz 1	52 34.558	29 37.082	1535
	2	Shiraz 2	52 35.822	29 44.275	1820
	3	Shiraz 3	52 35.784	29 44.287	1816
	4	Shiraz 4	52 35.811	29 44.269	1819
	5	Shiraz 5	52 34.601	29 37.103	1569
	6	Shiraz 6	52 34.449	29 37.465	1565
	7	Shiraz 7	52 33.704	29 37.946	1549
	8	Shiraz 8	52 34.586	29 39.490	1670
	9	Shiraz 9	52 32.642	29 40.263	1728
	10	Shiraz 10	52 35.789	29 44.353	1821
	11	Shiraz 11	52 35.785	29 44.351	1818
P. scoparia	12	Nourabad 3	51 20.875	30 4.695	1179
	13	Nourabad 4	51 39.730	29 48.513	934
	14	Nourabad 5	51 32.377	30 1.167	1086
	15	Nourabad 6	51 23.931	30 0.745	1285
	16	Nourabad 7	51 39.823	29 48.605	946
	17	Nourabad 8	51 21.011	30 6.513	1183
	18	Nourabad 9	51 31.694	30 1.252	1067
	19	Nourabad 10	51 58.427	30 01 08.4	1592
P. scoparia	20	Marvdasht 1	52 54.983	30 3.162	1728
	21	Marvdasht 2	52 54.800	30 6.249	1730
	22	Marvdasht 3	53 00.807	30 6.800	1812
	23	Marvdasht 4	53 12.117	30 5.742	1837
	24	Marvdasht 5	53 10.524	30 1.617	1828
	25	Marvdasht 6	53 12.643	29 59.116	1803
	26	Marvdasht 7	53 14.080	29 57.712	1765
	27	Marvdasht 8	53 6.493	29 48.754	1667
	28	Marvdasht 9	53 6.535	29 48.816	1663
	29	Marvdasht 10	53 8.662	29 47.443	1640
P. scoparia	30	Firuzabad 2	52 32.509	29 8.712	1725
	31	Firuzabad 3	52 34.486	28 58.157	1503
	32	Firuzabad 4	52 33.874	28 57.406	1530
	33	Firuzabad 5	52 32.400	28 55.816	1445
	34	Firuzabad 6	52 38.719	29 5.885	1917
	35	Firuzabad 7	52 23.202	28 53.222	1524
	36	Firuzabad 8	52 38.310	29 3.808	1732
	37	Firuzabad 9	52 32.801	29 9.124	1763
	38	Firuzabad 10	52 41.274	28 13.319	1275
	39	Firuzabad 11	-----	-----	1578
P. scoparia	40	Mian Jangal Fasa 1	52 46.117	29 26.327	1481
	41	Mian Jangal Fasa 2	52 50.130	29 19.653	1526
	42	Mian Jangal Fasa 3	--	--	2187
	43	Mian JangalFasa 4	53 24.351	29 9.542	1729
	44	Mian Jangal Fasa 5	53 23.894	29 9.939	1754
	45	Mian Janga lFasa 6	53 26.033	29 7.617	1720
	46	Mian Jangal Fasa 7	53 26.054	29 7.632	1716
	47	Mian Jangal Fasa 8	53 24.138	29 9.082	1756
	48	Mian Jangal Fasa 9	53 22.759	29 10.821	1815
	49	Mian Jangal Fasa 10	53 19.277	29 12.351	1825

to be continued...

Table 1 - List of the studied genotypes with indication of their regions and geographical coordinates of the collection sites

Species	No.	Population	E	N	Altitude (m)
P. scoparia	50	Eqlid 3	52 40.003	30 15.126	1701
	51	Eqlid 4	52 38.310	30 16.346	1742
	52	Eqlid 5	52 36.405	30 18.062	1800
	53	Eqlid 6	52 35.080	30 22.196	2321
	54	Eqlid 7	52 23.051	30 19.324	1843
	55	Eqlid 8	52 23.764	30 19.060	1750
	56	Eqlid 9	52 24.085	30 18.382	1715
P. scoparia	57	Lordegan 1	51 11.609	31 33.619	1752
	58	Lordegan 2	51 13.020	31 34.354	1962
	59	Lordegan 3	--	--	1948
	60	Lordegan 4	--	--	1963
P. elaeagnifolia	61	*P. elaeagnifolia* 1	52 35.806	29 44.098	1801
	62	*P. elaeagnifolia* 2	52 35.746	29 44.158	1804
	63	*P. elaeagnifolia* 3	--	--	2128
	64	*P. elaeagnifolia* 4	--	--	2570
	65	*P. elaeagnifolia* 5	52 34.880	30 22.732	2458
	66	*P. elaeagnifolia* 6	52 34.898	30 22.748	2455
P. eburnea	67	*P. eburnea*2	52 22.173	30 19.865	1912
	68	*P. eburnea*3	53 24.368	29 09.592	1732
	69	*P. eburnea*4	--	--	2280
	70	*P. eburnea*5	52 24.585	30 18.376	1711
	71	*P. eburnea*7	52 22.130	30 19.854	1902
	72	*P. eburnea*8	53 24.101	29 9.071	1763
P. dulcis	73	Mamaei	---	---	1910
	74	Ferragnes	---	---	1910
	75	Badam talk	---	---	1910

SimplySafe (EURx, Poland) and photographed with UV light (Nade Gel Documentation and Analysis System JS-6800, China). The size of produced fragments was defined according to size marker (Fermentas, Germany).

Data analysis

Marker results (reproducible distinct bands with high resolution) were dominantly scored in a data matrix. The matrix was used for calculation of population genetic variation indices.

The informativeness of primer pairs in genotyping and subsequent evaluation of genetic diversity and population structure was compared using the polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolving power (Rp). For each primer, the polymorphic information content (PIC) was estimated by PowerMarker v3.25 (Liu and Muse, 2005). Marker index for each primer was calculated as a product of polymorphic information content and effective multiplex ratio: MI = EMR * PIC (Varshney *et al*., 2007). The resolving power (Rp) of each primer was calculated as $Rp = \sum Ib$, where Ib shows the informative fragments. The Ib may be shown on a scale of 0/1 by the following formula; $Ib = 1 - (2 \times |0.5 - pi|)$ where pi is the proportion of populations containing the *ith* band (Prevost and Wilkinson, 1999).

Based on ISSR bands identified in the individuals, some basic parameters for genetic diversity including the total number of bands (TNB), the number of polymorphic bands (NPB), the percentage of polymorphic bands (PPB), mean Nei's gene diversity index (H), Shannon's information index (I), the observed number of alleles per locus (Na), the effective number of alleles per locus (Ne), the level of gene flow (Nm), population diversity (Hs), the total gene diversity (Ht), inter-population differentiation (Gst), genetic identity and genetic distance were calculated for each population using software POPGENE 1.32 (Yeh *et al*., 1999). Private bands (referring to the bands found only within one population) and major allele frequency were estimated by power marker software.

To illustrate the relationship among populations,

an unweighted pair group method with arithmetic mean (UPGMA) dendrogram was constructed based on Nei's genetic distance using POPGENE 1.32 (Yeh *et al.*, 1999). The dendrogram was generated using TreeView program.

To further understand the relationships among populations, a Bayesian clustering-based structure analysis was performed on the entire data set using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) to reveal the number of genetic pools. Two runs of analysis using the admixture model were performed. Initial runs were performed with a burn-in length of 50000 and 750000 MCMC (Markov Chain Monte Carlo) replicates for 10 times at each K from 1 to 10. The probable number of groups was estimated. The second run was 100000 for burn-in length and 300000 for MCMC replicates, 10 times for each K. To estimate the best K value. The Evanno test was performed on STRUCTURE results using ''Structure Harvester'' (Evanno *et al*., 2005). The results were summarized in a bar plot using DISTRUCT (Rosenberg, 2004).

3. Results

Informativeness of markers

The mean of PIC values was analyzed for all loci to evaluate markers efficiency. PIC value ranged from 0.845 (primer 4) to 0.973 (primer 1). The mean PIC value for all loci was 0.932. The highest EMR value of 38 (primer 1) and the lowest of 20 (primer 12), with an average EMR value of 29.25 per primer were obtained. The highest (36.97) and the lowest (19) MI values were observed with primers 1 and 12, respectively. The mean MI value was 27.211 per primer. The highest Rp value was observed with primer 1 (13.183) and the lowest with primer 4 (3.518) with an average Rp of 7.882 per primer (Table 2).

Genetic diversity

The 75 individuals of wild and domesticated almond assigned to 10 populations and were amplified with 12 selected primers (Table 2). A total of 353 bands were scored with an average band number of 29.33 per primer across 75 individuals. Among the 353 bands, 352 bands (99.69%) were polymorphic. The percentage of polymorphic bands (PPB) varied from 96.29% for primer 3 to 100% for the other primers (Table 2). At the population level, PPB ranged from 18.70% in *P. dulcis* to 58.07% in *P. scoparia* (Shiraz and Firuzabad populations) with a mean value of 49.66% (Table 3).

The N_a ranged from 19.63 for ISSR 3 to 20.00 for other ISSRs. Across the populations, N_a ranged from 11.870 for *P. dulcis* to 15.807 for *P. scoparia* (Shiraz and Firuzabad populations). The N_e ranged from 11.453 for ISSR 4 to 14.254 for ISSR 5 with an average of 13.003 alleles per locus. Across the populations, N_e

Table 2 - ISSR primers used in this study and their results

Primers	Primer sequences (5'-3')	Tm	Total number of alleles (a)	Number of polymorphic alleles (b)	% Polymorphism (b/a)*100	PIC	MI	EMR	MAF	Rp
1	GAC AGA CAG ACA GAC A	48	38	38	100	0.973	36.97	38.00	0.120	13.183
2	GTG CGT GCG TGC GTG C	58	30	30	100	0.952	28.56	30.00	0.173	4.932
3	GTG GTGGTGGTGTG-	61	27	26	96.29	0.948	23.72	25.03	0.186	8.132
4	CTC TCT CTC TCT CTC TTG	54	27	27	100	0.845	22.68	27.00	0.373	3.518
5	GAG AGA GAG AGA GAG	50	23	23	100	0.953	21.85	23.00	0.173	9.160
6	CAC CACCAC GC	38	33	33	100	0.947	31.02	33.00	0.200	9.946
7	ACA CAC ACA CAC ACA	54	23	23	100	0.855	19.55	23.00	0.360	4.889
8	GAA GAAGAAGAAGAA-	50	28	28	100	0.958	26.60	28.00	0.173	8.172
9	GTC GTCGTCGTCGTCGTC	61	32	32	100	0.873	27.84	32.00	0.333	4.692
10	GAG AGA GAG AGA CC	44	35	35	100	0.961	33.60	35.00	0.160	12.177
11	BDB ACA ACAACAACAA-	49	37	37	100	0.956	35.15	37.00	0.160	9.531
12	YHY GTG TGT GTG TG	42	20	20	100	0.959	19.00	20.00	0.133	6.252
Min.	---	---	20	20	96.29	0.845	19.00	20.00	0.120	3.518
Max.	---	---	38	38	100	0.973	36.97	38.00	0.373	13.183
Means	---	---	29.41	29.33	99.69	0.932	27.21	29.25	0.212	7.882
Total	---	---	353	352	---	---	326.54	351.03	2.544	---

Y = (C, T); D = (A, G, T); V = (A, C, G); B = (C, G, T); R = (A, G) Rp= resolving power; PIC= polymorphism information content; MI= marker index; EMR effective multiplex ratio; MAF= major allele frequency.

Table 3 - Genetic diversity within the populations of almond in Iran exhibited by inter simple sequence repeat (ISSR)

Population	No.	Observed no. of alleles (Na)	Effective no. of alleles (Ne)	Shannon's in formation index (I)	Nei's genetic diversity (H)	Percentage of polymorphic loci (PPB)	No. bands	No. private Bands
Shiraz	11	15.807	12.853	0.2749	0.1774	58.07	205	7
Nourabad	8	15.212	12.657	0.2570	0.1664	52.12	184	3
Marvdasht	10	15.581	12.723	0.2653	0.1708	55.81	197	6
Firuzabad	10	15.807	12.812	0.2737	0.1759	58.07	205	11
Mian Jangal Fasa	10	15.666	12.879	0.2738	0.1774	56.66	200	8
Eqlid	7	15.524	12.968	0.2816	0.1838	55.24	195	12
Lordegan	4	14.419	13.161	0.2652	0.1817	44.19	157	1
P. elaeagnifolia	6	14.703	12.577	0.2453	0.1607	47.03	167	6
P. eburnea	6	15.071	13.099	0.2778	0.1854	50.71	179	8
P. dulcis	4	11.870	11.496	0.1190	0.0831	18.70	66	0
Mean	7.5	14.966	12.722	0.2533	0.1662	49.66		

ranged from 11.496 for *P. dulcis* to 13.161 for *P. scoparia* (Lordegan population) (Tables 3 and 4). Across the populations, the highest values of I (0.2816) and H (0.1838) indexes were observed for *P. scoparia* (Eqlid populations). However, *P. dulcis* showed the lowest I (0.1190) and H (0.0831) values (Table 3). However, for studied accessions, the average values of Na and Ne were 14.966 and 12.722, respectively.

Genetic similarity and cluster analysis among populations

The dendrogram derived from UPGMA cluster analysis was generated for all populations (Fig. 1). Among seven distinct groups obtained by dendrogram, four groups represent populations of *P. scoparia*. The group I consisted of two populations of *P. scoparia* (Shiraz and Mian Jangal-e-Fasa) collected from Fars province. The group II was composed of the other three populations of *P. scoparia*(Nourabad, Marvdasht, and Firuzabad) sampled from Fars province. The Eqlid population of *P. scoparia*

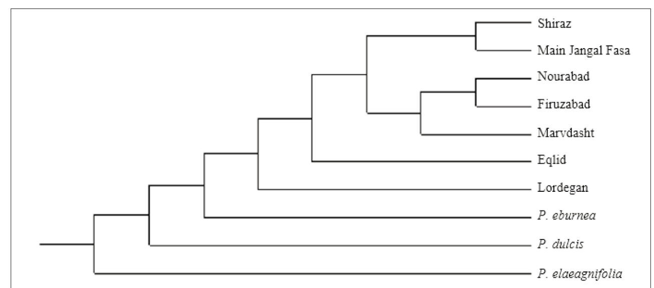

Fig. 1 - UPGMA dendrogram on the basis of Nei's (1978) evaluation of genetic distance among 10 populations of almond.

Table 4 - A summary of genetic parameters across inter-simple sequence repeat loci

Locus	Na	Ne	I	h	Ht	Hs	Gst	Nm
1	20.00	13.759	0.3872	0.2418	0.2412	0.2034	0.1568	2.6884
2	20.00	11.847	0.2547	0.1408	0.1427	0.1197	0.1615	2.5953
3	19.630	13.564	0.3529	0.2244	0.2273	0.1959	0.1385	3.1111
4	20.00	11.453	0.2178	0.1165	0.1183	0.0974	0.1771	2.3231
5	20.00	14.245	0.4103	0.2623	0.2538	0.2088	0.1774	2.3181
6	20.00	13.447	0.3652	0.2247	0.2234	0.1939	0.1319	3.2900
7	20.00	12.437	0.3061	0.1780	0.1775	0.1522	0.1428	3.0022
8	20.00	13.268	0.3392	0.2095	0.2092	0.1726	0.1751	2.3559
9	20.00	11.649	0.2255	0.1237	0.1234	0.1063	0.1387	3.1053
10	20.00	14.034	0.3857	0.2474	0.2426	0.2125	0.1244	3.5201
11	20.00	12.712	0.2869	0.1739	0.1761	0.1426	0.1903	2.1274
12	20.00	13.630	0.3593	0.2261	0.2216	0.1904	0.1408	3.0504
Mean	19.90	13.003	0.3242	0.1974	0.1964	0.1663	0.1546	2.7906

Na= Observed number of alleles; Ne= Effective number of alleles; I= Shannon's Information index; Nei's genetic diversity; Ht= Total gene diversity; Hs= Population diversity; Gst= Inter-population differentiation; Nm= Estimate of gene flow.

sampled in north of Fars province was separated in the group III. The group IV comprised Lordegan population of *P. scoparia* sampled from Charmahal and Bakhtiari province in central Zagros region. Groups V, VI and VII included populations of *P. eburnea*, *P. dulcis*, and *P. elaeagnifolia*, respectively.

The Nei's genetic distance ranged from 0.0077 to 0.0452 and genetic identity ranged from 0.9558 to 0.9923 (Table 5). The genetic identity between Nourabad and Firuzabad populations of *P. scoparia* was 0.9923 having the closest genetic relationship; however, the farthest genetic identity was 0.9558 between Lordegan population of *P. scoparia* and *P. elaeagnifolia* populations.

Population structure

The amount of gene flow (Nm) among populations was 2.7607, showing the moderate to high gene flow among populations studied herein. The genetic diversity within populations (*H*s) and the total genetic diversity (*H*t) of the species were 0.1663 and 0.1964, respectively (Table 6). The genetic differentiation among the populations (Gst) was 0.15 which shows that 15% of the total genetic variability was among populations and 85% was within populations.

The results of the structure analysis with ISSR markers are presented in figure 2. The structure plot suggested a lack of definite structure although

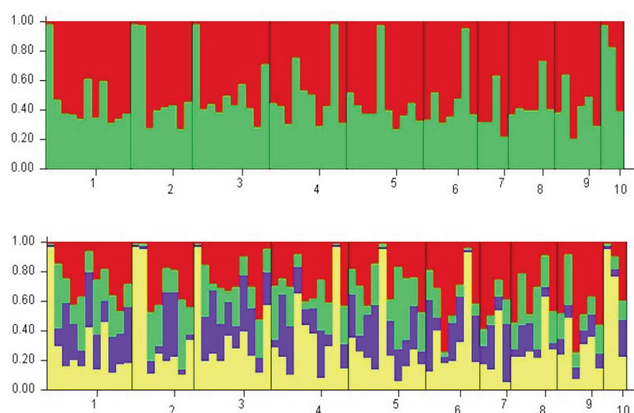

Fig. 2 - Population structure of almond populations for K = 2 and K = 4, showing a high degree of genotypic admixture among individuals. On the horizontal axis, the following population are illustrated: (1): *Prunus scoparia* (Shiraz); (2): *Prunus scoparia* (Nourabad); (3): *Prunus scoparia* (Marvdasht); (4): *Prunus scoparia* (Firuzabad); (5): *Prunus scoparia* (MianJangalFasa); (6): *Prunus scoparia* (Eqlid); (7): *Prunus scoparia* (Lordegan); (8): *Prunus elaeagnifolia*; (9): *Prunus eburnea*; (10): *Prunus dulcis*.

Evanno's test indicated that the most informative number of populations was K = 2 and K = 4.

Using the defined strategies of DNA purification with the selected primers, good patterns could be attained for the different accessions under study. Instance of patterns of amplification attained by ISSR

Table 5 - Genetic identity (above diagonal) and genetic distance (below diagonal) estimates between populations across all loci based on Nei (1978)

Population	Shiraz	Nourabad	Marvdasht	Firuzabad	Mian Jangal	Eqlid	Lordegan	*Prunus elaeagnifolia*	*Prunus eburnea*	*Prunus dulcis*
Shiraz	****	0.9862	0.9898	0.9899	0.9896	0.9773	0.9774	0.9693	0.9715	0.9722
Nourabad	0.0139	****	0.9910	0.9923	0.9882	0.9806	0.9806	0.9789	0.9792	0.9756
Marvdasht	0.0102	0.0091	****	0.9900	0.9841	0.9868	0.9756	0.9677	0.9709	0.9721
Firuzabad	0.0102	0.0077	0.0101	****	0.9894	0.9835	0.9783	0.9714	0.9777	0.9789
MianJangalFasa	0.0104	0.0119	0.0161	0.0107	****	0.9749	0.9779	0.9675	0.9725	0.9737
Eqlid	0.0230	0.0196	0.0132	0.0167	0.0254	****	0.9730	0.9600	0.9625	0.9728
Lordegan	0.0229	0.0196	0.0247	0.0219	0.0224	0.0273	****	0.9558	0.9718	0.9567
P. elaeagnifolia	0.0312	0.0214	0.0328	0.0290	0.0330	0.0409	0.0452	****	0.9713	0.9609
P. eburnea	0.0289	0.0210	0.0296	0.0225	0.0279	0.0382	0.0286	0.0291	****	0.9560
P. dulcis	0.0282	0.0247	0.0283	0.0213	0.0267	0.0275	0.0442	0.0399	0.0450	****

Table 6 - Assessment of the genetic variability among ten populations designated based on the ISSR analysis

	*H*t Total gene diversity	*H*s population diversity	Gst Inter-population differentiation	Nm Estimate of gene flow
Average	0.1964	0.1663	0.1533	2.7607
Standard deviation	0.0236	0.0168	---	-----

in various accessions of almond are shown in figure 3.

4. Discussion and Conclusions

Informativeness of markers

Due to highly variable nature and less investment in time and money than other marker systems, ISSR markers are widely used in population genetic studies (Harris, 1999). Moreover, Matesanz *et al.* (2011) reported that because of high polymorphism, only a few ISSR loci (as few as five to seven primer pairs) are enough to obtain reliable information on genetic diversity of populations.

The efficiency of a molecular marker system in distinguishing genotypes depends largely upon the polymorphism it can discover (Guo *et al.*, 2014). On the basis of high PIC values, MI, and Rp we conclude that ISSR markers used in this study were informative in the assessment of genetic diversity of almond accessions. The high PIC values with a mean of 0.932 show that all primers are informative, and this can be related to high genetic variation among accessions used in this research. Similar results were reported for sour cherry and *Prunus mira* (Najafzadeh *et al.,* 2014; Tian *et al.,* 2015). The variation may have been contributed by gene flow, natural hybridization, propagation by seed and human selection (Sefc *et al.,* 2000).

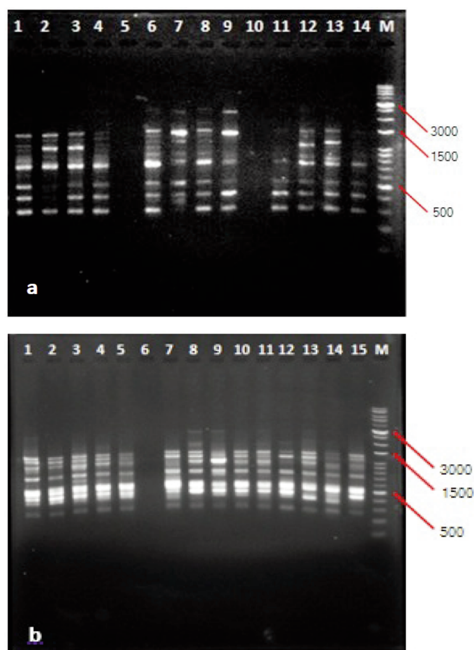

Fig. 3 - a) ISSR banding pattern generated using primer 11. Lane 1-14, Eqlid 7, Mian Jangal Fasa 6, Marvdasht 6, Eqlid 4, Nourabad 4, Firuzabad 2, *P. elaeagnifolia* 4, Nourabad 5, *P. elaeagnifolia* 2, Nourabad 10, Shiraz 8, Nourabad 9, Shiraz 2, Shiraz 6. b) ISSR banding pattern generated using primer 10. Lane 1-15, Shiraz 3, Shiraz 4, Marvdasht 8, Nourabad 7, Lordegan 1, Mamaei, Marvdasht 4, Shiraz 9, Badam talk, Shiraz 7, Mian Jangal Fasa 3, Mian Jangal Fasa 1, Eqlid 9, Marvdasht 3, Mian Jangal Fasa 4. M. 10 kb DNA ladder.

The Rp and MI measurements show distribution and number of alleles (bands) within the studied genotypes. Bands that are scored in the half of genotypes would possess optimal discriminatory power and with an increase in the number of bands, the Rp of a particular primer pair will be increased (Kayis *et al.*, 2010). Therefore, primers with the highest PIC, EMR, MI, and Rp values (ISSR1, ISSR10, and ISSR11) were generally the most effective in distinguishing between accessions and could be further used in almond genetic diversity studies. The similar results are reported in, *Prunu*s genus, sweet cherry, and sour cherry (Yılmaz *et al.*, 2009; Ganopoulos *et al.*, 2011; Najafzadeh *et al.*, 2014).

Genetic diversity

Information on genetic diversity and structure of wild almond populations is essential for their conservational programs. Moreover, narrow genetic background of the commercial cultivars of the genus *Prunus* restricts their cultivation in new regions with different environmental conditions. Therefore, genetic diversity among populations of this genus can be used to broaden the genetic background of commercial scion and rootstock cultivars and to overcome their distribution across different regions (Gradziel *et al.*, 2001).

Genetic variation depends on many factors such as mating system, genetic drift, gene flow, human activities, long-term evolutionary history, natural selection, and breeding systems (Schaal *et al.*, 1998; Hamrick and Godt, 1996).Populations of domesticated almonds used in this study possess restricted number of individuals and often are reproduced vegetatively. Thus, cultivated almonds shows lower levels of genetic diversity than the other species. However, higher genetic diversity was observed in certain individuals and populations of the wild almonds. This phenomenoncould be expounded by the fact that are propagated sexually whereas individuals of cultivated almonds aremainly reproduced asexually.

The PPB is a major genetic diversity index that showed high levels of genetic diversity among almond genotypes. Similar great genetic diversity

was reported by Sorkheh *et al.* (2017) in wild almond. The obtained PPB in this study was higher than values reported by Kumar *et al.* (2009) and Shuxia (2011) with *Prunus armeniaca* (96.5%) and *Prunus persica* (93%), respectively. Also, high genetic variation was shown by Rahemi *et al.* (2012) in wild almond, being similar to our results. One of the main reasons of the existing genetic variation is the process of self-incompatibility which is controlled by genes (Gouta *et al.,* 2010; Szikriszt *et al.,* 2011).The high number of generated alleles in our study may be due to use of several different genotypes that possessed high levels of genetic diversity.

The bands generated by each primer rests on the primer, sequence and the diversity size in special genotype (Shiran *et al.*, 2007). So, the number of bands differed in various genotypes. The private bands show the existence of special genes or sequences in native populations. The common bands show alleles which are shared among the cultivars studied. Thus, the private bands can be used in almond genetic fingerprinting and cultivar recognition.

Genetic similarity and cluster analysis among populations

Cluster analysis is widely used to study the genetic relationships among germplasms (Li *et al.*, 2010). The UPGMA dendrogram obtained in this study clearly distinguished species from each other and the clades were in accordance with morphological traits. Moreover, all populations were divided into their related taxa.

Prunus scoparia (Shiraz population)which seems to be mainly an artificial (cultivated) population and *P. scoparia* (MianJangal-e-Fasa population) both were separated into the same group. Therefore, we assume that some of the *P.scoparia* stands in Shiraz region were developed artificially through seed that may have originated from MianJangal-e-Fasa. Lordegan population lay in group IV, being closely related to Eqlid populations. It may be due to the geographic proximity and climatic resemblance between these two geographical locations. *Prunus eburnean* was grouped in cluster V, close to *P. scoparia* (Lordegan) populations. This close relationship is logical because both of them belong to Spartioides section within the genus *Prunus* (Kester and Gradziel, 1996). Moreover, close relationship between domesticated population of almond (*P. dulcis*) and *P. elaeagnifolia* could be explained in the same way since they both belong to Eu amygdalus section within the genus *Prunus* (Kester and Gradziel, 1996).

Genetic proximity between the genotypes or populations from different regions, for example Nourabad and Firuzabad (Table 5), could be explained by the geographical proximity of the regions, the exchange of plant material between sites and by the probable existence of common ancestors (El Hamzaoui *et al.*, 2014). Also, Noormohammadial *et al.* (2013) reported the gene exchange among *Prunus scoparia* populations which is similar to our results.

Molecular phylogeny results obtained in this study were similar to our findings using nut and kernel morphological characteristics to cluster the same subset of plant materials with some exceptions (Rahimi Dvin *et al.*, 2017). In that work, we found that *P. eburnea* and *P. scoparia* were placed close to each other and *P. elaeagnifolia* and *P. dulcis* formed the same clade.

The genetic distance among the studied almonds in this experiment is short, indicating that a high capacity for hybridization exists between genotypes and populations. The mating system can greatly affect genetic diversity both within and among populations. Generally, most of the genetic diversity in self-pollinated plants is distributed among populations, while in out crossed plants such as almond species, most of the genetic diversity is distributed within populations (Hamrick, 1989).

Population structure

Gene flow is defined as the gene movement within and between populations (Lowe *et al*., 2009). The estimate of gene flow (Nm) has been categorized as low (Nm<1), moderate (Nm>1) and extensive (Nm>4) (Kumar *et al*., 2014). The estimate of Nm (2.7607) was higher than 1, which indicates that the number of migrants per generation can prevent population differentiation caused by genetic drift. Moreover, we conclude that high genetic diversity and lack of differentiation is due to high amount of gene flow. Almond is an important food source for both human and animals and its seeds can be easily transported by birds and nomads (which is very common in the region) increasing the amount of Nm.

Genetic differentiation coefficient is an indicator of genetic diversity and structure of species (Zia *et al.*, 2014). It should be noted that Rosaceae species usually show low levels of genetic differentiation (Fineschi *et al.*, 2005). Based on Slatkin (1985), *Nm* >1 shows no significant genetic differentiation among populations. In this study, genetic differentiation

between populations had an average value of 0.15. Similar results were reported by Li *et al.* (2013) who obtained Gst of 0.18 with apricot. Many factors can influence on genetic differentiation which may occur independently in a population. For example, high dispersal rate of seeds must be involved in low genetic differentiation (Fanciulli *et al.*, 2000). Moreover, gametophytic incompatibility, prevents self-fertilization and encourages cross-pollination (Weinbaum, 1985) which retains high levels of genetic variability within seedling populations (Arulsekar *et al.*, 1986). As a consequence, populations of almonds which possess gametophytic incompatibilityshow low levels of genetic differentiation.

The genetic structure shows the history of populations with respect to their long-term evolution, mutation, recombination, genetic drift, gene flow, and natural selection (Slatkin, 1987; Schaal *et al.*, 1998). Therefore, providing information on the genetic diversity and structure of a crop is a prerequisite for the conservation and effective use of germplasms available for breeding (Laidò *et al.*, 2013).

Structure results demonstrated a high degree of admixture among individuals across 10 populations, consistent with moderate to high levels of gene flow across populations. Our results are similar to those of Mendigholi *et al.* (2013), who showed that the plots of structure exhibited the admixture of population and gene exchange which showed the existence of ancestral gene among *Prunus scoparia.*

The lack of population structure and moderate to high gene flow among the species in this study suggests the potential interbreeding among the populations. Nevertheless, a high individual genetic diversity purveys an optimistic prospect for the survival of the declining population with proper management interposition.

Results signified that ISSR primers which had been used herein had a significant distinctive power for the evaluation of the polymorphism in various almond populations. The obtained results present Iranian native almond species as a precious source of genetic diversity and recommends that they are an auspicious source of new genes for rootstock and cultivar breeding programs. Results also offer a contribution to the management and conservation of this valuable almond germplasm. Since the Iranian almond species and genotypes have not been selected for breeding programs, they are more probable to have a further diverse genetic background and may be employed in the selection of various genotypes so as to create new cultivars. It is expected that with extra experiments and analyses on morphological and physiological aspects, the most promising individuals could be identified and introducedfor direct utilization in afforestation, landscape development as well as nut and oil production or to be used by almond and stone fruits breeders in future breeding programs.

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Genetic parameters, correlations and path analysis in cowpea genotypes for yield and agronomic traits grown in *Cerrado*/Amazon Rainforest ecotone

D. Herênio Gonçalves Júnior (*), L. De Melo Rodrigues, J. Alves de Santana Filho, W. Nascimento Lima, A. Ferreira Alves
Laboratory of Plant Genetic Breeding (LMGV), Center for Agricultural Sciences and Technologies (CCTA), Brazil.

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(*) **Corresponding author:**
juniorherenio@gmail.com

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Abstract: The development of superior genotypes is the main objective of all plant breeding programs. To determine the genetic variability, heritability and correlations, 20 cowpea genotypes were grown in a randomized block design with four replications in *Cerrado*/Amazon Rainforest ecotone region. The data recorded were plant height, pod length, pod mass, pod grain mass, grain index, pod grain number and yield. Analysis of variance revealed significant differences between genotypes for all traits studied. The genotypic determination coefficient was high for all traits evaluated. Similarly, the accuracy parameter presented high estimates (>0.90). The magnitudes of the genotypic correlation coefficients were higher than the environmental and phenotypic correlations for most correlations, showing a greater influence of the genetic factor than the environmental factors. The direct and indirect effects provided greater reliability in the cause and effect interpretations between the studied traits, indicating that yield can be explained through the effects of the analyzed traits. The traits pod mass (0.9628) and pod grain mass (0.7835) showed the greatest favorable direct effect, showing a strong association between the analyzed characters and can be used in direct or indirect selection for yield in cowpea.

1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the oldest crops known to man and, because its moderate drought resistance, grows mainly in tropical climate areas (Egbadzor *et al.*, 2014). Recent studies suggest it originated from Central Africa over 4,000 years ago (Ogunkanmi *et al.*, 2014). According to Rocha *et al.* (2009), cowpea is a valuable legume, predominantly cultivated in Brazil, Africa and the United States. In the Brazilian North and Northeast regions, it is one of the main population's diet components, especially in rural areas (Santos *et al.*, 2014).

This crop still has low yields, despite the fact that its high adaptive potential to the conditions of tropical climate environments is verified

(Leite *et al.*, 2009). Teixeira *et al*. (2010) point out as causes the management techniques adopted and, mainly, the inefficiency of the technologies used and the use of traditional cultivars without breeding for yield. In Brazil there are cultivars with good commercial acceptance, but breeding programs aimed at evaluation and recommendation in specific environments are concentrated only in large producing centers (Oliveira *et al*., 2002; Barili *et al*., 2015).

One of the cowpea breeding programs basic goals is to obtain more productive genotypes. The availability of variance components estimates and genetic parameters such as coefficient of genetic variation, heritability and correlation coefficients for yield and their components are essential for the plant breeding programs development. These genetic parameters are characteristic of each population and may change in consequence of selection, changes in management, methods and estimation models, among other causes.

However, an important aspect about yield is that it is characterized as a complex variable, i.e., resulting from the expression and different components association (Santos *et al*., 2018). Correlation quantifies the association between any two variables. However, it does not allow inferences about cause and effect (Furtado *et al.*, 2002). The path analysis, proposed by Wright (1921), allows to partition the correlation coefficient into direct and indirect effects (path coefficient). For Cruz *et al.* (2014), this analysis can be defined as a standardized regression coefficient, being an expansion of the multiple regression analysis when complex interrelationships are involved.

In this sense, knowing the association between these traits allows the breeder to explore the possibility of indirect selection in cases of traits with complex inheritance and low heritability, such as yield. Correlation coefficient estimates make it possible to evaluate the magnitude and direction of the relationship between two traits and, consequently, the possibility of obtaining gains for one of them using indirect selection for the other trait. In some cases, indirect selection based on correlated response may be more effective and faster than direct selection of the desired trait (Cruz *et al*., 2014).

Thus, this research was conducted with the objective of estimating the genetic parameters for the yield and its components in 20 cowpea genotypes population cultivated in the Cerrado/Amazon Rainforest ecotone region in Brazil. As well as, investigate the associations between traits to direct selection strategies in breeding programs with this crop. Study results may assist in strategies for breeding and manipulation of traits by cowpea breeders in Brazil or other similar environments.

2. Materials and Methods

The experiment was carried out in Imperatriz city, Maranhão State, Brazil, in the experimental field of the *Centro de Difusão Tecnológica* (CDT) on premises of *Empresa Brasileira de Infraestrutura Aeroportuária* (INFRAERO) of geographic coordinates Latitude South 5°31'32'' and Longitude West 47°26'35'', and altitude of 123.30 meters. According to the Köppen climate classification, the region's climate is Aw, tropical savanna, with tropical wet and dry climate (Peel *et al*., 2007).

The survey of climate monitoring data for the region over the past 20 years was carried out. Data on annual total precipitation, maximum, minimum and average annual temperatures were collected. The data were obtained from an automatic climate monitoring station made available in the governmental meteorological database *Banco de Dados Meteorológicos para Ensino e Pesquisa* (BDMEP) administered by the Brazilian meteorology institute *Instituto Nacional de Meteorologia* (INMET). The time series graphs were produced using the ggplot2 package in the R software.

The treatments consisted in twenty erect habit cowpea genotypes from the Active Germplasm Bank (AGB) from the *Empresa Brasileira de Pesquisa Agropecuária* (EMBRAPA Meio Norte) cowpea genetic breeding program, located in Teresina, Brazil, 15 lines and 5 cultivars, respectively: LF-3; LF-21; LF-30; LF-48; LF-49; LF-62; LF-104; LF-143; LF-144; LF-148; LF-153; LF-154; LF-155; LF-159; LF-168; BRS-Guariba; BRS-Tumucumaque; BRS-Nova Era; BRS-Itaim; and BRS-Cauamé.

The soil physical and chemical characteristics were determined before the experiment beginning, from the superficial soil samples collected at random points in the experimental field up to 0.20 m depth. Soil texture was analyzed by the modified soil sedimentation Bouyoucos method after addition of a dispersing agent. Potential acidity was estimated from SMP pH after pH determination in calcium chloride 0,01 mol L^{-1} (Shoemaker *et al.*, 1961). Soil macronutrients and micronutrients analysis was performed to develop fertilizer recommendations.

The experimental design was a randomized complete block with 20 treatments and four replications.

The experimental plot consisted of two lines of 4.0 m and spacing of 0.50 m between lines and 0.20 m between plants, constituting a total experimental area of 220.00 m². Soil tillage was carried out in a conventional manner with one plow and two harrows. The digging and sowing operations were manual. From the chemical soil analysis, was performed the fertilization according to the requirements of the crop (Table 1). Irrigation was carried out by means of a sprinkler system sized to the crop and the region requirements, applying a daily water of 3.8 mm h^{-1}.

Invasive plants were controlled by hand weeding, performed weekly. Phytosanitary treatments were carried out through regular monitoring of pests and diseases, using the commercial insecticide Conect® when necessary. The harvest was performed when the pods of the plot were dry, totaling two harvests. The drying of the pods was completed in a forced air circulation oven, where the pods remained for two days at a temperature of 38°C.

In the useful area of each plot were recorded the following data: plant height (PH): average height in cm randomly measured in five plants of the plot; pod length (PL): average length in cm of five randomly harvested pods in the plot useful area; pod mass (PM): in grams, considering the five previously harvested pods; pod grain mass (PGM): in grams, considering the grains of the five pods submitted to the aforementioned evaluations; grain index (GI): refers to the dry grain mass in the dried pods. It is obtained by the expression:

$$GI = PGM/PM \times 100$$

seeds per pod (SPP): performed by counting the seeds in the five pods harvested for the previous samples; and yield: estimate considering the yield in all the useful plot area (m^2), extrapolating the value obtained for kg ha^{-1} correcting the value for grain mass to 13% moisture.

The collected data were initially submitted to the Shapiro-Wilk test to verify the data set normality and the Bartlett test to verify if the error has homogeneity of variance (homoscedasticity), not presenting the need for data transformation. Subsequently, one-way analysis of variance was performed to test the variability between genotypes, adopting the statistical model described in the equation below:

$$Y_{ij} = \mu + G_i + B_j + \varepsilon_{ij}$$

where:

Y_{ij} = observed trait value of the i-th genotype in the j-th block;

μ = general experimental mean;

G_i = effect of the i-th genotype considered fixed;

B_j = effect of the j-th block considered random;

ε_{ij} = random error associated to the i genotype and j block observations.

To understand the genotypic variability between the different traits measured, the components of phenotypic variance and genetic parameters were also estimated using the expressions suggested by Cruz *et al*. (2014):

a) Phenotypic variance: $\sigma^2_P = MS_g/b$

b) Environmental variance: $\sigma^2_E = MS_E/b$

c) Genotypic variance: $\sigma^2_G = (MS_g - MS_E)/b$

d) Genotypic determination coefficient: R^2 $(\sigma^2_G/\sigma^2_P) \times 100$

e) Intraclass correlation coefficient:

$ICC = \sigma^2_G/(MS_E+\sigma^2_G) \times 100$

f) Phenotypic coefficient of variation (%):

$$PVC = \frac{\sqrt{\sigma^2_P}}{\bar{x}} \times 100$$

g) Genotypic coefficient of variation (%):

$$GCV = \frac{\sqrt{\sigma^2_G}}{\bar{x}} \times 100$$

h) Environmental coefficient of variation (%):

$$ECV = \frac{\sqrt{MS_E}}{\bar{x}} \times 100$$

i) b quotient: $\frac{GCV}{ECV}$ ratio = $\sqrt{\frac{\sigma^2_G}{MS_E}}$

where: MS_g is the mean square of genotypes; MS_E is the mean square of error; b = number of blocks (replications) and $\bar{x}$ is the is the average of each trait.

j) Accuracy: $\hat{r} = (1 - 1/F)^{0.5}$

where Snedecor's F is the value of the variance ratio

Table 1 - Soil chemical characterization used in the field experiment

pH ($CaCl_2$)	OM (g Kg^{-1})	P (mg dm^{-3})	K (cmol dm^{-3})	Ca (cmol dm^{-3})	Mg (cmol dm^{-3})	Al (cmol dm^{-3})	H+Al (cmol dm^{-3})	SB (cmol dm^{-3})	CEC (cmol dm^{-3})	V (%)
4.8	18.4	13.5	0.26	1.66	0.69	0.00	1.70	2.61	4.31	60.5

OM= organic matter; P= phosphorus; K= potassium; Ca= calcium; Mg= magnesium; Al= Aluminium; H+Al= potential acidity; SB= sum of bases; CEC= Cation exchange capacity; V= base saturation.

for treatment effects (genotypes) associated with analysis of variance (ANOVA).

In the estimates of the correlations were used the expressions cited by Falconer (1987) and Ramalho *et al.* (1993):

a) Phenotypic correlation: $\Upsilon_{P(XY)} = \frac{COV_{P(XY)}}{\sqrt{\sigma^2_{PX} . \sigma^2_{PY}}}$

b) Genotypic correlation: $\Upsilon_{G(XY)} = \frac{COV_{G(XY)}}{\sqrt{\sigma^2_{GX} . \sigma^2_{GY}}}$

c) Environmental correlation: $\Upsilon_{E(XY)} = \frac{COV_{E(XY)}}{\sqrt{\sigma^2_{EX} . \sigma^2_{EY}}}$

where: Υ_{XY} is the correlation between the characters X and Y; COV_{XY} is the covariance between the characters X and Y; and, and σ^2_Y ad σ^2_X are the variances of the characters X e Y, respectively.

The unfolded of these correlations into direct and indirect effects of the six agronomic traits on yield was performed using the path analysis described by Cruz *et al*. (2014). The level of the multicollinearity of the *X'X* singular matrix was established by the product of the respective diagonal element of *X'X* by the component of the residual variance according to the methodology proposed by Montgomery *et al*. (2012). After verifying the multicollinearity of the phenotypic correlation matrix, this was implanted in direct and indirect effects, considering the following equation:

$Y = p_1X_1 + p_2X_2 + ... + p_nX_n + p_\varepsilon u$

where Y is the main dependent variable yield. X_1, X_2……, Xn are the independent variables. p_1, p_2, .. p_n are the path coefficients. The coefficient of determination was calculated by the expression

$R^2 = p^2_{1y} + p^2_{2y} + ... 2p_{2y}.p_{2nY2n}$

The estimates of the components of the phenotypic variance, genetic parameters, correlations between traits and path analysis were obtained using the computational application GENES (Cruz, 2013).

3. Results and Discussion

Timeless climate data and information

Over the past 20 years, the average annual air temperature has varied between 27.37 and 28.84°C (Fig. 1). The highest annual temperature observed was 35.22, in 2015. On the other hand, the lower annual temperature observed was 20.78°C, in 2018. The deviations observed for the maximum, minimum and average annual temperatures were ±0.57°C, ±0.70°C and ±0.41°C, respectively. Total annual precipitation ranged from 1961.30 mm in 2016, to 498.50 mm in 2000 (Fig. 1). Because this great difference between the results collected for total annual precipitation, there was a large deviation for this parameter, value equal to ±0358.38. The average of annual total precipitation over the last 20 years was 1395.85 mm.

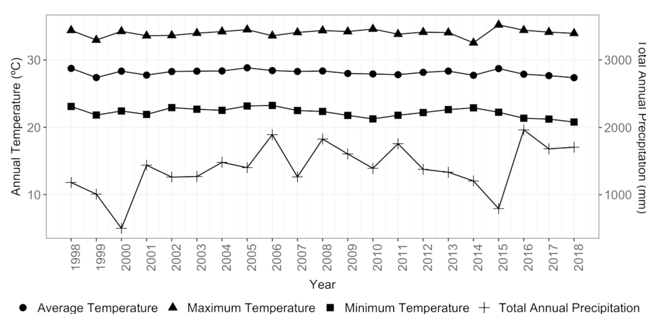

Fig. 1 - Annual maximum temperature, annual minimum temperature, average annual temperature and total annual precipitation of the last twenty years (1998-2018) in a region characterized by the Cerrado/Amazon Rainforest ecotone (INMET, 2019).

Estimates of genetic parameters

The analysis of variance showed a significant effect (p<0.01) between the genotypes according to the F test for all evaluated characteristics (Table 2), showing genetic variability presence in the population. Considering the existence of genetic variability in a population is a determining factor for any breeding program (Ramalho *et al*., 2012), at first, the germplasm under study is promising for selection or hybridization work with potential for new cultivars development. Similarly, Araméndiz-Tatis *et al*. (2018) also detected significant differences for the same traits evaluated in an assay where they estimated the genetic parameters of traits associated with yield in 42 white seed cowpea genotypes.

The relative standard deviation (RSD), which is used to estimate the experiments precision, presented values considered low for the traits PH, PL, PM, MGV and GI, which indicates excellent experimental precision. While for the characteristics SPP and Yield presented RSD equal to 12.24% and 21.86%, therefore, they are considered regular values, indicating good experimental precision (Cruz *et al*., 2014; Ferreira, 2018) (Table 2). Similar results for the same

Table 2 - Analysis of variance summary for the traits plant height (PH), pod length (PL), pod mass (PM), pod grain mass (PGM), grain index (GI), seeds per pod (SPP) and Yield in 20 cowpea genotypes evaluated in Cerrado/Amazon Rainforest ecotone region, Brazil, 2019

Variation source	DF	PH (cm)	PL (cm)	PM (g)	PGM (g)	GI (%)	SPP	Yield (Kg ha-1)
		Mean square						
Blocks	3	46.46	1.61	5.54	5.05	112.71	75.61	120831.01
Genotypes	19	319.08 **	4.28 **	34.85 **	13.43 **	112.71 **	238.99 **	136000.33 **
Error	57	6.46	0.70	0.99	0.55	16.19	21.72	20004.46
Mean	-	65.36	20.22	18.57	14.40	78.12	75.74	647.03
RSD (%)	-	3.89	4.14	5.35	5.17	5.15	6.15	21.86
I.V. (%)	-	1.95	2.07	2.68	2.59	2.58	3.08	10.93

DF= degrees of freedom; (**) significant at 1% probability of error by the F test; RSD= relative standard deviation; VI= variation index.

traits were obtained by Carvalho *et al.* (2012) and Correa *et al*. (2015).

The variation index (VI), another parameter related to the experimental precision, proposed by Gomes (1991), which is more adequate than the RSD, as it also considers the number of repetitions used in the experiment, besides the residual variation, presented low values for all traits, except for productivity that presented value of IV considered of medium magnitude.

The success of the selection depends on the existence and magnitude of the observed genetic variability for yield and its components in the material under breeding (Adewale *et al.*, 2010; Raturi *et al.*, 2015; Shereen and El-Nahrawy, 2018). Table 3 shows there was low phenotypic variation (s) for the traits PL, PM, PGM, while for PH, GI and SPP a certain phenotypic variation was observed. For yield, there is a high phenotypic variation (s).

The values estimated for genotypic variance (s) ranged from 0.89 for pod length (PL) to 78.15 for plant height (PH) (Table 3). Analyzing the genotypic variance (s) in relation to the phenotypic variance (s), it was observed there was a major contribution of genotypic variance (s) to the present phenotypic variability. These results were confirmed by the estimates of the genotypic determination coefficient (R^2) (Table 3).

Table 3 - Estimates of genetic parameters for traits plant height (PH), pod length (PL), pod mass (PM), pod grain mass (PGM), grain index (GI), seeds per pod (SPP) and Yield in 20 cowpea genotypes evaluated in Cerrado/Amazon Rainforest ecotone region, Brazil, 2019

Genetic parameters	Traits						
	PH (cm)	PL (cm)	PM (g)	PGM (g)	GI (%)	SPP	Yield (Kg ha^{-1})
σ^2_F	79.77	1.07	8.71	3.36	28.18	59.75	34000.08
σ^2_E	1.61	0.17	0.71	0.14	4.05	5.43	5001.11
σ^2_G	78.15	0.89	8.46	3.22	24.13	54.32	28998.96
R^2 (%)	97.98	83.15	97.17	95.87	85.64	90.91	85.29
ICC (%)	92.37	56.15	89.55	85.30	59.85	71.44	59.18
PCV (%)	13.66	5.12	15.89	12.73	6.80	10.21	28.50
GCV (%)	13.52	4.68	16.67	12.46	6.29	9.73	26.32
ECV (%)	3.89	4.14	5.35	5.17	5.15	6.15	21.86
b = GCV/ECV	3.48	1.13	2.93	2.41	1.22	1.58	1.20
$\hat{r}$	0.99	0.91	0.99	0.98	0.93	0.95	0.92
Mean	65.36	20.22	18.57	14.40	78.12	75.74	647.03

σ^2_F = phenotypic variance; σ^2_E = environmental variance; σ^2_G = genotypic variance; R^2= genotypic determination coefficient; ICC= intra class correlation coefficient; PCV= phenotypic coefficient of variation; GCV= genotypic coefficient of variation; ECV= environmental coefficient of variation; *b* quocient = GCV/ECV ratio; $\hat{r}$ = accuracy.

Cruz *et al*. (2014) mention that when the adopted statistical model considers genotypes as a fixed effect, as in the present study, heritability becomes the genotypic determination coefficient. The values of the genotypic determination coefficient (R^2) ranged from 83.15% for PL to 97.9% for PH. All evaluated traits presented high (R^2) estimates (>75%). This parameter provides indications of the expected performance of a given population for traits selection, which allows us to infer that the population in study is promising for the trait selection under study.

However, it is noteworthy that for complex inheritance characteristics such as yield, which are the expression result of many alleles and they are greatly influenced by the environmental conditions to which population undergoes, the high values of R^2 may be overestimated by genotype x environment interaction, since the present study was conducted in only one year and in a single environment.

Torres *et al.* (2015), in a study to determine the number of measurements required, evaluated 40 genotypes of prostrate and semi-prostrate cowpea types in the state of Mato Grosso do Sul, in ten assays, the R^2 ranged from 51.50% to 92.64%. While Shimelis and Shiringani (2010), in a study to determine the variance components and heritability in ten cowpea lines, obtained the genotypic determination coefficient of 55.00% for yield, lower than the value found in the present work.

Given the high value of R^2, it can be inferred that it is caused by the inherent genetic variability of the tested genotypes, because each of them contributes a distinct genetic identity (Teixeira *et al*., 2007). Fehr (1987) mentions that higher genotypic determination coefficients may be associated with lower environment variation and lower genotype-environment interaction. And according to Gomes (2009), there is low to medium accuracy in environmental control, since the relative standard deviations (RSD) were below 21% for all characters.

The intraclass correlation coefficient (ICC), which corresponds to the repeatability coefficient, indicates an estimate of the total measurement variability fraction owing to variations between individuals. The ICC ranged from 56.15% to 92.37% for pod length and plant height, respectively (Table 3). When characteristics have a lower intraclass correlation coefficient require a greater number of measurements (replications) to predict the real value of a given trait and vice versa. Therefore, it can be inferred for the superior genotypes selection, the number of measurements in the present study is satisfactory.

The coefficients of variation provide information about the variation nature and magnitude. They clarify if the variations are owing to genetic or environmental causes. Typically, the GCV values are bigger than ECV. If the differences between GCV and ECV are excessive, so the environmental effects will be more noticeable on the trait. Thus, in the observed results, the relative proportion (%) of the deviations from the mean because of genetic effects (GCV) were higher when compared to the environmental ones (ECV), for all traits (Table 3).

The genotypic coefficient of variation (GCV) ranged from 4.68% for pod length and from 26,32 to 26.32% for yield (Table 3). The highest estimates (GCV) were recorded for pod grain mass (12.46%), plant height (13.52%), pod mass (16.67%) and yield (26.32%), indicating that these traits offer greater selection perspectives to obtain genotypes much more aligned to the proposed, as they are erect habit and determined growth genotypes.

These results are consistent with those found by Lopes *et al.* (2017) for yield and pod mass; Regis *et al*. (2014) for pod grain mass and grain index, and Bhagasara *et al.* (2017) for yield in this species. The characteristics plant height, pod length and seeds per pod showed lower GCV and, therefore, present greater difficulties in the selection process and expected genetic advance. Fact in agreement with Correa *et al*. (2015) and Silva and Neves (2011). However, Gerrano *et al.* (2015), found higher GCV values for plant height (67.41%), pod length (19.97%) and seeds per pod (24.82%).

The *b* quotient is an auxiliary tool for the breeder. According to the interpretation of Cruz *et al.* (2014) for this parameter, when the value is greater than or equal to 1.00, it indicates that there is genetic variability within the population in study, which can therefore, be explored, and in the case, the trait is favorable to selection. The quotient b ranged from 1.13 for PL to 3.48 for PH (Table 3). Thus, it can concluded that the *b* quotient values found for all evaluated characteristics are favorable to selection in order to obtain more productive genotypes.

Genotype evaluation assays should be approached from a genetic and statistical point of view, not just from a statistical perspective. In the context of genotypic evaluation, accuracy is the most important statistical parameter. It has the property of informing about the correct ordering of genotypes for selection purposes and also about the effectiveness of inference about the genotypic value of each genotype (Resende, 2002).

Accuracy depends not only on the residual variation magnitude and the number of replication, but also on the proportion between the genetic and residual variations associated with the trait under evaluation. Accuracy refers to the correlation between the true genotypic value of genetic treatment and that estimated or predicted from the information from the experiments. As a correlation, it ranges from 0 to 1, and the appropriate accuracy values are those close to the unit or 100% (Henderson, 1984).

Therefore, for all evaluated characteristics in the present experiment, the observed values for accuracy are considered very high, as they are above 0.90. High accuracy variables indicate small absolute deviations between true genotypic values and those estimated from experimental information. Resende and Duarte (2007) emphasize the importance of achieving optimal selective accuracy greater than 0.90 for safe statistical inference.

Correlation between traits and path analysis

Correlation estimates indicate good signal agreement between phenotypic and genotypic correlations (Table 4). In general, genotypic correlations present values higher than their corresponding phenotypic and environmental correlations. Similar results were obtained by Andrade *et al.* (2010), Correa *et al*. (2015), Almeida *et al.* (2014), Gerrano *et al*. (2015), Teixeira *et al.* (2007) and Manggoel *et al.* (2012) in studies conducted with cowpea, evaluating yield components.

There was a significant (p≤0.01) and high magnitude positive phenotypic correlation (Υ_P) between the traits pod grain mass (PGM) and pod mass (PM), which was already expected, insofar that pod grain mass increase happens, it should also increase the pod mass, or vice versa. However, the traits grain index and seeds per pod presented negative phenotypic correlation at 1% probability (Table 4). For the other pairs of characteristics there were no significant phenotypic correlations.

Genotypic correlations (Υ_G) showed the same sign and, in most cases, values higher than their corresponding phenotypic correlations, indicating that the phenotypic expression is decreased because of environmental influences. Although the yield components were positively correlated with yield, the Υ_P and Υ_G estimates showed low magnitude and they

Table 4 - Estimates of the correlation coefficients phenotypic (Υ_P), genotypic (Υ_G) and environmental (Υ_E) between the traits plant height (PH), pod length (PL), pod mass (PM), pod grain mass (PGM), grain index (GI), seeds per pod (SPP) and Yield in 20 cowpea genotypes evaluated in Cerrado/Amazon Rainforest ecotone region, Brazil, 2019

Characteristics	Υ	PH	PL	PM	PGM	GI	SPP	Yield
PH	P	1	-0.47*	-0.14 NS	-0.06 NS	0.15 NS	-0.35 NS	0.18 NS
	G	1	-0.52*	-0.14 NS	-0.05 NS	0.18 NS	-0.37 NS	0.20 NS
	E	1	-0.04 NS	-0.07 NS	-0.09 NS	-0.16 NS	-0.02 NS	-0.07NS
PL	P		1	0.06 NS	0.14 NS	0.11 NS	-0.02 NS	0.05 NS
	G		1	0.07 NS	0.14 NS	0.12 NS	-0.00 NS	0.04 NS
	E		1	0.04 NS	0.14 NS	0.06 NS	0.21 NS	0.07 NS
PM	P			1	0.90 **	-0.64 **	0.32 NS	-0.14 NS
	G			1	0.91 **	-0.66 **	0.34 NS	-0.16 NS
	E			1	0.44 **	-0.47 **	0.10 NS	0.10 NS
PGM	P				1	-0.23 NS	0.18 NS	-0.03 NS
	G				1	-0.31 NS	0.19 NS	-0.04 NS
	E				1	0.55 **	0.04 NS	0.04 NS
GM	P					1	-0.39 NS	0.26 NS
	G					1	-0.43 NS	0.33 NS
	E					1	-0.12 NS	-0.12 NS
SPP	P						1	0.12 NS
	G						1	0.12 NS
	E						1	0.11 NS
Yield	P							1
	G							1
	E							1

NS= not significant; (*), (**) significant at 5% and 1%, respectively, by the t test.

were, mostly, non-significant.

Cruz *et al.* (2012) attribute the genetic correlations occurrence, mainly to the pleiotropy or to the genetic links between traits pairs, in the latter case, transient causes. In any case, genetic correlations favor the simultaneous selection of two or more traits by selecting only one of these. On the other hand, according to these authors, the selection of one trait may lead to an undesirable selection of another.

The negative estimates of correlation between pairs of traits indicate that improving one trait will decrease the other, and in these cases, the selection based on this one is not recommended. The characteristic plant height was phenotypically and genetically negatively correlated with pod length, indicating that the smaller the plant, the longer the pod length, which directly influences the yield. According to Falconer and Mackay (1996), genotypic and environment correlations of exchanged signals, as can be observed in some characteristics pairs (Table 4), reveal that the causes of genetic and environmental variation influenced the traits through different physiological mechanisms.

Given the complexity among the yield components that contribute to yield, the selection of cowpea genotypes is difficult. Thus, it is evident the need to unfold the correlations in direct and indirect effects, evaluating the importance degree of each of the explanatory variables in relation to the main or basic variable (Daros *et al.*, 2004).

Cruz *et al.* (2014) report that the parameter estimates under multicollinearity may assume absurd values or with no consistency to the studied biological phenomena. Thus, for greater reliability of the path analysis results, the phenotypic correlation matrix between characteristics was tested for multicollinearity by the condition number proposed by Montgomery *et al.* (2012).

The correlation matrix had a condition number equal to 993.97, that is, collinearity between the characters considered moderate to strong, presenting no problem for the path coefficients estimates. The coefficient of determination (R^2) and the residual effect indicate how much the explanatory variables determine the yield. The coefficient of determination was 0.2025 and the residual effect was 0.8930 (Table 5).

The direct effects magnitudes of the traits analyzed on yield were higher than the estimates magnitudes of their respective simple correlations with

Table 5 - Estimates of direct and indirect effects involving the main variable, Yield in kg ha^{-1}, and the explanatory variables: plant height, pod length, pod mass, pod grain mass, grain index, seeds per pod concerning to 20 cowpea genotypes evaluated in Cerrado/Amazon Rainforest ecotone region, Brazil, 2019

Characteristics	Association effects	Path coefficients
Plant height	Direct on Yield	0.3671
	Indirect via PL	-0.0805
	Indirect via PM	-0.1327
	Indirect via PGM	0.0434
	Indirect via GI	0.1162
	Indirect via SPP	-0.1302
	Total	0.1833
Pod length	Direct on Yield	0.1711
	Indirect via PH	-0.1727
	Indirect via PM	0.0612
	Indirect via PGM	-0.1058
	Indirect via GI	0.0845
	Indirect via SPP	0.0082
	Total	0.0466
Pod mass	Direct on Yield	0.9628
	Indirect via PH	-0.0506
	Indirect via PL	0.0109
	Indirect via PGM	-0.7015
	Indirect via GI	-0.4831
	Indirect via SPP	0.1191
	Total	-0.1425
Pod grain mass	Direct on Yield	0.7835
	Indirect via PH	-0.0203
	Indirect via PL	0.0231
	Indirect via PM	0.8621
	Indirect via GI	-0.1774
	Indirect via SPP	0.0664
	Total	-0.0295
Grain index	Direct on Yield	0.7593
	Indirect via PH	0.0561
	Indirect via PL	0.0190
	Indirect via PM	-0.6126
	Indirect via PGM	0.1831
	Indirect via SPP	-0.1444
	Total	0.2605
Seeds per pod	Direct on Yield	0.3702
	Indireto via PH	-0.1291
	Indirect via CV	0.0038
	Indirect via PM	0.3097
	Indirect via PGM	-0.1407
	Indirect via GI	-0.2963
	Total	0.1177
Coefficient of determination		0.2025
Residual variable effect		0.8930

yield (Table 5). Based on this information, it is possible to infer there are other traits influencing both the magnitude and the correlation direction between the yield components.

Considering the direct effects on yield, included in Table 5, the trait pod mass (0.9628) has the greatest effect, indicating a major contribution to the yield increase, surpassing the pod grain mass, which also had a high direct effect (0.7835). In contrast, pod length (0.1711) was the trait with the lowest effect. Important to mention there was no negative direct effect of any trait on yield.

Still in Table 5, it can be seen that although the character PM had a high direct effect on yield, in general, the indirect effects via PM on yield were low. Indicating that indirect truncation selection in the auxiliary character may not provide satisfactory gains in the main variable (yield). In these cases, the best strategy is the multi-trait selection (Cruz *et al.*, 2012).

Indirect effects on yield were relatively low, except for the trait PGM via PM, which pointed to an estimate of 0.8621. This result is indicative of the indirect selection viability via pod mass to obtain gains on the most important character. The trait PH had negative indirect effect via all the characteristics, except GI, which indicates that the plant height reduction induces the increase in the other characteristics, which is very important in this crop production system, considering that plants very high hinder crop handling and harvesting.

Considering the total effect, the traits have had the greatest effect on yield were as follows grain index (0.2605), plant height (0.1833), seeds per pod (0.1177) and pod length (0.0466) (Table 5). This result of the total effect in relation to the direct effects on yield was owing to the negative indirect effects via the other characteristics, which confirms the need to apply a multi-trait selection.

Considering that the existence of genetic variability in population is a determining factor for any breeding program (Ramalho *et al*., 2012), the germplasm under study is, initially, promising for selection or hybridization work with potential for the new cultivars development.

4. Conclusions

The study concluded there is a considerable degree of genotypic variation between important agronomic traits in cowpea. Genotypic variation contributed most of the phenotypic variation. Result corroborated by the high estimates of genotypic determination coefficient.

Thus, the genetic parameters estimates obtained for yield and agronomic traits, in the present study, will provide a basis for selection in order to obtain gains in the breeding for cowpea yield.

The path analysis indicated the pod mass and pod grain mass had the greatest favorable effect on yield in cowpea, and could also be used for indirect selection aiming at the development of new genotypes with high yield potential.

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Optimization of biosolids as a substrate for tomato transplant production

P.C. Otieno (*), S. Nyalala, J. Wolukau
Department of Crops, Horticulture and Soils, Egerton University, PO Box 536-20115, Egerton, Kenya.

Key words: biosolids, quality, seedling growth, *Solanum lycopersicum* L., substrate, tomato.

Abstract: The need to recycle waste and increasing pressure against peat extraction and importation, have led to increasing interest in substituting peat with organic wastes. Use of biosolids substrate would be a low cost alternative substrate to peat for commercial production of transplants. The objective of this study was to determine the effect of biosolids-forest soil mixture ratios on tomato 'Maxim F1', transplants emergence and growth. A randomized complete block design with four replications was used in this study. The treatments were: biosolids (BS) mixed with forest soil (FS) at rates of 0% 10%, 20%, 30%, 40%, 50% and 60% (v/v), tea compost (TC) and coco peat (CP). Five tomato seeds were planted in four 250 cm³ pots, grouped into four to form an experimental unit. Results showed that biosolids (BS) at rate of 30% registered significantly (*p*<0.05) higher seedling emergence (94%), leaf numbers (4.5), height (16.5 cm), collar diameter (6.3 mm), chlorophyll content (25 index units), root volume (2.0 cm³) and root/shoot dry matter (10.2 % and 16.3%, respectively) than the rest of the substrates except tea compost (TC). Sodium was significantly (*p* < 0.05) higher in BS at rates of 50% (350 mg kg⁻¹) and 60% (376 mg kg⁻¹) and this raised EC (4.5) and lowered pH of the media to 4.4. At 30% BS enhanced tomato transplant production to similar level as tea compost, hence recommended for commercial use.

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(*) **Corresponding author:**
pcotieno@egerton.ac.ke

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1. Introduction

Tomato (*Solanum lycopersicum* L.) can be established in the field by direct seeding or transplanting. Tomato production by transplanting has been done over the past two decades to increase resource use efficiency and reduce environmental impact on seeds planted (Restrepo *et al*., 2013). Cultivation from transplants has many advantages including earlier harvest; more efficient use of land, time, energy, and seeds; and healthy and homogenous production (Pascual *et al*., 2018). In comparison with direct sowing, transplanting is a more reliable method of ensuring higher plant survival, faster establishment, improved plant uniformity, early maturity, and reduced cost of production (Gogo *et al.,* 2012). The production of tomato seedlings, especially in sub-Saharan Africa with great expansion of open field and greenhouse crops, is a highly competitive business. Besides, uniform and rapid seed emergence and quality are

essential prerequisites to increasing tomato yield, quality, and profits (Wachira *et al*., 2014). In addition, tomato seeds especially F1 hybrids are expensive and farmers in developing countries cannot tolerate poor germination as a result of poor soil conditions (HCD, 2017). Use of ideal transplant substrates with appropriate physicochemical properties, is therefore critical (Sterrett, 2001).

In transplant production, the main purpose of a substrate is to satisfy the needs for good seedling growth within the limited space of a container and to prepare the seedlings for successful transplanting into the field (Pascual *et al*., 2018). The quality of growing media is one of the main factors influencing the success of horticultural nursery activity (Raviv and Lieth, 2008), and it is also directly linked to the quality of the materials utilized in growing media formulations (Reis and Coelho, 2007). The choice of appropriate substrate is therefore an important factor in promoting the optimum growth of plants. A number of potential substrates have been identified, of which Peat moss has long been the primary component of transplant and potting media for both vegetable and ornamental plants. This has been mainly due to its physical and chemical properties (Raviv *et al*., 1986): adequate free air space (FAS) at 0-10 cm water suction; high water content at low tension at 10-100 cm water suction; and high cation exchange capacity (CEC) minimizing loss of nutrients and facilitating adequate mineral nutrition (Colla *et al.,* 2007). However, peat also has some notable disadvantages; being conducive for the development of some soil-borne plant pathogens such as *Pythium* and *Rhizoctonia* (Hoitink and Kuter, 1986). Furthermore, Peat moss is normally harvested from wetland ecosystems at rates considered non-sustainable by wetland ecologists (Buckland, 1993). These drawbacks have motivated horticulturists throughout the world to seek alternatives like coir (coco peat) which has several qualities: high water-holding capacity, excellent drainage, absence of weeds and pathogens, renewable resource, with no ecological drawbacks to its use, acceptable pH, cation exchange capacity (CEC) and electrical conductivity (EC) and easier wettability (Cresswell, 1992). Under nursery conditions, coco peat and peat moss have been used as reliable media for organic production of lettuce transplants (Colla *et al*., 2007). However, coco peat has become more expensive and its properties are more variable (Chrysargyris *et al*., 2013). Thus, it is important to look for high quality, locally available and low-cost alternative substrates.

Among the organic substrates for transplants production, Vermicompost is a promising substitute for peat especially in the production of seedling, but not a sustainable solution for management of organic wastes (Ivanka and Tsvetanka, 2012). Use of biosolids from treated sewage, has been proven to be promising (Vyas, 2011; Giannakis *et al*., 2014). The effects of biosolids on seedling emergence and growth have been investigated by Chrysargyris and Tzortzakis (2015) and their results indicated that application of boisolids as a substrate in marigold (*Tagetes erecta* L.) and basil (*Ocimum basilicum* L.) seedlings production has potential. Similarly, use of organic urban waste compost for tomato (*Solanum lycopersicum* L.) transplant production has been reported to result in quality transplants in the seedbed (Herrera *et al*., 2008). Chrysargyris and Tzortzakis (2015) specified biosolids as an ideal component of mixed-peat substrates for eggplant (*Solanum melongena* L.) seedlings, at a rate less than 30% in a substrate mixture. In another study on cucumber transplants production, Mami and Peyvast (2010) recommended the use of biosolids at 5% and below on peat mixture. However, the use of biosolids as substrates depending on the ratios may have negative effects as a consequence of its high salt content, unsuitable physical properties (texture, structure, moisture content, porosity etc.), heavy metal toxicity, and variable quality and composition (Papamichalaki *et al*., 2014). The appropriate amount of biosolids added in growth medium needs to be determined to improve plant growth. Therefore this study investigated the the effect of biosolids-forest (BS: FS) soil mixing rates on tomato transplant emergence and growth.

2. Materials and Methods

Site description

This study was conducted in two trials at the Horticulture Research Field, Egerton University, Kenya during January to February and March to April, 2018. The site is located on latitude 0 23' S and longitude 35 35' E in the lower highland III (LH3) agro ecological zone at an altitude of 2238 m above sea level (Jaetzold *et al*., 2012). The experiments were done in an area measuring 1.2 m by 3.5 m within a plastic greenhouse size 8 m by 60 m and a height of 3 m. The greenhouse covering material was UV stabilized polythene sheet gauge 150 µm from Amiran, Co Ltd Nairobi Kenya. Greenhouse microclimatic condition, temperature and relative humidity averages were as

follows; day (6:00 AM-6:00 PM) and night (6:00 PM-6:00 AM) air temperatures inside the greenhouse during the experiment were 24.5±0.9°C and 13.3±4°C, respectively. Average day and night relative humidity inside the greenhouse were 55±6% and 80±6%, respectively.

Biosolids and forest soil sample collection, substrate preparation and analysis

Biosolids (BS) were collected from a lagoon pond at Egerton University Wastewater Treatment Plant and forest soil obtained from typically tropical forest. The substrates for transplants production were prepared by mixing the biosolids and forest soil (BS: FS at rates of 0, 10, 20, 30, 40, 50 and 60% (v/v). Samples from each rate, tea compost (TC) and coco peat (CP) as reference commercial substrates were comprehensively analysed in a laboratory to determine the physico-chemical characteristics of the substrates (Table 1). Porosity of each substrate was calculated from the ratio of the determined bulk density and of known particle density (2.65 g cm^{-3}) as given in the equation given below (Okalebo *et al.,* 2002):

Porosity (%) = 1 - (Bulk density /Particle density) × 100).

Experimental set up and design

The experimental design was randomized complete block design (RCBD), replicated four times. The treatments included seven BS: FS soil mixtures at different rates and two commercial substrates TC and CP. In the experiment, plastic pots (250 cm^3) were used for potting the substrates. An experimental unit composed of four pots, each planted with four tomato 'Maxim F1' seeds.

Transplants establishment and Irrigation schedule

Tomato seeds were planted in the pots in the evening and substrates watered to saturation point. After 24 hours each substrate was irrigated with 15 ml of water after every 12 hours for the first 15 days. The volume of water was increased to 20 ml for the next 10 days, then 25 ml for the remaining days of the experiment.

Determination of seedling emergence and growth

The number of emerging seedlings was recorded. Based on the number of planted seeds (20), seedling emergence percentages were computed progressively after 7, 9 and 11 days after planting (DAP). Germination percentage was determined using equation adopted by Atif *et al.* (2016), with modification

Table 1 - Physico-chemical characteristics of the substrate used for tomato transplant production

Characterization/substrates	FS	BS 10%	BS 20%	BS 30%	BS 40%	BS 50%	BS 60%	TC	CP
Bulk density (g cm^{-3})	1.7	1.6	1.5	1.3	1.3	1.3	1.3	1.3	1.2
Porosity (%)	35.9	39.6	43.4	50.9	50.9	50.9	50.9	50.9	54.7
Moisture content (%)	25.8	34	40.8	42.8	44.5	45.1	45.9	44.7	45.3
EC (mS m^{-1})	2.6	3.2	3.6	4.4	5.1	5.2	5.4	4.3	5.2
pH	7.4	6.2	6.6	6.5	6.4	5.6	5.4	7.4	7.4
Organic matter (g kg^{-1})	157.7	197.8	196.7	210	209.8	220	222.9	207.2	171.4
C:N	21.3	19.7	15.4	9.6	12.7	14.7	12.5	7.6	10.8
Total Carbon (mg g^{-1})	91.7	115.0	114.4	122.1	122.0	127.9	129.6	120.5	99.6
Total N (g kg^{-1}) **(0.1)** [y]	4.3	5.9	7.4	12.9	9.6	8.9	10.5	16.3	9.2
Total P (mg k g^{-1}) **(70)** [y]	69.1	83	90.3	101	95.9	79.3	70.3	116.1	33.8
K (mg kg^{-1}) **(700)** [y]	132.5	412.3	419.9	427.8	422.4	403.7	403.5	369.6	344.1
Ca (mg kg^{-1}) **(1000)** [y]	21.9	24	22.8	29.5	27	28.5	27.5	43.5	38.5
Mg (mg kg^{-1}) **(700)** [y]	131.1	126.1	117.7	119.1	113.8	47.7	37.2	126.6	114.6
Na (mg kg^{-1})	62.9	254.8	342.1	252.8	348.3	349.8	376.3	114.8	164.4
Mn (mg kg^{-1}) **(20)** [y]	69.6	530.4	524.8	539.4	553.9	551.9	544.8	167	29.8
Fe (mg kg^{-1})	27	2490	2473.9	2479.1	2471.5	1184.1	852.5	207.4	114.1
Zn (mg kg^{-1})	4.7	47.4	44	44	45.9	24.4	25.4	21.9	16.4
Cu (mg kg^{-1}) **(100)** [z]	4.4	12.2	12.7	10.3	12.7	13.1	13.3	14	6.5
Cd (mg kg^{-1}) **(1)** [z]	0.0023	0.0128	0.0115	0.0117	0.0122	0.0122	0.0122	0.0122	0.0121
Pb (mg kg^{-1}) **(150)** [z]	109.6	2.8	2.1	5.1	3.1	6	2.5	20.1	4.3

[y] Recommended levels of nutrient in soil for tomato production according to Sainju *et al*. (2003).

[z] Maximum ceiling values of heavy metals for agricultural land application according to NSW EPA (2000).

as given below:

$$G\ (\%) = (S_2 / S_1) \times 100$$

Where G is the germination percentage, S_1 is total seeds planted and S_2 seeds germinated.

Ten tomato seedlings were randomly selected and tagged for data collection on growth parameters. Seedling height, collar diameter, leaf number and leaf chlorophyll content were determined 14, 21 and 28 DAP. Seedling height was determined using a measuring tape from the ground level to the tip of the seedling. For stem diameter, a stainless hardened 150 mm LCD electronic digital vernier mark (Grainger, USA) was used. The unit of measurement was millimeters (mm). The stem diameter was measured on the main stem of the plant at 1 cm above the substrate. Number of leaves was determined by counting the true leaves.

Determination of transplant leaf chlorophyll content

This was determined using a chlorophyll content meter (CCM-200) plus; Opti-Sciences, Tyngsboro, MA). Estimate of chlorophyll content was in chlorophyll concentration index units (CCls). Three readings of chlorophyll content were taken on the third newly developed leaflet from the top of each tomato plant and means were computed for each replication. The Leaf chlorophyll was measured using SPAD chlorophyll meter (Minolta SPAD502 meter, Tokyo, Japan). Pengfei (2017) reported that SPAD values have a direct linear relationship with extracted leaf chlorophyll therefore, SPAD value was used to describe leaf chlorophyll index units (CCls) in the current study.

Determination of root volume and root/shoot dry weight

During seedling harvesting on the fourth week (28 DAP), four seedlings were randomly selected and carefully uprooted. The roots were washed clean in running tap water on a sieve of pore diameter of 1 millimeter. Separation of tomato transplant roots and shoot was done at the crown level. Root volume, was determined by scanning plant roots using Epson Expression 10000XL color image scanner and analyzed using Winrhizo software (LA 2100-Regent Instruments Inc.) as described by Mwamlima *et al.* (2019).

Separated shoot and root plant parts were dried in an oven to constant weights at 60°C for 24 h as described by Hossain *et al.* (2008). Mean weights of dried samples were taken as shoot and root biomass per plant. The roots and the shoots of the randomly selected four plants were also used to determine dry weight. This was done by oven drying the roots and shoots of the seedlings at 105°C until constant weight was achieved. Percentage dry root and shoot weight was then computed based on the initial fresh weight according to equation below (Atif *et al.*, 2016):

$$RDMA\ (\%) = DW\ (g) / FW\ (g) \times 100$$

where RDMA; root dry matter accumulation in percentage, DW; dry weight (g) and FW; fresh weight (g).

Data analysis

Data analysis was carried out using statistical package SAS version 9.1 (SAS Institute, Cary Inc., 2001). Data for the two trials were pooled and subjected to analysis of variance (ANOVA) at $p \leq 0.05$ and means for significant treatments separated using Tukey's Honestly Significant Difference (HSD) test at $p<0.05$. The model fitted for the experiment was $Y_{ij} = \mu + \beta_i + \alpha_j + \varepsilon_{ij}$, where, y_{ij} = tomato response, μ = overall mean, β_i = effect of the ith block, α_j = effect of the jth level of substrates ε_{ij} = random error term, i = 1, 2, 3, 4; j= 1, 2, 3 …9.

3. Results

Seedling emergence

The substrates tested influenced the emergence of tomato seedlings differently (Fig. 1). Tea compost (TC) and biosolids (BS) at 30% had the highest emer-

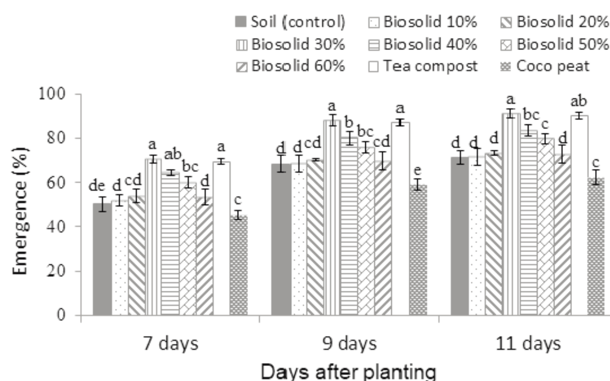

Fig. 1 - Effect of biosolids on emergence of tomato seedlings. Means ± standard deviation followed by the same letter within a day after planting are not significantly different according to Tukey's HSD test (p<0.05). FS = Forest soil; BS = Biosolids; TC = Tea compost; CP= Coco peat.

gence percentage compared to the rest of the substrates throughout the evaluation, while Coco peat (CP) had the lowest emergence percentage. From day 7 to day 11 after planting, BS at 30% was significantly (p<0.05) higher (90-95%) in seedling emergence and this was not significantly (p<0.05) different from that of TC (commercial substrate). At day 7, the control forest soil (FS) was not different from CP (another commercial substrate).

Plant height

Biosolids (BS) application rates influenced tomato seedling height during the growing period. A part from BS at the rate of 30% and TC, which produced the tallest transplants, there were no significant (p<0.05) difference among the rates of 20%, 40% 50% and 60% in plant height. Biosolids at 30% was consistently similar to tea compost (TC) in producing taller tomato seedlings 14, 21 and 28 days after planting (DAP). However, the shortest plants were obtained with forest soil (FS) and coco peat (CP) (Fig. 2).

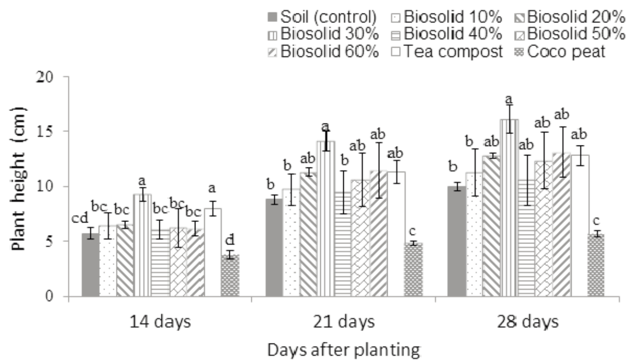

Fig. 2 - Effect of biosolids on tomato seedling height. Means ± standard deviation followed by the same letter within a day after planting are not significantly different according to Tukey's HSD test (p<0.05).

Leaf number

On tomato leaf number, BS at the rate of 30% was similar to TC in recording significantly (p<0.05) higher number of leaves per tomato plant throughout the period of the experiment (Fig. 3). However, there was no significant difference between BS rates within the range of 10% to 40% on 14, 21 and 28 DAP. The lowest tomato leaf number was obtained with FS and CP. Biosolids at 50 and 60% resulted in significantly (p<0.05) lower number of leaves than BS at 30% on 14 and 28 DAP.

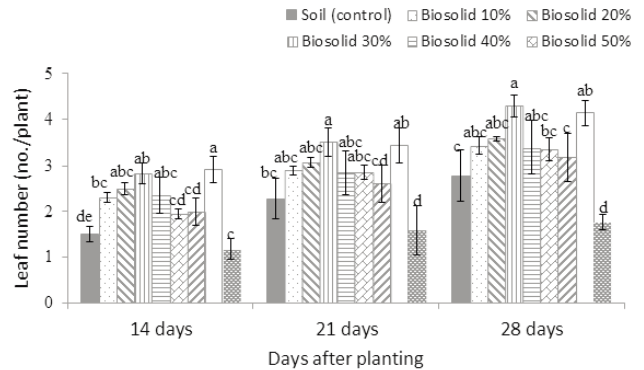

Fig. 3 - Effect of biosolids on tomato seedling leaf number. Means ± standard deviation followed by the same letter within a day after planting are not significantly different according to Tukey's HSD test (p<0.05).

Collar diameter

Application of biosolids at 30% resulted in the widest collar diameter of tomato seedlings throughout the period of the experiment (Fig. 4). At 14 DAP, all the treatments except tea compost recorded significantly (p<0.05) narrower collar diameter than BS at 30%.

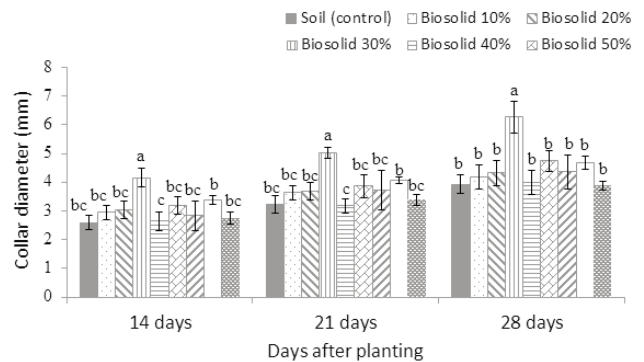

Fig. 4 - Effect of biosolids on tomato seedling collar diameter. Means followed by the same letter within a day after planting are not significantly different according to Tukey's HSD test (p<0.05).

Leaf chlorophyll content

Tomato transplants grown on biosolids at 30% had significantly (p<0.05) higher leaf chlorophyll content compared to the rest of the treatments (Fig. 5). Using coco peat (CP) resulted in transplants with the lowest chlorophyll content. However, there was no much difference in physical appearance of the leaf colour (Plate 1).

Root volume

Tomato transplants root volume was affected by use of biosolids (Fig. 6, Plate 2). Biosolids at 30% resulted in significantly (p<0.05) higher root volume than the forsest soil, coco peat and the other tested rates of BS. However, there was no significant difference in root volume between tomato transplants grown on biosolids at 30% and tea compost.

Root and shoot dry weight

There was similar response of transplants to different substrates in terms of roots and shoot dry weight (Fig. 7). Transplants grown on biosolids at 20% or 30% had significantly (p<0.05) higher root dry weight, which was similar to that obtained with tea compost. In addition, biosolids at 30% and tea compost similarly recorded significantly (p<0.05) higher shoot dry weight than all the other treatments. Forest soil and coco peat resulted in the lowest root and shoot dry weight.

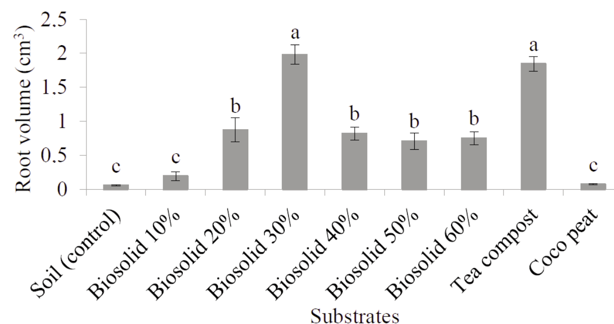

Fig. 6 - Effect of biosolids on tomato root volume. Means ± standard deviation followed by the same letter are not significantly different according to Tukey's HSD test (p<0.05).

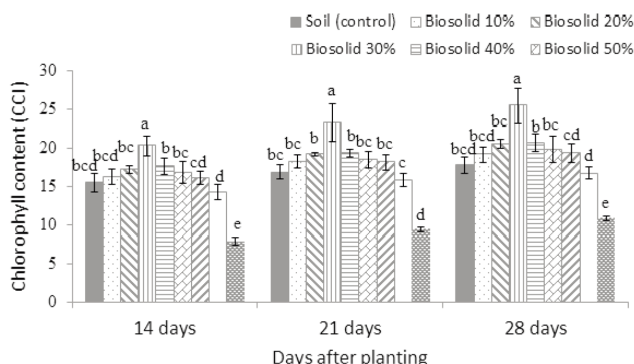

Fig. 5 - Effect of biosolids on tomato seedling leaf chlorophyll content. Means ± standard deviation followed by the same letter within a day after planting are not significantly different according to Tukey's HSD test (p<0.05).

4. Discussion and Conclusions

The response of tomato transplant growth parameters to various rates of biosolids (BS) in this study depended on the physico-chemical characteristics of the substrate. Although numerous authors have reported the beneficial effects of the addition of biosolids to peat mixes (Herrera *et al.*, 2008; Mami and Peyvast, 2010; Chrysargyris and Tzortzakis, 2015), limited number of studies, have reported the use of biosolids (BS) in forest soil (FS) mixture as a substrate. Our results show that use of BS at 30% can support tomato transplant. This can be attributed to its characteristic of higher availability of plant nutrients such as N, P, K, Mg, Fe, Mn, B and Mo (Table 1).

Biosolids application in the tested rates served

Plate 1 - Tomato leaf Chlorophyll observed in biosolids BS at 30% and 40% compared to at CP (Coco peat) substrate. Leaf colour appearance of tomato transplants grown on various substrates.

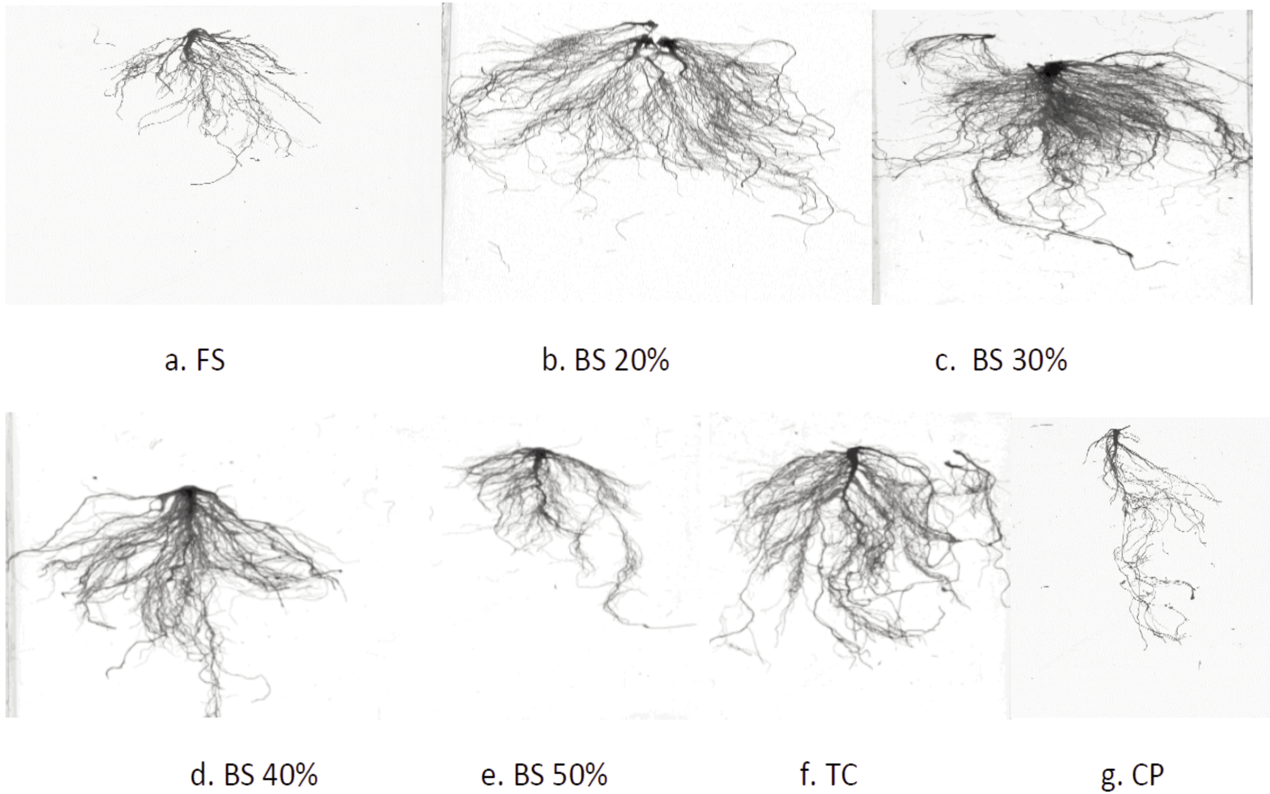

Plate 2 - Characteristics of tomato transplants root development in substrate tested, scanned from WinRhizo, for determination of root volume and density. Responses of roots development to different substrate, FS= Soil control; BS= Biosolids rates; TC= Tea compost; CP = Coco peat.

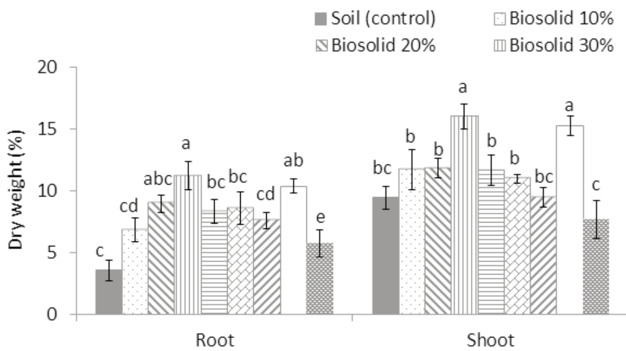

Fig. 7 - Effect of biosolids on tomato seedling root and shoot dry weight at week 5 after planting. Means ± standard deviation followed by the same letter within root or shoot are not significantly different according to Tukey's HSD test ($p<0.05$).

several purposes in the substrate. It improved the texture and water holding capacity, making conditions more favorable for root growth and increased emergence on tomato seedlings. The application of BS at 30% also supplied nutrients essential for plant growth, including N, P and K and Mg, as well as some essential micro nutrients like Zn, Fe, Cu, B and Mo (Table 1). As reported by Tzortzakis *et al*. (2012), nutrients in the biosolids offer several advantages over those in inorganic fertilizers because they are in organic form hence are released slowly to growing plants. Moreover in organic form, nutrients are less water soluble and therefore least likely to leach into groundwater or run-off into surface waters. This is in agreement with the findings of Zhao *et al*. (2010) in relation to the benefits associated with increased organic matter content in the soil. In line with this, Shiralipour *et al*. (1992) earlier reported that organic matter contribute to increased nutrient, total pore space, aggregate stability, erosion resistance, temperature insulation and reduced soil bulk density. These factors play a major role during germination and emergence of seedlings. Soil temperature and moisture content equally play a critical role during germination and emergence of tomato seedlings (Weaver *et al*., 1988). In concurrence with the pre-

sent study, findings by Chrysargyris and Tzortzakis (2015) revealed that biosolids enhance seed germination and emergence in eggplant transplants. The ability of biosolids to improve physical properties of a good media is related to increased organic matter content (Zhao *et al*., 2010). In regards to the current study, tomato seeds are small and therefore require fine and light media, a rhizophere created with BS at 30%, which possibly enhanced germination and emergence of the seedlings.

Plant height, leaf number and girth of the seedling were highest in seedlings grown in BS at 30% and apparently not very pronounced in TC (Fig. 3, 4). Abdel-Mawgoud (2007) reported plant growth and yield as a function of nutrients supply provided that all other conditions are met. In this study, there was clear positive trend of increasing plant height, leaf numbers with increased rates of BS. The results obtained with BS at 30%, may be attributed to its nutrient content as reported by Otieno *et al*. (2019). Enhancement of plant growth as a result of increasing nutrients in organic amendments has been reported by Sainju (2003). These results are in agreement with the work of Oyinlola and Jinadu (2012), where nitrogen rates in the soil increased tomato plant height. The nutrients not only encourage vegetative growth but also enhance photosynthesis, chlorophyll density and plant root respiration which result in greater plant growth when applied (Tan and Binger, 1986). The findings of this study suggest that the optimum rate of BS to use as soil amendment should not exceed 30% for transplant production. The difference between the BS at 30% and TC substrate seems to have been caused by the reduced level of K in the latter (Table 2). Potassium is an essential element during plant growth and development (Ortas, 2013). Since K is a vital element in many physiological processes, it may have been involved in transplant stem thickness. It is known that K plays a major role in physiological and biochemical processes such as enzyme activation; metabolism of carbohydrates and protein compounds Zhen *et al*. (1996). Besides, K has a significant role to play in the plant energy status for storage of assimilates and tissue water relation. Potassium is also needed in photosynthesis and the synthesis of proteins, hence its deficiency in plants will show as slow, stunted growth and in some crops, weak stems and lodging (Uchida, 2000).

Application of BS especially at 30% enhanced leaf chlorophyll as indicated by higher chlorophyll concentration index units (Fig. 5). One of the critical physiological developments responsible for seedling growth is photosynthesis. The quantity of chlorophyll per unit area is an indicator of photosynthetic capacity of a plant and this explains the better growth observed in tomato seedlings grown in BS at 30%. In other studies, Zuba *et al*. (2011) and Ilupeju *et al*. (2015), postulated that, the rates of organic amendment applied in growing media were linked to the nutrient element levels in the substrate. In regards to this study, plant nutrient availability may have enhanced the amount of chlorophyll in the plants, as exhibited by the presence of mineral elements such as N, P, Mg, Fe and Zn in biosolids in large quantities. These nutrient elements have been reported to be high in biosolids from organic part of municipal solid wastes (Chrysargyris and Tzortzakis, 2015). Other studies have also reported that biosolids are able to increase nutrient availability in soils (Shiralipour *et al*., 1992; Xu *et al*., 2012). In a related study on eggplant seedlings production, Chrysargyris and Tzortzakis (2015) observed that leaf chlorophyll content increased with addition of organic solid waste and similar results were earlier observed by Tzortzakis *et al.* (2012).

The underground part is very important in transplant life and determines whether it can survive when transferred to field environment or not. The roots in particular play a pivotal role in the plants life cycle (Somkuwar *et al*., 2012; Zhi *et al*., 2017). Roots are also known to provide an important link between soils and plants (McMichael *et al*., 2010; Xi *et al*., 2013). Furthermore, root systems have important physiological and biological functions for crop growth and yield (Liang *et al*., 2003; Yang *et al*., 2010). The ability of roots to develop perfectly depends on the medium or substrate status. Root growth is linked to the physico-chemical properties and nutrient availability in a substrate. The ability of a plant to absorb water and mineral nutrients from the substrate depends on its capacity to develop an extensive root system. In the present study, BS at 30% substrate significantly enhanced tomato transplant root growth and morphology (Table 1). In tomato, the tap root formed at an early stage extends deeply into the soil followed by secondary and tertiary, then delicate root hairs, which require water and air among the three phases (solid-liquid-gas) of the substrate (Manahan, 2000). The phases are very essential in water and plant nutrient absorption, based on porosity of the media as demonstrated by the BS at 30%.

Furthermore, the supply of O_2 is essential for root growth and metabolism. Generally, as roots grow through the soil they follow soil vailable pores and this is a contribution of the air space and the level of organic matter as evident in BS at 30%. This is also in agreement with Abad *et al.* (2001) and Pascual *et al*. (2018) who reported on the range of bulk density required for good root development in a substrate. Biosolids at 30% was therefore identified as an ideal substrate, in terms of producing many fibrous and dense root systems than the rest of the substrates (Plate 2 c, e). Additionally, based on the porosity of the studied substrates, it appears that BS at 30% not only created air space for the root development to enhance nutrient use efficiency, but also availed organic matter, which is connected to higher water holding capacity (Otieno *et al*., 2019). This is a critical factor for reducing irrigation schedule as in the case of the present study, making BS 30% a better substrate than the rest. The result of this study also suggests that the BS with rates as low as 10% may need frequent irrigation schedule. On the other hand, even though there was further increase in organic matter as the BS rates increased above 30%, increase in EC was observed (Table 1). This normally has a profound effect on the plant function, especially in reverse osmosis, which may subsequently affect continuous water flow and transpiration in the plant, leading to retarded growth (Mengel and Kirkby, 2001).

The enhanced shoot and root dry weight exhibited by BS at 30% was an indication of the potential of the BS as a soil amendment and its ability to improve the physico-chemical quality of the substrates for transplant development. Forest soil mixed with BS at 30% created room for root respiration and development. The plants had better chance for nutrient absorption hence increased dry matter compared to the other substrates tested. Phosphorous which occurred in high quantities in BS at 30% is involved in the formation of energy rich compounds, including adenosine triphosphate and adenosin diphosphate which in turn derive various bio-chemical reactions within the plant (Memon, 1996). As one of the vital plant macronutrient, phosphorus plays a vital role in the root and shoot development and this contributed immensely to the subsequent increase in shoot biomass of plants grown in BS at 30%. Biosolids analysis in this work also indicated the presence of Zn, Cu, Fe and Mn in significant quantities especially in BS 30%. As advocated by Atif *et al*. (2016), balanced presence of these essential micro elements may have promoted the growth of the seedlings in BS at 30%. These results are in agreement with work of Reis *et al*. (2017), who observed that addition of biosolids in soil resulted in significant increase in total root and shoot dry weight of *Leptospermum scoparium* in a pot experiment. Furthermore, Sainju *et al*. (2003) earlier reported that vigorous root growth stimulated by P helps in better utilization of water and other nutrients in the soil and promotes a sturdy growth of stem and healthy foliage which may subsequently contribute to roots and shoot dry matter.

The results in this study demonstrated that application of biosolids substrate was beneficial in the tomato transplants production. The influence of biosolids at 30% was significant and specifically on leaf number, plant height, chlorophyll content and root development. It is therefore a potential high quality, locally available and low cost substitute for peat and coir substrates in transplant production. Biosolids applied at moderate levels (30%) in forest soil mixture could not only improve the physic-chemical properties of the substrates but also reduce environmental pollution.

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AHS
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The salinity tolerance of pomegranate cultivars: Effects of salt stress on root and leaf mineral content

E. Jadidi [1], M. Tatari [2] (*), M. Ghasemnezhad, H.R. Salemi

[1] *Department of Horticultural Science, University Campus 2, University of Guilan, Rasht, Iran.*

[2] *Horticulture Crops Research Department, Isfahan Agricultural and Natural Resources Research and Education Center, AREEO, Isfahan, Iran.*

[3] *Department of Horticultural Science, University of Guilan, Rasht, Iran.*

[4] *Agricultural Engineering Research Department, Isfahan Agricultural and Natural Resouces Research and Education Center, AREEO, Isfahan, Iran.*

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Key words: dry and fresh weight, mineral composition, NaCl, salt stress.

(*) **Corresponding author:**
mtatari1@gmail.com

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All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

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Abstract: In this study, the effect of irrigation water salinity on rooted cuttings of eight pomegranate cultivars namely 'Malase Saveh' ('M-Saveh'), 'Malase Isfahan' ('M-Isfahan'), 'Robabe Ghermeze Shiraz' ('Robab'), 'Gabrie Yazd' ('G-Yazd'), Gabrie Torshe Yazd' ('GT-Yazd'), 'Zaghe Sefide Yazd' ('ZS-Yazd'), 'Zaghe Torshe Yazd' ('ZT-Yazd') and 'Malase Torshe Pishva' ('M-Pishva') was studied. Sodium chloride was added to irrigation water to get final concentrations of 3, 6, 9 and 12 dS/m. Leaf and root mineral content, leaf abscission and root characteristics were determined at the end of the experiment. The results showed that the salinity reduced significantly fresh and dry weight of root in all pomegranate cultivars. Water salinity up to 3 dS/m increased slightly roots fresh and dry weight of all cultivars and thereafter decreased. With increasing water salinity to 12 dS/m, accumulation of sodium and chloride both in roots and leaves increased, but nitrogen, phosphorus, magnesium and calcium contents decreased. The change of leaf potassium content was dependent to pomegranate cultivar. The lowest sodium and chloride accumulation in root was observed in 'ZT-Yazd', but 'M-Pishva' translocated the lowest sodium and chloride to leaf. The low ability of nitrogen absorption was found in 'M-Saveh', whereas 'M-Pishva' maintained the highest leaf nitrogen under salt stress conditions. The most potassium content of root was observed in GT-Yazd', while 'ZS-Yazd' and 'G-Yazd' translocated the highest potassium to leaves. Generally, the responses of pomegranate to absorption and translocation of elements to leaves under salinity conditions were completely dependent to cultivar. 'M-Pishva' and Yazd cultivars showed higher tolerance to salinity stress.

1. Introduction

High salt concentration in the soil is responsible for decreasing productivi-

ty in a wide range of agricultural crops in the world (FAO, 2008). Soil salinity also is one of the serious environmental stress that limits growth and productivity of horticultural crops. Different factors include the excessive application of chemical fertilizers, the use of saline water for irrigation and the high water levels led to soil salinity (Mastrogiannidou *et al*., 2016).

Irrigation with saline water affects the absorption of nutrient elements. The adverse effects depend on the salinity level, the type of salt, the plant species and the presence of other stress (Grattan and Grieve, 1999). Therefore, differences in adaptability potential in plant species to prevailing abiotic stress conditions can be attributed to their varied ability for nutrient uptake and consequently different content of macro and micronutrients in plant organs and tissues (Jamali *et al*., 2016).

Soil salinity indirectly decreases the absorption of nutrients in the plant by reducing root growth (Kiani and Abbasi, 2010). Karimi and Nasrollahpour-Moghaddam (2016) also found that salinity affected the accumulation of potassium content in the root and the transfer rate of sodium and calcium to shoot in Pistachio plants. In other research, it was found that pistachio rootstocks under salinity stress have restriction mechanisms for absorbing and transferring of sodium and chloride ions (Karimi and Maleki Kuhbanani, 2015). In olive, Rossi *et al*. (2015) found that roots distinctly play an important role in the regulation of apoplast to reduce permeation and ion transfer to shoot, which depends on plant genotype and sodium concentration.

Pomegranate is one of important commercial fruit in tropical and subtropical regions of the world and also in mediterranean climate, which is enriched of nutritional and antioxidant compounds (Parvizi and Sepaskhah, 2015). Other studies indicated that there is different levels of tolerance to abiotic stress conditions such as drought and salinity among different pomegranate cultivars (Tabatabaei and Sarkhosh, 2006; Okhovatian-Ardakani *et al*., 2010; Ibrahim, 2016). In general, tolerance to abiotic stress is complex and depends on both genetic and physiological properties. Karimi and Hasanpour (2014) showed that with increasing salinity level, the sodium, chlorine and potassium contents in roots and shoots of 'Robab' and 'Shishe Gap' pomegranate cultivars increased. 'Shishe Gap' cultivar showed good growth by restricting the absorption and transfer of chlorine under salt stress conditions (Karimi and Hasanpour, 2014).

It was also reported that the use of saline water reduced stem length, length and number of internode, leaf area and root development in pomegranate cultivars (Amiri *et al*., 2011). In contrast, Bhantana and Lazarovitch (2010) observed that there were no differences between 'Vanderfol' and 'SP-2' pomegranate cultivars in response to salt water stress. Some results showed a significant change in the anatomical structure of roots and leaves of the pomegranate, when exposed to salt levels of 800 to 4000 dS/m. These changes included an increase in the thickness of the cuticle layer, the formation of parenchymal cells, and an increase in the number and density of crystals in the parenchymal cells of the leaves and roots in pomegranates (Zarinkamar and Esfah, 2005).

Therefore, the aim of this study was the evaluation of the effect of irrigation water salinity on some root growth characteristics and absorption and transfer of root nutrient elements to shoot in eight pomegranate commercial cultivars.

2. Materials and Methods

Plant material and cultivation

This study was carried out during 2016-2017 in Isfahan Agricultural and Natural Resources Research and Education Center, Iran. Uniform rooted cuttings of eight pomegranate commercial cultivars including 'Malase Saveh' ('M-Saveh'), 'Malase Isfahan' ('M-Isfahan'), 'Robabe Ghermeze Shiraz' ('Robab'), 'Gabrie Yazd' ('G-Yazd'), Gabrie Torshe Yazd' ('GT-Yazd'), 'Zaghe Sefide Yazd' ('ZS-Yazd'), 'Zaghe Torshe Yazd' ('ZT-Yazd') and 'Malase Torshe Pishva' ('M-Pishva') were used as plant materials. Rooted cuttings were planted in 20-liter plastic pots (35×30cm). The soil mixture was clay loam and cow manure with 1:1 ratio (v/v). Physical and chemical properties of the soil mixture were analyzed at the beginning of the experiment (Table 1).

Salinity treatments

Four levels of water salinity (control, 3, 6, 9 and 12 dS/m) were used for irrigation of different pomegranate cultivars (Table 2). The source of sodium chloride used in this experiment was from the lake salt that was analyzed before application (Table 3). The salinity treatments were carried out from two months after planting in the pots for four months.

Table 1 - Some physical and chemical properties of soil mixture

Property	unit	value
pH	-	7.75
Saturation percentage	%	17
Field capacity	%	30
Permanent wilting point	%	7.25
Organic carbon	%	10.14
Nitrogen	%	0.096
Total neutralizing value	%	32.5
Silt	%	35
Clay	%	35
Sand	%	30
Tissue	-	Clay Loam
K	mg/kg	709
P	mg/kg	2.26
Zn	mg/kg	0.64
Mg	mg/kg	10.23
Fe	mg/kg	5.01
Cu	mg/kg	1.23
B	mg/kg	2.66
Na	Meq/L	4.53
Cl	Meq/L	7.19
Salinity	dS/m	3.24

In order to avoid salt stress shocks, initially, pomegranate plants were irrigated with water of 3ds/m electrical conductivity (EC). According to the climatic conditions of the region, the irrigation interval was applied in a Three-day interval which reduced to two days at very hot months. The ratio of electrical conductivity of the irrigation water and leach off were measured and the mean value was recorded as the leaching fraction (LF). The leaching coefficient was dependence to salinity level.

The amount of the consumed water by each pot during the growing season was measured. In order to determine the irrigation intervals and net irrigation requirement (In), the irrigation depth parameter (dn) was calculated. The depth of stored water in the soil and readily available water (RAW) were determined using the depth of root development and bulk density (Pb). In this way, soil moisture content at field capacity (FC) and wilting point (PWP) levels was measured. The obtained gross water requirement (Ig) depends on irrigation system efficiency. In order to prevent of the salt accumulation, over-irrigation was implemented to the pots (in the amount of 15% of the field capacity), so that the water was drained from the bottom of the pots.

Evaluated traits

At the end of the experiment, the plants were removed from the pot and the roots weighed after washing (root fresh weight). To determine root dry weight, samples were dried for 48 hours at 75°C and thereafter weighed using a digital scale (Smit *et al.*, 2000). In the end, the percentage of the root dry matter was obtained from the following formula:

Root dry matter = [(Fresh weight - dry weight)/Dry weight] x 100

The leaf abscission was counted at the end of the experiment (late September). The necrotic percentage of leaves at the end of the stress was visibly evaluated and recorded using the scoring method from 0

Table 2 - The chemical analysis of used water for salt stress treatment

EC dS/m	pH	K mg /L	Mg (mg /L)	Ca (mg /L)	Cl (mg /L)	Na (mg /L)
Control (0.38)	6.90	3.80	1.2	2.80	3	2.8
3	7.45	5.09	66	1.98	1320	770
6	7.84	12.49	162	4.86	3240	1890
9	7.90	19.66	255	7.65	5100	2975
12	8.20	25.45	330	9.9	6600	3850

Table 3 - Some chemical properties of used lake salt for salinity treatments

Co (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Fe (mg/kg)	Cl (%)	Na (%)	Mg (%)	Ca (%)	K (%)	P (%)
3	2	0	8	15	0	60	35	3	0.09	0.23	0

to 9. Zero means non-necrosis leaves, one for very low necrosis (between 0 to 10%), three for low necrosis (between 10 to 30%), five for moderate necrosis (between 30 to 50%), and seven for sever (between 50-70%), and nine means very severs necrosis (between 70-90%).

The content of mineral elements such as nitrogen, phosphorus, potassium, calcium, magnesium, chloride and sodium in leaf and root of pomegranate cultivars was determined just in control (without salt additions) and 12 dS/m salinity stress treatment at the end of the experiment.

The Kjeldahl method is used to determine the nitrogen content in leaf and root samples (Jones, 2001). Briefly, 0.3g of fine dry powder was digested in concentrated H_2SO_4 and distilled with NaOH (40%), and ammonium nitrogen was fixed in H_3BO_3 (2%) and titrated with 0.1N H_2SO_4.

The P content of samples was determined by the vanadate-molybdate colorimetric method (Chapman and Pratt, 1982). The absorbance of samples was measured at 470 nm in a UV/visible spectrophotometer (model PG Instrument+80, Leicester, UK).

Potassium (K) and sodium (Na) content was determined by the flame photometric method as described by Jones (2001). The digested extract was diluted by calcium chloride ($CaCl_2$) at 1:9 ratios (v/v) and the absorbance was measured at 766.5 nm (Jones, 2001).

Calcium (Ca) and magnesium (Mg) were measured using atomic absorption spectroscopy. Briefly, digested extracts were diluted with distilled water (1:9 v/v), then 4.75 mL of lanthanum nitrate [La (NO3)3] was added to 250 ml of the diluted extract. Finally, the absorbance was measured at 422.7 nm for Ca and 285.2 nm for Mg by atomic absorption (Jones, 2001). Chloride ion content was determined by titration.

Statistical analysis

This experiment was conducted as the factorial experiment based on a randomized complete block design with two factors. To evaluate each trait, three replications and five observations in each replication were considered. The first factor was eight pomegranate cultivars and the second factor was salinity treatment at five levels. Analysis of data was performed by ANOVA method using statistical software SAS (version 9.1) and means comparison using Tukey test.

3. Results

Fresh and dry weight of root

The results showed that with increasing salinity levels from 3 dS/m to 12 dS/m in irrigation water, both fresh and dry weight of roots in all pomegranate cultivars significantly decreased (Fig. 1). The lowest fresh and dry weight was found when plants irrigated with 12 dS/m, however, there was no significant difference between 12 and 9 dS/m. Overall, fresh weight of root in pomegranate cultivars in 6, 9 and 12 dS/m treatments decreased by 46.3, 57.4 and 66%, respectively, compared with control treatment (Fig. 1A), as well, root dry weight decreased by 45.4, 52.5 and 59 %, respectively (Fig. 1B).

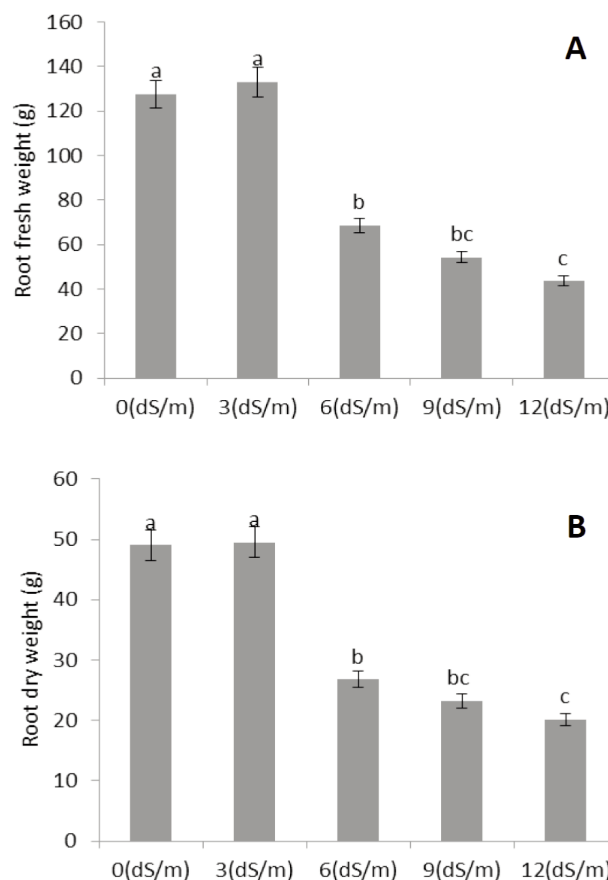

Fig. 1 - Effects of different levels of water salinity on fresh (A) and dry (B) weight of root in pomegranate cultivars.

The decreasing rate of fresh weight in the studied cultivars was completely dependent on the cultivar. The highest fresh weight of root was found in 'GT-Yazd', 'Robab' and 'M-Saveh' cultivars, respectively. The lowest fresh weight of root was observed in 'ZS-

Yazd' (Fig. 2).

Root dry matter also significantly decreased when pomegranate cultivars irrigated with 9 and 12 dS/m water (26.69 and 46.17%, respectively). In contrast, this trait slightly increased when plants irrigated with 3 dS/m saline water (Fig. 3).

Leaf abscission

Leaf abscission was significantly affected by water salinity levels and pomegranate cultivars. Overall, with increasing salinity level, leaf abscission was increased in all studied pomegranate cultivars (Fig. 4). The highest leaf abscission was recorded when plants irrigated with 9 and 12 dS/m water. In 3 dS/m, 'ZT-Yazd' and 'M-Pishva' had the lowest percentage of leaf abscission. The lowest percentage of leaf abscission in salinity of 6 ds/m was observed in 'M-Isfahan' and 'M-Pishva'. In 9 dS/m, 'G-Yazd' and 'Robab' showed the lowest leaf abscission. The lowest percentage of leaf abscission in salinity of 12 dS/m was observed in 'G-Yazd' and 'ZT-Yazd'. In this salinity level, leaf abscission was 100% in 'M-Saveh'.

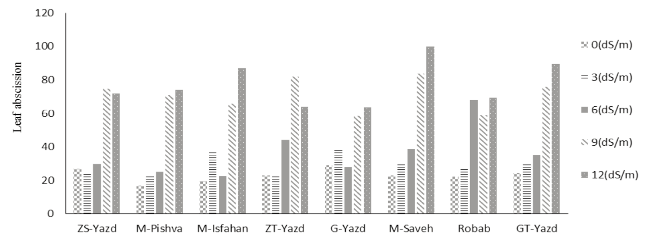

Fig. 4 - The interaction effects of salinity levels and cultivars on leaf abscission.

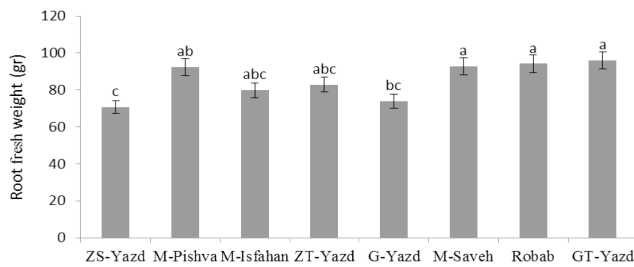

Fig. 2 - Effect of different pomegranate cultivars on root fresh weight.

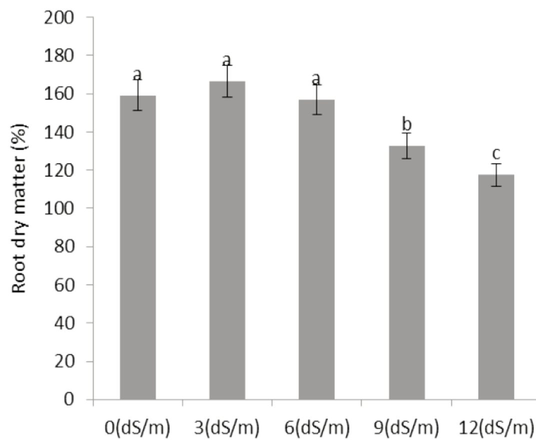

Fig. 3 - Effects of water salinity on root dry matter percentage of pomegranate cultivar.

Leaf necrosis

The leaf necrosis also affected by salinity level and pomegranate cultivars (Fig. 5). There was no difference in leaf necrosis between 3 dS/m and control treatments in 'ZT-Yazd' and 'M-Isfahan' cultivars. In 6 dS/m, the lowest leaf necrosis were observed in 'M-Pishva' and 'ZT-Yazd' with 1.6 and 1.8%, respectively. 'ZT-Yazd' and 'M-Pishva' had the least leaf necrosis in 12 dS/m with the average of 2.3% (Fig. 5).

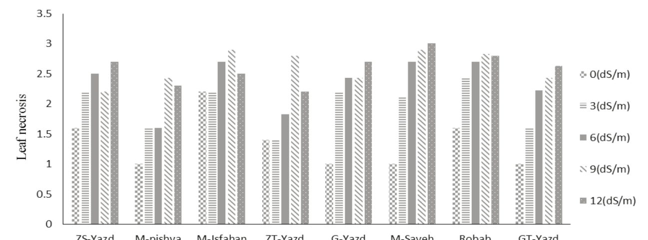

Fig. 5 - The interaction effects of salinity levels and cultivars on leaf necrosis.

Root and leaf Cl and Na content

Chloride and sodium contents of the root increased significantly when irrigated with 12ds/m saline water in compared with the control (Table 4).

The lowest root chloride content was found in 'ZT-

Table 4 - Effects of water salinity (12 ds/m) on root mineral elements of pomegranate

Salinity (dS/m)	Cl (mg/kg)	Na (mg/kg)	Mg (%)	Ca (%)	P (%)	N (%)
Control	16.325 b	24.798 b	0.228 a	1.071 a	0.173 a	1.213 a
12	51.430 a	90.434 a	0.198 b	0.721 b	0.140 b	0.962 b

Similar letters in each column indicate no significant difference at the 5% level of Tukey test.

Yazd' with 22.752 mg/kg. There was no significant difference among other pomegranate cultivars (Table 5).

In this study, pomegranate cultivars showed different levels of root sodium accumulation. The least sodium content was observed in 'ZT-Yazd' with 39.308 mg/kg. 'M-Saveh' and 'ZS-Yazd' showed the highest sodium accumulation of roots (74.118 and 62.947 mg/kg, respectively).

The interaction effect of saline water treatment and pomegranate cultivars on leaf sodium and chloride contents was significant. The highest leaf chloride content was found in 'M-Saveh' with 12 dS/m saline water (112.8 mg/kg) and the lowest ones was observed in 'M-Pishva' with an average of 46.72 mg/kg (Fig. 6).

The highest leaf sodium content was observed in 'M-Saveh' and 'GT-Yazd' with averages of 427 and 383.5 mg/kg, respectively (Fig. 7). In contrast, 'M-Pishva' showed the lowest leaf sodium content (141.5 mg/kg).

Root and leaf N content

The results showed that when pomegranate cultivars irrigated with 12 dS/m water, the nitrogen content of the roots reduced significantly to 20.69% compared with the control (Table 4). The response of pomegranate cultivars was different, when exposed to salinity stress (Table 5). The lowest roots nitrogen content was found in 'M-Saveh' (1%) but, no any significant difference was found among others cultivars (Table 5).

The leaf nitrogen content in pomegranate cultivars also decreased when irrigated with 12 dS/m saline water. The highest and the lowest leaf nitrogen content was found in 'M-Pishva' and 'M-Saveh' (in 12ds/m), respectively (Fig. 8).

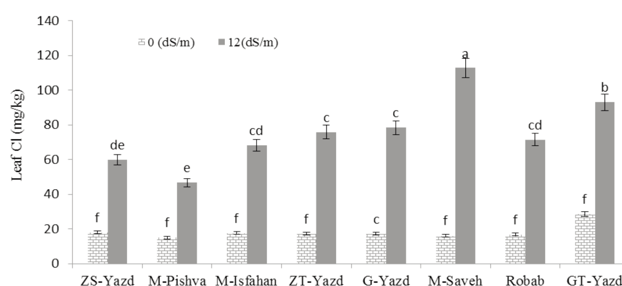

Fig. 6 - The interaction effects of water salinity and pomegranate cultivars on leaf chloride accumulation.

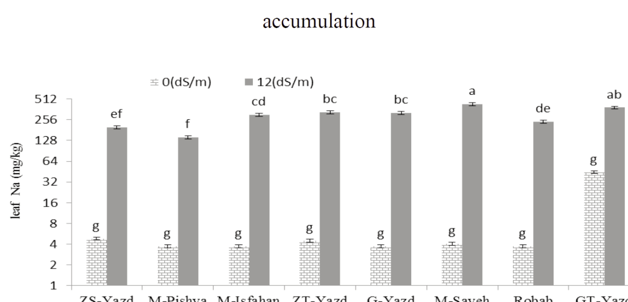

Fig. 7 - The interaction effects of water salinity and pomegranate cultivars on leaf sodium accumulation.

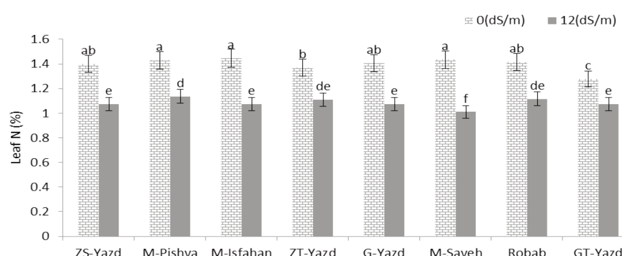

Fig. 8 - The interaction effects of water salinity and pomegranate cultivars on leaf nitrogen content.

Table 5 - The comparison of root mineral elements in eight pomegranate cultivars

Cultivars	Cl (mg/kg)	Na (mg/kg)	Mg (%)	Ca (%)	P (%)	N (%)
'ZS-Yazd'	36.178 a	62.947 ab	0.230 ab	0.883 ab	0.153 a	1.108 a
'M-Pishva''	38.848 a	58.632 b	0.213 b	0.910 ab	0.155 a	1.105 a
'M-Isfahan'	33.678 a	55.745 b	0.196 b	0.861 ab	0.163 a	1.111 a
'ZT-Yazd'	22.752 b	39.308 c	0.205 b	0.865 ab	0.151 a	1.106 a
'G-Yazd'	31.427 a	55.317 b	0.201 b	0.843 b	0.166 a	1.088 a
'M-Saveh'	39.583 a	74.118 a	0.256 a	0.906 ab	0.151 a	1.00 b
'Robab'	32.610 a	53.788 b	0.198 b	0.960 a	0.158 a	1.063 ab
'GT-Yazd'	35.943 a	61.072 b	0.205 b	0.940 a	0.156 a	1.123 a

Similar letters in each column indicate no significant difference at the 5% level of Tukey test.

Root and Leaf P content

The root phosphorus content significantly decreased in 12 dS/m salinity (19.07%) compared to control treatment. However, there was no significant difference among pomegranate cultivars in root phosphorus (Tables 4 and 5).

In the salinity of 12 dS/m, the leaf P content decreased compared with the control treatment. However, no significant difference was found among pomegranate cultivars for leaf phosphorus in the control treatment (Fig. 9).

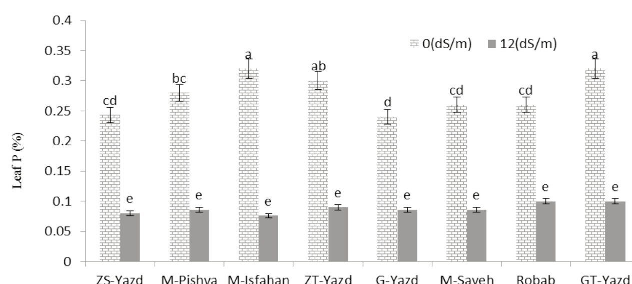

Fig. 9 - The interaction effects of water salinity and pomegranate cultivars on leaf phosphor content.

Root and leaf K content

The response of pomegranate cultivars to potassium accumulation of root was significantly different when irrigated with 12 dS/m water. 'ZS-Yazd', 'G-Yazd', 'M-Pishva' and 'GT-'Yazd showed increase potassium content of root while, potassium contents of 'Robab', 'M-Isfahan', 'M-Saveh' and 'ZT-Yazd' was decreased compared with the control. The lowest and the most potassium content of root were found in 'GT-Yazd' and 'M-Isfahan', respectively in salinity of 12ds/m (Fig. 10).

In contrast, leaf potassium content of all pomegranate cultivars decreased significantly when irrigated with 12 dS/m saline water (Fig. 11). The lowest content of leaf potassium was observed in 'M-Saveh', 'GT-Yazd' and 'ZT-Yazd' with 12 dS/m saline water treatment. The highest leaf potassium content in salinity conditions was found in 'ZS-Yazd' and 'G-Yazd'.

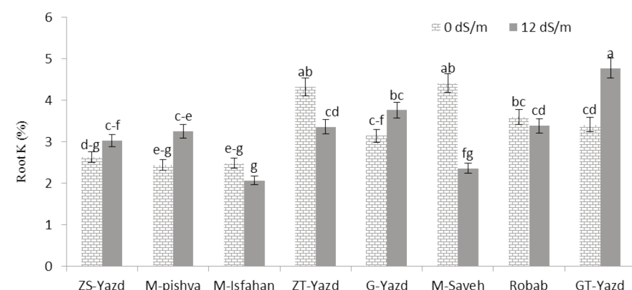

Fig. 10 - The interaction effects of water salinity and pomegranate cultivars on root potassium content.

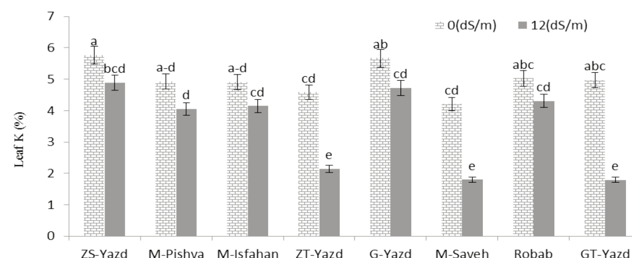

Fig. 11 - The interaction effects of water salinity and pomegranate cultivars on leaf potassium content.

Root and leaf Ca content

Root calcium content showed a significant reduction of 32.67% compared with the control when treated with saline water (Table 4). The lowest root calcium content was observed in 'G-Yazd' with an average of 0.84% (Table 5). In contrast, 'Robab' and 'GT-Yazd' cultivars showed the highest root calcium with averages of 0.96 and 0.94 %, respectively.

The leaf calcium content also decreased under salinity conditions (Fig. 12). The lowest leaf calcium content was recorded in 'G-Yazd' cultivar with an average of 0.843% and the highest ones were found in 'Robab' with 0.96% (Table 6).

Root and Leaf Mg content

The magnesium content of root also decreased when pomegranate plants irrigated with 12 dS/m saline water (13.15% compared with the control). The lowest of root magnesium content was found in 'M-Isfahan' and 'Robab' with averages of 0.196 and 0.198 %, respectively (Table 4 and 5). In contrast, 'M-Saveh' was able to absorb the most magnesium under salt stress condition compared with other cultivars.

Leaf magnesium content also decreased when treated with 12 dS/m irrigation water (Fig. 12). The least leaf magnesium content was found in 'M-Isfahan' with 0.196% (Table 6).

4. Discussion and Conclusions

Salinity reduces the ability of plants to water absorption and reduces the plant growth rate. The

salt will eventually rise to a toxic level via transpiration of leaves, causing leaf senescence and abscission (Munns, 2002). Previous studies also showed that salinity changed growth parameters in pomegranate (Karimi *et al*., 2011; Mastrogiannidou *et al*., 2016), and pistachio (Picchioni *et al*., 1990; Naieni *et al*., 2004; Saadatmand *et al*., 2007).

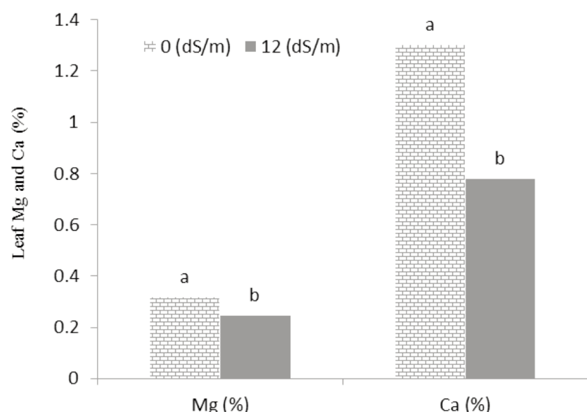

Fig. 12 - Effect of 12ds/m saline water treatment on leaf Mg and Ca content.

Table 6 - The comparison of root mineral elements in eight pomegranate cultivars

Cultivars	Leaf Mg (%)	Leaf Ca (%)
'ZS-Yazd'	0.230 ab	0.883 ab
M-Pishva'	0.213 b	0.910 ab
'M-Isfahan'	0.196 b	0.861 ab
'ZT-Yazd'	0.205 b	0.865 ab
'G-Yazd'	0.201 b	0.843 b
'M-Saveh'	0.256 a	0.906 ab
'Robab'	0.198 b	0.960 a
'GT-Yazd'	0.205 b	0.940 ab

Similar letters in each column indicate no significant difference at the 5% level of Tukey test.

The results of the current study showed that salinity affected the growth characteristics in pomegranate cultivars. Fresh and dry weight of roots as well as dry matter of root was decreased with increasing salinity levels. Reduction of fresh weight of root was completely dependent on pomegranate cultivar. For example 'GT-Yazd', 'Robab' and 'M-Saveh' cultivars maintained their growth characteristics more than other cultivars. Munns and Tester (2008) reported that in 'Malase Shirin' that was the salt tolerant cultivar, the growth rate increased after an increase in the salinity up to 40 mM, and the more. But in salt sensitive cultivars, increase in salinity decreased the plant growth characteristics (Munns and Tester, 2008). According to the previous studies, salinity tolerant plants, especially permanent species, have more potential to survive and maintain growth rate under salt stress conditions (Ferreira-Silva *et al*., 2008).

The results showed that fresh and dry weight of roots as well as dry matter percentage in mild salinity (3 dS/m) slightly increased but thereafter decreased. High levels of salinity reduced the rate of these traits. Amiri *et al*. (2011) showed that salinity levels of 40, 80 and 120 mM of sodium chloride in irrigation water of 'Robab' cultivar significantly reduced root fresh and dry weight. Also, Khoshbahkt *et al*. (2014) found that sodium chloride with 20, 40 and 60 Mm concentration in irrigation water caused a reduction in root fresh weight, and root dry weight in citrus. We also found that fresh weight of root was significantly affected by pomegranate cultivars.

Momenpour *et al*. (2015) also found a significant increase in almond leaf abscission when irrigated with saline water. Khoshbahkt *et al*. (2014) reported that with increasing sodium chloride in irrigation water, a significant increase was observed in the leaf abscission percentage of two citrus rootstocks. These results are consistent with the findings of other researchers, which the increase in salinity levels of irrigation water, led to an increase in necrotic leaflet growth (Momenpour *et al*., 2015). The result of the current study also showed that by applying salinity stress and increasing its concentration, in all studied cultivars, the percentage of leaf abscission increased. The least leaf abscission was observed in 'G-Yazd' and 'ZS-Yazd'. Reducing the number of leaves in the cultivars significantly reduces the plant photosynthesis level, which could be another reason for reducing plant growth characteristics in sensitive salinity cultivars.

According to the results, with increasing concentration of sodium chloride in irrigation water, leaf necrosis increased in the middle and the end of the stress period that are consistent with the findings of other researchers (Momenpour *et al*., 2015). In the concentrations of 6, 9 and 12 dS/m, 'M-Pishva', 'ZS-Yazd' and 'ZT-Yazd' showed the lowest percentage of leaf necrosis, respectively.

Previous studies showed that with increasing salinity level in irrigation water, accumulation of chloride and sodium ions increased in the pomegranate

root and leaf (Karimi and Hasanpour, 2014; Mastrogiannidou *et al*., 2016). Zarei *et al*. (2016) reported that salinity stress, increased the amount of root and leaf chlorine and sodium in fig cultivars. Khayyat *et al*. (2014) also reported that with increasing salinity level from 4.61 to 7.46 dS/m, shoot Cl content in pomegranate cv. 'Malas Mommtaz' increased, but it decreased in 'Shishe Kab'. In fact, 'Shishe Kab' could manage sodium transport into leaves better than 'Malas Mommtaz' cultivar. Salinity-tolerant plants transfer less sodium and chlorine to shoot than sensitive plants (Fernandez, 2014; Munns, 2002). We also find the significant difference in pomegranate cultivars for absorption of sodium and chloride ions and translocation to shoot. 'ZT-Yazd' showed the lowest content of root chloride and sodium in 12 dS/m. At the same concentration of salinity, the lowest leaf sodium and chloride content was observed in 'M-Pishva'.

The results showed that by increasing sodium chloride in irrigation water, nitrogen content of root and leaf reduced, which is in agreement with finding of Naeini *et al*. (2004) on 'Alak Torsh', 'Malas Torsh' and 'Malas Shirin' pomegranate cultivars.

Karimi and Hassanpour (2014) reported that under salinity stress, the root and leaf phosphorus content in 'Shisheh Gap' cultivar decreased. In the present study, there was a decrease in the content of root phosphorus under salinity. Momenpour *et al*. (2015) no significant difference was observed among almond pomegranate cultivars in the root and leaf phosphorus under salt stress conditions that were similar to the results of this study.

Mastrogiannidou *et al*. (2016) showed that increasing of sodium chloride concentration in irrigation water decreased root and leaf potassium content in 'Wonderful' pomegranate cultivar. However, Karimi and Hasanpour (2014) reported that with increasing sodium chloride in irrigation water, root and leaf potassium content in pomegranate increased. In our study, salinity reduced leaf potassium, but potassium changes of root were affected by pomegranate cultivar. GT-Yazd' and 'ZS-Yazd' showed the highest root and leaf potassium, respectively.

Salinity stress led to the reduction of leaf and root magnesium. Naeini *et al*. (2004) also reported that with increasing sodium chloride concentration in irrigation water, content of root and leaf magnesium decreased in 'Alake Torsh', 'Malase Torsh' and 'Malase Shirin' cultivars. In the current study, M-Isfahan showed the lowest root and leaf magnesium. The highest leaf and root magnesium was observed in 'M-Saveh'.

Increasing salinity was associated with a decrease in leaf and root calcium. Aboutalebi *et al*. (2008) reported that by increasing sodium chloride, the root calcium in four citrus species decreased and in one of them increased. In the present study, 'Robab' had the highest leaf and root calcium in salinity conditions. Sarafi *et al*. (2017) indicated that 'Wonderful' and 'Ermioni' cultivars had the different abilities for P, K, Ca and Mg uptake under salt stress conditions. Differences in the absorption of elements were reported between 'Robab' and 'Shishe Kab' by Hasanpour *et al*. (2015).

'ZT-Yazd' showed the lowest leaf abscission and necrosis. 'M-Pishva' showed less leaf necrosis than other cultivars in salinity conditions. 'ZT-Yazd' and 'M-Pishva' showed the least amount of chlorine and sodium accumulation in root and leaf, respectively. It seems that the mechanism of these two cultivars is different to salinity tolerance, so 'ZT-Yazd' transmits excess chlorine and sodium to the leaves, but 'M-Pishva' transmits less chlorine and sodium to the leaves and shows the accumulation of these elements in the root. Perhaps for this reason, the amount of leaf nitrogen in 'M-Pishva' was higher than other cultivars in salinity conditions. However, in severe salinity conditions, in some cultivars, the potassium content of root decreased, but the potassium content of root in these two cultivars, along with 'G-Yazd' and 'GT-Yazd' increased. It seems that there is a wide variation among the studied cultivars that leads to different reactions to salinity. In general, 'M-Pishva' and Yazd cultivars showed higher tolerance to salinity stress.

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Identification of promising tomato breeding lines with determinate growth by selection index

M. Viera Nascimento [1] (*), M.C. Ribeiro Ávila [1], M. Fiori de Abreu-Tarazi [1], A.P. Oliveia Nogueira [2], L.F. Cardoso Campos [1], A. Dos Reis Nascimiento [1]

[1] *Department of Horticulture, School of Agronomy, Federal University of Goiás, Goiania, Goiás, Brazil.*

[2] *Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil.*

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(*) **Corresponding author:**
nascimento_mariana1@hotmail.com

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Abstract: Source of important vitamins, fibers, and minerals, the tomato (*Solanum lycopersicum* L.) stands out in the world agricultural scenario for its economic and social relevance and versatility. The Brazilian market is dominated by multinationals companies, and this market segment obtains cultivars from other countries, with genetics accurate to climatic conditions and cultivation method very different from those used in Brazil. As a result, the local cultivation of tomatoes plants becomes dependent on market variations and has required a material that has limited production efficiency. This study aimed to estimate genetic parameters from agronomic traits and to select industrial tomato lines using the selection index. A randomized block experimental design with three replications was used. Eighty-five industrial tomato lines from the germplasm bank of the Vivati Plant Breeding Ltda were evaluated. Each plot had 12 plants. The two central plants of each plot were evaluated. The evaluations were carried out using adapted morphological descriptors described in the guidelines for carrying out the distinguishability, homogeneity, and stability (DHE) tests of the Ministry of Agriculture, Livestock, and Supply of Brazil (MAPA). The genotypic determination coefficient (H^2) of the traits related to fruit pericarp thickness, fruit firmness, fruit yield, average cycle, average number of fruits per plant, and soluble solids was high. The base index and the classic index presented the largest gain from selection for the fruit yield trait. Rank summation index and genotype-ideotype distance index had the highest total selection gain values. The tomato lines PXT-601 and PXT-610 stood out as superior genotypes by the methods of direct selection and by selection indexes.

1. Introduction

Tomato is grown in different regions of the world and stands out as the most produced vegetable in the world, second only to potatoes in the cultivated area (Geraldini *et al.*, 2018). Part of the success of tomatoes comes from its diversity in food and nutritional aspects that help human health. The fruit is rich in vitamins A and C and lycopene, substances that help prevent cancer of the gastrointestinal tract (Simão and Rodriguez,

2008).

Brazil is in ninth place in the world tomato production ranking. At the top is China, accounting for 31% of production, followed by India with 11%, and the United States with 8% of global production (Dossa and Fuchs, 2017). In 2017, 36.688 hectares of tomato were grown in Brazil, 47.40% of the production was destined for fresh consumption, and 52.60% for processing industries (Marcomini and Molena, 2018).

The Brazilian national market is dominated by multinationals and acquires imported cultivars, with genetic characteristics adapted to climatic conditions and cultivation systems very different from those found in Brazil. As a result, the Brazilian cultivation of tomato becomes dependent on market swings. It obtains cultivars with productive potential restricted if compared to the yields reached in the environment that they were developed. Also, the plants may suffer losses by climate intolerances and plant diseases, when facing the Brazilian growing conditions.

Due to the economic importance of the crop, tomatoes produced for processing industries have been the focus of research, especially in genetic breeding with the aim of produce cultivars that possess genes able to assist in the adaptation and tolerance to biotic and abiotic stresses, which can result in important contributions to the sector (Parmar *et al*., 2017).

In a breeding program, the objective is to enhance the main phenotypic trait and conserve or improve the expression of secondary traits at the same time (Nogueira *et al*., 2012). However, the direct selection of quantitative traits is influenced by the environment, which may cause unfavorable changes in other traits (Vasconcelos *et al*., 2010).

One way to improve this process is to use the simultaneous selection of a group of important agronomic traits, that is, to use the selection indexes. These indexes relate information of different traits and make it possible to perform a selection effectively, which increases the probability of success in a plant breeding program (Cruz *et al*., 2012; Vianna *et al*., 2013; Rezende *et al*., 2014).

Considering the importance of the industrial processing of tomatoes and the market demand for cultivars that meet the requirements of this industrial chain, it is indispensable to know the relationship between agronomic traits and the study of the indexes. This makes it possible to obtain the best prediction of gains and yields and greater efficiency in the selection process. Given the above, this aimed to estimate genetic parameters for agronomic traits and to select industrial tomato lines using the selection index.

2. Materials and Methods

The study was conducted in the experimental area of Vivati Plant Breeding Ltda, in Abadia de Goiás Unit, Goiás, Brazil, at 16°45’26” S, 49°26’15” W, and 898 m of altitude. The climate, according to Koppen, is classified as tropical humid, characterized by rainy summer with high temperatures and dry winter, with an average annual rainfall of 1.575 mm.

The genotypes analyzed in this study are owned by Vivati Plant Breeding Ltda, which use their own selection and maintain methods. The seeds were sown in 450-cell polystyrene trays, filled with a substrate composed of coconut fiber, rice husk, and peat and covered with vermiculite. The trays were kept in a greenhouse for 35 days when the seedlings had from two to three true leaves, and they were able to transplant to the field.

The soil preparation was carried out with a tractor and rotary tiller. Seedbeds were prepared with 1.0 m wide, 0.20 m high, with 1.0 m spacing between beds. At the transplanting, 1.500 kg ha^{-1} of the NPK formulation 04-30-10 was applied. As topdressing fertilization, 20 kg ha^{-1} of MAP, 75 kg ha^{-1} of ammonium sulfate, 100 kg ha^{-1} of ammonium nitrate, and 200 kg ha^{-1} of potassium chloride were divided into four applications with a 20-day interval after transplanting.

The seedlings were manually transplanted to the field 35 days after sowing (DAS), with 0.40 m between plants and 1.0 m between rows. Irrigation was performed by a drip system, supplying the water requirement based on the parameters for crop irrigation management.

Weed control was performed weekly to avoid competition. Insecticide baits were placed throughout the field to identify the insect infestation rate and help the decision of pesticide application. Phytosanitary control was carried out whenever necessary, to maximize fruit production (FAO, 2006).

The tomato lines were characterized by morphological traits contemplated in the guidelines for performing the distinguishability, homogeneity, and stability (DHE) assays by the MAPA, which were modified by the authors. A randomized block experimental design with three replications was used. Eighty-

five industrial tomato lines were evaluated. Each plot had 12 plants. The two central plants of each plot were evaluated. The descriptors analyzed are shown in Table 1.

It was estimated the genotypic determination coefficient (H^2), according to the estimator below:

$$H^2 = \frac{\hat{\emptyset}g}{QMT/_r}$$

$$\hat{\emptyset}g = \frac{(QMT - QMR)}{r}$$

Where:
H^2 = genotypic determination coefficient;
∅ = quadratic genetic component;
QMT = mean square of genotypes;
QMR = mean square of the residue; and
ϒ = number of replications.

Genotypes were grouped based on the Scott-Knott test at the 1% and 5% probability level. Subsequently, the selection gains estimates were reached by the aid of the selection index methodologies cited by Cruz (2006): direct and indirect selection; classic index proposed by Smith (1936) and Hazel (1943); rank summation index of Mulamba and Mock (1978); base index of Williams (1962); and genotype-ideotype distance index (GID). The selection criterion applied was to increase the traits: fruit pericarp thickness (FPT), fruit firmness (FF), yield (YLD), average number of fruits per plant (NFP), and soluble solids (SS).

The index proposed by Smith (1936) and Hazel (1943) was established by the selection index (I) and the genotypic aggregate (H) described below:

$$I = b_1y_1 + b_2y_2 + ... + b_ny_n = \sum_{i=1}^{n} b_i\, y_j = y'b$$

$$H = a_1g_1 + a_2g_2 + ... + b_ny_n = \sum_{i=1}^{n} a_i\, g_i = g'a$$

where:
n = number of traits evaluated;
b = vector of dimension 1 xn of the selection index weighting coefficients to be estimated;

Table 1 - Descriptors for industrial tomatoes (adapted from MAPA, 2005) and details on their analysis

Traits	Trait description	Description code	Comments
01. Fruit pericarp thickness	Slim	S	The analysis was performed using a digital caliper, measuring the diameter (mm) from the outer wall to the inner wall of the pericarp
	Average	A	
	Thick	T	
02. Fruit: firmness	Soft	S	The analysis was performed by subjecting the fruits to pressure at one point in the middle region, measuring the resistance of the pulp to penetration, using Instrutherm model PTR-300 digital penetrometer, and obtaining the values expressed in Newton (N)
	Medium	M	
	Firm	F	
03. Maturation cycle	Precocious	P	It was evaluated from the transplanting of seedlings
	Medium	M	
	Late	L	
04. Yield	Low	L	It was determined by the weight and number of fruits per plant
	Average	A	
	High	H	
05. Number of fruits per	Low	L	It was counted all fruits of each plant, including the green and damaged ones
	Average	A	
	High	H	
06. Soluble solids	Low	L	The analysis was performed by transferring a drop of the fruit juice to the Hanna Instruments model HI 96801 digital refractometer prism and then reading it, expressed in °Brix
	Average	A	
	High	H	

y = nxp dimension matrix (plants) of phenotypic values of traits;
a = is the 1 xn dimension vector of previously established economic weights;
g = nxp dimension matrix of unknown genetic values of the n traits considered.

The vector b = P -1 Ga, where P -1 is the inverse of the matrix, of dimension nxn of phenotypic variance and covariance between traits. G is the nxn dimension matrix of genetic variance and covariance between traits.

The expected gain for trait j was expressed by:

$$\Delta g_{j(i)} = DS_{j(i)} h_j^2$$

Where:
Ag j(i) = gj (i): expected gain for trait j, with selection based on index I;
DS j(i) = selection differential of trait j, with selection based on index I;
h^2j = heritability of trait j.

In the rank summation index of Mulamba and Mock (1978), the orders of each genotype were summed, resulting in the selection index, as described below:

$$I = r_1 + r_2 + ... + r_n$$

Where:
I = index value for a given individual or family;
r_n = an individual's rank (or rank) from the jth trait;
n = number of traits considered in the index.

The weights were given by:

$$L = p_1 r_1 + p_2 r_2 + ... + p_n r_n$$

Where:
pj = economic weight attributed to the jth trait.

For the base index of Williams (1962), the following index was used as selection criteria:

$$I = a_1 y_1 + a_2 y_2 + ... + a_n y_n = \sum_{i=1}^{n} a_i y_i = y'a$$

Where:
y = are the means;
a = are the economic weights of the traits studied.

For the index of genotype-ideotype distance (Cruz, 2006), the mean and maximum and minimum values for each variable were calculated. Xij was considered as the mean phenotypic value of the ith genotype concerning the ith trait. As well, we considered the value Yij representing the transformed mean phenotypic value and Cj as a constant relative to the average genotype depreciation. Thus, we had: LIj as the lower limit to be presented by the genotype, relative to the characteristic j, LSj as the upper limit to be presented by the genotype and VOj as the optimal value to be presented by the genotype, under selection.
If LIj <Xij <LSj, then Yij = Xij;
If Xij <LIj, Yij = Xij + VOj - LIj - Cj;
If Xij> LSj, Yij = Xij + VOj - LSj + Cj.

In the methodology, it was considered Cj = LSj - LIj. The Cj value ensured that any value of Xij within the range of variation around the optimum resulted in a value of Yij of magnitude close to the optimal value (VOj), as opposed to the values of Xij outside this range. Thus, the Xij transformation was performed to ensure the depreciation of phenotypic values out of range. The Yij values obtained by transformation were later standardized and weighted by the weights assigned to each characteristic, obtaining the Yij values, as described below:

$$y_{ij} = \frac{y_{ij}}{\sqrt{a_j S(Y_j)}}$$

Where:
S (Yj) = standard deviation of the mean phenotypic values obtained by the transformation;
aj = weight or economic value of the characteristic.

Then, we calculated the GID index values expressed by the distances between the genotypes and the ideotype, as illustrated:

$$I_{GID} = \sqrt{\frac{1}{n} \sum_{j=1}^{n} (y_{ij} - vo_j)^2}$$

From these indexes, the best genotypes were identified, and the selection gains were calculated. All genetic and statistical analyzes were processed through the Computational Program in Genetics and Statistics - GENES Program (Cruz, 2016).

3. Results and Discussion

Genetic variability was found for all traits by the F-test at 1% or 5% probability level, which evidenced the ability to perform the selection of superior tomato lines. It was verified by values of coefficient of variation (CV) ranging from 1.36% to 29.03% for MC and NFP, respectively. The highest CV values were observed in trait NFP (29.03%), SS (18.28%), and YLD (18.05%) (Table 2).

The genotypic coefficient of determination (H^2) allows us to define the estimate of genetic gain to be achieved and to establish the most appropriate strategy to be used in the breeding program (Baldissera *et al.*, 2014). H^2 values change according to each charac-

Table 2 - Mean square, coefficient of variation, and genetic parameters of agronomic traits and yield of 85 industrial tomato lines

Source of Variation	DF	Mean square					
		FPT	FF	YLD	CM	NFP	SS
Blocks	2	0.65	0.59	488.22	2.89	1663.12	37.75
Lines	84	2.08 **	0.56 **	698.53 **	8.16 **	1664.94 *	0.82 *
Residue	168	1.35	0.23	338.89	2.29	1175.83	0.55
CV (%)	-	15.96	16.69	18.05	1.36	29.03	18.28
CVg/CVe	-	0.42	0.68	0.59	0.92	0.37	0.41
H^2	-	34.84	58.30	51.48	71.93	29.38	33.29

FPT= fruit pericarp thickness, FF= fruit firmness, YLD= yield, MC= maturation cycle, NFP= number of fruits per plant, SS= soluble solids, H^2= genotypic coefficient determination, CV= coefficient of variation, CVg= coefficient of genetic variation, CVe= coefficient of experimental variation. ** and * significant by F-test at 1% and 5% probability, respectively.

teristic and are classified as high when they are higher than 0.7 (Alvares *et al.,* 2016).

The highest H^2 values were found for the maturation cycle (71.93%), fruit firmness (58.30%), and yield (51.48%). These values allow us to reach success by the phenotypic selection, which can be proven by the results found in the CVg/CVe ratio, which were close to 1.0 for these traits. The lowest H^2 values were observed for the number of fruits per plant (29.38%) and soluble solids (33.29%).

The medium and high results of the heritability coefficient and coefficient of genetic variation are related to higher selective accuracy, higher genetic variability, and the probability of successfully choosing genotypes with optimal agronomic traits (Storck and Ribeiro, 2011).

The CVg/CVe ratio was close to 1.0 only for the medium cycle. The CVg/CVe ratio can be accepted as an indicator of the obtaining of more relevant genetic gains in the selection of superior genotypes (Cruz *et al.*, 2012).

The constitution of tomato fruits for the industry has been remodeled through genetic improvement, to select cultivars with desirable characteristics for processing. As a general rule, the desired tomato lines are those that combine higher yield with quality, and that meet the needs of the industry, which currently are firm fruits, with a high content of soluble solids, a shorter cycle, a higher number of fruits per plant and higher fruit pericarp thickness (Iglesias *et al.*, 2015; Peixoto *et al.*, 2017).

Fruit pericarp thickness ranged from 5.36 to 9.04 mm (Table 3). Only 3.7% of the tomato lines had a

Table 3 - Fruit pericarp thickness (FPT), fruit firmness (FF), yield (YLD), maturation cycle (MC), number of fruits per plant (NFP), and soluble solids (SS) of 85 industrial tomato lines

Lines	Traits					
	FPT mm	FF N	YLD t ha^{-1}	MC days	NFP n° plant^{-1}	SS °Brix
PXT-102	5.5 b	1.79 b	103.79 a	109 b	123.67 a	4.17 a
PXT-104	6.55 b	1.98 b	93.96 b	107 b	111 b	4.57 a
PXT-106	7.59 a	2.39 b	108.19 a	111 a	130.83 a	3.07 b
PXT-107	6.11 b	2.31 b	111.50 a	109 b	109 b	4.07 a
PXT-108	7.20 a	2.67 b	123.94 a	110 b	96.33 b	4.13 a
PXT-109	7.91 a	2.30 b	85.28 b	110 b	92.33 b	3.77 b
PXT-111	7.75 a	2.58 b	68.98 b	107 b	74.83 b	4.43 a
PXT-113	7.15 a	3.15 a	85.76 b	112 a	99.17 b	4.77 a
PXT-114	7.40 a	2.38 b	72.87 b	110 b	112.83 b	3.03 b
PXT-115	8.08 a	2.55 b	86.27 b	107 b	106.00 b	4.20 a
PXT-116	7.36 a	1.94 b	117.45 a	113 a	107.17 b	3.67 b
PXT-117	8.08 a	2.65 b	119.54 a	112 a	130.83 a	4.40 a
PXT-118	6.90 b	2.70 b	110.33 a	110 b	141.17 a	3.77 b
PXT-120	7.17 a	2.13 b	114.81 a	113 a	128.17 a	4.73 a
PXT-121	6.00 b	2.63 b	115.12 a	112 a	135.17 a	3.17 b
PXT-122	7.39 a	2.40 b	106.26 a	113 a	129.33 a	4.10 a
PXT-123	8.58 a	2.68 b	103.88 a	112 a	143.33 a	4.30 a
PXT-124	6.15 b	2.41 b	113.87 a	113 a	77.67 b	3.87 b
PXT-125	5.69 b	3.14 a	93.54 b	113 a	159.00 a	4.80 a
PXT-126	6.15 b	3.05 a	115.37 a	109 b	155.83 a	4.83 a

Means followed by the same letters belong to the same group by the Scott-Knott test at 5% probability level.

To be continued...

Table 3 - Fruit pericarp thickness (FPT), fruit firmness (FF), yield (YLD), maturation cycle (MC), number of fruits per plant (NFP), and soluble solids (SS) of 85 industrial tomato lines

Lines	Traits					
	FPT mm	FF N	YLD t ha^{-1}	MC days	NFP n° plant^{-1}	SS °Brix
PXT-401	5.37 b	3.08 a	95.68 b	113 a	173.83 a	3.60 b
PXT-402	5.90 b	2.74 b	107.19 a	112 a	139.00 a	4.57 a
PXT-403	6.37 b	3.33 a	122.89 a	112 a	146.00 a	4.00 b
PXT-404	6.74 b	2.11 b	118.68 a	113 a	93.67 b	3.37 b
PXT-405	5.50 b	2.88 b	117.25 a	112 a	115.50 b	4.30 a
PXT-406	6.37 b	3.30 a	100.62 a	110 b	156.17 a	4.40 a
PXT-407	6.43 b	2.85 b	103.61 a	111 a	99.33 b	3.80 b
PXT-408	6.01 b	2.62 b	90.30 b	112 a	126.00 a	4.87 a
PXT-409	6.03 b	3.22 a	99.00 b	111 a	100.33 b	4.87 a
PXT-410	7.42 a	2.80 b	106.20 a	112 a	112.00 b	4.63 a
PXT-411	7.81 a	2.46 b	101.26 a	113 a	96.83 b	4.00 b
PXT-412	7.58 a	2.65 b	110.89 a	113 a	80.92 b	4.63 a
PXT-413	7.95 a	3.12 a	111.25 a	113 a	116.83 b	3.10 b
PXT-501	9.04 a	2.85 b	90.56 b	110 b	120.00 a	4.63 a
PXT-502	8.44 a	3.35 a	116.72 a	110 b	101.67 b	3.57 b
PXT-503	7.72 a	3.35 a	100.34 a	113 a	124.67 a	4.17 a
PXT-504	7.10 a	3.48 a	95.05 b	112 a	122.83 a	4.17 a
PXT-505	7.44 a	2.84 b	113.92 a	108 b	127.00 a	4.37 a
PXT-506	6.79 b	3.06 a	114.68 a	111 a	110.83 b	3.97 b
PXT-551	6.66 b	2.97 a	95.56 b	112 a	128.33 a	4.07 a
PXT-552	7.16 a	2.68 b	112.40 a	111 a	109.00 b	3.47 b
PXT-553	6.92 b	2.99 a	112.46 a	111 a	146.83 a	3.63 b
PXT-554	8.69 a	2.67 b	83.15 b	112 a	69.50 b	4.47 a
PXT-555	6.96 b	2.65 b	104.99 a	108 b	154.17 a	4.10 a
PXT-556	8.49 b	3.06 a	101.46 a	109 b	91.83 b	4.63 a
PXT-557	6.92 b	3.08 a	104.24 a	112 a	96.67 b	3.60 b
PXT-558	7.53 a	3.69 a	102.49 a	109 b	164.00 a	4.23 a
PXT-559	7.31 a	3.77 a	103.30 a	112 a	98.76 b	3.26 b
PXT-560	6.88 b	2.50 b	109.27 a	109 b	93.00 b	4.50 a
PXT-561	8.22 a	2.70 b	85.59 b	109 b	102.50 b	3.63 b
PXT-562	7.73 a	2.88 b	102.51 a	110 b	94.00 b	5.03 a
PXT-563	7.22 a	3.05 a	111.74 a	113 a	90.50 b	4.07 a
PXT-564	6.95 b	2.50 b	131.20 a	113 a	98.33 b	3.26 b
PXT-565	7.12 a	3.07 a	104.29 a	111 a	114.83 b	4.10 a
PXT-566	8.44 a	3.38 a	114.83 a	111 a	128.17 a	4.43 a
PXT-567	8.35 a	2.84 b	91.36 b	112 a	148.83 a	3.83 b
PXT-568	7.23 a	3.42 a	105.30 a	111 a	122.50 a	3.80 b
PXT-569	8.06 a	2.87 b	76.93 b	112 a	121.83 a	4.13 a
PXT-570	8.02 a	3.34 a	88.84 b	113 a	139.33 a	3.40 b
PXT-571	7.23 a	2.80 b	90.29 b	112 a	94.17 b	3.96 b
PXT-572	8.14 a	2.76 b	83.28 b	110 b	104.33 b	4.76 a
PXT-601	6.4a b	3.31 a	137.92 a	112 a	154.66 a	4.26 a
PXT-602	7.59 a	2.76 b	122.97 a	110 b	114.17 b	5.26 a
PXT-603	6.19 b	3.08 a	98.68 b	112 a	136.67 a	3.66 b
PXT-604	7.60 a	2.98 a	117.62 a	109 b	105.33 b	3.37 b
PXT-605	7.82 a	2.94 a	77.11 b	111 a	116.17 b	3.60 b
PXT-606	7.67 a	2.82 b	99.15 b	112 a	144.50 a	4.03 a
PXT-608	8.82 a	2.79 b	93.17 b	110 b	84.33 b	3.03 b
PXT-609	7.29 a	2.78 b	97.48 b	109 b	104.17 b	3.77 b
PXT-610	7.69 a	3.08 a	145.98 a	113 a	132.67 a	4.27 a

Means followed by the same letters belong to the same group by the Scott-Knott test at 5% probability level.

To be continued...

Table 3 - Fruit pericarp thickness (FPT), fruit firmness (FF), yield (YLD), maturation cycle (MC), number of fruits per plant (NFP), and soluble solids (SS) of 85 industrial tomato lines

Lines	Traits					
	FPT mm	FF N	YLD t ha^{-1}	MC days	NFP n° plant^{-1}	SS °Brix
PXT-611	7.94 a	2.84 b	104.42 a	113 a	110.67 b	4.00 b
PXT-613	7.75 a	3.03 a	92.45 b	109 b	106.50 b	4.40 a
PXT-614	7.46 a	3.31 a	99.55 b	112 a	107.00 b	3.50 b
PXT-615	7.37 a	2.67 b	83.69 b	110 b	79.33 b	3.73 b
PXT-616	7.69 a	3.91 a	98.75 b	111 a	122.50 a	4.87 a
PXT-617	8.27 a	3.04 a	85.38 b	113 a	92.83 b	3.97 b
PXT-618	8.68 a	3.04 a	114.23 a	110 b	96.33 b	4.57 a
PXT-619	6.61 b	3.57 a	97.11 b	111 a	152.00 a	4.17 a
PXT-651	7.45 a	3.37 a	56.63 b	109 b	125.83 b	3.60 b
PXT-652	8.16 a	4.10 a	74.64 b	109 b	157.67 a	3.57 b
PXT-653	8.39 a	3.31 a	82.29 b	112 a	103.83 b	2.93 b
PXT-654	6.84 b	3.43 a	84.09 b	111 a	166.17 a	3.87 b
PXT-655	7.55 a	2.35 b	108.04 a	109 b	124.17 a	4.27 a
PXT-656	6.90 b	3.43 a	114.19 a	110 b	149.50 a	3.90 b
PXT-687	6.70 b	2.95 a	98.99 b	109 b	113.83 b	4.77 a

Means followed by the same letters belong to the same group by the Scott-Knott test at 5% probability level.

high thickness of the pericarp. According to Vieira *et al.* (2019), the thickness of the pericarp, together with the resistance of the epidermis and the texture of the placenta tissue, influences the firmness of the fruit (the relationship between the volume of the pericarp and volume of the locular material).

Only 3.7% of the tomato lines had high values of fruit firmness. Firmer fruits present less degradation of the cell wall and increase the resistance of the fruits during the transport process. The fruit firmness ensures resistance to mechanical damage during mechanized harvesting and bulk transport. Fruits that are not firm are more susceptible to the transformation and breakage of the skin, releasing cellular juice and causing fermentation and deterioration of the fruits before the arrival in the industry (Vieira *et al.*, 2019). The fruit firmness is extremely important for the industry, because, between the harvest and the unloading process in the industry, there are many losses, due to a large number of disintegrated fruits, related to excessive compression (Moura and Golynski, 2018).

One of the main characteristics to be used in the selection of the ideal genotype for the tomato processing industry and mainly for the producers is fruit yield. Among the tomato lines evaluated, again, 3.7% of them obtained high values, above 131 t ha^{-1}. The average yield of the state of Goiás, where the tomato lines were evaluated, were 85 and 94 t ha^{-1} in the 2017 and 2018 harvests, respectively (Globo Rural, 2018).

The average cycle ranged from 106 to 113 days. Only 5.88% of tomato lines evaluated had a short cycle. Most cultivars marketed by seed companies have a cycle between 95 and 125 days (Kelley *et al.*, 2010), which demonstrates that all tomato lines evaluated are classified between the short and middle cycles. The use of short-cycle genotypes is desirable in breeding programs, as it allows for a shorter stay in the field, where they will be subject for a shorter time to effects of biotic and abiotic factors such as disease and drought stress (Gatut-Wahyu *et al.*, 2014).

The number of fruits per plant ranged from 69.50 for PXT-554 to 173.83 for PXT-401. Cultivars with a low number of fruits per plant are not recommended because they have lower yield during the harvesting process (Santos, 2015).

High soluble solids content is one of the main characteristics that an industrial tomato material must-have. According to Figueiredo *et al*. (2015), the higher the soluble solids content, the higher the efficiency of industrial production, and the lower the energy expenditure during the pulp concentration procedure. In practice, for each addition of a °Brix in the pulp, there is a 20% increase in industrial production. Values above 4.5°Brix are higher than the Brazilian average. Among 85 tomato lines evaluated, 23.17% is above this value, reaching the maximum value of 5.23 °Brix.

Direct selection resulted in higher individual gains (Table 4). This selection is directed only for one trait of interest and comprises the obtention of maximum gains of a single trait for which selection is practiced. According to how this trait is associated with others, favorable or unfavorable results may occur in traits of secondary importance (Cruz, 2016).

Direct selection for FPT, NFP, and SS resulted in direct gains for fruit firmness, with values of 1.97%, 8.69%, and 2.93%, respectively. Noteworthy was the direct selection for the number of fruits per plant, which resulted in the largest indirect gain for fruit firmness.

The indexes of selection consist of an alternative that allows the simultaneous selection to perform effectively by combining different traits (Rosado *et al*., 2012). In general, the index of the rank summation index of Mulamba and Mock (1978) showed the largest gain of yield (7.89%) and soluble solids (4.02%), followed by the Smith (1936) and Hazel (1943) index, with 7.20% of the gain of yield. However, these two indexes had low selection gain values for the other traits (Table 5).

Table 4 - Genetic gain estimates obtained for five traits evaluated by direct and indirect selection for 85 industrial tomato lines

Traits	Genetic gain (%)				
	FPT	FF	YLD	NFP	SS
FPT	**6.21**	0.43	-0.43	-3.25	-1.94
FF	1.97	**14.41**	-3.34	8.69	2.93
YLD	-1.41	-1.51	**12.05**	-0.67	-1.47
NFP	-2.32	5.05	-0.05	**10.26**	0.2
SS	-0.12	-0.23	-0.44	1.06	**6.81**
Total	4.33	18.15	7.79	16.09	6.53

FPT= fruit pericarp thickness, FF= fruit firmness, YLD= yield, NFP= number of fruits per plant, SS= soluble solids.

The rank summation index of Mulamba and Mock (1978) had the highest gain for all the traits and the highest total gain, with values of 22.92%. The genotype-ideotype distance index obtained the second-highest total gain value, with 22.54%. These indices presented a balanced distribution of selection gains. In the research carried out by Rosado *et al.* (2012), the authors reported that the rank summation index of Mulamba and Mock (1978) was the most appropriate, allowing for a balanced distribution of selection gains for a larger number of yellow passion fruit progenies.

Table 5 - Genetic gain estimates obtained for five traits by selection by the classical index proposed by Smith (1936) and Hazel (1943), rank summation index of Mulamba and Mock (1978), base index of Williams (1962), and genotype-ideotype distance index for 85 industrial tomato lines

Traits	Genetic gains (%)			
	Smith (1936) and Hazel (1943)	Mulamba and Mock (1978)	Williams (1962)	Genotype-ideotype distance
FPT	-4.55	1.88	-2.98	2.31
FF	5.34	5.12	5.83	7.07
YLD	7.20	7.89	6.71	6.69
NFP	7.22	4.01	8.77	4.12
SS	0.29	4.02	0.57	2.35
Total	15.50	22.92	18.90	22.54

FPT= fruit pericarp thickness, FF= fruit firmness, YLD= yield, NFP= number of fruits per plant, SS= soluble solids.

The top ten genotypes, selected by all selection methods used in this study and their values of fruit pericarp thickness (Table 6), fruit firmness (Table 7), yield (Table 8), number of fruits per plant (Table 9), and soluble solids (Table 10) are shown in the tables below. The lines PXT-601 and PXT-610 were selected in all selection methods applied, verifying the superiority of these genotypes.

4. Conclusions

The rank summation index of Mulamba and Mock (1978) and the classical index proposed by Smith (1936) and Hazel (1943) applied to agronomic traits of eighty-five industrial tomato lines turned out to the largest selection gain for the yield trait.

Rank summation index of Mulamba and Mock (1978) has the highest total genetic gain values.
The lines of tomato PXT-601 and PXT-610 stand out as superior genotypes by the direct selection method and selection indexes.

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Table 6 - Fruit pericarp thickness (FPT) in mm from ten superior genotypes selected by direct selection for fruit pericarp thickness, and classic index proposed by Smith (1936) and Hazel (1943), rank summation index of Mulamba and Mock (1978), base index of Williams (1962), and genotype-ideotype distance index (GID)

Selection indexes							
Williams (1962) and direct selection of fruit pericarp thickness		Smith (1936) and Hazel (1943)		Mulamba and Mock (1978)		Genotype-ideotype distance	
Lines	FPT	Lines	FPT	Lines	FPT	Lines	FPT
PXT-601	6.44	PXT-601	6.44	PXT-566	8.44	PXT-566	8.44
PXT-610	7.69	PXT-403	6.37	PXT-610	7.69	PXT-558	7.53
PXT-126	6.15	PXT-610	7.69	PXT-558	7.53	PXT-616	7.69
PXT-403	6.37	PXT-126	6.15	PXT-616	7.69	PXT-601	6.40
PXT-558	7.53	PXT-401	5.37	PXT-601	6.44	PXT-117	8.08
PXT-401	5.37	PXT-656	6.90	PXT-117	8.08	PXT-656	6.90
PXT-656	6.90	PXT-405	5.50	PXT-126	6.15	PXT-610	7.69
PXT-555	6.96	PXT-121	6.00	PXT-602	7.59	PXT-123	8.58
PXT-553	6.92	PXT-406	6.37	PXT-618	8.68	PXT-503	7.72
PXT-406	6.37	PXT-619	6.61	PXT-123	8.58	PXT-618	8.68

Table 7 - Fruit firmness (FF) in Newton from ten superior genotypes selected by the direct selection for fruit firmness, and classic index proposed by Smith (1936) and Hazel (1943), rank index of Mulamba and Mock (1978), base index of Williams (1962), and genotype-ideotype distance index (GID)

Selection Indexes							
Williams (1962) and direct selection of fruit firmness		Smith (1936) and Hazel (1943)		Mulamba and Mock (1978)		Genotype-ideotype distance	
Lines	FF	Lines	FF	Lines	FF	Lines	FF
PXT-601	3.31	PXT-601	3.31	PXT-566	3.38	PXT-566	3.38
PXT-610	3.08	PXT-403	3.33	PXT-610	3.08	PXT-558	3.69
PXT-126	3.05	PXT-610	3.08	PXT-558	3.69	PXT-616	3.91
PXT-403	3.33	PXT-126	3.05	PXT-616	3.91	PXT-601	3.31
PXT-558	3.69	PXT-401	3.08	PXT-601	3.31	PXT-117	2.65
PXT-401	3.08	PXT-656	3.43	PXT-117	2.65	PXT-656	3.43
PXT-656	3.43	PXT-405	2.88	PXT-126	3.05	PXT-610	3.08
PXT-555	2.65	PXT-121	2.63	PXT-602	2.76	PXT-123	2.68
PXT-553	2.99	PXT-406	3.30	PXT-618	3.04	PXT-503	3.35
PXT-406	3.30	PXT-619	3.57	PXT-123	2.68	PXT-618	3.04

Table 8 - Yield (YLD), in Mg ha-1, of ten superior genotypes selected by direct selection for yield, and classic index proposed by Smith (1936) and Hazel (1943), rank summation index of Mulamba and Mock (1978), base index of Williams (1962), and genotype-ideotype distance index (GID)

Selection Indexes							
Williams (1962) and direct selection of yield		Smith (1936) and Hazel (1943)		Mulamba and Mock (1978)		Genotype-ideotype distance	
Lines	YLD	Lines	YLD	Lines	YLD	Lines	YLD
PXT-601	137.92	PXT-601	137.92	PXT-566	114.83	PXT-566	114.83
PXT-610	145.98	PXT-403	122.89	PXT-610	145.98	PXT-558	102.49
PXT-126	115.37	PXT-610	145.98	PXT-558	102.49	PXT-616	98.75
PXT-403	122.89	PXT-126	115.37	PXT-616	98.75	PXT-601	137.92
PXT-558	102.49	PXT-401	95.68	PXT-601	137.92	PXT-117	119.54
PXT-401	95.68	PXT-656	114.19	PXT-117	119.54	PXT-656	114.19
PXT-656	114.19	PXT-405	117.25	PXT-126	115.37	PXT-610	145.98
PXT-555	104.99	PXT-121	115.12	PXT-602	122.97	PXT-123	103.88
PXT-553	112.46	PXT-406	100.62	PXT-618	114.23	PXT-503	100.34
PXT-406	100.62	PXT-619	97.11	PXT-123	103.88	PXT-618	114.23

Table 9 - Number of fruits per plant (NFP) of ten superior genotypes selected by direct selection for number of fruits per plant, and classic index proposed by Smith (1936) and Hazel (1943), rank summation index of Mulamba and Mock (1978), base index of Williams (1962), and genotype-ideotype distance index (GID)

Selection Indexes							
Williams (1962) and direct selection of number of fruits per plant		Smith (1936) and Hazel (1943)		Mulamba and Mock (1978)		Genotype-ideotype distance	
Lines	NFP	Lines	NFP	Lines	NFP	Lines	NFP
PXT-601	154.67	PXT-601	154.67	PXT-566	128.17	PXT-566	128.17
PXT-610	132.67	PXT-403	146.00	PXT-610	132.67	PXT-558	164.00
PXT-126	155.83	PXT-610	132.67	PXT-558	164.00	PXT-616	122.50
PXT-403	146.00	PXT-126	155.83	PXT-616	122.50	PXT-601	154.66
PXT-558	164.00	PXT-401	173.83	PXT-601	154.67	PXT-117	130.83
PXT-401	173.83	PXT-656	149.50	PXT-117	130.83	PXT-656	149.50
PXT-656	149.50	PXT-405	115.50	PXT-126	155.83	PXT-610	132.67
PXT-555	154.17	PXT-121	135.17	PXT-602	114.17	PXT-123	143.33
PXT-553	146.83	PXT-406	156.17	PXT-618	96.33	PXT-503	124.67
PXT-406	156.17	PXT-619	152.00	PXT-123	143.33	PXT-618	96.33

Table 10 - Soluble solids (SS), in °Brix, from ten superior genotypes selected by direct selection for soluble solids, and classic index proposed by Smith (1936) and Hazel (1943), rank summation index of Mulamba and Mock (1978), base index of Williams (1962), and genotype-ideotype distance index (GID)

Selection Indexes							
Williams (1962) and direct selection of soluble solids		Smith (1936) and Hazel (1943)		Mulamba and Mock (1978)		Genotype-ideotype distance	
Lines	SS	Lines	SS	Lines	SS	Lines	SS
PXT-601	4.27	PXT-601	4.27	PXT-566	4.43	PXT-566	4.43
PXT-610	4.27	PXT-403	4.00	PXT-610	4.27	PXT-558	4.23
PXT-126	4.83	PXT-610	4.47	PXT-558	4.23	PXT-616	4.87
PXT-403	4.00	PXT-126	4.83	PXT-616	4.87	PXT-601	4.26
PXT-558	4.23	PXT-401	3.60	PXT-601	4.27	PXT-117	4.40
PXT-401	3.60	PXT-656	3.90	PXT-117	4.40	PXT-656	3.90
PXT-656	3.90	PXT-405	4.30	PXT-126	4.83	PXT-610	4.27
PXT-555	4.10	PXT-121	3.17	PXT-602	5.27	PXT-123	4.30
PXT-553	3.63	PXT-406	4.40	PXT-618	4.57	PXT-503	4.17
PXT-406	4.40	PXT-619	4.17	PXT-123	4.30	PXT-618	4.57

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Short note

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Improvement of *in vitro* germination of *Cycas revoluta* zygotic embryos using gelrite as gelling agent

J. Benjelloun, A. Taoufyq, Z. El Abidine Triqui, Q.L. Alami, R. Layachi, A. Smouni, S. Bouzroud (*), A. Guedira

Laboratoire de Biotechnologie et Physiologie Végétales, Centre de Biotechnologie Végétale et Microbienne Biodiversité et Environnement, Faculté des Sciences, Université Mohammed V de Rabat, Rabat, Morocco.

Key words: *Cycas revoluta*, gelrite, germination, *in vitro*, regeneration, zygotic embryos.

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(*) **Corresponding author:**
sarah.bouzroud@gmail.com

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All relevant data are within the paper and its Supporting Information files.

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Abstract: An efficient *in vitro* germination protocol for *Cycas revoluta*, a widespread ornamental tree, has been established using zygotic embryos as explants with a focus on mineral composition of the culture media, the gelling agent and cytokinine type. A high percentage of germination, 73% was obtained with SH medium instead of 27% with MS medium. A 100% of germination was obtained with the combination of SH medium and gelrite as gelling agent. The addition of cytokinines prompt shoot formation. An optimum shoot induction occurred using 0.5 mg/l of BAP where an average of 14.1 shoot were produced per explants while 2.2 shoots were formed in the presence of 2iP. A 100% of rooting was observed in the presence of 0.5 mg/l of 2iP whereas only 30% of shoots obtained on the SH medium with 0.5 mg/l of BAP were able to develop roots.

1. Introduction

Cycas revoluta is taxonomically known as the most primitive species among the living cycads (Stevenson, 1990; Jones, 1994). *Cycas revoluta* is one of the widespread ornamental trees, grown in temperate, subtropical and tropical regions more precisely in Miyazaki and Kagoshima Prefectures in Kyushu District down to the Ryukyu Islands, Okinawa Prefecture in Japan (Khalighi, 2001; Zarchini *et al*., 2011).

Cycas revoluta is propagated either from seeds, which remain viable for only a short time, or from vegetative offshoots (Demiray *et al*., 2017). As slowly growing plants, they require 3 to 10 years to attain reproductive maturity (Rinaldi, 1999). Germination of *Cycas revoluta* seeds is hard and time consuming (Zarchini *et al*., 2011). Physical dormancy of seed causes delay in seed germination (Frett, 1987). Seeds can take 3 to 9 months to initiate germination before they can continue to germinate for periods of a year or more. *C. revoluta* seeds also demonstrates rapid loss of viability and low morphogenic potential, which hinder its conservation as well as

favor an effective and rapid mass propagation (Naderi *et al*., 2015). The delay in seed germination along with the slow growth of *Cycas* plants increase the cost of production (Frett, 1987; Litz *et al.*, 2005; Demiray *et al.*, 2017). Thus, conventional methods are not quiet efficient for large-scale propagation of this species. Therefore, other propagation methods are needed (da Silva *et al*., 2014). The use of *in vitro* techniques to accelerate seed germination is a suitable way to conserve many of the endangered species.

Several attempts have been made to establish an efficient protocol for *Cycas revoluta* propagation (Rinaldi and Leva, 1995; Rinaldi, 1999; Naderi *et al.*, 2015; Demiray *et al.*, 2017). However, the results were not satisfying. The present study focuses on developing an efficient *in vitro* germination protocol from mature zygotic embryos (ZEs) with a focus on the gelling agent, the mineral composition of the culture media and the presence of 2-isopetynyl adenine (2iP) or 6-Benzylaminopurine (BAP).

2. Materials and Methods

Plant material

Seeds collected from 50 years old female mature plants grown in Faculty of Sciences garden, University Mohammed V in Rabat (Morocco) were used in this study.

Seed sterilization and zygotic embryos isolation

Seeds were soaked in water for 48 hours in order to soften the sacrotesta, the orange external layer. Once removed, seeds were then flamed with ethanol for 2 minutes in order to eliminate the sclerotesta. Following removal of the sclerotesta, the megagametophytes were surface sterilized for 20 minutes by soaking in 30% dilution of NaOCl containing 2-3 drops of Tween-20, followed by 3-4 rinses with sterile distilled water. After surface sterilization, megagametophytes were pooled, longitudinally bisected and the ZE was excised from each megagametophyte.

Zygotic embryos culture

ZEs 1.7 to 2-cm long were placed in culture jars containing 120 ml of culture medium with 2 explants per jar. Two basal mediums Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) were tested for *in vitro* germination. Both media were supplemented with 30 g/L of sucrose and solidified with 0.8% of bacteriological agar type E (BIOKAR Diagnostic) or 0.3% of gelrite (SIGMA-Aldrich). BAP or 2iP (0.5 mg/L) were added to the culture medium. The pH was adjusted to 5.8 with either 1N HCl or KOH prior to autoclaving, at 108 kPa and 120°C for 20 min. Cultures were incubated in a culture room at 25±2°C and in the dark for 21 days, thereafter under a photoperiod of 16h of light/8h of darkness.

Statistical analysis

Percentages of germination and plant development were compared using Z test or a fixed model of analysis of variance (ANOVA) depending on the condition. Thirty biological replicates were performed for each condition. In case of significant difference between groups, a Least Significant Difference LSD test was used for means separation. Shoot number, shoot length and number of leaves per shoot obtained from each embryo was analyzed by Z test, at risk of 0.05.

3. Results and Discussion

Effects of the culture medium on germination and regeneration

Nitrogen formulation influences seed germination and callus induction in cycads (Rinaldi, 1999; Demiray *et al.*, 2017). Investigating seed responsiveness to SH and MS medium revealed significant differences in germination (GP) and development percentages (PDP) between the two tested mediums. The results revealed that the highest GP (73%) was obtained with SH medium while the GP was around 57% with MS medium (Table 1). A higher regeneration percentage (around 27%) was also observed with SH medium while only 13% of seeds were able to regenerate (Table 1). Rinaldi and Leva (1995) have found that SH or MS medium, both containing ammonium, promoted shoot formation. Rinaldi (1999) also reported that the percentage of responding explants and the number of regenerated shoots were significantly higher on SH medium than on MS medium. However, the presence of nitrate as a sole source of nitrogen, as in the Klimaszewska and Keller medium (Klimaszewska and Keller, 1985), did not promote shoot regeneration (Rinaldi and Leva, 1995;

Table 1 - Effect of medium culture on germination and plant development of *Cycas revoluta* ZEs.

Medium	Germination (%)	Plant development (%)
SH	73 (*)	27 (*)
MS	57 (**)	13 (**)

* indicate the statistical significance ($p<0.05$) using Z test.

Rinaldi, 1999). This difference might be due to the differences in ammonium amounts found in SH and MS media.

Effect of gelling agents on zygotic embryos development

The gelling agent influences the germination of *Cycas revoluta* ZEs. We found that the germination percentage reached 100% with gelrite used as gelling agent in SH medium while only 73% of ZEs were able to germinate in SH medium supplemented with Agar. Moreover, the highest PDP was obtained with SH medium solidified by gelrite (Fig. 1).

The use of gelrite as gelling agent was previously used to stimulate the growth and the development of *in vitro* cultured plants. In *Sequoia sempervirens*, Fira and Clapa (2008) have reported a higher shoot multiplication with gelrite. Similar finding was also stated in oil palm in which gelrite was proven to be better than Agar giving the highest conversion rate of polyembryoids into plantlets (Palanyandy *et al*., 2020). The micropropagation of Cowpea cultivars (*Vigna unguiculata* L. Walp) was highly assessed using gelrite instead of agar (Aasim *et al.*, 2009). Veramendi *et al*. (1997) have proposed gelrite as a great alternative to agar for micropropagation and microtuberization of *Solanum tuberosum*. Scholten and Pierik (1998) explained this beneficial effect by the inorganic composition and the dynamical interaction between gelling agent-medium-tissue. Later, Puchooa *et al.* (1999) linked the positive effect of gelrite on *in vitro* plant culture to the chemical composition of this gelling agent; with a high content of copper, iron, magnesium, zinc and calcium compared to Agar. In addition to that, Buah (1999) explained the beneficial effect of gelrite on growth of banana by the fact that gelrite provide a better availability of water. Moreover, the diffusion of phenols and other inhibitive molecules in the media culture is facilitated by the use of gelrite (Huang and Chi, 1988; El Abidine Triqui *et al.*, 2008). Thus, it is assumed that the low regeneration rate of seedlings obtained with Agar is probably due to the accumulation of inhibitive compounds in this gelling agent; following this, explants can no longer absorb the mineral salts that are essential for their development. Previous works have reported the positive effect of gelrite on seed germination and development in several plant species (Asif *et al*., 2001; Yamazaki and Miyoshi, 2006; Pech y Aké *et al.*, 2007), however none of them was related to *Cycas revoluta*. This work can be qualified as one of the pioneers highlighting the positive effect of gelrite on *Cycas revoluta* ZEs development.

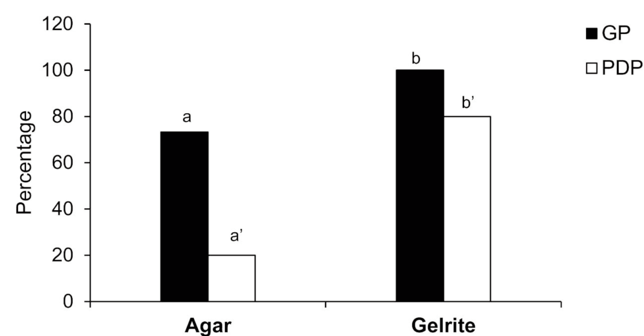

Fig. 1 - Effect of gelling agent (agar or gelrite) on germination (PG) and plant development (PDP) percentages. Bars with different letters indicate the statistical significance (p<0.05) according to LSD test.

Effect of cytokinines on shoot development

Even though germination percentage reached 100% with SH medium solidified with gelrite (Fig. 2), the number of shoots per ZE was significantly lower (Table 2).

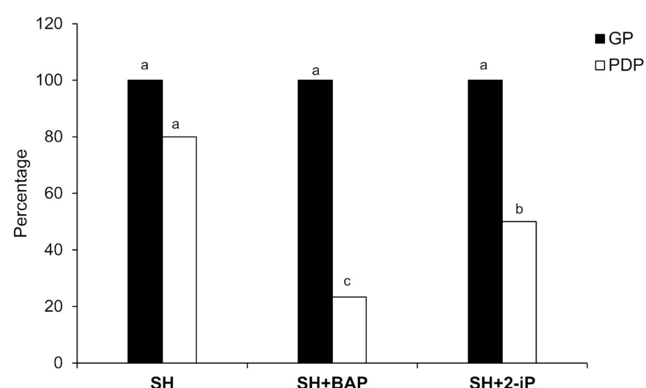

Fig. 2 - Effect of plant regulators (BAP and 2-iP) on germination (GP) and plant development (PDP) percentages of *Cycas revoluta* zygotic embryos cultured on SH medium. ZEs were cultured on SH medium solidified with gelrite and supplemented with 0.5 mg/l of BAP or 0.5 mg/l of 2iP. Bars with different letters indicate the statistical significance (p<0.05) according to LSD test.

Table 2 - Effect of phytohormones on the number of shoot/ explant, number of leaves/ shoot and shoot length of *Cycas revoluta* ZEs.

	SH	SH + BAP	SH + 2-iP
Number of shoot/ explant	1.6 (***)	14.1(*)	2.2 (**)
Shoot length	0.7 (**)	0.52 (**)	2.73 (*)
Number of leaves/shoot	1.81 (**)	9.13 (*)	3.6 (**)

* indicate the statistical significance (p<0.05) using Z test.

The addition of phytohormones, likely BAP and 2iP, increased shoot number in *Cycas revoluta* ZEs. The average of shoots developed per explant was around 14.1 in the presence of BAP and 2.2 when ZE were cultured in SH medium supplemented with 2iP (Table 2). However, shoot length was significantly higher in the presence of BAP with 9.3 leaves per shoot versus 1.7 and 3.6 when cultured in the absence of phytohormones or with BAP added to the media culture (Fig. 3 d, e, f). Shoot differentiation was previously obtained from zygotic embryos of *Cycas revoluta* in the media with BAP (0.5 mg/l) and 2.4-D (1 mg/l) (Rinaldi and Leva, 1995). Naderi *et al*. (2015) found that BAP stimulated shoot regeneration from *Cycas* ZEs while other phytohormones like 2.4-D or Kinetin alone or in combination with BAP failed.

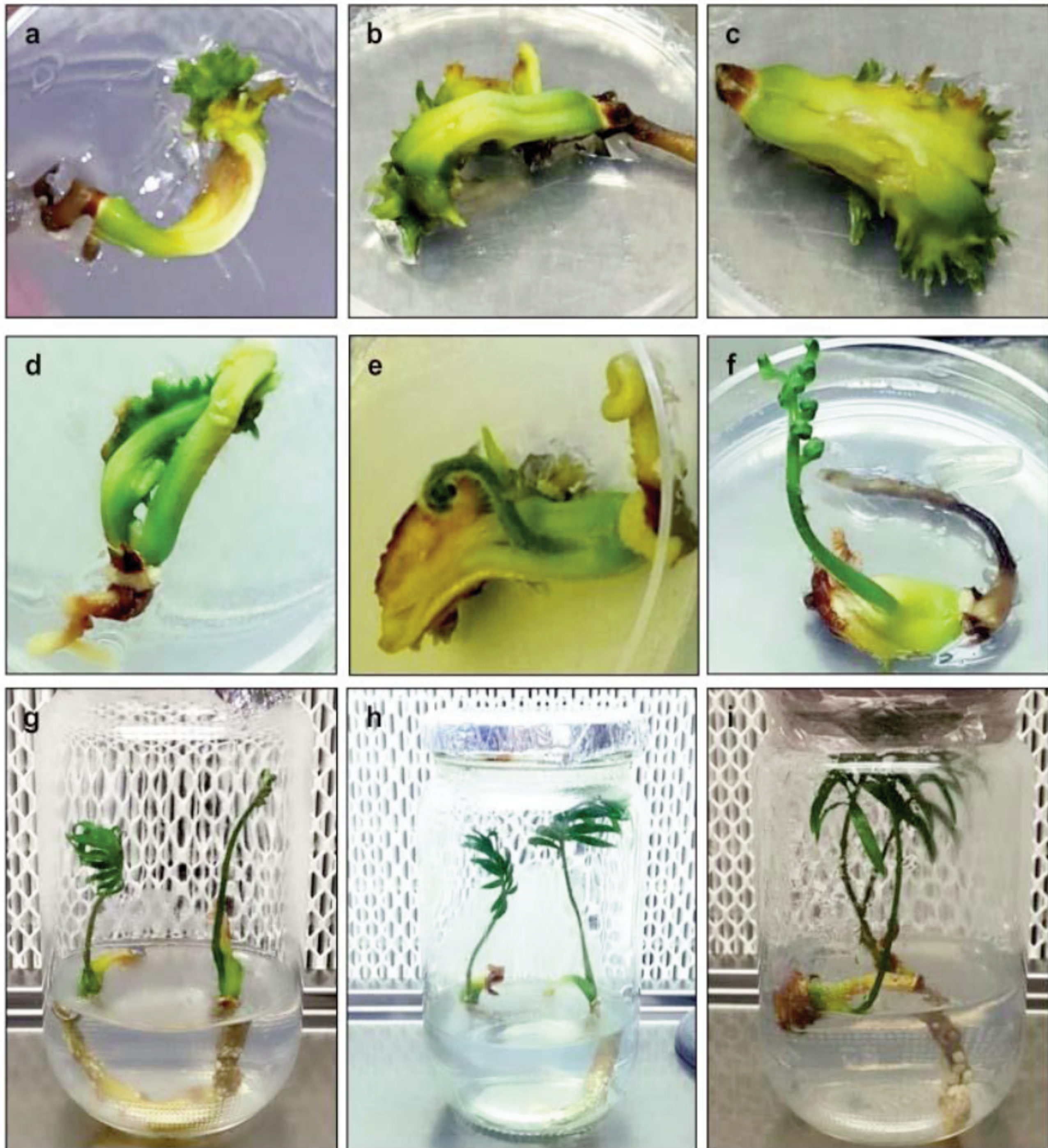

Fig. 3 - Effect of phytohormones on zygotic embryos development. (a) shoot induction in SH basal medium, (b, c) shoot regeneration in SH medium supplemented with 0.5 mg of BAP and 0.5 mg of 2iP respectively, (d, e, f) shoot elongation SH, SH+BAP and SH+2iP Prespectively, (g, h, i) rooted plants after 3 months of culture in SH, SH+BAP and SH+2iP, respectively.

The combination of BAP (0.2 mg/l) and 2,4-D (0.02 mg/l) induced adventitious shoots from mature *C. revoluta* ZEs (Motohashi *et al.*, 2008). Shoot elongation was although promoted by the presence of 2iP. Our data showed that shoot length developed in SH medium supplemented with 2iP was significantly higher than those obtained in the presence of BAP. Root development was obtained in all growth conditions. We noticed that the shoots developed on the SH medium supplemented with 0.5 mg/l of 2iP were all rooted (100%) whereas only 30% of shoots obtained on the SH medium with 0.5 mg/l of BAP developed roots instead of 50% on SH medium (free from plant growth regulators) (Data not shown). In addition to the high rooting percentage obtained in the presence of 2iP, we also noticed that the presence of 2iP promoted the development of primary and/or secondary roots with likely meristematic structures identified as nodules (Fig. 3i). This finding was previously reported by Dhiman *et al*. (2000), who suggested that such nodules are meristematic zones with distinct organogenic potential, which can probably be evolved to embryos or seedlings depending on the culture conditions.

4. Conclusions

This study aims to improve an efficient *in vitro* germination protocol from mature zygotic embryos of *Cycas revoluta*. Based on our results, we found that SH medium was beneficial for seeds germination rather than MS medium. Moreover, the use of gelrite, instead of Agar, enable us to obtain a 100% of seed germination. An average of 14.1 shoots per zygotic embryo was thus obtained with the addition of 0.5 mg/L BAP to the culture media. Taken together, this protocol represents a useful and potential commercial method for *Cycas revoluta* mass propagation.

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Supplementary materials

Phenolic fingerprint in wild growing pomegranate fruits from Azerbaijan

OPEN ACCESS

A. Zeynalova, E. Novruzov, P. Bartolini, C. Brunetti, B. Maserti

[1] *Institute of Botany, Azerbaijan National Academy of Sciences, Badamdar 40, Baku, AZ1004, Azerbaijan.*

[2] *Institute for Sustainable Plant Protection (IPSP), CNR, Via Madonna del Piano, 50019 Sesto Fiorentino (FI), Italy.*

***) Corresponding author:**
cecilia.brunetti@ipsp.cnr.it

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Abstract: The demand for pomegranate (*Punica granatum* L.) juices worldwide increasing due to its documented health-promoting effects which likely derive from phenolic compounds. This study reports the phenolic composition of the juices obtained from eight wild-growing pomegranate accessions collected in eight areas of Azerbaijan, characterized by different climate and soil composition. The anthocyanins found in all the accessions were cyaniding derivatives and pelargonidin derivatives, while only two accessions contained also delphinidin-3,5-O-diglucoside. The main hydrolysable tannins contained in the juices were punicalagin and ellagic acid derivatives. These bio-active metabolites found in the juices varied qualitatively and quantitatively among the eight accessions, thus constituting specific traits for selecting promising accessions that can be used as a nutritious food source. The different phenolic profiles might be determined both by genotype and the growing environmental conditions, or by their interaction. Our results suggest that some of the studied wild-growing pomegranate accessions might have a commercial value because of their richness in bioactive metabolites and might constitute a suitable source of genes for breeding programs.

Table S1 - Soil characteristics of the eight Azerbaijan regions where the accessions were collected

Collection site	Code	Type of soil	Granulometric composition of soils [1]	Salinization of soil (%) [2]	Humus content (%) [3]	N (%)	Total iodine (mg/kg)	Co (mg/kg) 0-18 cm	Average boron content (mg/kg)	CEC (meq/100 g)	Ch:Cf ratio	pH
Khizi District (200 m.a.s.l)	Pg 1	Grey brown salt marshes	3S 3D 4C (average clay alumina)	<0.25	1.11-3.14	0.24	2.5-5.7	32.0	58.0	22-30	*	7.5-8.2
Siyazan district (230 m.a.s.l)	Pg 2	Saline soils	3S 4D 3C (average clay loamy soil)	0.25-0.5	0.5-5.8	*	2.4-3.8	30.0	83.0	ott-20	Cf > Ch	7.3-7.5 (salted with neutral salts)
Shekı Regıon (280 m.a.s.l)	Pg 3	Chestnut (Gray-brown) Light chestnut	5S 3D 2C (heavy loamy sandstone)	<0.25	2.0-3.0 1.14-1.85	0.16-0.28 0.13-0.17	4.2-10.5 4.2-6.8	15.0 10.0	24.5 26.2	25-40 21-37	* *	7.2 7.2-7.5
İsmaıllı dıstrıct (540 m.a.s.l)	Pg 4	Mountain brown soil	3S 4D 3C (average clay loamy soil)	<0.25	7-ago	0.47-0.82	2.5-5.7	16.0	74.0	39.7	0.8-1.2	7.0-7.3
Yevlakh district (60 m.a.s.l)	Pg 5	Saline earth serozems	3S 4D 3C (average clay loamy soil)	0.15-0.22	1.40-1.87	0.11-0.12	1.5-4.3	2.4	81.0	18-27	0.5-0.6	8.1-8.3
Agsu mountaın pass	Pg 6	Salt marshes	3S 4D 3C (average clay loamy soil)	0.5-1.1	4.3-6.6	*	2.4-3.8	25.0	58.0	47 -54	Cf > Ch	08/10/2020 (containin soda)
Agsu dıstrıct (190 m.a.s.l)	Pg 7	Salt marshes	3S 4D 3C (average clay loamy soil)	0.5-1.1	4.3-6.6	*	2.4-3.8	25.0	58.0	47-54	Cf > Ch	8-ott
Gokchay dıstrıct (170 m.a.s.l)	Pg 8	Light brown soils	3S 4D 3C (average clay loamy soil)	<0.25	3.0-4.0	0.16	2.5-5.7	12.0	74.0	*	*	7.0-7.5

* Information not found.
[1] Correlation between elements of granulometric composition of soils: sand, dust, clay.
[2] The salt content of the dry residue (%) in 0-100 cm layer.
[3] In the upper 0-19 cm of soil layer.
CEC - Cation Exchange Capacity.
Ch:Cf ratio - Humic and fulvic acids in humus composition.

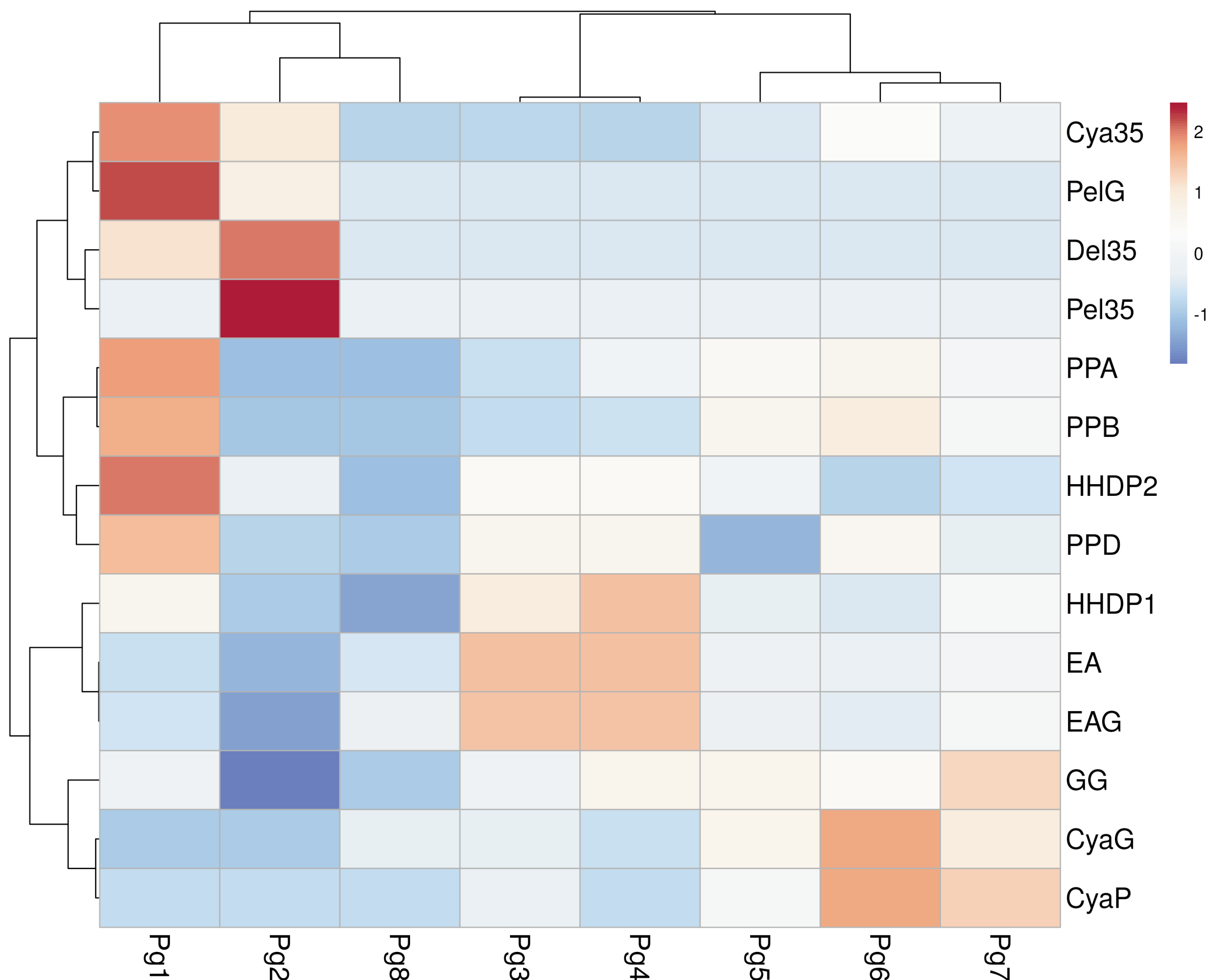

Fig. S1 - Heat map showing the concentration of phenolic compound in wild-growing pomegranate in each studied area.
Cya35= cyanidin-3,5-O-diglucoside; PelG: pelargonidin-3-O-glucoside;
Del35= delphinidin-3,5-O-diglucoside;
Pel35= pelargonidin-3,5-O-diglucoside;
PPA= punicalagin isomer α;
PPB= punicalagin isomer β;
HHDP2= HHDP-hex-deriv 2;
PPD= sum of punicalagin derivative;
HHDP1= HHDP-hex-deriv1;
EA= ellagic acid;
EAG= ellagic acid glucoside;
GG= galloyl-glucose;
CyaG= cyanidin-3-O-glucoside;
CyaP= cyanidin-3-O-pentoside.

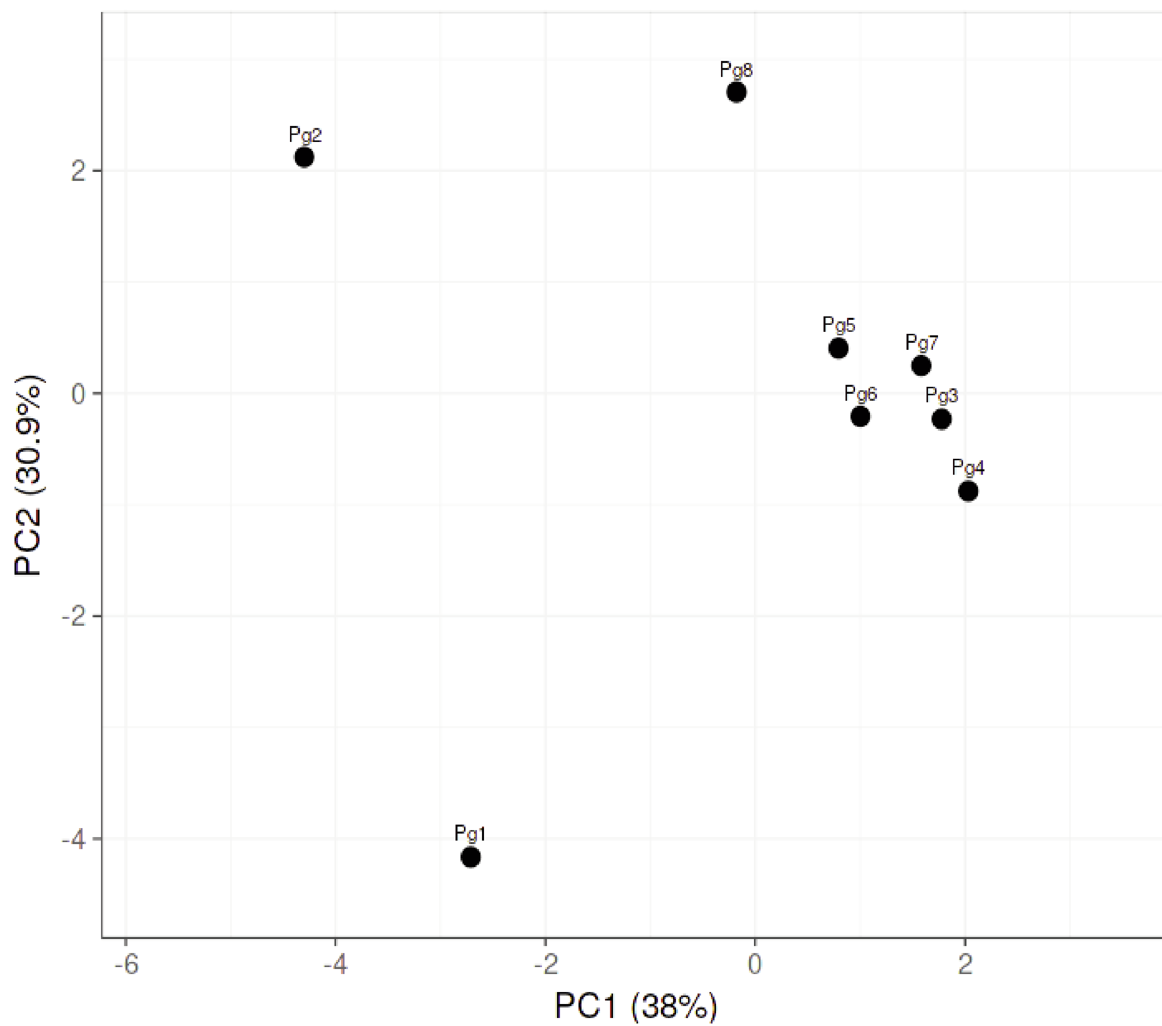

Fig. S2 - PCAbi-plot for the studied areas of wild-growing pomegranates based on the concentration of phenolic compounds.