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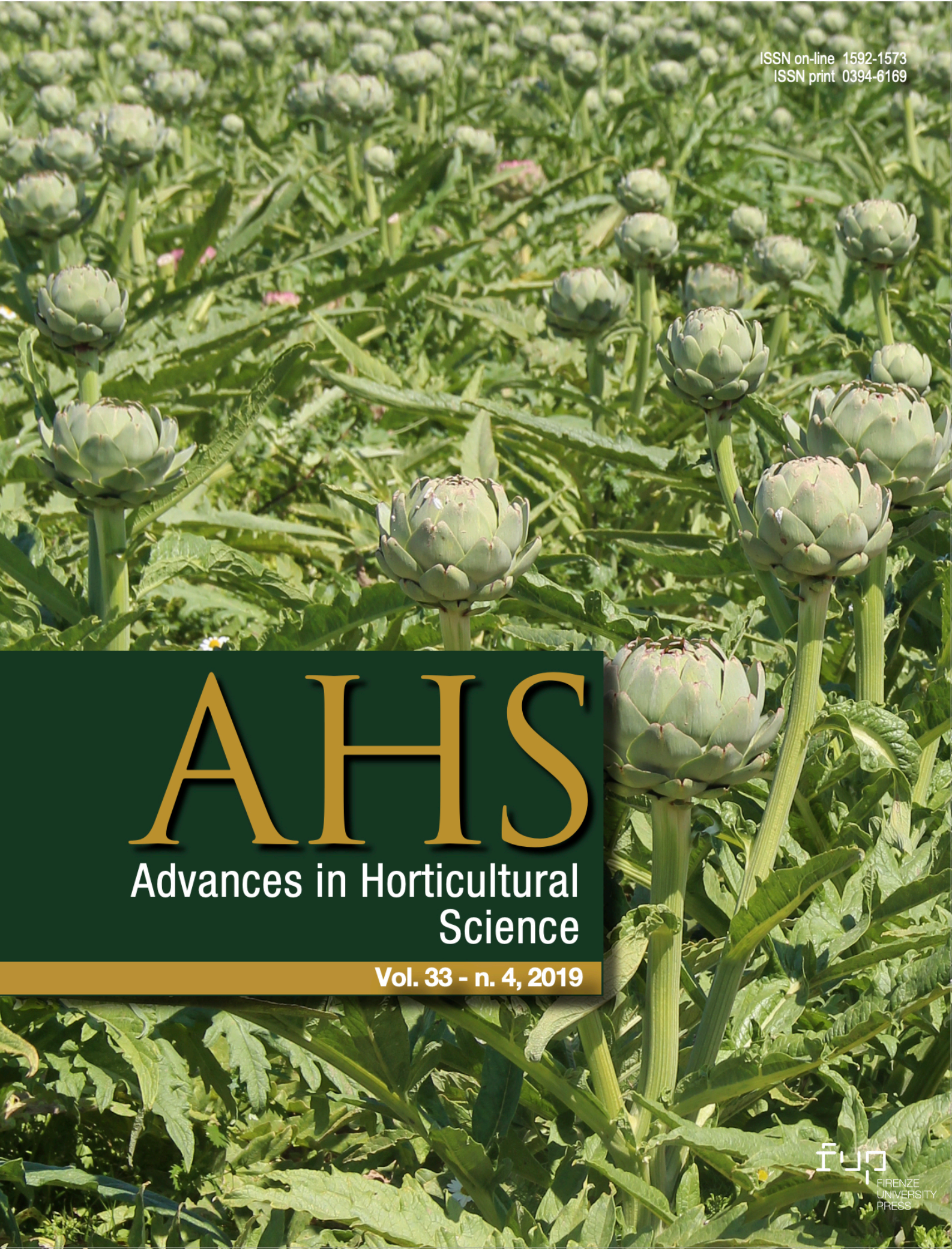

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Advances in Horticultural Science

Economic analysis of crisp lettuce production in different planting spacing and soil cover

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Key words: economic indicators, *Lactuca sativa,* production costs, vegetables.

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Abstract: The objective of this study was to estimate and evaluate the economic indicators of lettuce production, cultivated using different soil cover and plant spacing. The experiment was conducted in subdivided parcels, with four replications. The treatments were composed by a combination of three soil cover (uncovered soil, straw and plastic cover) and three planting spaces (0.25 x 0.20, 0.25x0.25 and 0.25x0.30 m). The productivity and economic indicators were evaluated for a production area of 1000 m^2. For the different treatments, a total operating cost of USD 781.80 to USD 663.30 1000 m^{-2} was obtained. It was observed that for the cultivation of lettuce in soil covered by straw, from the rubbing, and spacing of 0.25x0.25 m the economic indicators were raised. With a productivity of 687.70 boxes 1000 m^{-2}, for this treatment was obtained gross revenue of USD 1,828.99, operating profit of USD 1,135.41 and a profitability index of 62.08%. Thus, lettuce cultivation provides positive profitability regardless of the spacing or type of cover used and the combination between the 0.25 x 0.25 m planting spacing and the use of straw as a soil cover culminates in higher monetary gains.

1. Introduction

The consumption of vegetables has been increasing continuously due to the dietary habits adopted by the population (Maziero *et al.*, 2017), which consequently influences the demand for higher yields (Silveira *et al.*, 2015). Among the products sought by consumers, lettuce is the world leader in terms of acceptance, increasing its importance to the productive sector due to the large volume of commercialization (Vieira and Barreto, 2006).

Considering the participation of family farms in the vegetable produc-

tion scenario, one of the main productive obstacles is the investment required. Most of the costs are related to inputs purchase, as fertilizers, seedlings and seeds (Rezende *et al.*, 2005, 2009; Batista *et al.*, 2013). This burden of the production process is necessary, in order to obtain higher quality products, increasingly demanded by the consumer market.

Through the research, it is seeking the application of techniques that assist the rural producers in obtaining greater monetary returns, encouraging them to continue in the agricultural activity. Simple changes in management can be effective strategies for reducing production costs. In this sense, for the lettuce crop, it is observed that the use of straw as a cover is an effective way of preserving the soil physicochemical characteristics, favoring plants development and increasing the profitability (Vendruscolo *et al.*, 2017 a). In addition, due to intense soil movement applied to vegetable production (Ziech *et al.*, 2014), the cover contributes to the preservation of organic matter and erosion reduction (Cividanes, 2002; Souza and Resende, 2006).

Materials that are easily accessible to the rural producer, such as straw from grazing, can be used as mulch, replacing other materials such as plastic, for example, which has an effective participation in the costs of producing vegetables (Vendruscolo *et al.*, 2017 b), also representing a source for environmental contamination (Chang *et al.*, 2013). In this sense, it is important to generate information on the economic feasibility of techniques applied to agriculture, based on data with high reliability.

The presence of a superficial straw layer is beneficial from the point of view of soil quality maintenance (Cardoso *et al.*, 2012), favoring the development of plants of economic interest (Torres *et al.*, 2015; Vendruscolo *et al.,* 2017 a). It also favors the maintenance of soil moisture by controlling the direct evaporation of the surface (Carneiro *et al.*, 2014), reducing the need for large volumes of water in irrigation. The decrease in weed competition due to the suppression of spontaneous plants is another advantage of soil cover (Moraes *et al.*, 2013), which implies the less need for herbicides or even manual weeding. In this sense, it was verified, for the radish culture, that the maintenance of the cultural remains of silk flower on the soil surface increased the number of commercial roots produced (Oliveira *et al.*, 2015).

In addition to the soil cover, other management techniques can assist producers in obtaining higher yields and superior quality of their product. The plant population used in agricultural crops is decisive in creating an environment conducive to its development and should be established for the specific conditions of a given locality, in order to avoid excessive competition for resources such as water, light, nutrients and carbon dioxide essential to their development (Taiz *et al.*, 2017). In addition, the unnecessary expense of purchasing seedlings, one of the inputs with the largest share in production costs (Vendruscolo *et al.*, 2017 a).

Specifically for lettuce, it is observed that larger plant spacings can generate plants with higher weight (Vasconcelos *et al.*, 2017), which may be related to the broad development of the aerial part observed in commercial genotypes. It is also verified that population density variation can be an effective tool for controlling weeds in different crops (Carvalho and Guzzo, 2008; Bajwa *et al.*, 2017; Li *et al.*, 2018), decreasing spending on hand and acquisition of agrochemicals.

In view of the information above, the objective of this study was to estimate and evaluate the economic indicators of lettuce cultivation, using different soil cover and plant spacing.

2. Materials and Methods

The study was conducted in an experimental area located at the Goiás Federal University, in Goiânia, Goiás, Brazil. For the locality the following average climatic indicators are verified: annual precipitation of 1,575 mm and average monthly temperature of 22.9°C, the predominance of Aw climate, characterized by a tropical climate with rainy season of October to April and a period with precipitations below 100 mm monthly between May and September. The average climatic parameters of air temperature and humidity were obtained at an evaporimetric station at 300 m distance from the experimental area (Fig. 1).

The soil was classified as Latossolo Vermelho, following the methodology proposed by Santos *et al.* (2013). The soil chemical analysis (depth of 0-0.2 m), before the implantation of the experiment revealed the following nutrient content: Ca^{2+}: 2.8 $cmol_c$ dm^{-3}, Mg^{2+}: 1.8 $cmol_c$ dm^{-3}, K^{+}: 0.37 $cmol_c$ dm^{-3}, P (Mehlich I): 25.8 mg dm^{-3}, organic matter: 3.0 g dm^{-3}, Al^{3+}: 0.0 $cmol_c$ dm^{-3}, H+Al: 2.8 $cmol_c$ dm^{-3}, pH ($CaCl_2$): 5.3, 7.8 $cmol_c$ dm^{-3} of CTC, 64.0% of V, according to Donagemma *et al.* (2011). The soil granulometric analysis, according to da Silva (2009), presented 44 g

kg^{-1} of clay in 0-0.2 m layer (da Silva, 2009).

Previously to the planting, an initial fertilization was carried out, which consisted of the equivalent application of 320 kg ha^{-1} of simple superphosphate and liming in order to raise the bases saturation to 80%. The limestone was incorporated and the beds were confectioned with 1.00 m wide and spaced 0.50 m apart.

The experiment was conducted in a subdivided

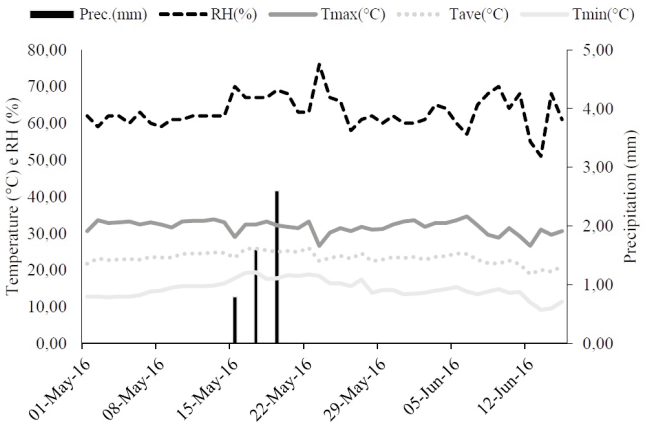

Fig. 1 - Summary of climatic conditions of relative air humidity and maximum, average and minimum temperature during the period of conduction of the study.

plots design, with four replications for each treatment. The treatments were composed by a combination of three soil cover (uncovered soil, straw and plastic cover) and three planting spaces (0.25x0.20, 0.25x0.25 and 0.25x0.30 m), making a total of nine treatments. Each plot had dimensions of 1.0x1.25 m (1.25 m^2). For evaluation, the central plants of the two internal lines were used and the remaining plants were used as a border.

Irrigation was carried out by drippers spaced twenty centimeters apart in three polyethylene tapes suitable for this purpose, positioned between the planting lines. The acquisition of the drip tapes was included in the calculation of the costs, considering the implantation of this system in the production area and the useful life of this material. However, the amounts spent on the purchase of other materials, such as pumps, pipes and others irrigation materials were not considered.

After the irrigation tapes placement, the soil covers were installed according to the treatments. For that, a straw from the grass (*Zoysia japonica*) was distributed over the plots until a layer of 5 cm was obtained. The plastic cover consisted of the placement of double-sided polyethylene canvas (black and white), with the white face facing upwards.

The seedlings of crisp lettuce, cv. Vanda, were purchased in a commercial seedling producer, with a surplus of 10% of the quantity required for the replacement of dead plants. The seedlings transplanting proceeded on May 1, 2016. For this purpose, pits were opened amidst the covers, with sufficient size for seedling insertion.

Cover fertilization was carried out in three applications during the cycle, based on the recommendations for the crop (Trani *et al.*, 2014), applying 60 kg ha^{-1} of urea (45% N) and 50 kg ha^{-1} KCl (60% K_2O). During the lettuce cultivation, there was no application of fungicides, insecticides or herbicides. Plants were harvested at 45 days after transplanting.

Treatments were considered as commercial crops with the purpose of determining the production costs of a productive cycle of crisp lettuce. In this way, the total operational cost (TOC) structure was obtained as proposed by Martin *et al.* (1998) by adding up the effective operating cost (EOC), which is composed of the expenses of the operations and inputs used, other expenses involved (OE), and costing interest per year (CIY). A rate of 5% of total EOC expenses was considered for other expenses (OE) that involves costs with administration, technical assistance and other fees to be paid for the activity, while costing interest (CIY) used was 6.5% per year, over 50% of EOC, estimated as an annual interest rate (Martin *et al.*, 1998).

Before the calculation of TOC, the base total operating cost (TOC base) was obtained, for which the costs related to the experimental variables were not considered, remaining constant for all treatments. For monetary amounts, the presentation was made in Reais (R$) and US dollars (USD). The conversion was made considering the quotation on June 15, 2018 (USD 1.00 = R$ 3.76). Each economic data was based on a single production mean, which was composed by the four replications.

The average prices, received by the producers, were obtained from the Goiás State Supply Centers (2018) website. The average price paid to producers in the first half of June of 2018 was USD 2.66 per box of 4.8 kg, for calculation purposes, the same was used in the present study.

The average region labor force in 2018 was USD 18.62 day^{-1}. Thus, labor costs were obtained through the index generated by the need for manual operations for each operation, multiplied by the daily value. For inputs, the cost was calculated based on the average product value in the region, obtained in the first half of 2018, and the amount of material used.

The profitability of each treatment was obtained through estimates of gross revenue, obtained multiplying the quantity produced (4.8 kg boxes) for the average price received by the producers, the difference between gross revenue and total operating cost represents the profitability index: the proportion of the gross revenue that represents the final amount after covering the production total operational cost. Equilibrium price was also obtained as the minimum price necessary to be obtained to cover the TOC at a given level of production total operating cost, considering the average productivity obtained by the producer, and the equilibrium productivity, given the minimum productivity required to cover TOC at a given level of production total operating cost.

3. Results and Discussion

The production of crisp lettuce in an area equivalent to 1000 m^2 presented a base total operating cost (TOC base) of USD 454.64 (Table 1), which was formed by the cost of mechanized operations (68.02%), manual operations (20.47%), inputs (3.88%), other expenses (4.62%) and interest expenses (3.00%). The effective participation of mechanized operations was due to the acquisition of dripping tapes, which had a 96.75% participation in this item and 65.81% over TOC base. In addition, manual operations and inputs had a share of 20.47% and 3.88%, respectively, in the TOC base.

The small inputs participation is due to the non-insertion of the seedlings acquisition value. However, when this cost is added to the inputs amount, the participation on the TOC and the TOC itself increases (Table 2). The seedlings value also have a participation of 42.47%, 38.84% and 36.47% for treatments composed of 0.25x0.20 m, 0.25x0.25 m and 0.25x0.30 m of planting spacing, respectively. These results related to the seedlings acquisition corroborate with those obtained by Rezende *et al.* (2005), who verified a participation of 55.20% of the inputs on TOC and by Rezende *et al.* (2009), which obtained a burden of approximately 49.30% on TOC due to expenditures on inputs and other materials.

When the plastic cover was used, it was verified that the acquisition of the cover added to its placement, participated in an average of 9.63%, while the straw had a participation around 2.66%, relative to its distribution on the beds. During the lettuce cycle, weed control burdened in 7.76% and 2.66%, respectively, the treatments without cover and straw cover,

Table 1 - Estimated base total operational cost for crispy lettuce in 1,000 m^2

Description	Specification	Quantity	Unit cost R$	Cost R$	Unit cost USD	Cost USD
A - Mechanized operations						
Tillage	HM Tp 65cv. 4x2 + grade aradora 14 x 26"	0.30	52.95	15.89	14.08	4.22
Disk harrow	HM Tp 65cv. 4x2 + grade niveladora 28x22"	0.10	52.43	5.24	13.94	1.39
Beds preparation	HM Tp 65cv. 4x2 + roto-encanteirador	0.35	47.75	16.71	12.70	4.44
Irrigation	Irrigation equipment	1.00	1,125.00	1,125.00	299.20	299.20
Subtotal A				1,162.84	0.00	309.27
B - Manual operations						
Beds preparation	Man-day	0.50	70.00	35.00	18.62	9.31
Seedling transplant	Man-day	1.00	70.00	70.00	18.62	18.62
Fertirrigation	Man-day	3.00	70.00	210.00	18.62	55.85
Harvest	Man-day	0.50	70.00	35.00	18.62	9.31
Subtotal B				350.00	0.00	93.09
C - Inputs						
Limestone	kg	13.00	0.09	1.16	0.02	0.31
Single superphosphate	kg	32.00	1.34	42.88	0.36	11.40
KCl (60% K_2O)	kg	5.00	2.00	10.00	0.53	2.66
Urea (45% N)	kg	6.00	2.05	12.30	0.55	3.27
Subtotal C (R$)				66.34	0.00	17.64
Effective Operational Cost (A+B+C)				1,579.10		419.99
D - Other expenses				78.96		21.00
E - Costing Interest per year				51.32		13.65
Base Total Operating Cost (A+B+C+D+E)				1,709.46		454.64

respectively (Table 2).

The acquisition costs and placement of the plastic cover, together with the greater seedlings acquisition costs caused by the smaller planting spacing, resulted in the higher TOC. This was 15.15% higher than the lowest TOC, obtained with the combination of straw coverage and larger planting spacing (0.25x0.30 m) (Table 2).

According to the average price received by producers during the first half of June 2018 (USD 2.66 box 4.8 kg^{-1}), it was found that gross revenue, operating profit and profitability index obtained varied according to the planting spacing and cover used (Table 3).

Treatment composed of the straw cover and planting spacing of 0.25x0.25 m resulted in higher values for the three variables. This result is due to the lower straw cost and its action on the maintenance of the physical and chemical quality of the soil (Collier *et al.*, 2011; Cardoso *et al.*, 2012), as well as weed control through suppression of their development (Moraes *et al.*, 2013). In contrast, the lowest profitability index was obtained using the plastic cover and the planting spacing of 0.25x0.20 m. Under these conditions, the low productivity in addition to the cover value were the main factors that contributed to the variables decrease in 23.44%, 45.53% and 28.85% of gross revenue, operating profit and profitability index, respectively, compared with the best treatment.

The lower equilibrium yield was obtained for the treatment composed by 0.25x0.30 m planting spacing and straw cover. This result was 15% below the treatment composed of a plastic cover and 0.25x0.20 m planting spacing, for which the highest equilibrium production was verified (Table 4). For this treatment, the highest equilibrium price was also obtained with an increase of 47.23%, compared to the lowest equilibrium price, obtained with the use of straw and planting spacing of 0.25x0.25 m.

In general, the use of straw as soil cover favors the economic return with the lettuce crop. In addi-

Table 2 - Participation of the cost variation factors over the total operational cost, in 1,000 m^2, for the cultivation of crisp lettuce in different planting spacing and soil cover

Cover	Planting spacing (m)	Cost in R$						Cost in USD					
		Seedlings cost	Cover cost	Cover placement	Weed control	OE + CIY	TOC	Seedlings cost	Cover cost	Cover placement	Weed control	OE + CIY	TOC
Control	0.25x0.20	870.00	0.0	0.00	210.00	89.10	2,878.50	231.40	0.00	0.00	55.90	23.70	765.60
Control	0.25x0.25	690.00	0.0	0.00	210.00	74.30	2,683.60	183.50	0.00	0.00	55.90	19.70	713.70
Control	0.25x0.30	585.00	0.0	0.00	210.00	65.60	2,570.00	155.60	0.00	0.00	55.90	17.40	683.50
Straw	0.25x0.20	870.00	0.0	70.00	70.00	83.30	2,802.70	231.40	0.00	18.60	18.60	22.20	745.40
Straw	0.25x0.25	690.00	0.0	70.00	70.00	68.50	2,607.90	183.50	0.00	18.60	18.60	18.20	693.60
Straw	0.25x0.30	585.00	0.0	70.00	70.00	59.80	2,494.20	155.60	0.00	18.60	18.60	15.90	663.30
Plastic	0.25x0.20	870.00	196.40	70.00	0.00	93.80	2,939.60	231.40	52.20	18.60	0.00	24.90	781.80
Plastic	0.25x0.25	690.00	196.40	70.00	0.00	78.90	2,744.70	183.50	52.20	18.60	0.00	21.00	730.00
Plastic	0.25x0.30	585.00	196.40	70.00	0.00	70.20	2,631.10	155.60	52.20	18.60	0.00	18.70	699.70

Table 3 - Productivity, gross revenue, operating profit and profitability index, obtained with the cultivation of crisp lettuce in different planting spacings and soil cover, in 1,000 m^2

Cover	Planting spacing (m)	Produtivity (Boxes 4.8 Kg)	Gross revenue		Operation profit		Profitability index (%)
			R$	USD	R$	USD	
Control	0.25x0.20	592.96	5,929.57	1,577.01	3,051.09	811.46	51.46
Control	0.25x0.25	640.17	6,401.70	1,702.58	3,718.07	988.85	58.08
Control	0.25x0.30	641.27	6,412.66	1,705.49	3,842.69	1,021.99	59.92
Straw	0.25x0.20	663.33	6,633.27	1,764.17	3,830.57	1,018.77	57.75
Straw	0.25x0.25	687.70	6,877.01	1,828.99	4,269.15	1,135.41	62.08
Straw	0.25x0.30	553.92	5,539.20	1,473.19	3,045.01	809.84	54.97
Plastic	0.25x0.20	526.50	5,265.02	1,400.27	2,325.46	618.47	44.17
Plastic	0.25x0.25	564.50	5,644.95	1,501.32	2,900.25	771.34	51.38
Plastic	0.25x0.30	557.81	5,578.11	1,483.54	2,947.06	783.79	52.83

tion, it can be easily acquired in the rural property by scrubbing areas with grasses and acts in the prevention of physical and chemical soil erosion (Cardoso *et al.*, 2012), as well as acting on inhibition of weed development (Moraes *et al.*, 2013), favoring the development of the culture of interest.

These facts are supported by a study about the lettuce cultivation on the vegetal residue of different species, which demonstrates that plants cultivated on sorghum and millet straw favor the crop profitability indexes. In this study, it was also observed the importance of choosing the species that will compose the straw cover, since there are effects of allelopathy (Vendruscolo *et al.*, 2017 a, b). The straw deposition on the soil surface, in the cultivation of American lettuce and cabbage, also favored the development of these crops, especially when using *Brachiaria* or millet straw (Torres *et al.*, 2015).

4. Conclusions

The insertion of these techniques, with the use of vegetal soil cover, in addition to culminating in higher financial returns, due to lower production costs and increased productivity, also represents a technique of greater environmental viability. It is observed that crops with high demand for technologies tend to generate large amounts of slow degradation residues, such as plastics, inferring in environmental contamination (Chang *et al.*, 2013). Thus, we concluded that the crisp lettuce crop provides positive profitability, regardless of the planting spacing or cover type used. For the conditions observed during this study, the combination of the 0.25x0.25 m planting spacing and the straw as a soil cover culminates in higher financial returns.

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Table 4 - Production and equilibrium price obtained with lettuce cultivation in different spacing and soil cover, in an area of 1,000 m^2

Cover	Planting spacing (m)	Production (Boxes 4.8 kg)	Equilibrium price	
			Boxes 4.8 kg^{-1} (R$)	Boxes 4.8 kg^{-1} (USD)
Control	0.25x0.20	287.85	4.85	1.29
Control	0.25x0.25	268.36	4.19	1.11
Control	0.25x0.30	257.00	4.01	1.07
Straw	0.25x0.20	280.27	4.23	1.12
Straw	0.25x0.25	260.79	3.79	1.01
Straw	0.25x0.30	249.42	4.50	1.20
Plastic	0.25x0.20	293.96	5.58	1.48
Plastic	0.25x0.25	274.47	4.86	1.29
Plastic	0.25x0.30	263.10	4.72	1.25

* In each variable, data followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at 5% level. Data represent the mean value ± S.D. the mean of four replicates.

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Effect of foliar application of boric acid on fruit quality and yield traits of mango

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Key words: acidity, boron concentration, flowering, *Mangifera indica* L., TSS.

Abstract: Imbalance uptake of boron disturbs the process of pollination that eventually decrease the flowering, fruit setting and yield. Its deficiency also deteriorates the quality of fruit by increasing fruit acidity. Therefore, source, balanced application, method of application and optimum uptake of B is an important aspect and need keen scientific attention. So, a field study was conducted with the hypothesis that foliar application of B would be an effective technique to improve the yield and quality of Mango cv. Summer Bahisht (SB) Chaunsa. The source of B was boric acid (BA) applied twice as foliar spray i.e., 0, 0.1, 0.2 and 0.3%. Results confirmed that as compared to control, a significant improvement in fruit weight at ripening and harvesting stages (36.9%), fruit length (21.9%), fruit width (10.1%), flower (22.1%) and fruit terminals m^{-2} (40.0%) confirmed the effectiveness of T_4 (BA= 0.3%). A significant improvement in average yield (78.6%) validated the efficacious functioning of boric acid (0.3%). In conclusion, boric acid is an important and effective source of B to improve the quality and yield of Mango cv. SB Chaunsa. Similarly, BA (0.3%) is a better option than 0.2 and 0.1% BA to improve the quality and yield of mango.

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

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1. Introduction

Mango (*Mangifera indica* L.) belongs to the genus *Mangifera* and family *Anacardiaceae* which has 74 genera and 600 species (Mitchell and Mori, 1987; Tian *et al.,* 2010). It is known as 'King of Fruits' due to its sweetness, fragrance and nutritional status (Sharma and Singh, 2009). Mango is becoming popular in the western countries and it is originated from Indian sub-continent (Yadav and Singh, 2017). However, in recent years, the productivity of Mango has been reduced due to deficiency of micronutrients especially boron (B) and other environmental stresses (Saran and Kumar, 2011; Adak *et al.,* 2017; Ahmad *et al.,* 2018).

Boron deficiency is quite common after zinc micronutrient especially in

arid and semi-arid regions (Zhang *et al.,* 2015). The deficiency of B is ubiquitous in acidic sandy, alkaline soils and sandy soils (Camacho-Cristóbal *et al.,* 2018). Boron deficiency is one of the key factors for a reduction in the yield of fruit crops (Davarpanah *et al.,* 2016; Karlidag *et al.,* 2017). Its deficiency symptoms show their effect on younger plant parts, while toxic effects are on older parts of plants (Fernández-Escobar *et al.,* 2016). The deficiency of B in mango plant results in deformed leaves hooked and pre-matured dieback of inflorescence, loss of apical dominance, death of apical bud, swelling at internode and retorted growth (Litz, 2009).

Contemporary agricultural practices include the additional supply of fertilizers that enhanced the growth of plants and resulted in an improvement of yield (Barker and Pilbeam, 2006). Micronutrients especially B plays an indispensable role in the growth and development of fruit trees (Davarpanah *et al.,* 2016). Mango fruit drop before maturity is a common issue in most of the mango growing areas (Murti *et al.,* 2008). On the other hand, the potential of foliar application of B has been well documented as an effective amendment for the improvement in mango fruit formation (Saran and Kumar, 2011). Boron is intrinsic for fruit trees in forming higher germinating pollen and elongated pollen tube which sets fruit (El-Sheikh *et al.,* 2007). It is also indirectly involved in the activation of plant hormones and dehydrogenase enzyme (Marschner, 2012). Boron has low adsorption capacity and leaches at a high rate in soil (Raja *et al.,* 2005).

Foliar application of fertilizers is more convenient and effective as compared to soil application (Fernández *et al.,* 2013). It has been observed that foliar application of fertilizers moreover confers quick response and alleviate the deficiency symptoms leading to fruitful returns (Obreza *et al.,* 2010). Scientists have also documented that foliar application of B at different rates enhance mango fruit setting panicle^{-1}, fruit weight and volume (Zhong and Dong, 2000; Bibi *et al.,* 2019). Therefore, the current study was conducted with the aim to examine the effectiveness of the various application of boric acid as a source of B on growth and yield of mango. It is hypothesized that foliar application of boric acid as a source of B would be an effective technique for improvement in growth and yield of mango.

2. Materials and Methods

Experimental site

The study was planned to evaluate the influence of foliar applied boron on yield and quality of mango cultivar Summer Bahisht Chaunsa during the year 2016-17 at experimental mango orchard near Bahauddin Zakariya University, Multan (Fig. 1). Fifteen to twenty years old mango trees were selected for the experimental purpose. The study was carried out in accordance with RCBD design (Randomized Complete Block Design) with four treatments and four replications.

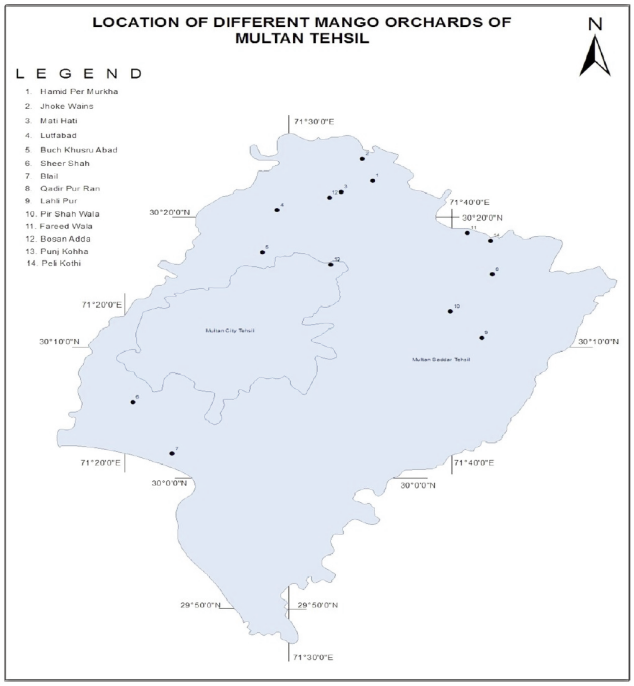

Fig. 1 - GPS locations of area.

Treatment plan

There were four different levels of boric acid (BA) i.e., T_1 (control) = no boric acid, T_2 = 0.1% BA, T_3 = 0.2% BA and T_4 = 0.3% BA which were applied as foliar application. Each tree was taken as one replica of each treatment (consist of four trees). All treatments were applied twice in a year i.e., firstly at inflorescence stage and secondly at the marble stage (pea size of fruit).

Physio-chemical analysis

Soil sampling. The data regarding physio-chemical composition was recorded at harvest stage on ripened fruit. Composite soil samples (0-30 cm) were collected from experimental mango orchard. This depth for soil sampling was selected because the majority of mango feeding roots are present in the depth of 0-30 cm.

Sample preparation. Soil samples were initially air dried, after that grinded and finally sieved through 2 mm sieve in the laboratory of Department of Soil

Science, Faculty of Agricultural Sciences and Technology Bahauddin Zakariya University Multan, Pakistan for determination of different physio-chemical characteristics of the soil.

Soil characterization. For determination of soil texture, hydrometer method was used (Bouyoucos, 1962). Soil pH and electrical conductivity (EC) was assessed by using JENWAY 3510 pH and BANTE DDS-12DW Microprocessor EC meter (Abid *et al.,* 2017). Soil cation exchange capacity (CEC) was calculated according to Richards (1954) and Rhoades (1982). For determination of soil organic matter (SOM) Walkley Black method was followed (Jackson, 1975). Olsen *et al.* (1954) method was adopted for the analysis of soil available P while for soil extractable K Rowell (1994) method was used. Calcium Carbonate (lime) was determined according to Allison *et al.* (1965). All characteristics of the soil are provided in Table 1.

Boron determination in soil and leaves

Leaves samples for B determination were collected twice during 2016-17. Samples were collected prior to foliar application of boric acid and after spraying the mango orchard. Boron (B) concentration from soil was assessed by HCl extraction as described by Ponnamperuma *et al.* (1981), while for leaves samples method of Gaines and Mitchell (1979) was followed.

Growth and yield attributes

Different mango parameters were recorded by selecting 15 panicles and labelled on each tree prior to measurements in an experimental mango garden. Flower terminal m^{-2}, fruit terminal m^{-2}, total number of flowers per panicles was observed with respect to each treatment and average was computed among all the treatments. The total number of flowers from the selected panicles (in case of each tree) was calculated to calculate the average one. Fruit weight (FW) after harvest and ripening was measured by selecting 15 fruits from each treatment.

Yield of fruits

The average yield $tree^{-1}$ was assessed at the time of harvest by weighing out all fruits of tree regarding each treatment and their average was calculated by multiplying average fruit weight with total number of fruits. Fruit weight after harvest was noted soon after harvesting when fruit was not ripen. When the fruit was ripened, again the weight of fruit was noted and referred to as fruit weight after ripening.

Maturity days

Maturity days were recorded by estimation of days from flowering period to harvest stage.

Length and diameter of fruits

Fruit length and diameter were assessed by digital Vernier Caliper after randomly selecting 5 fruits from each treatment.

Total soluble solids

The total soluble solid percentage was calculated by using a digital refractometer as described by AOAC (2005).

Acidity of fruits

The acidity of mango was estimated followed by the method as proposed in Souza et al (2015), one drop (mango juice) was retained on the mirror regarding digital refractometer and reading was observed in case of Brix on the screen.

Sugar contents

Total sugar contents were recorded according to the titrimetric method as described by Raganna (1986). The method proposed by Rusk (1961) was used to estimate the vitamin C content from the juice of mango pulp. Pulp recovery (%) was determined by the following formula

Pulp recovery = (Peel weight – stone weight / Fruit weight) × 100

Statistical Analysis

For statistical analysis standard statistical procedure was adopted (Steel *et al.,* 1997). The significance of treatments was analyzed through analysis of variance (ANOVA). Tukey's test was applied at $p \leq 0.05$ for comparison of treatments by using statistical software "Statistix 8.1".

Table 1 - Pre-experimental characteristics of soil

Textural class	Depth (cm)	pH	ECe ($dS\ m^{-1}$)	SAR $(mmol\ L^{-1})^{1/2}$	$CaCO_3$ (%)	Organic matter (%)	Boron concentration in soil ($mg\ kg^{-1}$)	Boron concentration in leaves ($mg\ kg^{-1}$)	Phosphorus in soil ($mg\ kg^{-1}$)	Potassium in soil ($mg\ kg^{-1}$)
Loam	0-30	8.4	2.04	2.3	6	0.31	0.36	20.49	13	218

3. Results

Fruit weight

Effect of various foliar application rates of boric acid remained significant ($p \leq 0.05$) for fruit weight at ripening and harvesting stages. No significant change in fruit weight was observed at ripening and harvest stages among control T_3 and T_2. However, the application of T_4 was significantly better as compared to control for fruit weight at ripening and harvesting stages (Table 2). The maximum increase of 36.9% in fruit weight was noted where T_4 was applied as compared to control at ripening and harvesting stages.

Fruit length and width

Effect of various foliar application rates of boric acid remained significant ($p \leq 0.05$) for fruit length and width. Application of T_2, T_3 and T_4 were statistically alike to each other but performed significantly better as compared to control for fruit length and width. The maximum increase of 21.9 and 10.1% in fruit length and width was noted respectively where T_4 was applied as compared to control.

Average yield

Effect of various foliar application rates of boric acid remained significant ($p \leq 0.05$) for average yield. Application of T_3 and T_2 did not differ significantly for the average yield of mango fruit as compared to control. However, the application of T_4 was significantly better as compared to control for average yield (Table 2). The maximum increase of 7.86% in average yield was noted where T_4 was applied as compared to control.

Peel and stone weight

Effect of various foliar application rates of boric acid was significant ($p \leq 0.05$) for stone and peel yield. Application of T_3 and T_2 did not differ significantly for stone and peel weight of mango fruit as compared to control. However, the application of T_4 significantly decreased stone and peel yield as compared to control (Table 2). The maximum decrease of 22.2 and 15.0% in peel and stone yield was noted where T_4 was applied as compared to T_3 and T_2 respectively.

Flower and fruit terminal m^{-2}

Effect of various foliar application rates of boric acid was significant ($p \leq 0.05$) for flower and fruit terminals m^{-2}. It was observed that T_4 and T_2 did not differ significantly but differed significantly better as compared to control for flower terminals m^{-2}. Application of T_3 also performed significantly better for flower terminals m^{-2} as compared to control (Table 3). For fruit terminals, m^{-2} T_3 and T_4 were statistically alike to each other but differed significantly as compared to control. Application of T_4 also differed significantly better for fruit terminals m^{-2} as compared to control (Table 3). The maximum increase of 22.1 and 40.0% in flower and fruit terminals m^{-2} was noted respectively where T_4 was applied as compared to control.

Total number of flowers, male and hermaphrodite flower

Effect of various foliar application rates of boric acid was significant ($p \leq 0.05$) for total number of flowers,

Table 2 - Effect of various levels of boric acid on yield attributes of mango

Treatments	Fruit weight after ripening (g)	Fruit weight after harvest (g)	Fruit length (cm)	Fruit width (cm)	Average yield (kg $tree^{-1}$)	Peel weight (g)	Stone weight (g)
T_1 (control)	250.72 b	270.93 b	10.46 b	5.74 b	132.25 b	40.48 ab	51.18 ab
T_2 0.1% boric acid	281.62 b	301.74 ab	11.82 a	6.10 a	135.75 b	47.20 a	58.30 a
T_3 0.2 % boric acid	304.29 ab	334.28 ab	12.01 a	6.11 a	138.43 ab	47.72 a	51.27 ab
T_4 0.3 % boric acid	343.34 a	371.16 a	12.75 a	6.32 a	142.65 a	37.15 b	49.53 c

Mean values followed by the different letter in the same column are statistically different ($p \leq 0.05$).

Table 3 - Effect of various levels of boric acid on flowering related attributes of mango

Treatments	Flower terminal m^{-2}	Fruit terminal m^{-2}	Total number of flowers $panicle^{-1}$	Male flower (%)	Hermaphrodite flowers (%)	Maturity days
T_1 (control)	21.50 c	7.32 c	895.43 c	548.475 b	346.525 ab	195 b
T_2 0.1% boric acid	25.08 ab	8.75 b	933.54 b	618.45 ab	314.55 b	197 a
T_3 0.2 % boric acid	24.25 b	9.25 a	1017.5 a	633.225 ab	384.275 a	196 b
T_4 0.3 % boric acid	26.25 a	10.25 a	1001.75 a	672.525 a	329.225 b	198 a

Mean values followed by the different letter in the same column are statistically different ($p \leq 0.05$).

male and hermaphrodite flower. Application of T_3 and T_4 did not differ significantly with each other but remained significant for total number of flowers in mango as compared to control (Table 3). However, the application of T_2 also significantly improved total number of flowers in mango as compared to control. In the case of male flowers, only T_4 remained significantly better from control (Table 3). For hermaphrodite flowers, application of T3 remained significantly better as compared to control. The maximum increase of 11.9, 22.6, and 22.2% in total number of flowers, male and hermaphrodite flower was noted where T_4, T_4 and T_3 were applied as compared to control, control and T_2 respectively.

Maturity days

Effect of various foliar application rates of boric acid was significant ($p \leq 0.05$) for fruit maturity days. Application of T_2 and T_4 remained statistically alike to each other but remained significantly better as compared to control for maturity days. No significant change was noted among T_3 and control for maturity days. The maximum increase of 1.51% in maturity days was noted where T4 was applied as compared to control.

Acidity and boron concentration

The foliar application was effective in case of quality traits of mango. It was observed that with the application of boric acid treatments acidity of fruit was decreased. Minimum acidity was noted in T_3 treatment while T_2 and T_4 treated plots exhibited the same response for the acidity of fruit (Fig. 2). The pulp recovery, vitamin C, total soluble solids and total sugar contents tend to increase with an increasing application rate of foliar boric acid (0.3% boric acid) (Fig. 3).

Boron concentration in leaves

Foliar application of B was found effective in term of improvement in leaves B concentration (Fig. 4). Higher application of foliar B 0.3% (T_4) enhanced the B concentration in leaves as compared to T_1. Lowest B concentration was noted in T_1. No significant change was noted among T_4 and T_3 for B concentration in leaves. Similarly, T3 and T2 were statistically alike to each other for B concentration in leaves.

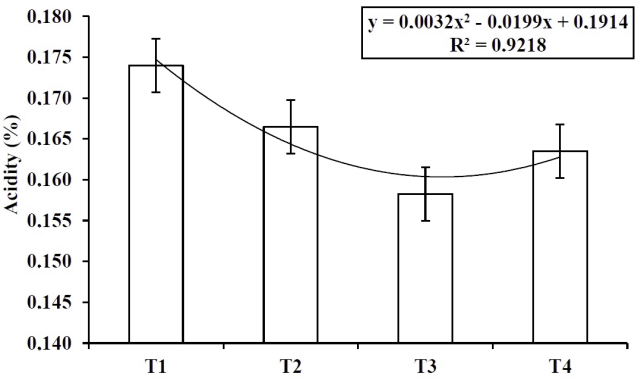

Fig. 2 - Effect various levels of boric acid on acidity of mango fruit.

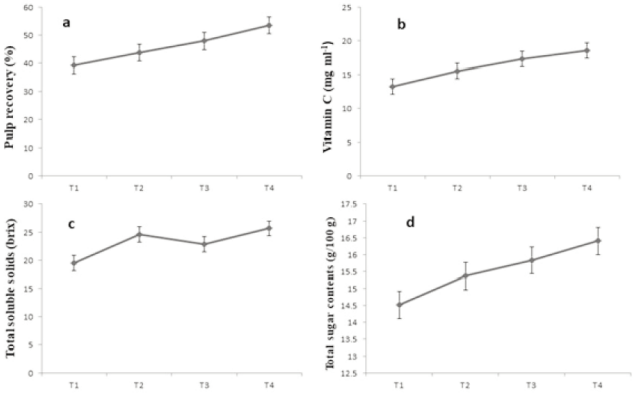

Fig. 3 - Effect of various levels of boric acid on pulp recovery, vitamin C, total soluble solids and total sugar contents in mango fruits

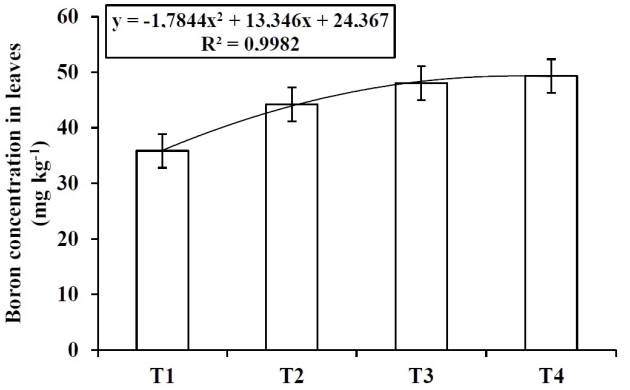

Fig. 4 - Effect of various levels of boric acid on boron concentration in mango leaves.

4. Discussion and Conclusions

The current study depicted the beneficial role of foliar application of boric acid as a source of B for improving mango yield and quality traits. Similarly, the effective role of foliar application has been reported previously (Anees *et al.,* 2011; Singh *et al.,* 2017; Ahmad *et al.,* 2018; Oldoni *et al.,* 2018). Our results are in accordance with Silva *et al.* (2014) that supply of boron is intrinsic to enhance the length and diameter of mango fruit with foliar application. Moreover, it was revealed that boron is less mobile in the plant, therefore, it piles up in older plant leaves and becomes unable for the sturdy growth of fruit development (Oldoni *et al.,* 2018). Contrarily

foliar application was reported to supplement boron supply to the growing plant organs which ultimately enhanced mango fruit diameter (Bhatt *et al.,* 2012). It was further strengthened by the findings of Singh *et al.* (2017) that B application increases fruit size due to the better mobilization of food material from production sites to storage organs and rapidly fruit development. It was noted that foliar application was significantly effective for improvement in mango fruit weight. Our results were inlined with findings of Dutta (2004). The possible reason behind the increase in fruit weight might be due to increased cell expansion and cell division. Moreover, boron contribution in hormonal metabolism and boron is a key player in the rapid mobilization of sugar and water in fruits (Haq *et al.,* 2013). Increase in yield after boron application was correlated to an increase in carbohydrates metabolism (Perica *et al.,* 2001 a). The higher yield was also associated with the greater number of flower formation due to boron absorption and they also set a greater number of fruits (Usenik and Stampar, 2007; Sarrwy *et al.,* 2012). The similar result regarding an increase in fruit yield was also reported in almond (Nyomora *et al.,* 1999), Kinnow (Ullah *et al.,* 2012), guava (Rawat *et al.,* 2010), persimmon (Khayyat *et al.,* 2007) and peach (Ali *et al.,* 2014). Mango flowering is influenced by physiological events taking place throughout the course of its growth. We observed that improvement in flowering is due to the synthesis of flower promoters synthesized in leaves and their translocation to sprouts via phloem (Ramírez and Davenport, 2012). Foliar application increases bud formation by the synthesis of essential hormones and metabolite translocation to the bud of the tree (Usenik and Stampar, 2002). Perica *et al.* (2001 b) reported the similar findings that boron foliar spays resulted in a higher percentage of perfect flowers. It was well acknowledged that boron concentration obtained higher in pollen grains and flowers as compared to leaves. This improved flowering is due to the readily available boron required for reproductive organs (Dell and Huang, 1997; Blevins and Lukaszewski, 1998). Therefore, these results are in agreement with other fruits, where boron concentration at bud initiation, reproductive tissues, flowers and fruits were favourable (Nyomora *et al.,* 1999). It was well documented the role of boron application considerably enhanced the emerging flowers and fruits (Perica *et al.,* 2001 b). Mango quality traits were improved due to boron application was reported (Ahmad *et al.,* 2018). Increase in sugar contents due to boron was also reported from earlier studies (Hassan, 2000; Shaaban, 2010). It is due to the development of storage and accumulation in sugar content with the conversion of polysaccharide and starch into simple sugar (Kahlon and Uppal, 2005). It is evident that boron is responsible for declining acidity of fruit (Baiea *et al.,* 2015). This result was further supported by (Anees *et al.,* 2011; Sarker and Rahim, 2012; El-Razek *et al.,* 2013). Other quality traits improvement reports were found accordingly with our results (Bhatt *et al.,* 2012; Haq *et al.,* 2013). The higher rate of boron accumulation in leaves is due to the direct absorption by the leaves (Khan *et al.,* 2012). Foliar application increased boron concentration with higher application rate (Perica *et al.,* 2001 b). This result was endorsed by previous studies in olive (Hegazi *et al.,* 2018), apricot (Karlidag *et al.,* 2017) and strawberry (Kitir *et al.,* 2018).

It was concluded that foliar application of boric acid is an effective source to alleviate B deficiency in mango trees. In addition, the application of boric acid also improves the yield and fruit quality of mango. Furthermore, increasing application rate 0 to 0.3% was concluded fruitful for the overall yield and quality traits. However, more investigations are yet recommended to introduce exact application rate of boric acid to alleviate boron deficiency or its toxicity in mango trees.

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Prostrate or upright growth habit in tomato cultivars: contributory roles of stem diameters and fruit weight under fertilizer application

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Key words: aerial stem, basal stem, cultivars, growth habit, NPK 15:15:15.

Abstract: For increased production of fresh market tomato fruits, identification of cultivars that combine upright growth habit (UGH) and increased fruit weight under increased fertilizer rates is essential. Six tomato cultivars comprising five improved types namely Tropimech, Buffalo, Roma VF, Roma Savana, UC 82 and a local cultivar Kerewa were evaluated to identify cultivars that combined average fruit weight with UGH under different rates of NPK 15:15:15 fertilizer (0, 30, 50, and 80 kg ha^{-1}). Aerial and basal stem diameter (ASD and BSD), and weight per fruit (WPF) cumulatively accounted for the largest significant variation (47.17 **) in growth habit of the tomato cultivars with ASD being the most determinant of all. Increased fertilizer rates resulted in increased morphological and yield parameters but promoted prostrate growth habit in tomato cultivars. At 30 kg ha^{-1}, Roma VF and Roma Savanna combined UGH with ample yield per plant while at 50 and 80 kg ha^{-1} of fertilizer, UC 82 consistently maintained the most UGH with higher yield. Growing UC 82 at 50 kg ha^{-1} of fertilizer is recommended for better UGH and higher yield. Consideration should be given to ASD, BSD, and WPF in future improvements of tomato for UGH and yield.

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1. Introduction

One of the challenges confronting tomato production is the inability of the crop to maintain an upright growth habit at full fruit formation due to increased fruit weight and weak stem. This attributes manifest more in most improved tomato cultivars that are bred purposely for high yield and do not exempt the local ones. Maintenance of upright growth habit in tomato cultivars at maturity is an important attribute in harvesting high percentage of fresh tomato fruits considering the prevalence of diseases

that occur with contact of fruits with bare soil (Santamaría and Toranzos, 2003). Tomato fruit is perishable and the contact with soil can promote rotting and scorching and increase disease prevalence that can lead to low quality fruits and greater yield loss (Naika *et al*., 2005). Tomato plant are often staked or trellised in order to minimize the contact of fruit with soil however, the cost incurred on staking during production of fresh market tomato fruits are among the highest for any vegetable crop (Davis and Estes, 1993; Kemble *et al*., 1994; Frasca *et al*., 2014). Identifying tomato cultivars with most upright growth characteristics can prevent scorching and rottenness often associated with tomato fruit at maturity and could contribute to increased fruit quality and fruit yield and reduce cost of staking in tomato production.

The permanent displacement of plant from its upright position otherwise termed 'lodging' is one major cause of yield loss in most crops (Crook and Ennos, 1995). It usually occurs due to inability of plants to withstand strong winds and poor anchorage of plant roots to its substratum. Terminal and axillary weights from fruits can contribute to plant inability to stay erect. The height of the plant as well as thickness of the basal stem are some of the contributing factors to lodging in plants (Zhang *et al.*, 2014). In tomato, variations in stem diameter occur along the height of the crop. The basal section of the stem which is usually thinner though could be more lignified is unable to support the uppermost part which is characterized by robust stem and heavy fruit weights at fruiting stage in most cultivars. The variation in stem diameter usually observed along the stem of tomato cultivars could be a factor contributing to lodging in tomato. Assessing the extent of this variation along the height of the crop along with other morphological traits could provide information on morphological traits that should be considered in future improvement of tomato cultivars for upright growth and yield.

Plant response to fertilizer application occurs through expansion of various plant parts culminating eventually in surface area expansion. Application of inorganic fertilizer at high dosage rates can increase yield but may also increase susceptibility of the crop to lodging due to reduced structural carbohydrates content and lignin deposition resulting in weakening of the stem (Zhang *et al*., 2014; Zhang *et al.,* 2016). Conversely, poor fertilization culminating in nitrogen deficiency can instigate stem diameter variation attributable to reduced transpiration and assimilate loadings which ultimately affects storage tissue concentration (De Swaef *et al.*, 2015). In tomato, application of inorganic fertilizers such as calcium nitrate at different rates increased plant height and fruit yield (Montagu and Goh, 1990; Souri and Dehnavard, 2018). This could also induce bioaccumulation of salt in plant tissue at higher rates which often affect growth of tomato (Romero-Aranda *et al.,* 2006; Tuna *et al.,* 2007; Caruso *et al.,* 2011). Finding a balance in upright growth, increase in dosage rates of nitrogen fertilizer applied and ample fruit yield is therefore a necessity in ensuring maximum gains from tomato cultivation. Little studies have explored the contribution of stem diameter variation and fruit weight on upright growth of improved tomato cultivars. Past breeding objectives in tomato has focused mainly on yield, shelf-life, taste and nutritional quality (Bai and Lindhout, 2007), however evaluation of morphological traits that could contribute indirectly to these traits is also necessary. Documented findings should assist in identifying cultivars that combine high yield with upright growth under different rates of fertilizer application. The following questions were raised and addressed in this study (i) Does variation in stem diameter contribute to growth habit of tomato cultivars? (ii) Do tomato cultivars maintain similar growth habit at physiological maturity when maximum weight of fruits is achieved and when fruits are turning red? (iii) Do fertilizer rates influence growth habit of tomato cultivars? and (iv) Does fruit weight, number of fruits or both contribute to upright growth habit of tomato cultivars?

2. Materials and Methods

Experimental site

The experiment was conducted at Ilaraa, a tomato production area located in the derived savanna zone of Yewa North Ogun state, Nigeria (7.4°N, 2.7°E, 188 m asl). The area is characterized by erratic rainfall at early and later part of the year which usually remains stable around June to July. The soil is well drained and is characterized by high proportion of sand with relatively lower proportion of clay and silt and hence supports good growth of tomato. The commonly grown cultivar, Kerewa, is supplied in commercial quantities to adjoining markets and Lagos, a neighbouring state.

Soil sample collection and preparation

Prior to establishment of the trial, the experimen-

tal field was divided into three rectangular blocks each with 3 m x 6 m in dimension. Core soil samples were taken at 0-15 cm along the diagonals of each block after which they were bulked to form composite samples. The soil samples were air dried and sieved with 2 mm sieve after which they were subjected to laboratory soil analysis.

Laboratory analysis of soil sample

The pH of the soil was determined in 1:2 soil water ratios with a glass electrode pH meter (McLean, 1982). Particle size distribution was determined by hydrometer method (Gee and Bauder, 1986). Exchangeable bases (Ca, Mg, K and Na) in the soil were extracted using ammonium acetate method (Thomas, 1982). Following extraction of exchangeable bases, Ca and Mg were determined with Buck Scientific 210 VGP model, Atomic Absorption Spectrophotometer (AAS), while K and Na were read on flame photometer. The exchangeable acidity was determined by titration method (Anderson and Ingram, 1993). Effective Cation Exchange Capacity (ECEC) was estimated by the summation of exchangeable bases and exchangeable acidity (Anderson and Ingram, 1993). Base saturation of the soil was calculated as the fraction of the exchangeable bases and ECEC expressed in percentage. Total nitrogen was determined by Kjeldahl method (Bremner, 1996). Organic carbon was determined by the wet oxidation method as described by Walkley-Black (Nelson and Sommer, 1996). Available phosphorus was determined by Bray-1 method (Bray and Kurtz, 1945).

Experimental methods

The experimental protocol was based on the factorial combination of six cultivars and three fertilizer rates plus a non-fertilized control. Six tomato cultivars comprising five improved types namely Roma VF, Roma Savana, UC 82, Tropimech -with determinate growth habit, Buffalo and the local cultivar-Kerewa, which are both an indeterminate cultivars that require staking. Buffalo has a large fruit with thick flesh and few seeds. Tropimech has an egg-shaped fruit and high fruit setting. UC 82 in addition to having determinate growth is an early maturing cultivar with square to egg-shaped red fruit. The seeds of the tomato cultivars were all obtained from Agro service station and were nursed for 3 weeks. The seedlings were later transplanted onto a prepared field at a spacing of 50x50 cm between plants along and between rows. The plot dimension was 1 m x 1.5 m with six plants. Spacing between plots and replicates was 50 cm and 1 m, respectively (Fig. 1). Fertilizer as N:P:K 15:15:15 in the form of elemental nitrogen (N), phosphate (P_2O_5) and potash (K_2O) respectively, was applied at the rates of 30, 50 and 80 kg ha^{-1} at 8 weeks after planting while plot with no fertilizer application served as the control. The experimental treatments were distributed in the field according to the Randomized Complete Block Design (RCBD) with three replicates. Harvesting of tomato fruits commenced at physiological maturity, when assimilates had been fully partitioned into the fruits as confirmed by fully formed fruits, and when fruits were turning red. The fruits were counted and weighed using Electronic Compact Scale (ATOM A-110C), China.

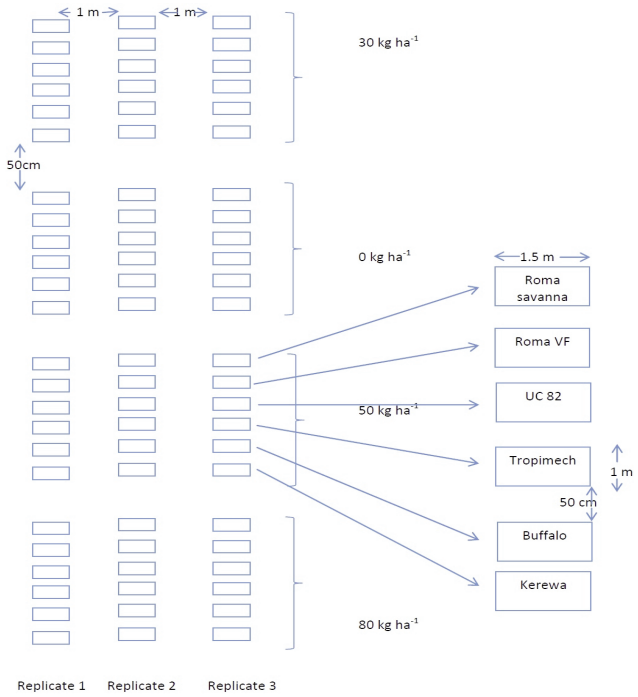

Fig. 1 - Plot layout of the field showing the arrangement of plots within the field. Plots in group of six represent the cultivars arranged randomly within each group.

Data collection

At physiological maturity of tomato fruits, plant basal stem diameters was measured at 5 cm above the soil mark while aerial diameter was measured near the last leaf at the top using plant leaf thickness gauge (Model YH-1 Top instrument). Height measurement was performed on the primary stem axis from soil mark to the base of the last leaf. The growth habit of the tomato cultivars was assessed using visual scoring on a scale of 1 to 5:1 represents upright growth, 2 for 30° deviation, 3 for 45° deviation, 4 for 70° deviation and 5 for fully prostrate stem (Ozminkowski *et al.*, 1990). Fruit production per plant

was obtained by harvesting all fruits on a plant at physiological maturity which were later counted and weighed. Total fruit weight per plant was obtained by weighing all fruits harvested within a plot and divided by the total number of plants per plot. Stem diameter variation (mm cm^{-1}) was computed using the following formula:

$$\text{Stem diameter variation (mm cm}^{-1}) = \frac{\text{ASD - BSD}}{\text{PH}} \times 100$$

Where ASD = aerial stem diameter; BSD = Basal stem diameter; and PH = Plant height. These parameters were selected for measuring stem diameter variation (SDV) based on their ability to measure difference in terminal stem diameters (ASD and BSD) in relation to average distance between the two as measured by plant height (PH).

Statistical analyses

Data of growth and yield parameters were subjected to Analyses of Variance (ANOVA) using Genstat 12th Edition (Payne *et al.*, 2009) package. The means relevant to the variables significantly affected by the experimental treatments were separated using Fischer's Protected Least Significant Difference at $p \leq 0.05$. Correlation and stepwise regression analyses of the morphological and yield parameters were conducted to study the association among the variables and to identify the traits with largest contribution to growth habit of tomato at physiological maturity. Multivariate analysis was also conducted to identify cultivars that combined an upright growth habit with high yield among the tomato cultivars. In correlation, regression and principal component analyses (PCA) carried out, logarithm transformation was conducted on the data before analyses. The mean of the transformed values for each of the variable across fertilizer rates were first computed for each cultivar before conducting PCA. The PCA was conducted using correlation matrix method.

3. Results

The routine soil analysis of the experimental site is presented in Table 1. The pH of the soil (6.9) is neutral and has higher proportion of sand (886.0 g kg^{-1} equivalent to 88.6% of 1 kg soil sample) as compared with that of silt and clay (69.33 and 44.67 g kg^{-1} respectively) amounting to 6.9 and 4.5% respectively of 1 kg soil sample. Exchangeable Ca and Mg contents of 3.72 and 1.48 cmol kg^{-1}, respectively were moderately high. However, potassium (0.08 cmol kg^{-1}) level in the soil was very low compared to critical level of 0.15 cmol kg^{-1}. Exchangeable sodium (0.10 cmol kg^{-1}) was also low while the ECEC of 5.43 cmol kg^{-1} was lower than critical level of 8.0 cmol kg^{-1} for low soil fertility in tropical soils. There was low exchangeable acidity (0.05 cmol kg^{-1}) and high base saturation percentage of 99.12 %. Total nitrogen (0.70 g kg^{-1}) and organic carbon (6.43 g kg^{-1}) were very low. Available phosphorus (26.02 mg kg^{-1}) was very high and in several folds higher than critical level of 8.0 mg kg^{-1} in non-degraded soils.

Table 1 - Pre-experimental characteristics of soil

Physicochemical properties	Soil composition
pH	6.92
Sand (g kg^{-1})	886.00
Silt (g kg^{-1})	69.33
Clay (g kg^{-1})	44.67
Texture	Sandy
Ca (cmol kg^{-1})	3.72
Mg (cmol kg^{-1})	1.48
K (cmol kg^{-1})	0.08
Na (cmol kg^{-1})	0.10
Al+H (cmol kg^{-1})	0.05
ECEC_(cmol kg^{-1})	5.43
BS_%	99.12
N (g kg^{-1})	0.70
OC (g kg^{-1})	6.43
Av_P (mg kg^{-1})	26.02

The effects of fertilizer rates and cultivars on morphological and yield parameters of tomato are presented in Table 2. Significant effects ($p \leq 0.01$) of fertilizer rate and cultivar were observed on all the parameters with the exception of basal stem diameter (BSD) and weight of fruit per plant (WFPP) which were not significantly affected by cultivar. Increased application rate of NPK 15:15:15 fertilizer from 30 to 80 kg ha^{-1} resulted in a significant increase in all morphological parameters of the tomato cultivars. A more enhanced prostrate growth habit (PGH) was however observed in tomato plants with increased rate of fertilizer. The control recorded 2.17 growth habit score (GHS) while fertilizer application rate of 50 kg ha^{-1} and 80 kg ha^{-1} recorded 3.33 and 3.17 in GHS, respectively. Among the cultivars, the widest aerial stem diameter, ASD (8.46 mm) and stem diameter variation (SDV) (5.75 mm cm^{-1}) were observed in Buffalo, while Kerewa had the highest number of fruit per plant, NFPP (5.61) but the least upright plant with mean GHS of 3.42. The highest weight per fruit

(WPF 25.65 g) was however observed in UC 82. No significant interaction between fertilizer rate and cultivar was observed for all the parameters studied.

Table 3 shows the correlation coefficients among the morphological and yield parameters of the tomato cultivars. Significant correlations were observed among most of the parameters with correlation between NFPP and WFPP being the highest (0.79**) and followed by correlation between ASD and SDV (0.76**). The lowest significant correlation was observed between plant height (PH) and SDV (0.24*). The correlation of WPF was significant (0.50**) only with WFPP. Among the yield parameters, WPF did not show significant correlation (0.05 NS) with SDV. Growth habit score had positive significant correlation with all the morphological and yield parameters with the exception of WPF (-0.02 NS).

The percentage variations (adjusted R^2) of subsets regression of morphological and yield parameters on growth habit of tomato cultivars across fertilizer application rates are presented in Table 4. Under each parameter contribution to growth habit i.e. one term, ASD accounted for the highest (44.89**) significant variation among the growth and yield parameters while WFPP contributed the least (9.82**) significant variation. The WPF did not solely account for significant variation in growth habit of the tomato cultivars. Across the various subsets of regression i.e. two terms and above, ASD in most cases, was consistent in its significant contribution to variations in growth habit of tomato cultivars. The highest significant variation (47.17**) in GH of tomato cultivars was obtained when three terms consisting ASD, BSD, and WPF were included in the regression model while other cumulative inclusions accounted for lesser significant variation in growth habit of tomato cul-

Table 2 - Effects of fertilizer rate and cultivar on morphological and yield parameters of improved tomato cultivars

Sources of variation	Levels of variation (kg)	Aerial stem diameter (mm)	Basal stem diameter (mm)	Plant height (cm)	Stem diameter variation (mm cm^{-1})	Number of fruit plant^{-1}	Weight fruit^{-1} (g)	Weight of fruit plant^{-1} (g)	Growth habit
Fertilizer Rates (F) (kg ha^{-1})	0	5.66	5.32	31.96	0.01	2.78	18.46	49.68	2.17
	30	6.89	5.87	41.01	0.03	3.96	22.40	83.35	2.83
	50	7.52	5.91	42.68	0.04	4.27	21.70	84.29	3.33
	80	7.70	6.23	42.62	0.03	5.20	25.77	126.80	3.17
	LSD	0.64	0.50	4.31	0.01	0.14 t	4.43	28.49	0.10 t
	Significance	**	**	**	**	*	*	**	**
Cultivars (C)	Roma VF	6.12	5.68	39.51	0.01	3.03	24.63	76.25	2.33
	UC 82	6.82	5.69	36.39	0.03	3.78	25.65	93.99	2.58
	Roma Savana	6.45	5.83	38.73	0.01	2.98	20.72	64.87	2.42
	Buffalo	8.46	6.08	39.17	0.06	5.58	19.08	107.79	3.33
	Tropimech	6.64	5.45	43.66	0.03	3.26	25.90	85.36	3.17
	Kerewa	7.19	6.25	39.96	0.02	5.61	16.53	87.93	3.42
	LSD	0.78	0.61	5.28	0.01	0.17 t	5.43	34.90	0.12 t
	Significance	**	NS	*	**	*	**	NS	**
F x C		NS	NS	NS	NS	NS	NS	NS	NS

** and * = significant at p≤0.01 and 0.05 probability level respectively; NS= non-significant. Least significant difference (LSD) with 't' are from transformed data.

Table 3 - Correlation analysis showing the relationships among growth and yield parameters of improved tomato cultivars grown in Ilaraa

Parameters	Aerial stem diameter, ASD (mm)	Basal stem diameter, BSD (mm)	Plant height (cm)	Stem diameter variation (mm cm^{-1})	Number of fruit plant^{-1}	Weight fruit^{-1} (g)	Weight of fruit plant^{-1}, (g)	Growth habit score
Arial stem diameter								
Basal stem diameter	0.69**							
Plant height	0.52**	0.36**						
Stem diameter variation	0.76**	0.10 NS	0.24*					
Number of fruit per plant^{-1}	0.55**	0.57**	0.34**	**0.28***				
Weigh fruit^{-1}	0.12 NS	0.14 NS	0.13 NS	0.05 NS	-0.14 NS			
Weigh of fruit plant^{-1}	0.56**	0.59**	0.38**	0.27*	**0.79****	0.5**		
Growth habit score	0.68**	0.35**	0.38**	0.61**	0.40**	-0.02 NS	0.33**	

** and * = significant at p≤0.01 and 0.05 probability level respectively; NS= non-significant. Values in bold are the highest and lowest significant correlations values from the parameters involved.

Table 4 - Adjusted R2 of subsets linear regression of morphological and yield parameters against growth habit of tomato cultivars across rates of fertilizer

One term	Two terms	Three terms	Four terms	Five terms	Six terms
44.89 (1**)	47.00 (1**, 2 NS)	47.17 (1**, 2 NS, 6 NS)	46.88 (1**, 2 NS, 5 NS, 7 NS)	46.24 (1**, 2 NS, 3 NS, 5 NS, 7 NS)	45.46 (1**, 2 NS, 3 NS, 5 NS, 6 NS, 7 NS)
36.13 (4**)	46.21 (1**, 4 NS)	47.09 (1**, 2*, 5 NS)	46.85 (1**, 2*, 5 NS, 6 NS)	46.20 (1**, 2 NS, 3 NS, 5 NS, 6 NS)	45.41 (1 NS, 2 NS, 3 NS, 4 NS, 5 NS, 7 NS)
14.78 (5**)	45.28 (1**, 6 NS)	46.42 (1**, 4 NS, 6 NS)	46.83 (1**, 2*, 6 NS, 7 NS)	46.17 (1**, 2 NS, 3 NS, 6 NS, 7 NS)	45.37 (1 NS, 2 NS, 3 NS, 4 NS, 5 NS, 6 NS)
13.36 (3**)	44.40 (1**, 7 NS)	46.37 (1*, 2 NS, 3 NS)	46.59 (1*, 2 NS, 3 NS, 6 NS)	46.16 (1*, 2 NS, 4 NS, 5 NS, 7 NS)	45.37 (1*, 2 NS, 4 NS, 5 NS, 6 NS, 7 NS)
10.65 (2**)	44.23 (1**, 3 NS)	46.28 (1*, 2 NS, 4 NS)	46.44 (1*, 2 NS, 4 NS, 6 NS)	46.13 (1*, 2 NS, 4 NS, 5 NS, 6 NS)	45.34 (1 NS, 2 NS, 3 NS, 4 NS, 6 NS, 7 NS)
9.82 (7**)	44.21 (1**, 5 NS)	46.22 (1**, 2 NS, 7 NS)	46.40 (1**, 2* ,3 NS, 5 NS)	46.11 (1**, 2 NS, 5 NS, 6 NS, 7 NS)	45.09 (1*, 3 NS, 4 NS, 5 NS, 6 NS, 7 NS)
<0.00 (6 NS)	43.52 (2**, 4**)	46.07(1**, 3 NS, 4 NS)	46.38 (1*, 2 NS, 4 NS, 5 NS)	46.10 (1*, 2 NS, 4 NS, 6 NS, 7 NS)	44.08 (2 NS, 3 NS, 4**, 5 NS, 6 NS, 7**)
	41.18 (3**, 4**)	46.00 (1*, 4 NS, 5 NS)	46.37 (1**, 3 NS, 4 NS, 6 NS)	45.85 (1*, 3 NS, 4 NS, 5 NS, 7 NS)	44.61 (1 NS, 2 NS, 3 NS, 4 NS, 5 NS, 6 NS, 7 NS) for 7 terms

** and * = significant at p≤0.01 and 0.05 probability level respectively; NS= non-significant. Adjusted R2 presented are the first few highest ranked adjusted R2 from each subset. The bold-italic adjusted R2 in the column for six terms represents the adjusted R2 when all terms are included in the regression model. Values in parenthesis represent the parameters and their significant level of contribution to the adjusted R2 in each subset. 1= Aerial Stem Diameter; 2= Basal Stem Diameter; 3= Plant height; 4= Stem Diameter Variation; 5= Number of fruits per plant; 6= Weight per fruit; and 7= Weight of fruit per plant.

tivars.

The comparison of growth habit and WFPP (yield) of tomato cultivars at each fertilizer rate for identification of cultivar with best combination of high yield with the most upright growth is presented in figure 2. For the control (0 kg ha^{-1}), the logarithm of WFPP exceeded that of the growth habit for all the tomato cultivars except Kerewa. With application of fertilizer, overlapping relationship between growth habit score and WFPP were observed among the cultivars. At 30 kg ha^{-1} of fertilizer, Roma VF and Roma Savana maintained higher yield than their respective growth habit while at higher doses, UC 82 consistently maintained higher yield combined with the most upright growth habit. Across the fertilizer rates, growth habit score of Buffalo, Tropimech and Kerewa exceeded their respective WFPP.

Figure 3 shows the principal component biplot of the cultivars using all morphological parameters. The lines in the biplot represent the direction of increase of the variables with the side of the line with the label having the highest value for the variable. The two axes of the biplot accounted for 79.9% variation among the cultivars. At one side of the plot are Tropimech, Buffalo and Kerewa with the cultivars having the highest SDV, PGH, NFPP, WFPP and PH while UC 82, Roma VF and Roma Savana are at the other side where the values for these traits are lower. The cultivar, UC 82 however falls within the center of polygon while other cultivars are at the vertices position.

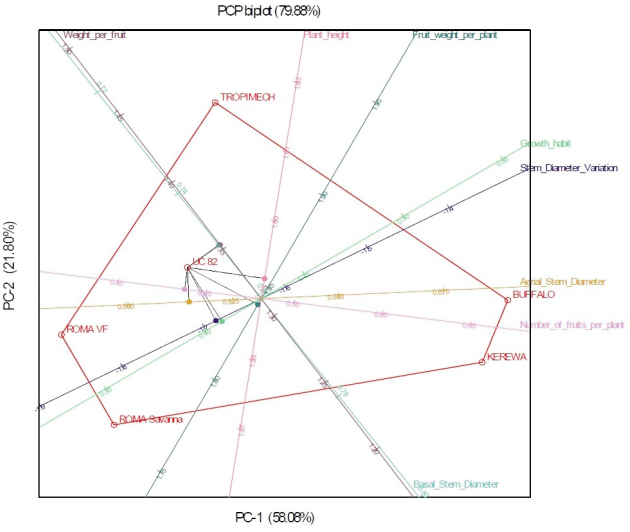

Fig. 3 - Principal Component's biplot showing the differential performance of the tomato cultivars for the morphological and yield parameters.

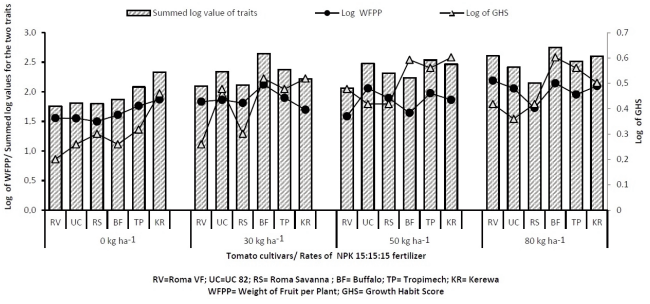

Fig. 2 - Comparison of growth habit and weight of fruit per plant for identification of tomato cultivar with better combination of traits under different fertilizer rates.

4. Discussion and Conclusions

Stem diameter variation measurement is not common in tomato cultivars assessment but its measurement along the height of the crop provides useful information on the susceptibility of tomato to prostrate growth at maturity. The need to improve tomato cultivars for upright growth while maintaining higher yield, particularly under the tropical humid condition where fruits get in contact with moist soil due to the weight of fruits, is quite imperative.

Among the various morphological and yield parameters examined, the ASD and BSD, and WPF were among the most contributory traits that cumulatively influenced the growth habit of tomato with ASD of the tomato cultivars being the most influential on growth habit of the tomato cultivars. The non-significant cumulative contribution of NFPP to growth habit of the tomato cultivars underscores the importance of WPF present on a plant. The likely concentration of heavy-weight fruits at one side of the plant could have promoted prostrate growth habit of the tomato cultivars. The mean fruit weight of tomato fruits has been regarded as a strong varietal character in determining the marketable yield of tomato (Moreno and Moreno, 2008; De Sio *et al*., 2018). This therefore implied that WPF in addition to stem diameter of both basal and aerial stems but most importantly the aerial stem should be focused on in future improvement of tomato cultivars, particularly where infection and damage by soil borne pathogens are prevalent.

Improved tomato cultivars though varied in their individual growth responses, are similar in response to increased fertilizer rates as also reported in previous research (Ercolano *et al*., 2015). Virtually all the morphological and yield parameters showed increase response with increase rate of fertilizer with the highest performance observed at 80 kg ha^{-1}. The low level of nitrogen observed in the soils could have elicited the responses of the tomato cultivars to doses of fertilizer applied. The low levels of N and organic carbon in soil with low clay has been attributed to leaching of nitrates and high rate of mineralization of organic matter that characterize very highly degraded tropical soils such as the type in the experimental site (Snakin *et al.,* 1996). Nitrate leaching in sandy soils can be prevented by using organic or organo-mineral forms of nitrogen fertilizer, which also reduces the accumulation of this anion in plant edible parts (Caruso *et al.,* 2011). The low N content in the soil in the present research can also be attributed to low ECEC of the soil which was a reflection of higher sand particle and low activity clay of the soil which encouraged leaching of cations (Udoh *et al.*, 2013). The low exchangeable acidity and high base saturation also observed in the soil is an indication that the soil's exchange site was dominated by basic cations.

The increase in vegetative growth such as ASD and BSD, observed with higher dosage rate of fertilizer would have been the cumulative effect of increase nitrogen content of the fertilizer. The increase in basal stem diameter (BSD) observed with increase fertilizer rates did not however translate to upright growth in most of the improved tomato cultivars selected as there were corresponding increases in fruit weight and robustness of the ASD. In rice, reduced structural carbohydrates content and lignin deposition is usually associated with increased fertilizer application and can contribute to PGH (Zhang *et al.,* 2016). This could also have been responsible for PGH exhibited by the tomato cultivars in the present research. Increased fruit yield observed in the tomato cultivars with increased fertilizer rates can be attributed to increased dosage rate of phosphorus and potassium in the fertilizer that complimented soil available phosphorus, as both stimulate yield increase in crop (Vance *et al.*, 2003). In previous investigations (Amalfitano *et al*., 2017; Morano *et al.,* 2017), the increase of macronutrients supply to plants led to yield increase up to a crop system dependant threshold. High level of available P in this soil may have been caused by the pH level of the soil. At soil pH of 6.92, phosphorus is more likely to be available than at lower pH of 4 to 4.5 where P is more likely to be fixed (DeForest *et al.,* 2012).

Plotting the log values of traits against their summed log values as obtained in figure 3 can be an alternative way of selecting cultivars of plant with good combination of traits. The plot of log of WFPP and growth habit against their summed log values at each rate of fertilizer applied provided the opportunity to identify cultivars with better performance for these two traits under a given fertilizer rate. The plots indicated upright growth habit for lower logarithm values of growth habit and higher yield for higher logarithm value of WFPP. It was revealed that under no fertilizer application, higher WFPP of all the cultivars except Kerewa combined with upright growth habit whereas this varied among the cultivars under application of fertilizers. With NPK 15:15:15 fertilizer, most of the cultivars especially Buffalo, Tropimech and Kerewa maintained PGH in combination with higher yield. This corroborated the earlier

findings that increased fertilizer application rates can increase yield but also increase the susceptibility of plant to lodging (Zhang *et al.,* 2014). Roma VF and Roma Savanna were however identified as cultivars that combined average yield with the most upright growth among the cultivars at 30 kg ha^{-1} of fertilizer. At higher doses i.e. 50 kg ha^{-1} and 80 kg ha^{-1}, UC 82 consistently maintained the most upright growth combined with higher yield among the cultivars. However, higher rates of chemical fertilizer that can cause increased accumulation of salt in soil and tissue of tomatoes as reported by Widders and Garton (1992) and Tuna *et al.* (2007) may therefore make it necessary to recommend application of 50 kg ha^{-1} of the fertilizer for upright growth habit and higher yield and for possible reduction in accumulation of the salt in tissues of tomato.

The inclusion of other morphological parameters in the multivariate principal component analyses further revealed the cultivars performance for other morphological traits. The selected cultivar in addition to combining high yield with most upright growth was able to maintain a reduced variation in basal and aerial stem diameter with a reduced height coupled with a reduced NFPP. These important traits are to be considered in the breeding of cultivars for upright growth habit and high yield in future breeding programs for tomato. The positioning of the tomato cultivar UC 82 at the center of the polygon further indicated the average performance of the cultivar for the important traits considered in this study while the polygon with other cultivars positioned at its vertices identified cultivars with exceptional performance for some specific traits. Tropimech for example maintained a closer relationship with PH and WFPP in the biplot which confirmed the highest value it recorded for these traits. Furthermore, the positioning of Buffalo and Kerewa in the direction of ASD and NFPP also indicated their higher performance for these traits while Roma VF positioned at opposite end of these vertices had lower performance for the traits. Based on the average performance of UC 82 among the cultivars for all the traits, the cultivar is therefore recommended for upright growth habit and high yield under application of 50 kg ha^{-1} of N:P:K 15:15:15 fertilizer.

Stem diameter variation along the height of tomato plants is important to the upright growth habit of tomato cultivars. Improved tomato cultivars however varied in their susceptibility to prostration at maturity. The robustness of the aerial stem diameter, the thin nature of the basal stem as well as WPF at harvest are some of the factors responsible for this growth habit. Cultivars with robust basal stem combined with reduced variation in stem diameter and appreciable yield will be a promising cultivar for producing fresh tomato fruits in future. Application of 50 kg ha^{-1} of NPK 15:15:15 fertilizer can also reduce the susceptibility of the crop to prostrate growth at maturity. Studies that consider reducing the spacing of the identified cultivar to achieve optimum yield ha^{-1} in comparison with others with higher yield and PGH is suggested to justify the reduced yield that upright growth of stem could cause. The growing of the tomato cultivars in locations with different agroecology is recommended for a better varietal selection.

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Micropropagation of two near threatened orchid. Part 1: *Catasetum pileatum* cv. Alba

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Key words: *in vitro* multiplication, orchid propagation, ornamentals, plant growth regulators.

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Abstract: Many orchid species are threatened. In this study, a reliable and efficient protocol was outlined for *in vitro* propagation of *Catasetum pileatum* cv. Alba, a near threatened orchid species with the proper usage of plant growth regulators (PGRs). Protocorms as explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of kinetin (Kn; 0.00, 0.20, 0.50, 1.00, 2.00, 3.00 and 5.00 mg l^{-1}) and indole-3-butyric acid (IBA; 0.00, 0.10, 0.20, 0.50 and 1.00 mg l^{-1}), either individually or in combination. The frequency of protocorm-like bodies (PLBs) regeneration significantly relied on concentrations of PGRs used. A combination of 1.00 mg l^{-1} Kn and 1.00 mg l^{-1} IBA was found to be suitable for maximum PLB regeneration (8.63 per explant) and the largest number of leaf (12.70 per explant). The highest rooting frequency with 7.40 roots per explant was achieved on protocorms grown in medium enriched with 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA. Plantlets were transplanted to pots filled with a mixture of peat moss, leca and perlite (1:1:1) and transferred to the greenhouse. The plantlets were successfully acclimatized in the greenhouse with a survival rate of 80% exhibiting normal developmental patterns.

1. Introduction

Many orchid species in all over the world are threatened. These species have been listed in the red data book of the International Union for Conservation of Nature and Natural Resources (IUCN, 2011) because of immethodical collection, illegal trade and biodiversity loss and they have been included in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where the international trade is strictly controlled (Chugh *et al*., 2009; Swarts and Dixon, 2009; Reed *et al*., 2011). *Catasetum pileatum,* a rare and near threatened orchid, is a lowland species, where it occurs as a showy epiphyte. This ornamental species is an impressive plant even out of flower. Orchids are precious as pot and cut flowers not only because of their exotic beauty but also for their long shelf life (Chugh *et al*., 2009).

In vivo propagation of orchids is a slow process and resulted in traits segregation. Also, propagation of orchids by sexual means like seed caus-

es the production of heterozygous plants. Therefore, establishment of protocols for *in vitro* proliferation of orchids is important and a proper alternative procedure for propagation of orchids. *In vitro* techniques can be used for conservation of rare and endangered plant species and production of large number of plantlets in short period of time (Engelmann, 2011). Of course, micropropagation of orchids deals with some problems such as high cost of production, low rate of shoot proliferation, poor rooting frequency and phenotypic variations (Bhattacharyya *et al*., 2016).

Different PGRs such as α-naphthaleneacetic acid (NAA), IBA, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), 6-benzyle amino purine (BAP), 6-benzyladenine (BA) and Kn have been used for tissue culture of threatened and endangered orchids (Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Medium composition for *in vitro* culture of orchids by PLBs is cultivar and species-specific and depends on several factors especially PGRs (Luo *et al*., 2009). Various explants such as seeds, leaves (foliar explants), nodes, PLBs, protocorms, tubers, shoot tips and floral stalk buds have been used for micropropagation of threatened and endangered orchids (Vij and Aggarwal, 2003; Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Baker *et al*., 2014; Chen *et al*., 2015; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Among all explants, PLBs and protocorms are more efficient because these can be rapidly multiplied on solid or liquid culture media, and maximum PLBs can be provided in a short period of time (Luo *et al*., 2003).

Asymbiotic seed germination and the use of PLBs induced from vegetative organs are two efficient propagation methods for large-scale propagation of orchids (Zeng *et al*., 2012). These protocols have been established for many orchid species, and different media, primary and secondary metabolites, and PGRs have been used for germination and propagation (Arditti and Ernst, 1993; Roy *et al*., 2011). Many protocols for *in vitro* propagation of orchids, especially those at risk of extinction, using PLBs as explants and various PGRs, have been reported (Teixeira da Silva *et al*., 2005, 2006; Firoz Alam *et al*., 2010; Sinha *et al*., 2010; Baker *et al*., 2014). This investigation is the first to report on *in vitro* multiplication of *Catasetum pileatum* cv. Alba, a near threatened orchid species, for developing a protocol by protocorms as explants and Kn and IBA as PGRs.

2. Materials and Methods

Plant material

Healthy and sterilized protocorms (0.7 cm long) of *Catasetum pileatum* cv. Alba grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) were prepared from the Plant Biotechnology Laboratory, Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran (Fig. 1A). The protocorms were used as explants and cul-

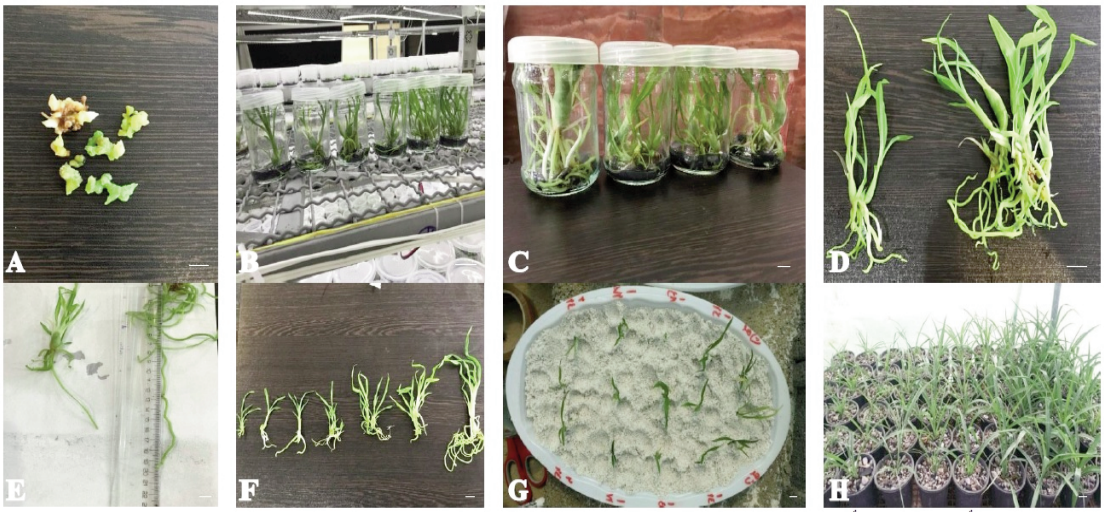

Fig. 1 - Micropropagation of *Catasetum pileatum* Alba through protocorms. (A) PLBs produced on medium containing 1.00 mg l^{-1} Kn + 1.00 mg l^{-1} IBA. (B) Developing PLBs on media enriched with different concentrations of Kn and IBA. (C) Micropropagated shoots from PLBs on medium containing Kn and IBA. (D) Leaves produced on PGRs-free medium (left) and medium containing 1.00 mg l^{-1} Kn + 1.00 mg l^{-1} IBA (right). (E) Roots produced on PGRs-free medium (left) and medium containing 0.50 mg l^{-1} Kn + 0.50 mg l^{-1} IBA. (F) Plantlets produced on media enriched with different concentrations of Kn and IBA. (G) Plantlets transplanted in pots filled with perlite. (H) Greenhouse acclimatized plantlets grown in pots filled with a mixture of leka, peat moss and perlite (in ratio of 1:1:1) (Scale bar = 1 cm).

tured on culture media poured into the culture bottles for *in vitro* propagation (Fig. 1B).

Culture medium and culture conditions

The explants were cultured on MS medium containing 3% sucrose and 0.8% Agar-agar. The medium was enriched with various PGRs. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media containing culture tubes were autoclaved at 104 kPa and 121°C for 20 min.

To evaluate the effect of PGRs on PLBs regeneration, shoot multiplication and root induction, the explants were cultured on MS medium containing different concentrations of kinetin (Kn; 0.00, 0.20, 0.50, 1.00, 2.00, 3.00 and 5.00 mg l^{-1}) and indole-3-butyric acid (IBA; 0.00, 0.10, 0.20, 0.50 and 1.00 mg l^{-1}), either individually or in combination. For each treatment, three replicates and for each replicate, three explants were taken (totally; 35 treatments, 105 replicates and 345 explants). Following establishment, cultures were maintained at 24±2°C, 70-80% RH, and 16-h photoperiod of 50-60 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

Assessment of characteristics

After 60 days, the effect of PGRs on advanced PLBs development was assessed by using PLBs number, plantlet height, leaf number, root length, root number and viability percentage. PLBs germination percentage was calculated by the following formula:

$$\% \text{ sprouted PLBs} = \frac{\text{Number of sprouted PLBs}}{\text{Total PLBS}} \times 100$$

Hardening and acclimatization

In vitro rooted plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove substrate residual and transplanted to plastic dishes containing perlite for 15-20 days (Fig. 1G). Then, plantlets were transferred to plastic pots (18 cm height × 12 cm diameter) filled with a potting mixture of Leca (Light Expanded Clay Aggregate), peat moss and perlite (in ratio of 1:1:1) (Fig. 1H). All the pots were then transferred to the greenhouse with temperature of 24±2°C to 20±2°C day/night, light intensity of 3500 Lux, RH of 80-90% and 14-h photoperiod) for acclimatization. The pots were covered with polyethylene bags to retain moisture inside and were opened gradually during 2 weeks. Survival rate (%) was recorded after 60 days from transfer to greenhouse conditions. Plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). The number of surviving plants was recorded after 4 weeks from transfer.

Experimental design and data analysis

The experiments were established in a completely randomized design with three replicates per treatment (totally; 345 explants). PGRs-free MS medium was used as control in the experiments. Data were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at $P < 0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

3. Results

Assessment of suitable conditions for PLBs regeneration

The main goal of this study was the maximum regeneration of PLBs during clonal proliferation. LSD test showed significant differences among different concentrations of Kn, also reciprocal effect of Kn and IBA for PLBs number ($p<0.01$). LSD also showed that the effect of IBA was no significant on PLBs number when applied individually (Table 1). The effects of Kn and IBA in the MS medium on the PLBs multiplication and growth are shown in figure 1 and Tables 2, 3 and 4. In the current study the formation of PLBs and shoot buds from the explants was observed within 45

Table 1 - Analysis of variance of the effect of different concentrations of Kn and IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

Source of variations	df	Plantlets height	Number of PLBs per protocorm	Leaf number	Root number	Root length	Viability percentage
Kn	6	50.8**	10.61**	30.88**	0.870**	23.52**	457**
IBA	4	7.16 NS	1.66 NS	3.916**	15.57**	24.04**	258*
Kn × IBA	24	6.29**	2.60**	4.32**	0.647**	7.73**	167*
Error	70	3.42	1.126	1.72	0.241	0.973	83.8
cv (%)	-	21.5	19.97	20.2	9.714	10.91	11.01

*, **: Significant at the 0.05 and 0.01 probability level, respectively, NS: Not significant at p=0.05.

days of culture establishment. The regeneration of PLBs are closely related with the concentration of both Kn and IBA when used in combination, however, Kn had more important role than IBA when these are applied individually. When the explants were cultured in medium supplemented with Kn individually, formation of more PLBs was observed than when explants were cultured in medium containing IBA individually (Tables 2 and 3). Among the tested Kn concentrations, 1.00 mg l^{-1} proved beneficial in inducing the highest frequency of 6.76 per explant generating PLBs (Table 2). When the explants were grown in medium enriched with Kn and IBA (1.00 mg l^{-1} from both of them) the largest number of PLBs (8.63 per explant) was observed (Table 4). Minimum PLBs regeneration (with average of 4.00 PLBs per explant) was obtained in media without Kn.

Assessment of suitable conditions for plantlets height and leaf number

The effect of PGRs in the MS medium on the plantlets growth (plantlets height and leaf number) is shown in figure 1C and Tables 1-4. After 60 days of culture of protocorms on media fortified with different concentrations of PGRs, plantlets height and leaf number were measured. Plantlets growth was significantly affected by the composition of the medium. Growth of plantlets from the explants without the addition of Kn to the culture medium was relatively poor. The plantlets growth rate ranged from 5.00 to 17.00 cm on the regeneration media (Table 4). MS medium fortified with 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA was the most appropriate medium for plantlet height (16.76 cm per explant) (Table 4). Treatment with 1.00 mg l^{-1} Kn in combination with 0.50 mg l^{-1} IBA produced plantlets with 13.36 cm long. Among all levels of Kn and IBA used individually, Kn at 1.00 mg l^{-1} was noted better induction of plantlet on explants than the other levels (Tables 2 and 3). The plantlets produced on medium without Kn was least (Tables 3 and 4).

MS medium fortified with 1.00 mg l^{-1} Kn and 1.00 mg l^{-1} IBA was the most appropriate medium for leaf number (12.70 per explant) (Fig. 1D, Table 4). The media containing 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA was suitable for leaf number, too (Table 4). Among all concentrations of IBA and Kn used individually (Tables 2 and 3), maximum leaf number (9.36 per explant) was produced in medium containing 1.00 mg l^{-1} Kn. Thus, the optimal concentration of Kn was

Table 2 - Mean comparison of the effect of different concentrations of Kn on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

Kn (mg l^{-1})	Plantlets height (cm)	Number of PLBs per protocorm	Leaf number	Root number	Root length (cm)	Viability percentage
0.00	6.22 d	4.01 d	4.75 d	5.32 a	8.68 bc	75.30 c
0.20	7.27 cd	4.80 c	5.58 cd	5.12 ab	10.28 a	85.30 b
0.50	9.72 b	5.41 bc	6.73 b	5.08 abc	10.68 a	84.00 b
1.00	11.84 a	6.76 a	9.36 a	5.38 a	9.14 b	93.30 a
2.00	9.02 b	5.18 bc	6.32 bc	4.95 bc	9.26 b	80.00 bc
3.00	8.38 bc	5.73 b	6.57 b	4.75 c	6.93 d	82.00 bc
5.00	7.62 c	5.27 bc	6.11 bc	4.81 bc	8.30 c	82.00 bc

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

Table 3 - Mean comparison of the effect of different concentrations of IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

IBA (mg l^{-1})	Plantlets height (cm)	Number of PLBs per protocorm	Leaf number	Root number	Root length (cm)	Viability percentage
0.00	7.96 c	5.00 b	6.15 b	4.33 d	8.22 c	83.80 ab
0.10	8.66 abc	5.43 ab	6.20 ab	4.62 cd	9.32 b	88.09 a
0.20	9.15 ab	5.37 ab	6.17 b	4.80 bc	10.34 a	83.80 ab
0.50	9.14 ab	5.68 a	6.92 ab	6.53 a	9.62 b	81.40 b
1.00	8.00 bc	5.05 ab	7.00 a	5.00 b	7.70 c	78.50 b

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

1.00 mg l^{-1}. Also, the optimal concentrations of IBA were 1.00 and 0.50 mg l^{-1}. These concentrations in combination with each other recorded maximum leaf production. This means that the PGRs acted synergistically.

Assessment of suitable conditions for root characteristics and acclimatization

Advanced root development was significantly affected by the composition of the medium, when measured through root length and root number. All treatments of PGRs, individually and in combination had significant effects (P<0.01) on root growth (Table 1). Root length was highest (14.06 cm per explant) in 0.50 mg l^{-1} Kn along with 0.50 mg l^{-1} IBA (Fig. 1E), however, this medium was not significantly different compared to 0.50 mg l^{-1} Kn along with 0.20 mg l^{-1} IBA medium with induction of 12.90 cm long for root (Table 4). All other media were significantly different and gave lower root length growth rates.

Among all treatments, 1.00 mg l^{-1} Kn plus 0.50 mg l^{-1} IBA was found to be the most effective for root for-

Table 4 - Mean comparison of the effect of different concentrations of Kn and IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

Plant growth regulators (mg l^{-1})		Plantlets height (cm)	Number of PLBs per protocorm	Leaf number	Root number	Root length (cm)	Viability percentage
Kn	IBA						
0.00	0.00	5.63 jk	3.56 jk	5.20 f-h	5.26 d-g	5.73 o	73.33 e-g
0.00	0.10	6.60 h-k	3.83 ijk	4.46 h	4.13 k-n	6.83 mno	80.00 c-g
0.00	0.20	5.10 k	4.46 e-k	4.73 gh	4.93 e-j	11.23 cd	86.60 a-e
0.00	0.50	5.20 k	4.63 e-k	4.66 gh	7.00 a	11.80 bc	70.00 fg
0.00	1.00	6.60 h-k	3.56 jk	4.70 gh	5.30 c-g	7.83 k-m	66.60 g
0.20	0.00	6.50 i-k	5.36 c-h	5.40 e-h	4.56 g-n	10.90 c-g	90.00 a-d
0.20	0.10	8.73 c-i	5.93 b-f	5.56 d-h	4.43 h-n	12.93 ab	100.00 a
0.20	0.20	6.83 f-k	4.30 e-k	5.53 d-h	4.80 f-l	10.13 d-h	73.33 e-g
0.20	0.50	6.70 g-k	5.36 c-h	5.80 c-h	7.26 a	9.43 f-j	83.30 b-f
0.20	1.00	7.60 d-k	4.06 h-k	5.60 c-h	4.53 g-n	8.00 j-m	80.00 c-g
0.50	0.00	9.43 c-i	4.66 e-k	6.13 b-h	4.40 h-n	7.63 l-n	86.60 a-e
0.50	0.10	9.50 c-i	4.16 g-k	7.73 bc	5.10 d-i	9.76 d-i	83.30 b-f
0.50	0.20	10.30 cd	7.36 ab	6.46 b-h	4.30 j-n	12.90 ab	86.60 a
0.50	0.50	10.60 bc	5.73 b-h	6.13 b-h	6.63 ab	14.06 a	86.60 a-e
0.50	1.00	8.80 c-i	5.13 d-h	7.20 b-f	5.00 d-j	9.06 h-l	76.66 d-g
1.00	0.00	9.53 c-i	6.00 b-f	8.00 b	3.86 mn	9.33 g-k	93.30 abc
1.00	0.10	9.86c-e	6.63bcd	6.56b-h	4.86e-k	9.13h-l	96.60ab
1.00	0.20	13.36 b	5.53 c-h	7.40 b-e	5.63 cde	11.00 c-f	100.00 a
1.00	0.50	16.76 a	7.10 abc	12.16 a	7.40 a	8.23 i-m	96.60 ab
1.00	1.00	9.66 c-g	8.63 a	12.70 a	5.16 d-h	8.00 j-m	80.00 c-g
2.00	0.00	8.56 c-j	4.66 e-k	6.40 b-h	4.36 i-n	8.96 h-l	76.66 d-g
2.00	0.10	10.40 b-d	6.20 b-e	6.80 b-g	4.76 f-l	9.50 e-j	86.60 a-e
2.00	0.20	9.90 c-e	5.10 e-j	5.86 b-h	4.4 6 h-n	11.06 c-e	70.00 fg
2.00	0.50	7.50 d-k	5.33 c-h	5.90 b-h	5.76 cd	9.70 d-i	76.66 d-g
2.00	1.00	8.73 c-i	4.60 e-k	6.66 b-g	5.40 c-f	7.10 mno	90.00 a-d
3.00	0.00	7.83 c-k	5.50 c-h	5.86 b-h	3.83 n	7.66 l-n	76.66 d-g
3.00	0.10	7.23 e-k	5.93 b-e	6.80 b-g	4.46 h-n	7.60 l-n	90.00 a-d
3.00	0.20	9.40 c-i	6.93 a-c	7.66 bcd	4.56 g-n	6.20 no	80.00 c-g
3.00	0.50	9.60 c-h	5.90 b-g	6.56 b- h	6.06 bc	6.96 mno	86.60 a-e
3.00	1.00	7.86 c-k	4.40 f-k	5.96 b-h	4.83 f-k	6.23 no	76.660 d-g
5.00	0.00	8.23 c-k	5.26 c-j	6.10 b-h	4.03 l-n	7.33 mn	90.00 a-d
5.00	0.10	8.33 c-j	5.33 c-h	5.50 e-h	4.63 f-m	9.50 e-j	80.00 c-g
5.00	0.20	7.16 e-k	4.93 d-k	5.53 d-h	4.93 e-j	9.86 d-h	90.00 a-d
5.00	0.50	7.63 c-k	5.83 b-g	7.26 b-f	5.63 cde	7.16 mno	70.00 fg
5.00	1.00	6.76 g-k	5.00 e-k	6.16 b-h	4.83 f-k	7.66 l-n	80.00 c-g

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

mation (7.133 per explant) (Table 4). Root production in this medium was not significantly higher than following media. The root number (7.26 and 7.00 per explant) produced in media containing 0.20 mg l^{-1} Kn plus 0.50 mg l^{-1} IBA and 0.50 mg l^{-1} IBA without Kn, respectively was noticeable (Table 4). In most cases, minimum root number was recorded in media without IBA. Among all concentrations of IBA and Kn used singly (Tables 2 and 3), maximum root number (6.53 per explant) was produced in medium fortified with 0.50 mg l^{-1} IBA.

The *in vitro* rooted plantlets (Fig. 1F) were successfully acclimatized in the greenhouse. Pots were filled with leca, peat moss and perlite (in ratio of 1:1:1) (Figs. 1G and H). Acclimatization was achieved in 4-6 weeks, and at this stage plants attain the height of about 10-15 cm. Acclimatization of micropropagated plantlets to the natural conditions requires several anatomical, morphological and physiological changes especially in xylem, leaves and photosynthesis. The hardened plantlets (Fig. 1H) are maintained in the Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran with 80% field establishment rate.

Assessment of suitable conditions for viability percentage

Significant difference was observed between the Kn ($P<0.01$), IBA and combination of Kn and IBA ($P<0.05$) levels and viability percentage of protocorms (Table 1). It has been observed that among the combinations and concentrations of PGRs, 0.20 mg l^{-1} Kn with 0.10 mg l^{-1} IBA and 1.00 mg l^{-1} with 0.20 mg l^{-1} IBA were the most effective for viability percentage (100.00) (Table 4). Viability percentage in these two media was significantly higher than that of other media. Least viability percentage (66.60) was observed in PLBs cultured on medium containing 1.00 mg l^{-1} IBA without Kn (Table 4).

4. Discussion and Conclusions

Current study revealed that the addition of external PGRs in appropriate concentrations induced PLBs formation from the protocorm explants cultured in the MS medium. In orchids, PLB regeneration from explants such as shoot and root tips and leaf and stem segments is a proper method of *in vitro* proliferation (Seeni and Latha, 2000; Dohling *et al*., 2012). Consonant with our findings, Roy *et al*. (2011) demonstrated that the frequency of PLBs regeneration of orchid *Vanda coerulea* significantly relied on kinds and concentrations of PGRs used. These researchers showed that a combination of 1.00 mg l^{-1} NAA and 0.85 mg l^{-1} BAP was found to be suitable for maximum PLB regeneration. Luo *et al*. (2008) showed that 0.50 mg l^{-1} Kn was proper for PLB formation of orchid *Dendrobium densiflorum*. The regeneration and proliferation of multiple PLBs are closely related with the type and concentration of cytokinins used. Among all cytokinins used for *in vitro* PLBs regeneration of orchids, BA, BAP, TDZ and Kn have the most application (Luo *et al*., 2008; Chugh *et al*., 2009; Firoz Alam *et al*., 2010; Roy *et al*., 2011; Panwar *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). When the explants were grown in medium enriched with both of Kn and IBA the maximum PLBs was obtained. Contrary to our finding, BAP individually was better than in combination with NAA for the maximum PLBs formation of orchid *Oncidium* (Kalimuthu *et al*., 2007). Study on *Vanda coerulea* revealed that when the protocorms were cultured on BAP or NAA alone, at any concentration recorded low proliferation rate. This means that the PGRs acted synergistically (Roy *et al*., 2011). Baker *et al*. (2014) reported the highest PLBs regeneration of orchid *Catasetum* on MS medium containing a combination of 0.50 mg l^{-1} BA plus 0.50 mg l^{-1} NAA. Present study demonstrated that the production of more than seven PLBs was observed in the media supplemented with 0.50 mg l^{-1} kn along with 0.20 mg l^{-1} IBA and 1.00 mg l^{-1} Kn along with 0.50 mg l^{-1} IBA. Similar findings were found by Panwar *et al*. (2012) through study on orchid *Eulophia nuda*.

The combination, concentrations and the ratio between the PGRs are critically important for the formation of shoots and PLBs in orchids (Dohling *et al*., 2007; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Investigation on orchid *Eulophia nuda* showed that the maximum shoot multiplication was achieved on MS medium containing 2.00 mg l^{-1} BA and 1.00 mg l^{-1} Kn after 4 weeks of cultures (Panwar *et al*., 2012). The type, concentrations and different combinations of PGRs plays an important role during micropropagation of many orchid species (Arditti and Ernst, 1993; Panwar *et al*., 2012).

In orchids, the use of protocorm and PLB as the explants is the most appropriate and simplest method for *in vitro* propagation. Protocorm contains meristematic cells and can differentiate to a new shoot. Therefore, protocorm can be used to enhance proliferation and simultaneous production of orchid plantlets (Teixeira da Silva *et al*., 2005). Protocorms

are being applied by many researchers as explants for *in vitro* propagation of many rare and endangered orchid species (Deb and Temjensangba, 2006, Teixeira da Silva *et al*., 2006; Roy *et al*., 2011; Dohling *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). PLB production was induced from many explants such as protocorm, shoot tip, node, root tip, leaf and stem segments during *in vitro* propagation of orchids (Dohling *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017).

The regeneration and propagation of multiple shoots are closely related with the type and concentration of cytokinins used (Amoo *et al*., 2014). The differentiation of multiple shoots from PLBs has been reported in some orchids such as *Cymbidium*, *Dendrobium*, *Catasetum*, *Phalanoepsis*, *Habeneria* and *Satyrium* (Talukdar, 2001; Sheelavanthmath and Murthy, 2001; Mahendran and Bai, 2009; Hossain *et al.*, 2010; Baker *et al*., 2014; Kaviani *et al*., 2017). Cytokinins have a wide range of functions including regulatory role on various physiological and developmental processes (Werner *et al*., 2001). In *Dendrobium huoshanense* C.Z. Tang et S.J. Cheng, Kn was reported to be more effective for plantlet regeneration from PLBs than BAP, N-benzyl-tetrahydropyranyladenine (BPA), isopentenyl adenine (2-iP), TDZ and Zeatin (Zt) (Luo *et al*., 2009). The best response appeared on the medium enriched with 4.50 mg l^{-1} Kn. Kn was also used for shoot proliferation of some other orchids (Saiprasad *et al*., 2004; Malabadi *et al*., 2005; Martin and Madassery, 2006; Panwar *et al*., 2012). Contrary to our findings, study on *Dendrobium nobile* revealed that when explants were cultured in MS medium supplemented with BAP alone, formation of PLBs was done but direct shoot formation was not observed (Bhattacharyya *et al*., 2016). Study of Mahendran and Bai (2009) demonstrated that among the cytokinins used for multiple shoot induction of *Satyrium nepalense* D. Don. TDZ was found to be superior. In this study, protocorm developed multiple shoots directly on the medium supplemented with cytokinins. In most of the orchids the presence of cytokinins singly promoted optimal shoot proliferation (Mahendran and Bai, 2009). In the present study, addition of external PGRs (both of Kn and IBA) in suitable concentrations induced plantlets growth and leaf formation from PLBs cultured in the MS medium without callus formation. The main advantage of direct organogenesis without an intervening callus phase is that somaclonal variation is reduced (Roy *et al*., 2011). When the media was supplemented with IBA singly the response was poor. Similar finding was reported by Roy *et al*. (2011) worked on *Vanda coerulea*. Bhattacharyya *et al*. (2016) showed that when the explants were grown in medium containing cytokinin and auxin, a higher rate of response frequency (92.6%) of shoot buds and PLBs was observed in all PGRs combinations. In *Eulophia nuda* Lindl., maximum shoot multiplication and elongation were obtained on MS medium containing 2.00 mg l^{-1} BA and 1.00 mg l^{-1} Kn (Panwar *et al*., 2012). PGRs in orchids act more efficiently when used in combination (Seeni and Latha, 2000; Roy *et al*., 2011).

Similar with our findings, IBA resulted in a better rooting efficiency over NAA in terms of rooting frequency and number of roots induced per shoot in *Satyrium nepalense* D.Don. and *Dendrobium nobile* (Mahendran and Bai, 2009; Bhattacharyya *et al*., 2016). As, maximum rooting efficiency (86% or 5.4 roots/shoot) was obtained in medium supplemented with 2.00 mg l^{-1} of IBA after 8 weeks of culture (Bhattacharyya *et al*., 2016). The highest number of roots per shoot (6.40) was achieved at 2.00 mg l^{-1} IBA (Mahendran and Bai, 2009). The effectiveness of IBA in rooting has been shown for some other orchids like *Vanilla planifolia* (Giridhar *et al*., 2001), *Cymbidium alofolium* (L.) SW. and *Dendrobium nobile* Lindl. (Nayak *et al*., 2002), *Cymbidium pendulum* (Nongdam *et al*., 2006), *Satyrium nepalense* (Mahendran and Bai, 2009), *Vanda teres* (Firoz Alam *et al*., 2010) and *Eulophia nuda* Lindl. (Panwar *et al*., 2012). A maximum 90% response for root formation and highest number of roots (5.50) with length (5.30 cm) per shoot tubers was calculated on IBA (0.50 mg l^{-1}) treated shoots of *Eulophia nuda* Lindl. (Panwar *et al*., 2012). Study of Baker *et al*. (2014) on micropropagation of *Catasetum* demonstrated that the largest number of root (7.16) and root length (193.40 mm) were obtained on MS medium supplemented with 0.50 mg l^{-1} BA together with 0.50 mg l^{-1} NAA.

Orchids are among the most beautiful ornamental plants. *Catasetum pileatum* cv. Alba is a rare and near endangered orchid. Many of orchids are threatened, rare, vulnerable, endangered, indeterminate or in danger of extinction. Therefore, it is necessary to develop the suitable methods for conservation and large-scale production of these plants that can be used for their re-introduction.

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Micropropagation of two near threatened orchid. Part 2: *Phalaenopsis amabilis* Blume var. Grandiflora

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Key words: *in vitro* multiplication, orchid propagation, ornamentals, plant growth regulators.

Abstract: *Phalaenopsis* is one of the most popular orchids in the world, through the development of many artificial hybrids. In this research, a reliable and efficient protocol is presented for *in vitro* proliferation of *Phalaenopsis amabilis* Blume cv. Grandiflora. Protocorm-like bodies (PLBs) were cultured on Murashige and Skoog (MS) medium containing different concentrations of kinetin (Kn; 0.00, 0.50, 1.00, 2.00 and 3.00 mg l^{-1}) and indole-3-butyric acid (IBA; 0.00, 0.10, 0.20, 0.50 and 1.00 mg l^{-1}), either individually or in combination and activated charcoal (AC; 0.00, 0.50 and 1.00 g l^{-1}). A combination of 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn on medium containing 1.00 g l^{-1} AC was found to be suitable for maximum leaf number (6.16±0.503 per explant). The highest rooting frequency with 7.13±0.153 roots per explant was achieved on medium enriched with 0.50 mg l^{-1} IBA and 0.50 mg l^{-1} Kn on medium containing 1.00 g l^{-1} AC. The largest number of callus (9.10±0.611) was induced on explants cultured in medium containing 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn on medium without AC. The plantlets were successfully acclimatized in the greenhouse with a survival rate of 95% exhibiting normal developmental patterns.

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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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1. Introduction

Phalaenopsis Blume, known as moth orchid, is a genus of approximately 60 species native to tropical rainforests of South and South-East Asia, Australia and New Guinea (Winkelmann *et al*., 2006). *Phalaenopsis* as a cut and pot flowering plant is one of the most popular orchids in the trade and hobbyists through the development of many artificial hybrids. They are epiphytic plants, and consist of only a few leathery leaves (Sinha *et al*., 2010).

Large scale natural clonal propagation is not possible in *Phalaenopsis*. Therefore, the establishment of protocols for *in vitro* proliferation of orchids is the only method for high frequency regeneration of these plants. *In vitro* techniques can be used for storage of rare and endangered plant species and production of large number of plantlets in short period of time (Engelmann, 2011). *In vitro* multiplication of orchids deals with some problems such as high cost of production, low rate of shoot prolifer-

ation, poor rooting frequency and genetic variations (Bhattacharyya *et al*., 2016). Several methods for *in vitro* propagation of *Phalaenopsis* through callus induction and cell suspension culture were developed (Tanaka, 1992; Arditti and Ernst, 1993; Tokuhara and Mii, 2001; Sinha *et al*., 2010). Park *et al*. (2002) developed an efficient *in vitro* propagation method for *Phalaenopsis* by using protocorm-like bodies (PLBs) derived from leaf explants. Medium composition for *in vitro* culture of orchids by PLBs is species-specific and depends on several factors (Luo *et al*., 2009). Kuo *et al*. (2005) reported a protocol for regenerating a *Phalaenopsis* cultivar by direct somatic embryogenesis. This method was not so efficient and feasible for commercial propagation because of low frequency regeneration of different cultivars of *Phalaenopsis* hybrid. On the other hand, many protocols for *in vitro* propagation of orchids, especially those in danger of extinction, using PLBs as explants and various PGRs, have been reported (Sinha *et al*., 2010; Teixeira da Silva, 2006; Baker *et al*., 2014; Kaviani *et al*., 2017).

Various PGRs like α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), 6-benzyle amino purine (BAP), 6-benzyladenine (BA) and kinetin (Kn) have been applied for micropropagation of rare and endangered orchids (Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Many explants such as seed, leaf, node section, protocorm, PLB, tuber, shoot tip and inflorescence have been used for *in vitro* proliferation of endangered orchids (Sinha *et al*., 2010; Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Baker *et al*., 2014; Chen *et al*., 2015; Bhattacharyya *et al*., 2016). PLB is more efficient because of maximum multiplication in a short period of time (Luo *et al*. 2003). This study describes an efficient and reliable protocol for high frequency regeneration and callus induction of *Phalaenopsis amabilis* Blume var. Grandiflora, a rare and near endangered orchid species by PLBs.

2. Materials and Methods

Source of explant

Leaves (0.5-1 cm long) were excised from young *Phalaenopsis amabilis* Blume var. Grandiflora plants growing in the greenhouse of Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran. The leaves were washed under running tap water for 15-20 min and rinsed thoroughly with distilled water. These were surface sterilized with $HgCl_2$ (0.1% w/v) for 10 min followed by NaOCl (20%) for 15 min with 1 drop of Tween 20, then rinsed with sterile distilled water. Finally, leaves were sterilized in ethanol 75% for 1 min and washed 3-4 times with sterilized distilled water and finally excised to segments of 5-7 mm as primary explants for culture in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% agar. The medium was supplemented with 0.20 mg l^{-1} NAA along with 3.00 mg l^{-1} BAP (appropriate types and concentrations of PGRs obtained before for maximum production of PLBs; data not shown). Healthy and sterilized PLBs (Fig. 1A) produced in the Plant Biotechnology Laboratory, Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran, were excised and used as secondary explants for *in vitro* propagation.

Culture medium and growth conditions

The explants (PLBs) were cultured in MS medium containing 3% sucrose and 0.8% agar. The medium was enriched with various PGRs and activated charcoal (AC). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media contained in culture bottles were autoclaved at 104 kPa and 121°C for 20 min.

To evaluate the effect of PGRs and AC on shoot multiplication (i.e. increasing the number of leaves and their development) and root induction, the explants were cultured on MS medium containing different concentrations of kn (0.00, 0.50, 1.00, 2.00 and 3.00 mg l^{-1}) and IBA (0.00, 0.10, 0.20, 0.50 and 1.00 mg l^{-1}), either individually or in combination. Medium was supplemented with or without activated charcoal (AC; 0.00, 0.50 and 1.00 g l^{-1}). For each treatment, three replicates and for each replicate, three specimens (explants) were taken (totally; 75 treatments, 225 replicates and 675 specimens or explants). Following establishment, cultures were maintained at 24±2°C, 70-80% RH, and 16-h photoperiod of 50-60 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

After 60 days, the effect of PGRs and AC on advanced PLBs development was assessed by measuring number of leaves per explant, leaf length, leaf width, number of roots per explant, root length, number of explants with callus and viability percentage.

Plant development and acclimatization

In vitro rooted plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove adherent nutrient and transplanted to plastic pots (18 cm height × 12 cm diameter) filled

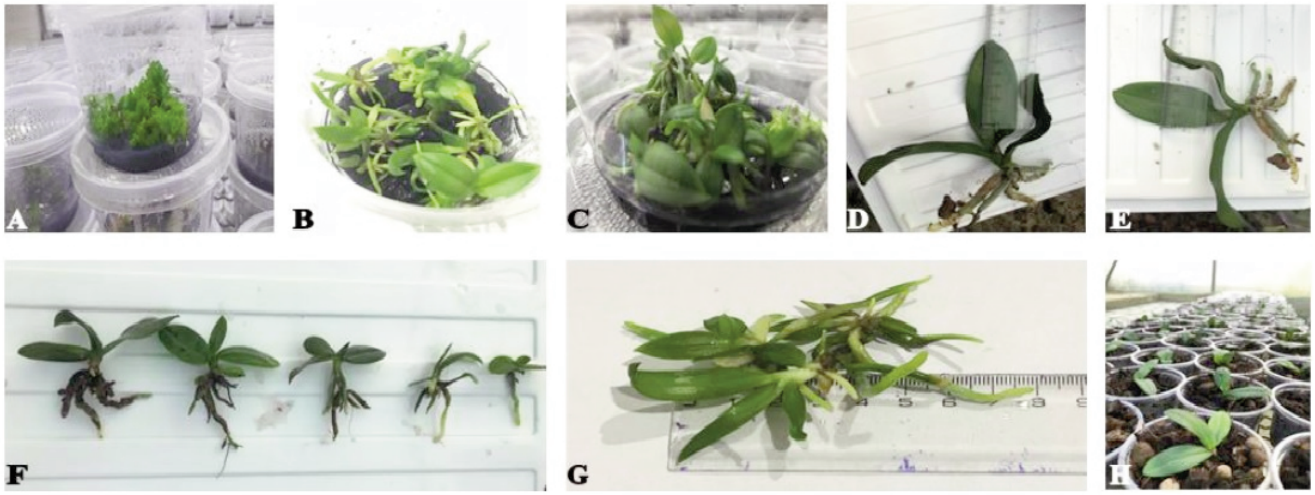

Fig. 1 - Micropropagation of *Phalaenopsis amabilis* Blume cv. Grandiflora through protocorm-like bodies (PLBs). (A) PLBs produced on MS medium containing 0.20 mg l^{-1} NAA + 3.00 mg l^{-1} BAP. (B) Micropropagated shoots from PLBs on medium containing 0.50 mg l^{-1} IBA + 2.00 mg l^{-1} Kn. (C) Plantlets produced on medium supplemented with 0.20 mg l^{-1} IBA + 2.00 mg l^{-1} Kn + 1.00 g l^{-1} AC. (D) Length of leaf obtained on medium containing 0.50 mg l^{-1} IBA together with 2.00 mg l^{-1} Kn and 1.00 g l^{-1} AC. (E) Width of leaf obtained on medium containing 0.50 mg l^{-1} IBA together with 1.00 mg l^{-1} Kn 1.00 g l^{-1} AC. (F) Plantlets produced on media containing 1.00 g l^{-1} AC together with different concentrations of IBA and Kn. From left to right: 0.10 mg l^{-1} IBA + 0.50 mg l^{-1} Kn, 0.50 mg l^{-1} IBA + 0.50 mg l^{-1} Kn, 0.20 mg l^{-1} IBA + 1.00 mg l^{-1} Kn, 0.10 mg l^{-1} IBA + 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} Kn without IBA. (G) Number and length of roots obtained on medium enriched with 0.50 mg l^{-1} IBA plus 0.50 mg l^{-1} Kn and 1.00 g l^{-1}. (H) Greenhouse acclimatized plantlets in pots filled with leca, peat moss and perlite (in ratio of 1:1:1).

with a potting mixture of leca (Light Expanded Clay Aggregate), peat moss and perlite (1:1:1). All the pots were then transferred to the greenhouse with temperature of 24±2°C to 20±2°C day/night (light intensity of 3500 Lux, RH of 80-90% and 14-h photoperiod) for acclimatization. The pots were covered with polyethylene bags to retain moisture inside and were opened gradually during 2 weeks. Plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). The number of surviving plants was recorded after 12 weeks of transfer.

Experimental design and data analysis

The experiments were established in a completely randomized design with three replicates per treatment (totally 675 explants). PGRs-free MS medium was used as control in the experiments. The results were expressed as mean±SD. Data were subjected to analysis of variance (ANOVA) (except for acclimatization records) and means were compared by the LSD test at $P<0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

3. Results

The effect of PGRs and AC on the leaf growth (number, length and diameter) and root growth (number and length) of *Phalaenopsis amabilis* Blume var. Grandiflora is shown in Tables 1-4 and figure 1. PLBs produced on MS medium containing 0.20 mg l^{-1} NAA + 3.00 mg l^{-1} BAP (appropriate types and concentrations of PGRs obtained before for maximum production of PLBs; data not shown), were used as primary explants (Fig. 2A). These PLBs were produced after 60 days of culture of leaf explant on this medium (Fig. 2B). These PLBs were used as secondary explants and cultured on media supplemented with

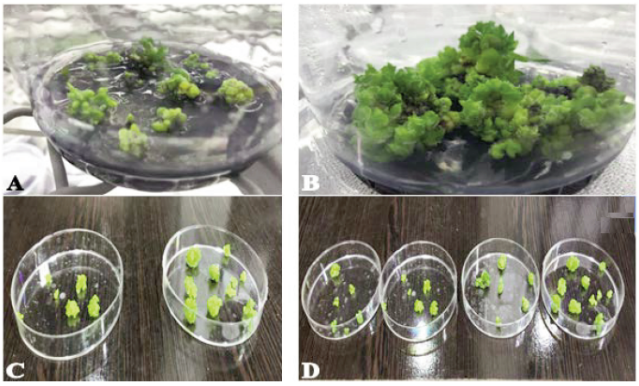

Fig. 2 - (A) PLBs and callus formation. (B) PLBs development and multiplication in MS medium containing 0.20 mg l^{-1} NAA + 3.00 mg l^{-1} BAP. (C) Callus formation from the explants cultured in media enriched with 0.50 mg l^{-1} IBA + 3.00 mg l^{-1} Kn (left) and 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn (right). (D) Callus formation from the explants cultured in media enriched with different concentrations of IBA and Kn (from left to right: 0.50 mg l^{-1} IBA + 3.00 mg l^{-1} Kn, control, 1.00 mg l^{-1} IBA + 1.00 mg l^{-1} Kn and 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn).

different concentrations of PGRs and AC. ANOVA showed significant differences between various concentrations of PGRs and AC on most measured parameters (Table 1).

Effect of PGRs and AC on multiplication parameters

Advanced shoot development was significantly affected by the composition of the medium. The media containing 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn along with 0.50 g l^{-1} AC, and without AC were suitable for leaf number (Tables 2, 3, Figs. 1B, C). The highest leaf length (4.66±0.702 cm per explant) and leaf width (3.13±0.603 cm per explant) were obtained in media enriched with 0.50 mg l^{-1} IBA along with 2.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA along with 1.00 mg l^{-1} Kn, respectively (Table 4, Figs. 1D, E). MS medium enriched with 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn along with 1.00 g l^{-1} AC was the most appropriate medium for leaf number (6.16±0.503 per explant) (Table 4). Among all concentrations of IBA, Kn and AC used

Table 1 - Effect of different concentrations of Kn, IBA and AC on the studied parameters of *in vitro* grown *Phalaenopsis amabilis* Blume cv. Grandiflora

Source of variations	df	Mean of squares						
		Leaf number	Leaf length	Leaf width	Root number	Root length	Callus number	Viability percentage
AC	2	6.526 **	5.563 **	6.881 **	6.38 **	5.87 **	3.402 ns	76.00 ns
IBA	4	14.71 **	8.818 **	4.708 **	23.50 **	48.60 **	5.224 **	1000 **
Kn	4	48.81 **	9.746 **	9.030 **	5.54 **	11.13 **	49.2 **	1026 **
AC × IBA	8	0.206 ns	1.139 **	0.417 *	3.29 **	2.35 **	9.66 **	291 **
AC × Kn	8	1.238 **	1.560 **	0.3782 ns	1.63 **	1.434 *	12.87 **	297 **
IBA × Kn	16	7.043 **	4.208 **	2.805 **	6.35 **	4.43 **	20.60 **	651 **
AC × IBA × Kn	32	1.326 **	1.587 **	0.697 **	2.89 **	2.066 **	10.90 **	662 **
Error	150	0.453	0.304	0.197	0.597	0.602	1.517	99.66
CV	–	19.09	20.83	25.01	15.79	17.6	25.47	12.47

*, **: Significant at the 0.05 and 0.01 probability level, respectively, NS: Not significant at p=0.05.

Table 2 - Effect of different concentrations of Kn and IBA without AC on the studied parameters of *in vitro* grown *Phalaenopsis amabilis* Blume cv. Grandiflora

PGRs (mg l^{-1})		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Callus number	Viability percentage
IBA	Kn							
0.00	0.00	2.66±0.681 d-g	1.73±0.153 h	1.16±0.306 fg	4.03±0.513 bc	3.33±0.603 hi	4.76±2.454 cd	70.00±10.000 bc
0.00	0.50	3.76±0.624 bcd	2.03±0.208 e-h	0.93±0.153 g	4.33±0.929 a-c	3.10±0.624 i	5.03±0.854 cd	63.30±10.000 c
0.00	1.00	2.93±0.265 c-g	2.13±0.520 d-h	1.20±0.265 e-g	4.00±0.529 bc	3.53±1.206 f-i	5.10±1.539 cd	73.30±10.000 a-c
0.00	2.00	2.20±0.493 fg	2.86±0.503 b-e	1.56±0.153 d-g	4.86±1.358 a-c	4.73±0.400 bcdef	4.53±0.737 cd	90.00±15.275 a
0.00	3.00	2.83±0.208 d-g	2.40±0.208 c-h	2.00±0.100 c-e	3.76±0.416 c	3.30±0.231 hi	7.96±2.166 ab	80.00±10.000 ab
0.10	0.00	2.06±0.200 g	1.83±0.321 f-h	1.66±0.100 c-g	4.80±0.889 a-c	4.20±0.794 b-i	3.63±1.332 cd	96.60±15.275 a
0.10	0.50	3.20±0.929 c-g	2.30±0.802 c-h	1.56±0.361 d-g	4.56±0.100 a-c	3.93±0.493 c-i	3.16±0.954 d	80.00±15.275 ab
0.10	1.00	3.10± 0.643 c-g	2.43±0.781 c-h	2.43±0.265 a-c	5.16±0.800 a-c	5.13±0.702 bcd	5.73±2.452 bc	70.00±10.000 bc
0.10	2.00	4.10±0.624 a-c	2.26±0.153 c-h	2.86±0.794 ab	4.53±1.415 a-c	4.43±0.451 b-h	3.76±3.694 cd	73.30±20.000 a-c
0.10	3.00	3.00±0.300 c-g	1.80±0.153 gh	1.26±0.351 d-g	4.06±0.493 bc	4.20±1.012 b-i	4.60±1.193 cd	83.30±10.000 ab
0.20	0.00	2.56±0.300 efg	2.40±0.854 c-h	1.16±0.361 fg	4.96±1.450 a-c	5.30±0.351 b	3.56±0.781 cd	73.30±10.000 a-c
0.20	0.50	3.76±0.306 bcd	2.43±0.321 c-h	1.80±0.529 c-f	5.40±0.700 ab	4.20±0.462 b-i	9.10±0.611 a	73.30±10.000 a-c
0.20	1.00	3.73±0.723 b-e	3.10±0.700 a-d	2.03±0.500 cd	5.40±0.493 ab	6.60±0.458 a	4.76±1.858 cd	83.30±10.000 ab
0.20	2.00	5.10±0.889 a	2.76±0.493 b-g	3.06±0.153 a	5.63±1.015 a	5.10±0.586 bcd	3.40±0.473 d	80.00±10.000 ab
0.20	3.00	3.73±0.153 b-e	3.66±0.794 ab	1.96±0.529 c-f	4.90±0.651 a-c	4.96±1.365 bcde	4.60±0.651 cd	80.00±15.275 ab
0.50	0.00	2.83±0.153 d-g	2.16±0.306 d-h	1.33±0.814 d-g	4.90±0.954 a-c	5.23±0.361 b	4.73±0.896 cd	90.00±5.774 a
0.50	0.50	3.46±0.300 b-e	2.33±0.153 c-h	1.70±0.058 c-g	5.20±0.721 a-c	4.40±1.250 b-h	3.93±0.404 cd	80.00±10.000 ab
0.50	1.00	3.66±0.361 b-e	2.90±0.854 a-e	2.06±0.200 b-d	4.50±0.971 a-c	5.20±1.552 bc	4.86±1.973 cd	80.00±10.000 ab
0.50	2.00	4.60±0.361 ab	3.63±0.721 ab	1.33±0.351 d-g	4.56±0.635 a-c	4.66±0.961 b-g	3.40±0.833 d	80.00±10.000 ab
0.50	3.00	3.20±0.503 c-g	2.20±0.458 c-h	1.30±0.265 d-g	5.06±1.250 a-c	3.40±0.513 g-i	4.73±1.249 cd	80.00±10.000 ab
1.00	0.00	2.86±0.503 d-g	2.26±0.306 c-h	1.56±0.100 d-g	4.56±1.457 a-c	3.73±1.350 e-i	4.43±1.058 cd	80.00±10.000 ab
1.00	0.50	3.13±0.458 c-g	2.13±0.764 d-h	1.66±0.208 c-g	4.63±1.358 a-c	4.20±1.286 b-i	5.73±3.134 bc	90.00±10.000 a
1.00	1.00	3.03±0.833 c-g	2.83±0.306 b-f	1.96±0.751 c-f	4.33±0.681 a-c	3.86±0.987 d-i	4.93±1.102 cd	73.30±10.000 a-c
1.00	2.00	4.56±0.723 ab	3.20±0.351 a-c	1.63±0.800 c-g	4.70±0.651 a-c	3.86±1.845 d-i	4.03±2.364 cd	80.00±5.774 ab
1.00	3.00	3.26±0.854 c-e	3.90±0.208 a	1.40±0.850 d-g	4.56±0.404 a-c	4.06±0.416 b-i	4.20±0.513 cd	90.00±15.275 a

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

Table 3 - Effect of different concentrations of Kn and IBA along with 0.50 mg l^{-1} AC on the studied parameters of *in vitro* grown *Phalaenopsis amabilis* Blume cv. Grandiflora

PGRs (mg l^{-1})		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Callus number	Viability percentage
IBA	Kn							
0.00	0.00	3.40±0.950 cd	1.66±0.872 f	1.73±1.210 b-e	3.86±0.907 gh	3.20±0.493 g	4.00±0.624d-g	90.00±10.000 a
0.00	0.50	2.66±0.907 d	1.76±0.252 ef	0.80±1.250 f	3.23±1.286 h	3.33±0.351 efg	5.60±1.054 bcd	80.00± 0.000 ab
0.00	1.00	3.63±0.819 bcd	2.60±0.265 a-f	1.30±0.208 c-f	4.10±0.751 f-h	3.16±0.346 g	6.70±1.136 abc	80.00±20.817 ab
0.00	2.00	2.90±1.106 d	3.10±0.306 abc	1.60±0.513 b-e	5.13±0.889 b-g	3.23±0.458 fg	3.00±0.666 g	70.00±15.275 b
0.00	3.00	2.90±0.917 d	2.33±0.961 b-f	1.33±0.513 c-f	4.80±0.907 b-g	3.33±0.954 efg	3.73±1.159 d-g	70.00±10.000 b
0.10	0.00	3.20±0.351 d	2.33±0.265 b-f	1.13±0.115 ef	5.80±1.002 a-e	5.06±1.212 a-d	3.26±1.332 fg	90.00±5.774 a
0.10	0.50	2.66±0.777 d	2.10±0.265 def	1.60±0.100 b-e	6.16±0.473 abc	3.76±0.751 d-g	5.50±0.721 bcd	90.00±10.000 a
0.10	1.00	2.86±0.702 d	2.43±0.721 b-f	2.06±0.208 abc	5.83±0.200 a-e	4.23±0.557 c-g	6.56±0.656 abc	90.00±10.000 a
0.10	2.00	5.13±0.666 a	2.10±0.611 def	2.30±0.265 a	5.96±1.682 a-d	3.86±0.100 c-g	3.40±1.353 efg	90.00±15.275 a
0.10	3.00	3.33±1.193 d	2.53±0.666 b-f	1.56±0.306 b-f	4.90±0.917 b-g	4.70±1.266 a-e	3.53±1.159 d-g	73.30±10.000 ab
0.20	0.00	2.96±1.277 d	2.90±0.458 a-d	1.20±0.265 def	3.73±0.520 gh	5.96±1.106 ab	3.90±0.656 d-g	70.00±10.000 b
0.20	0.50	3.26±0.473 d	2.63±0.436 a-f	1.93±0.451 a-d	6.10±1.411 abc	4.63±1.229 b-f	5.36±0.666 b-e	70.00±15.275 b
0.20	1.00	3.80±1.044 bcd	2.23±0.351 c-f	2.26±0.289 ab	7.06±0.777 a	6.06±1.710 a	5.43±1.514 b-e	76.60±20.817 ab
0.20	2.00	5.80±1.002 a	2.16±0.557 c-f	1.83±0.814 b-e	5.56±0.557 a-f	5.26±0.902 abc	8.56±3.029 a	70.00±15.275 b
0.20	3.00	3.83±0.800 bcd	3.23±0.611 ab	1.73±1.150 b-e	4.50±1.436 d-h	4.00±0.800 c-g	3.16±0.624 fg	90.00±10.000 a
0.50	0.00	3.10±1.539 d	2.10± 0.950 def	1.36±0.681 c-f	5.40±1.234 b-f	4.40±1.290 c-g	7.30±0.702 ab	70.00±10.000 b
0.50	0.50	4.63±1.629 abc	2.70±0.153 a-e	1.63±0.586 b-e	6.23±1.320 ab	3.80±2.022 d-g	4.33±0.643 d-g	73.30±10.000 ab
0.50	1.00	2.86±0.709 d	2.73±1.210 a-e	1.90±0.611 a-f	4.93±0.700 b-g	4.53±0.709 c-g	5.16±1.229 c-f	80.00±10.000 ab
0.50	2.00	4.76±0.473 ab	3.56±0.709 a	1.46±0.709 c-f	4.66±0.624 c-h	4.00±0.954 c-g	4.76±0.833 c-g	86.60±10.000 ab
0.50	3.00	3.53±0.361 bcd	2.93±1.595 a-d	1.70±0.458 b-e	4.13±1.007 f-h	4.56±0.900 b-g	4.90±0.436 c-g	86.6±10.000 ab
1.00	0.00	2.86±0.681 d	3.03±0.306 a-d	1.56±0.252 b-f	4.73±0.794 b-h	3.66±1.234 d-g	3.20±2.219 fg	70.00±10.000 b
1.00	0.50	3.36±0.900 cd	2.36±0.755 b-f	1.66±0.208 b-e	4.53±0.361 d-h	4.56±0.436 b-g	5.60±3.005 bcd	90.00±10.000 a
1.00	1.00	3.40±0.794 cd	2.93±0.300 a-d	1.93±0.755 a-d	4.43±0.987 e-h	4.53±1.405 c-g	4.63±3.233 c-g	70.00±15.275 b
1.00	2.00	4.73±0.379 ab	2.90±0.153 a-d	1.46±0.200 c-f	4.13±1.493 f-h	4.26±0.624 c-g	3.93±0.666 d-g	73.30±10.000 ab
1.00	3.00	3.76±0.907 bcd	2.73±0.100 a-e	1.63±0.451 b-e	4.20±1.795 f-h	4.20±0.755 c-g	3.93±0.153 d-g	90.00±11.547 a

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

individually, maximum leaf number (4.52±0.33 per explant) was produced in medium containing 2.00 mg l^{-1} Kn (data not shown). Production of leaf was relatively high by all PLBs grown on medium containing 2.00 mg l^{-1} Kn in combination with all concentrations of IBA with or without AC (Tables 2, 3, 4). Thus, the optimal concentration of Kn was 2.00 mg l^{-1}. Also, the optimal concentrations of IBA were 0.20 and 0.50 mg l^{-1}. These concentrations in combination with each other recorded maximum shoot and root production. Media supplemented with 1.00 g l^{-1} AC was most suitable for *in vitro* leaf growth since it resulted in the largest leaf number and development (Table 4, Figs. 1D, E, F).

Advanced root development was significantly affected by the composition of the medium, when measured through root length and root number. All treatments of PGRs and AC, individually and in combination had significant effects ($P<0.01$) on root growth (Table 1). Root length was highest (6.66±0.709 cm per explant) in presence of 0.20 mg l^{-1} IBA plus 2.00 mg l^{-1} Kn and 1.00 g l^{-1} AC medium (Table 4). However, no statistically significant difference in root length was detected between this and half-concentration of IBA and Kn (Fig. 1F). All other media differed significantly and gave lower root length growth rates. Among all treatments, 0.50 mg l^{-1} IBA plus 0.50 mg l^{-1} Kn and 1.00 g l^{-1} AC was found to be the most effective for root formation (7.13±0.153 per explant) (Table 4, Fig. 1G). However, the root number (7.06±0.777 per explant) produced in medium containing 0.20 mg l^{-1} IBA plus 1.00 mg l^{-1} Kn and 0.50 g l^{-1} AC was noticeable (Table 3). In most cases, minimum root number was recorded in media without IBA. Among all concentrations of IBA, Kn and AC used individually, maximum root number (5.28±0.564 per explant) was produced in medium containing 0.20 mg l^{-1} IBA (data not shown).

The *in vitro* rooted plantlets were successfully acclimatized in the greenhouse (Fig. 1H). Pots were filled with leca, peat moss and perlite (in ratio of 1:1:1). Acclimatization was achieved in 4-6 weeks, and at this stage plants attain the height of about 12-16 cm. Acclimatization of micropropagated plantlets to the natural conditions requires several anatomical, morphological and physiological changes especially in xylem, leaves and photosynthesis. The hardened plantlets were maintained in the Hyrcan Agricultural

Sciences and Biotechnology Research Institute, Amol, Iran with 95% field establishment rate.

Effect of PGRs and AC on viability percentage

Significant differences were found in viability percentage among the different concentrations of PGRs alone and in combination with each other, also with AC concentrations. The rate of produced plantlets was highest when IBA at 0.10 mg l^{-1} alone was added to the media (Table 2). Least viability percentage (63.30±10.00) was observed in PLBs cultured on media containing 0.50 mg l^{-1} Kn without AC and 1.00 mg l^{-1} IBA along with 1.00 mg l^{-1} Kn with 1.00 g l^{-1} AC (Tables 2, 4).

Effect of PGRs and AC on callus production

LSD test did not show significant differences among different concentrations of AC for callus production. A combination of 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn induced highest callus production (9.10±0.611) (Figs. 2C, D), which differed significantly from the other tested combinations, being this rate two or three-fold higher than in the other treatments (Tables 2, 3, 4). There was no any direct correlation between increasing PGRs and AC concentrations and increase in callus production. In most cases, minimum callus formation was observed in the explants cultured on media without IBA or Kn with or without AC (Tables 2, 3, 4, Figs. 2C, D).

4. Discussion and Conclusions

The present investigation demonstrated that the addition of external PGRs in proper concentrations induced leaf formation from the PLBs explants cultured in the MS medium. The regeneration of multiple shoots (leaves in some orchids like *Phalaenopsis amabilis*) has been reported to be closely related with the type and concentration of cytokinins used (Amoo *et al*., 2014). Development of multiple shoots from PLBs has been successfully achieved in some orchids such as *Cymbidium*, *Dendrobium*, *Catasetum*, *Phalanoepsis*, *Habeneria* and *Satyrium* (Talukdar, 2001; Sheelavanthmath and Murthy, 2001; Mahendran and Bai, 2009; Baker *et al*., 2014; Kaviani *et al*., 2017). In *Dendrobium huoshanense*, Kn was reported to be more effective for plantlet regeneration from PLBs than other cytokinins (Luo *et al*., 2009). Kn was also used for shoot multiplication of

Table 4 - Mean comparison of the effect of different concentrations of Kn and IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

PGRs (mg l^{-1})		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Callus number	Viability percentage
IBA	Kn							
0.00	0.00	3.30±0.896 e-h	2.30±0.529 ef	1.40±0.462 d	4.50±0.802 c-f	3.46±0.436 de	5.80±0.208 a-c	90.00±15.275 a
0.00	0.50	3.10±0.493 e-h	2.56cdef ± 0.656	1.60±0.451 d	3.90±0.529 f	3.20±1.153 e	4.56±1.422 bc	73.30±10.000 a-c
0.00	1.00	3.40±0.666 d-g	2.73±0.802 cdef	2.20±0.608 a-d	4.83±1.464 b-f	4.16±1.210 c-e	5.66±0.451 a-c	80.00±10.000 a-c
0.00	2.00	3.43±0.473 d-g	2.33±0.361 def	1.90±0.737 bcd	4.90±0.917 b-f	3.73±1.069 c-e	4.43±1.779 bc	70.00±11.547 bc
0.00	3.00	3.06±1.453 e-h	2.20±1.405 ef	2.30±0.153 a-d	4.33±0.321 def	4.53±0.208 b-e	3.86±0.265 c	73.30±10.000 a-c
0.10	0.00	3.00±0.611 e-h	2.30±0.416 ef	1.50±0.321 d	5.36±0.493 b-e	5.10±0.458 a-d	3.83±0.666 c	80.00±15.275 abc
0.10	0.50	2.83±0.945 gh	3.16±1.401 cde	2.00±0.208 a-d	4.73±0.764 b-f	4.53±0.643 b-e	6.90±1.007 ab	90.00±10.000 a
0.10	1.00	3.26±1.277 e-h	2.23±0.794 ef	2.10±0.200 a-d	5.86±0.451 abc	6.13±0.436 ab	5.80±1.097 a-c	73.30±10.000 a-c
0.10	2.00	5.03±1.290 ab	3.46±0.551 bc	2.63±0.400 ab	5.76±0.208 abcd	5.13±1.168 a-d	5.20±0.889 a-c	90.00±5.774 a
0.10	3.00	2.90±1.650 fgh	3.06±0.200 cdef	2.06±0.513 a-d	4.73±0.200 b-f	4.50±0.624 b-e	4.26±1.217 bc	90.00±10.000 a
0.20	0.00	2.26±0.850 h	2.90±0.208 cdef	1.43±0.351 d	6.10±0.950 ab	4.70±0.473 b-e	3.80±1.332 c	90.00±10.000 a
0.20	0.50	3.80±0.929 b-g	3.20±0.416 cde	1.90±0.416 bcd	4.40±0.473 def	4.06±0.666 c-e	6.90±0.300 ab	80.00±10.000 a-c
0.20	1.00	3.23±0.513 e-h	2.60±0.794 cdef	2.56±0.896 abc	6.10±0.700 ab	6.66±0.709 a	5.03±1.350 bc	76.60±10.000 a-c
0.20	2.00	6.16±0.503 a	2.73±0.907 cdef	2.86±0.058 b	4.46±0.643 c-f	5.40±0.361 a-c	4.36±1.790 bc	70.00±10.000 bc
0.20	3.00	4.20±0.208 b-f	3.43±0.666 bcd	2.00±0.208 a-d	4.93±0.850 b-f	5.20±0.850 a-c	3.80±1.836 c	90.00±10.000 a
0.50	0.00	3.00±0.153 e-h	2.20±0.651 ef	1.80±0.058 bcd	5.36±0.252 bcde	4.66±0.850 b-e	6.16±1.159 a-c	76.60±10.000 ac
0.50	0.50	4.63±0.702 bcd	2.70±0.306 cdef	1.56±0.987 d	7.13±0.153 a	4.40±1.124 c-e	5.26±0.586 a-c	86.60±11.547 ab
0.50	1.00	3.73±0.436 b-g	2.80±0.737 cdef	3.13±0.603 a	4.10±0.416 ef	3.86±0.493 c-e	4.46±1.909 bc	73.30±10.000 a-c
0.50	2.00	4.93±0.814 abc	4.66±0.702 a	1.43±0.551 d	4.76±0.681 b-f	4.36±0.751 c-e	4.03±0.889 c	80.00±5.774 a-c
0.50	3.00	3.66±0.404 c-g	2.90±0.300 cdef	1.76±0.306 c	4.50±0.854 c-f	4.40±0.252 cde	5.10±0.751 a-c	90.00±10.000 a
1.00	0.00	3.06±0.569 e-h	3.00±0.300 cdef	2.06±0.231 a-d	5.00±0.971 b-f	3.96±0.862 c-e	4.13±0.751 c	80.00±15.275 a-c
1.00	0.50	3.33±1.114 d-g	2.53±1.124 cdef	1.66±0.709 cd	4.86±0.757 b-f	5.06±0.529 a-d	7.73±1.210 a	70.00±10.000 bc
1.00	1.00	3.86±0.404 b-g	1.96±0.700 f	2.06±0.265 a-d	4.96±0.451 b-f	4.86±0.850 b-e	4.90± 1.253 bc	63.30±10.000 c
1.00	2.00	4.30±0.416 bcde	2.26±0.777 ef	1.86±0.115 bcd	4.76±0.557 b-f	4.30±0.361 c-e	4.56±0.929 bc	90.00±10.000 a
1.00	3.00	4.10±0.458 b-g	4.46±0.321 ab	1.70±0.458 bcd	4.83±0.651 b-f	3.80±0.624 c-e	3.80±0.458 c	90.00±10.000 a

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

some other orchids (Saiprasad *et al*., 2004; Malabadi *et al*., 2005; Panwar *et al*., 2012). In *Satyrium nepalense*, protocorm developed multiple shoots directly on the medium supplemented with cytokinins. In most of the orchids the presence of cytokinins alone promoted optimal shoot proliferation (Mahendran and Bai, 2009). BA is known to promote seedling leaf formation in *Paphiopedilum* (Huang *et al*., 2001; Chen *et al*., 2015). Bhattacharyya *et al*. (2016) reported that when the explants were grown in medium containing cytokinin and auxin, a higher rate of response frequency of shoot buds and PLBs was observed in all PGRs combinations. Also according to Seeni and Latha (2000) and Roy *et al*. (2011), PGRs in orchids act more efficiently when used in combination. Therefore, cytokinin and auxin are supposed to act synergistically.

Effectiveness of AC on shoot multiplication and leaf growth has been demonstrated in some orchids (George and Ravishankar, 1997; Thomas and Michael, 2007; Hossain *et al*., 2010; Roy *et al*., 2011; Zeng *et al*., 2012; Panwar *et al*., 2012). Study on *Paphiopedilum wardii* evidenced that the plantlet growth *in vitro* was significantly affected by AC along with PGRs (Zeng *et al*., 2012). Roy *et al*. (2011) showed that healthy plantlets of *Vanda coerulea* were induced from PLBs when cultured on medium fortified with 3.00 g l^{-1} AC, 5.36 µM NAA and 3.80 µM BAP. In our study, there was no difference between 0.50 and 1.00 g l^{-1} AC for leaf growth (Tables 3, 4). Supplementation of AC in the media significantly influenced plantlet growth (shoot multiplication and root growth) over the control (Roy *et al*., 2011). This finding confirmed our results on the effect of AC on leaf growth parameters. In fact, a combination of 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn on medium containing 0.50 and 1.00 g l^{-1} AC was found to be suitable for maximum leaf number.

This positive effect of AC on shoot multiplication has been attributed to the ability of AC to absorb phenolic compounds released by the plantlets into the media and regulate the pH level (Pan and van Staden, 1998; Eymar *et al*., 2000). A positive linear relationship was found between AC concentration and plantlet growth of *Vanda coerulea* Griff ex. Lindl. (Blue Vanda) (Roy *et al*., 2011). In *Paphiopedilum spicerianum*, 1.0 mg l^{-1} NAA, 10% banana homogenate and 0.50 g l^{-1} AC was the most effective to promote seedling formation (Chen *et al*., 2015). AC might also act as a growth promoter that inhibits harmful effects of some compounds produced during seedling formation (Roy *et al*., 2011).

Our study showed the positive effect of IBA on root formation. In *Satyrium nepalense* and *Dendrobium nobile*, IBA resulted in a better rooting efficiency over NAA in terms of rooting frequency and root number (Mahendran and Bai, 2009; Bhattacharyya *et al*., 2016). According to these authors, maximum rooting efficiency (86% or 5.4 roots/shoot) was obtained in medium fortified with 2.00 mg l^{-1} IBA in *Dendrobium nobile* (Bhattacharyya *et al*., 2016), while the highest number of roots per shoot (6.40) was achieved at 9.84 mM IBA in *Satyrium nepalense* D. Don. (Mahendran and Bai, 2009). The effectiveness of IBA in rooting has been shown for some other orchids like *Vanilla planifolia* (Giridhar *et al*., 2001), *Cymbidium aloifolium* (L.) SW. and *Dendrobium nobile* Lindl. (Nayak *et al*., 2002), *Cymbidium pendulum* (Nongdam *et al*., 2006), *Satyrium nepalense* (Mahendran and Bai, 2009), *Vanda teres* (Firoz Alam *et al*., 2010) and *Eulophia nuda* Lindl. (Panwar *et al*., 2012). A maximum 90% response for root formation and highest number of roots (5.50) with length of 5.30 cm per shoot was obtained on IBA (2.46 mM) treated shoots of *Eulophia nuda* Lindl. (Panwar *et al*., 2012). Study of Baker *et al*. (2014) on micropropagation of *Catasetum* demonstrated that the largest number of root (7.16) and root length (193.40 mm) were obtained on MS medium supplemented with 0.50 mg l^{-1} BA along with 0.50 mg l^{-1} NAA. Our results are in line with previous findings, as maximum root length and root number were obtained in medium containing both IBA and Kn.

Our study showed the positive effect of AC on root growth. Similarly, Roy *et al*. (2011) evidenced a positive influence of AC on root growth of *Vanda coerulea*. Some other researches demonstrated that the presence of AC in the media stimulated rooting in *Vanilla planifolia* (George and Ravishankar, 1997), *Cymbidium sinense* (Chang and Chang, 2000) and *Paphiopedilum spicerianum* (Chen *et al*., 2015). Addition of AC in rooting medium maintained the pH level, increased the nitrogen uptake and stimulated the rooting of *in vitro* shoots (Eymar *et al*., 2000; Panwar *et al*., 2012).

In the present work, minimum callus formation was obtained frequently on media without IBA or Kn with or without AC. In *Eulophia nuda* and on medium containing higher concentration of BA, the explants produced callus at the base of shoots while lesser number of shoots were differentiated on medium with lower BA concentration (Panwar *et al*., 2012). Also, in medium with higher concentration of BA

combined with Kn lower shoot production with more callus induction was observed.

In conclusion, *Phalaenopsis amabilis* Blume var. Grandiflora is a scarce and near threatened orchid. Many of orchid's species and cultivars are threatened, rare, vulnerable, endangered, indeterminate or in danger of extinction. Thus, it is necessary to develop convenient methods for the conservation and large scale production of these plants to be used for re-introduction, as well as commercial propagation.

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Effects of foliar application of glycine and glutamine amino acids on growth and quality of sweet basil

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Abstract: Amino acids have diverse roles in plant metabolism, and recently amino acid based fertilizers have been used largely in crop production systems. Despite most of these new fertilizers are formulated for foliar application; however, morphophysiological responses of crops to amino acids application have not yet been well documented. In the present study, foliar application of glycine or glutamine in different concentrations of 0 (distilled water), 250, 500, 1000 mg·L^{-1}, as well as a treatment of 250 mg·L^{-1} glycine + 250 mg·L^{-1} glutamine were evaluated on growth of sweet basil (*Ocimum basilicum* L.) plants. The results showed that foliar application of glycine or glutamine at 1000 mg·L^{-1} showed no improvement in comparison with control for all traits except for leaf L-ascorbic acid concentration that showed the highest value under these amino acids treatments. However, foliar application of these amino acids at 250 and particularly 500 mg·L^{-1} showed promising effect on sweet basil growth. Plant shoot fresh and dry weight, leaf area, leaf SPAD value, and leaf chlorophyll content were improved by foliar application of 500 mg·L^{-1} glycine or glutamine in comparison with control plants. Foliar application of amino acids increased leaf nitrogen (glutamine 250 and 500 mg·L^{-1}), potassium (glycine 250 mg·L^{-1}), magnesium (glutamine 250 or 500 mg·L^{-1}, glycine 250 mg·L^{-1}, glycine + glutamine; 250 + 250 mg·L^{-1}), iron (glycine and glutamine at 500 mg·L^{-1} and glutamine at 250 mg·L^{-1}), and zinc (glycine and glutamine at 250 or 500 mg·L^{-1}), whereas the increase in leaf nutrients caused by other treatments was not significantly different from control plants. Leaf calcium concentration was not changed by amino acid treatments. The results indicate that foliar application of moderate to low concentrations of glutamine or glycine can improve sweet basil growth.

1. Introduction

Basil (*Ocimum basilicum* L.) is a high marketable value vegetable, which is consumed as a fresh aromatic ingredient and included in cooked dishes. Increasing importance is being given to this produce due to its remarkable quality and nutritional features (Morano *et al.,* 2017).

Chemical fertilizers have an inevitable role in food production of agricultural crops; however, during last decades the soil fertility and quality have been adversely affected by many fertilization strategies (Souri and Hatamian, 2019). This is mainly due to the low efficiency of applied fertilizers which can even cause yield reduction upon increasing supply (Ercolano *et al.,* 2015). In some situations and particularly under adverse climatic conditions, foliar instead of soil application (Golubkina *et al.,* 2018), or chelated forms instead of simple chemical forms of fertilizers (Garcia *et al.,* 2011; Marschner, 2012; Souri and Aslani, 2018) can improve nutrient uptake and use efficiency of applied fertilizers. Each year, agriculture production needs a large amount of expensive nitrogenous fertilizers that generally have low uptake efficiency rates and result in both plant (Caruso *et al.,* 2011) and environmental pollution (Souri and Hatamian, 2019). Amino acids are being incorporated for more than two decades into fertilizer formulations to improve fertilizers use efficiency (Abdul-Qados, 2009; Souri, 2016).

Foliar application of amino acids can have beneficial effects on plant growth and production (Sadak *et al.,* 2015; Shams *et al.,* 2016; Hussain *et al.,* 2018; Souri and Aslani, 2018). It has been shown that foliar application of arginine amino acid has improved shoot growth and grain yield of wheat in saline and non saline conditions (Abdul-Qados, 2009; Rizwan *et al.,* 2017). Better growth and higher biomass production via application of various amino acid chelates of iron or zinc has been also reported on some agronomic and horticultural crops (Khan *et al.,* 2012; El Sayed *et al.,* 2014). Similarly, foliar application of a mix of amino acids at 500 and 700 ppm increased plant height, plant fresh and dry weights, leaf N concentration, yield of leaves and leaf soluble carbohydrates in celery (Shehata *et al.,* 2011).

Glycine is the simplest amino acid and is used largely for production of chelated fertilizers in form of aminochelates (Souri, 2016), whereas effect of foliar application of glutamine has not been well documented so far. In the present study, the effect of foliar application of different concentrations of glycine and glutamine has been evaluated on growth characteristics of sweet basil plants.

2. Materials and Methods

This study was conducted under greenhouse conditions and from 20 May till the end of July 2017. The soil used in experiment had a loamy texture and moderate levels of nutrients. The soil physiochemical characteristics are presented in Table 1. 4 L black plastic pots (30 cm height and 20 cm diameter) were filled with about 4 kg dry soil and watered at 80% of its soil field capacity (FC). Three days later 40 seeds of sweet basil (*Ocimum basilicum* L.) from a local population (Karaj landrace) were sown in 1 cm depth of the soil. Two weeks after germination plants were reduced to 10 uniform seedlings per pot. The plants were irrigated daily based on 80% soil field capacity. The temperature was on average 28±5°C, the light intensity 200 $\mu mole.m^{-2}.s^{-1}$, and the air humidity about 70-75%.

Different concentrations of two amino acids of glycine and glutamine including 0, 250, 500 and 1000 $mg{\cdot}L^{-1}$, as well as a treatment of 250 $mg{\cdot}L^{-1}$ glycine + 250 $mg{\cdot}L^{-1}$ glutamine were sprayed on sweet basil plants. Treatments and pots were arranged in completely randomized design and each treatment contained three replications. Each pot represented one replication and contained 10 plants. Each concentration of glycine or glutamine was sprayed on plant leaves five times during two-month growth period, and the first spray was done two weeks after seedling emergence. The remaining sprays were done in one week interval. Distilled water was sprayed on control plants. Spray treatments were done in the early morning, one hour after sunrise and with a portable sprayer by which the upper and lower surface of leaves were sprayed. A total amount of 80-90 mL solution of each treatment was sprayed on plants per pot.

Plants were harvested two months after emergence and before flowering stage. The average height of ten plants in each pot was measured by a ruler before harvest. The leaf SPAD value was measured by a portable SPAD meter (Model SPAD-502

Table 1 - Physico-chemical characteristics of soil used for the experiment

Texture	EC ($dS.m^{-1}$)	pH	O.C. (%)	Total N ($mg.kg^{-1}$)	Available P ($mg.kg^{-1}$)	Available K ($mg.kg^{-1}$)	Fe ($mg.kg^{-1}$)	Zn ($mg.kg^{-1}$)	Lime (%)
Loamy-Sand	1.79	7.62	0.78	750	11.61	385	5.5	1.6	7.3

Plus Illinois, USA) performing 30 readings (on middle part of leaves) per pot. The plant leaf area of three randomly selected plants per pot was measured by leaf area meter (Delta-T Devices Ltd, England). Plants were cut at soil surface and shoot fresh weight was measured by a precise digital scale. Fresh shoots of each pot were transferred to an oven at 65°C for 48 h, and thereafter plant dry weight was measured accordingly. Leaf chlorophyll and carotenoids were determined using acetone extraction of 0.5 g fresh leaves following the methods of Khan *et al.* (2012) and Kałużewicz *et al*. (2018). For determination of leaf L-ascorbic acid (vitamin C), 5 g of fresh leaf tissue were crushed in a porcelain mortar in 10 mL metaphosphoric acid 6%, and then the juice was transferred into a 25 mL tube and centrifuged at 4000 rpm for 10 min. 5 mL of the supernatant was transferred into an Erlenmeyer flask, and added with 20 mL of metaphosphoric acid 3%. The extract was then titrated using di-chloro phenol indophenol reagent until appearance of a rosa color. The amount of vitamin C (mg 100 g^{-1} FW) was calculated according to a standard curve of L-ascorbic acid concentrations of 0, 25, 50, 2100 and 200 mg·L^{-1} following Souri and Aslani (2018). Nitrogen concentration of leaves was determined by the Kjeldahl method, K using flame photometry, Mg, Ca, Fe and Zn by atomic absorption spectrophotometer.

Data were analyzed by analysis of variance using SPSS 16 and comparison of means was performed using LSD test at P≤0.05 probability level.

3. Results and Discussion

In the present study a soil with moderate levels of nutrient elements was used and no further application of chemical fertilizers was applied. The results showed that plant height was significantly increased by foliar application of 250 mg·L^{-1} glutamine compared to control plants (Table 2). Application of both amino acids of glycine and glutamine significantly increased the leaf concentration of chlorophyll a (at 250 or 500 mg·L^{-1}) and chlorophyll b (at 500 mg·L^{-1}) compared to control plants (Table 2). Total chlorophyll concentration in leaves was significantly increased than control by glutamine or glycine at 500 mg·L^{-1} or glutamine at 250 mg·L^{-1} (Table 2). Leaf carotenoids concentration increased and decreased upon foliar application of 1000 mg·L^{-1} glycine and 1000 mg·L^{-1} glutamine compared to control plants, respectively (Table 2). The plant leaf SPAD value (Fig. 1) was not significantly changed under different concentrations of glutamine or glycine compared to control plants. However, foliar application of 1000 mg·L^{-1} glycine reduced leaf SPAD value of plants compared to some amino acid treatments (Fig. 1). This could be due to damage of high glycine concentration to basil leaves, as the same finding was also reported by

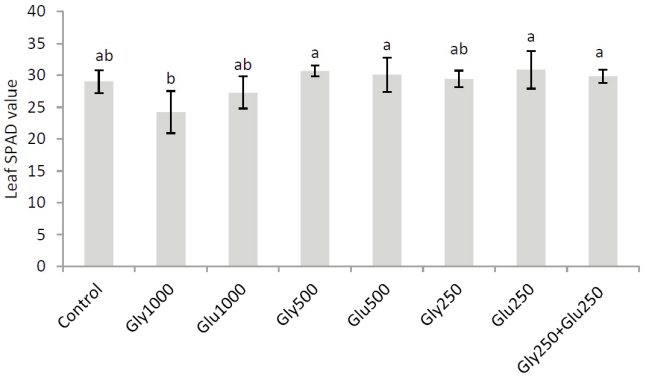

Fig. 1 - Leaf SPAD value of sweet basil plants under foliar application of glycine or glutamine amino acids. Comparison of means was performed at P≤0.05 probability level of LSD test.

Table 2 - Plant height and leaf pigments concentration of sweet basil plants under foliar application of glycine and glutamine amino acids

Treatments	Plant height (cm)	Chl a (mg.g^{-1} FW)	Chl (mg.g^{-1} FW)	Total Chl (mg.g^{-1} FW)	Carotenoids (mg.g^{-1} FW)
Control (distilled water)	46±5 bc	10.5±1.4 d	5.6±0.8 b	16.1±1.3 bc	1.7±0.3 b
Gly1000	44±4 c	11.9±3.2 cd	6.0±1 b	18.0±4.1 bc	2.3±0.1 a
Glu1000	43±10 c	10.3±1 d	5.2±0.9 b	15.5±1.4 c	1.2±0.1 c
Gly500	55±15 abc	18.3±1.3 ab	9.0±2 a	27.3±3.4 a	1.5±0.2 bc
Glu500	56±10 ab	19±2.4 a	9.2±1.3 a	28.2±3.6 a	1.4±0.1 bc
Gly250	53±11 abc	17.4±0.7 ab	7.8±0.9 ab	25.2±1.4 a	1.6±0.5 b
Glu250	62±13 a	15.2±2.7 bc	5.0±0.9 b	20.2±3.6 b	1.4±0.3 bc
Gly250+Glu250	50±30 b	13.1±0.5 c	5.2±3.7 b	18.3±3.6 bc	1.5±0.3 bc

- 250, 500 and 1000 indicates their concentration as mg·L^{-1}.
- Comparison of means was performed at P≤0.05 probability level of LSD test.

Fahimi *et al*. (2016) on cucumber plants. The increase in plant height and leaf greenness can be due to beneficial effects of glycine and particularly glutamine on leaf pigmentation (Sadak *et al.,* 2015; Fahimi *et al.,* 2016). Amino acids can have stimulation effect on plants growth, and in this respect, it has been shown that application of amino acids can enhance chlorophyll content and leaf greenness of plants (Kielland, 1994; Souri *et al.,* 2017; Kałużewicz *et al.,* 2018), probably due to higher protein biosynthesis and reduction in chlorophyll degradation rates (Rainbird *et al.,* 1984; Souri and Hatamian, 2019). Better nutrients use efficiency and their enhanced translocation play also an important role in this regard (Marschner, 2012). Application of aminolevulinic acid (ALA), as a common precursor to tetrapyrrole compounds found in chlorophyll and hemes, improved spinach plant growth and increased the concentration of photosynthetic pigments (Smoleń *et al.,* 2010).

In the present study, plant leaf area (Fig. 2) was increased by foliar application of both glycine and glutamine at 500 mg·L^{-1} compared to control plants. Both glycine and glutamine at either concentration of 250 or 500 mg·L^{-1} increased shoot fresh weight (Fig. 3); whereas plant shoots dry weight was increased only under foliar application of 500 mg·L^{-1} glycine or glutamine compared to control plants. Leaf area expansion is a function of interacting various external and internal factors that results in increased cell division or particularly cell enlargement. Various amino acids like glutamine and glycine are required components for normal cell growth and expansion (Marschner, 2012; Souri, 2016). The increase in plant biomass caused by foliar application of moderate levels of glutamine or glycine could be due to the latter stimulation effects on plant growth. The beneficial effects of foliar application of amino acids on plant growth, yield and quality of the product has been shown for some crops (Keutgen and Pawelzik, 2008; Amin *et al.,* 2011; Khan *et al.,* 2012; Sadak *et al.,* 2015). In addition, amino acids can enhance nutrient uptake and act as precursors for certain plant hormones (Marschner, 2012; Souri and Hatamian, 2019). Foliar application of different concentrations of a mix 17 different amino acids after 45 and 60 days from sowing showed promising effects on bean plant growth and productivity under salinity conditions (Sadak *et al.,* 2015). A similar effect was also obtained in bean plants under field conditions (Souri and Aslani, 2018). Foliar application of zinc-lysine significantly increased the photosynthesis, grain yield, enzyme activities and Zn contents in different wheat plant tissues (Rizwan *et al.,* 2017). Multiple sprays of a mix of amino acids (0.5 mL·L^{-1}) at different growth stages of grapevines particularly at flowering stage, increased growth characteristics and various components of fruit yield and quality compared to control (Khan *et al.,* 2012).

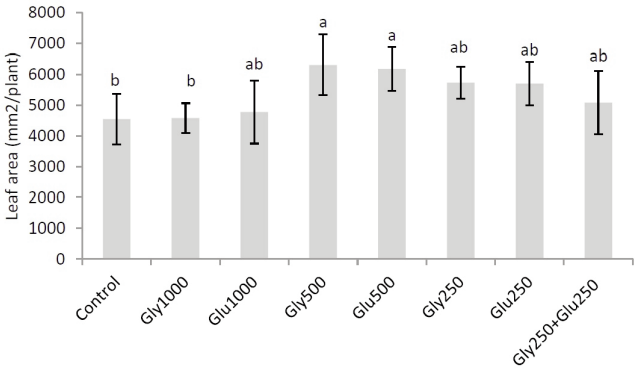

Fig. 2 - Plant leaf area of sweet basil under foliar application of glycine or glutamine amino acids. Comparison of means was performed at P≤0.05 probability level of LSD test.

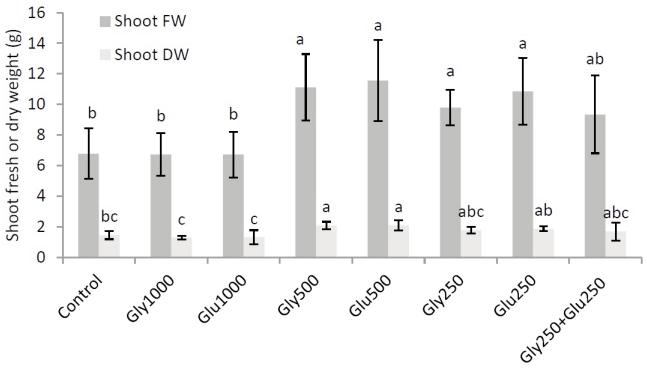

Fig. 3 - Shoot fresh and dry weights of sweet basil plants under foliar application of glycine or glutamine amino acids. Comparison of means was performed at P≤0.05 probability level of LSD test.

Leaf L-ascorbic acid (vitamin C) was not significantly affected by foliar application of glycine or glutamine compared to control plants; however, the highest leaf ascorbic acid content was recorded in plants treated with 1000 mg·L^{-1} glycine or glutamine (Fig. 4). Foliar application of amino acids significantly increased leaf nutrient concentration (except calcium) under some specific concentrations of glycine or glutamine (Table 3). Leaf nitrogen was significantly increased by glutamine spray either at 250 or 500 mg·L^{-1}, whereas leaf potassium concentration was significantly increased by glycine at 250 mg·L^{-1} compared to control plants. Foliar application of glutamine either at 250 or 500 mg·L^{-1}, and foliar application of glycine at 250 mg·L^{-1} significantly increased

leaf magnesium concentration compared to control plants. Leaf iron concentration was significantly increased by foliar application of glutamine either at 250 or 500 mg·L^{-1}, or by foliar application of glycine at 500 mg·L^{-1} compared to control plants. Similarly, leaf zinc concentration was significantly increased by foliar application of glycine or glutamine either at 250 or 500 mg·L^{-1} compared to control plants (Table 3).

Foliar spray of 250-500 mg·L^{-1} of both amino acids significantly increased leaf nutrient concentrations. The most significant effect of amino acids on plants is reportedly the increased plant nutrient uptake (Koksal *et al.,* 1999; Abdul-Qados, 2009; Garcia *et al.,* 2011). Various amino acids can act as ligand for nutrients particularly micronutrient metal ions, so they can protect metal nutrients from harmful reactions (Souri and Hatamian, 2019). The structure of amino acids allows the molecule to act as acid or base, depending on medium pH, thus helping to improve the fertility management of soils particularly under adverse climatic conditions. Moreover, amino acids or their fertilizer products can be considered as "semi-intelligent fertilizers" (Souri, 2016). They are natural ligands with a friendlier effect on environmental issues and best candidates to partially replace fertilizer application, and to relief chemical fertilizers pressure in many cropping systems.

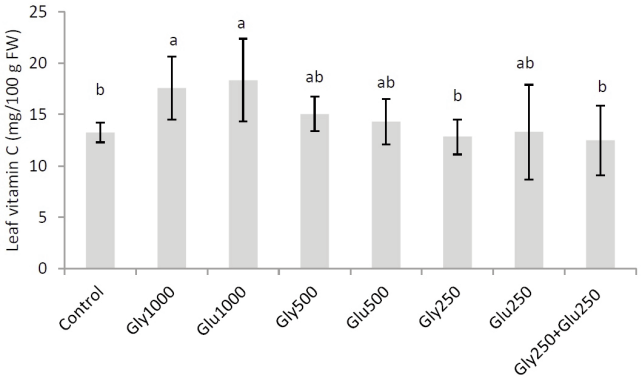

Fig. 4 - Leaf L-ascorbic acid (vitamin C) concentration of sweet basil plants under foliar application of glycine or glutamine amino acids. Comparison of means was performed at P≤0.05 probability level of LSD test.

Amino acids are also a preferential source of nitrogen for plant nutrition. It has been shown that partial replacement of nitrate by amino acid application can have beneficial effects on plant growth and production (Marschner, 2012; Sadak *et al.,* 2015; Souri *et al.,* 2017). Amino acids are the intermediate compounds in nitrogen assimilation and play key roles in plant cell metabolism, as they are the main form of nitrogen translocation through phloem to growing parts (Marschner, 2012; Kolota *et al.,* 2013). Nitrate in the soil is very vulnerable to leaching and gaseous emissions (Kolota *et al.,* 2013); whereas the mandatory application of reduced forms of nitrogen under organic farming (Caruso *et al.,* 2012) or the advisable supply of ammonium and amino acids can prevent these effects and reduce nitrate accumulation in plant tissues (Cao *et al.,* 2010; Souri *et al.,* 2017). Total concentration of amino acids and leaf protein concentration significantly increase by foliar application of single or a mix of amino acids (Marschner, 2012; Sadak *et al.,* 2015). Moreover, it has been shown that foliar or soil application of amino acids or their chelated nutrients can improve quality parameters of plants (Koksal *et al.,* 1999; Keutgen and Pawelzik, 2008; Shaheen *et al.,* 2010; Smoleń *et al.,* 2010; El Sayed *et al.,* 2014; Souri *et al.,* 2017). Application of glutamic acid enhanced some quality parameters of Chinese chive plants (Cao *et al.,* 2010). Similarly, foliar spray of proline and tryptophan enhanced growth, yield and fruit quality and

Table 3 - Leaf nutrient concentrations of sweet basil plants under foliar application of glycine and glutamine amino acids

Treatments	N (% DW)	K (% DW)	Mg (% DW)	Ca (% DW)	Fe (mg.kg^{-1} DW)	Zn (mg.kg^{-1} DW)
Control (distilled water)	2.1±0.2 b	1.5±0.2 b	0.21±0.04 d	1.5±0.2 ab	49±6 c	33±3 c
Gly1000	2.13±0.4 b	1.6±0.1 ab	0.19±0.03 d	1.3±0.4 b	45±7 c	33±2 c
Glu1000	2.4±0.3 ab	1.5±0.3 b	0.23±0.04 cd	1.5±0.2 ab	52±10 bc	35±2 bc
Gly500	2.3±0.4 ab	1.7±0.2 ab	0.27±0.04 bcd	1.6±0.2 ab	61±10 ab	40±4 ab
Glu500	2.7±0.5 a	1.8±0.4 ab	0.36±0.06 a	1.9±0.5 a	66±8 a	42±6 a
Gly250	2.5±0.2 ab	1.9±0.3 a	0.29±0.05 abc	1.7±0.2 ab	53±6 abc	41±4 ab
Glu250	2.6±0.3 a	1.8±0.3 ab	0.33±0.04 ab	1.7±0.3 ab	62±5 ab	42±5 a
Gly250+Glu250	2.5±0.3 ab	1.7±0.1 ab	0.30±0.06 abc	1.8±0.1 a	57±9 abc	38±6 abc

- 250, 500 and 1000 indicates their concentration as mg·L^{-1}.
- Comparison of means was performed at P≤0.05 probability level of LSD test.

minimized cracked fruit percentage of pomegranates (El Sayed *et al.,* 2014). It has also been shown that under heavy metal pollution of soil, application of amino acids can reduce the heavy metal content of plant tissues (Bashir *et al.,* 2018).

In the present study, foliar application of glutamine resulted in better growth traits than glycine spraying. This effect can be mainly due to the well-known role of glutamine in nitrogen assimilation and plant metabolism (Marschner, 2012). Enhancement of glutamine synthetase has been shown to reduce the severity of ammonium toxicity in tomato plants (Forde and Clarkson, 1999; Marschner, 2012). In addition to the amino and carboxyl groups, amino acids have a side chain or R group attached to the α-carbon. Each amino acid has unique characteristics arising from the size, shape, solubility, and ionization properties of its R group. As a result, the side chains of amino acids exert a profound effect on the structure and biological activity of proteins (Souri, 2016; Souri and Hatamian, 2019). Among various amino acids used as foliar spray on bean plants, it was found that glutamic acids and arginine were the most effective on plant growth biostimulation (Sadak *et al.,* 2015).

It has been revealed that amino acids are effective components in nutrient use efficiency as well as in detoxification of toxins and heavy metals in plants that can significantly increase their antioxidant capacity and tolerance to stressful conditions. For many decades, soil fertility and quality was adversely affected by continues application of chemical fertilizers and adverse climatic conditions. Fertilization strategies have actively contributed in enhancing soil salinity that nowadays is a global challenge in many countries. Biostimulation effect of amino acids on nutrient uptake and plant growth can reduce fertilizer application rates and detrimental effects of fertilization on environment and food quality. However, in the present study low to moderate levels of glycine or glutamine (250-500 mg·L^{-1}) had beneficial effects on sweet basil growth, while both amino acids at 1000 mg·L^{-1} were less or not effective on plant growth characteristics.

4. Conclusions

In the present study, the growth of sweet basil plants was improved by foliar application of 250 or 500 mg·L^{-1} of glycine or glutamine amino acids, whereas application of 1000 mg·L^{-1} of these two amino acids resulted in no difference from control plants. There were significant better effect on plant height, leaf SPAD value, leaf area, shoot fresh and dry weights, and nutrient uptake with foliar application of glycine and particularly glutamine at 500 mg·L^{-1}. Leaf vitamin C was highest in plants treated with 1000 mg·L^{-1} amino acid spray. Foliar application of glutamine was more effective than glycine on many growth traits and nutrients uptake. Our results suggest that amino acids are effective to improve the nutrient uptake and accordingly the plant growth, yield and quality under the view of a modern and sustainable agriculture.

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AHS
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Plant regeneration by organogenesis from bulbous explants in *Fritillaria imperialis* L., a wild rare ornamental species at the risk of extinction

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Key words: crown imperial, micropropagation, plant growth regulators, threatened ornamentals, tissue culture.

Abstract: *Fritillaria imperialis* L. (Liliaceae) is a rare and endangered ornamental plant grown in mountain regions and Zagros altitudes, Ilam province, Iran. This species is in danger of extinction due to invasive collection. Plant regeneration was done by organogenesis from bulb scales as explants cultured on Murashige and Skoog (MS) media fortified with different concentrations of kinetin (KIN, 0.00, 0.50, 1.00 and 2.00 mg l^{-1}) and α-naphthaleneacetic acid (NAA, 0.00, 0.50, 1.00 and 2.00 mg l^{-1}), either individually or in combination. The largest number of leaf (3.80), root (5.86) and callus (8.16) per explant was regenerated on the medium containing 0.50 mg l^{-1} KIN and 1.00 mg l^{-1} NAA. Maximum viability percentage (96.66%) was obtained in medium supplemented with 1.00 mg l^{-1} KIN. *In vitro* regenerated plantlets were cultivated in plastic pots containing peat moss and perlite (1:1). The plantlets were successfully acclimatized in an adaptation greenhouse with a survival rate of 95% exhibiting normal developmental patterns.

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

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1. Introduction

Fritillaria (Liliaceae) is a genus of about 100 species of bulbous perennials found in a range of habitats, from woodland to open meadows and high screes, distributed throughout the temperate regions of the N. hemisphere, particularly the Mediterranean, S.W. Asia and W. North America. Each bulb of *Fritillaria* has 2 or more scales, and sometime abundant basal bulblets. *Fritillaria imperialis* L. (crown imperial or tears of Mary) is a perennial plant with high medicinal and ornamental importance (Wang *et al*., 2005). The 14 important species of *F. imperialis* L. are native to Iran (De Hertogh and Le Nard, 1993). Wild populations of *F. imperialis* are mostly found in high altitudes (>2,000 m) of western parts of Iran, particularly in three provinces, Chahar Mahal-va-Bakhtiari, Kohkyluyeh-va-Bouyrahmad and Ilam. The leaves are usually lance-shaped or linear and the flowers, borne in spring or early summer, are usually pendulous and solitary, or in terminal racemes or umbels, and have 6 tepals. *F. imperialis*

L. has been used either as pot plant for designing landscape or cut flower. Thanks to attractive red and yellow flowers, this plant reveals a great commercial potential (De Hertogh and Le Nard, 1993). The bulbs of *F. imperialis* contain alkaloids, non-alkaloid and high starch content (Li *et al*., 2000; Wang *et al*., 2005).

In Iran, wild populations of two important species, *F. imperialis* and *F. persica*, are at the risk of extinction, because of many harvesting, lack of protecting rules, changing the pastures to dry farmlands, and pest and pathogens invasions. The natural proliferation rate of *Fritillaria* is relatively low that hampers the large-scale cultivation of this plant. *F. imperialis* cannot efficiently propagate by traditional methods such as cutting, bulb scale and seed, because of small numbers of scales (3-5) per bulb and restricted amount of meristematic cells (De Hertogh and Le Nard, 1993). Plant proliferation takes 5-7 years through the seed, also seeds have physiological dormancy. In addition, seedlings are weak, survival rate and growth are low, and produced plants by seed are not true-to-type; because of cross-pollinate nature of *Fritillaria* (De Hertogh and Le Nard, 1993; Baskin and Baskin, 2004). Limited availability of bulblets from nature is another problem. These limitations suggest the need to develop alternative propagation methods for commercial production of these elite species.

The application of biotechnology especially *in vitro* proliferation is a suitable method for reproduction of rare and endangered species with difficult propagation and mass production of valuable genotypes (Vetchinkina *et al*., 2012). This method is becoming increasingly important for conservation of rare and endangered plant species (Almeida *et al*., 2005). *In vitro* propagation is an effectively alternative means for rapid multiplication of species, in which conventional methods have problems and limitations. Tissue culture using bulb scale segments, the most commonly used explants in tissue culture of bulbous plants including Liliaceae family (Mirici *et al*., 2005), and other explants such as foliar and flower explants has been reported for some cultivars of *F. imperialis* (Paek and Murthy, 2002).

Two basic morphogenetic ways leading to the regeneration of the whole plant from somatic tissues are organogenesis and embryogenesis. Both ways of morphogenesis can occur as direct (without passing callus phase) and indirect (with passing callus phase). The wild population of *F. imperialis* is highly heterozygote and non-uniform, because of its self-incompatibility nature. There are a few reports on direct and indirect organogenesis in *F. imperialis* (Witomska and Lukaszewska, 1997; Witomska, 2000; Paek and Murthy, 2002; Subotić *et al*., 2010; Kizil and Khawar, 2014). This paper describes a protocol for rapid *in vitro* multiplication of *F. imperialis* L. by bulb scales as explant and KIN and NAA as plant growth regulators (PGRs) that could be helpful for large-scale production for field culture.

2. Materials and Methods

The bulbs of *Fritillaria imperialis* L. were harvested from natural habitat (mountain regions and Zagros altitudes, Ilam province, Iran) (Figs. 1, 2A) and used as the starting material for the establishment of *in vitro* culture. Bulbs were transferred to the Plant Tissue Culture and Biotechnology Laboratory, Amol, Mazandaran Province located in the northern part of Iran. In laboratory, bulbs (Fig. 2B) were washed under running tap water (Fig. 2C) for half an hour to remove mud and dirt. The bulbs were put into a vessel filled with water and a few drops of dish-washing liquid for 10 min (Fig. 2D). Then, these were washed in running tap water for half an hour, again. Cleaned bulbs were decontaminated with a fungicide (0.10 mg l^{-1} benomile + carbendazim, rural T.S.) for 20 min followed by once washing for 10 min. in distilled water. The bulbs were sterilized by immersing in 0.01 g l^{-1} mercuric chloride (HgCl) for 15 min with continuous stirring using magnetic stirrer, then by 20% sodium hypochlorite (NaOCl) solution (commercial bleach) for 15 min followed by three times rinsing (each for 10 min.) in sterile distilled water and finally

Fig. 1 - *Fritillaria imperialis* L. growing at its natural habitat (mountain regions and Zagros altitudes, Ilam province, Iran).

the bulbs were dipped in 70% ethanol for 60 sec. The scales were rinsed in sterile distilled water for three times.

Dried bulbs (by placing on paper for 5 min.) were vertically cut into 10 × 10 mm under aseptic conditions to obtain twin scales as explants to induce callus and shoots for *in vitro* propagation (Fig. 2E). Outer scales directly in contact with disinfectant during sterilization were removed before obtaining double scale explants (Fig. 2F).

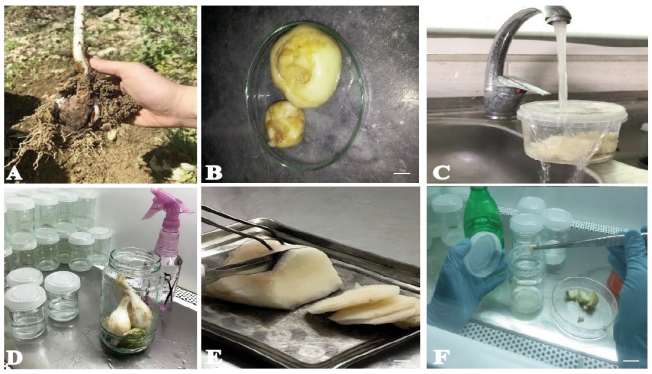

Fig. 2 - Sterilization of bulbs and preparation of scales explants. A) *Fritillaria imperialis* L. eradicated from the soil. B) Separated bulbs. C) Washing the bulbs under running tap water. D) Bulbs ready for sterilization. E) To cut cleaned bulbs as slices (scales). F) Cultivation of scales as explant into the culture vessels (scale bar = 10 mm).

The medium used was MS (Murashige and Skoog, 1962) with 3.00% sucrose and 0.80% Agar-agar. The pH of the medium was adjusted to 5.80. All media were autoclaved at 104.00 kPa and 121°C for 20 min. The scales were cultured on media fortified with NAA and KIN (0.00, 0.50, 1.00 and 2.00 mg l^{-1} from each) to induce shoots from bulb scales. Callus was formed on bulb scales cultured in media containing PGRs. *In vitro* regenerated shoots were grown in these media to find the suitable regeneration potential for shoot and root production. All experiments were performed in 280 ml-jam glasses (6 cm diameter × 10 cm height) and each glass contained 50 ml medium. All cultures were incubated at 24-26°C, 70-80% RH under light intensity of 50 µmol m^{-2} s^{-1} from white fluorescent lamps with 16 h light photoperiod per day. Data were recorded after 65 days of culturing. The parameters: leaf length, leaf diameter, leaf number, root length, root number, callus number and viability percentage were assessed.

In vitro produced plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove adherent nutrient and transferred to plastic pots containing peat moss and perlite (1:1). All the pots were then transferred to the adaptation greenhouse with temperature of 24±2°C to 20±2°C day/night, light intensity of 8000 Lux, RH of 80-85% and 14-h photoperiod for acclimatization. After 30 days all plantlets were acclimatized.

The experiments were carried out in a completely randomized design with three replicates per treatment and four scales per replicate (totally; 192 explants). PGRs-free MS medium is used as control in the experiments. The results are expressed as mean ± SD of the experiments. Data pertaining to plantlets growth and development were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at $P < 0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

3. Results

The twin bulb scale explants (10×10 mm) of *F. imperialis* L. cultured on MS media containing different concentrations of KIN and NAA showed variation in the frequency of callus formation, shoot regeneration and root formation (Fig. 3, Tables 1-4).

Leaf induction and proliferation

Callus was induced on bulb scales (Fig. 3A). Leaves were produced from callus through indirect organogenesis (Figs. 3B, C). The minimum leaf length (1.26 cm) from twin scale explants was measured on MS

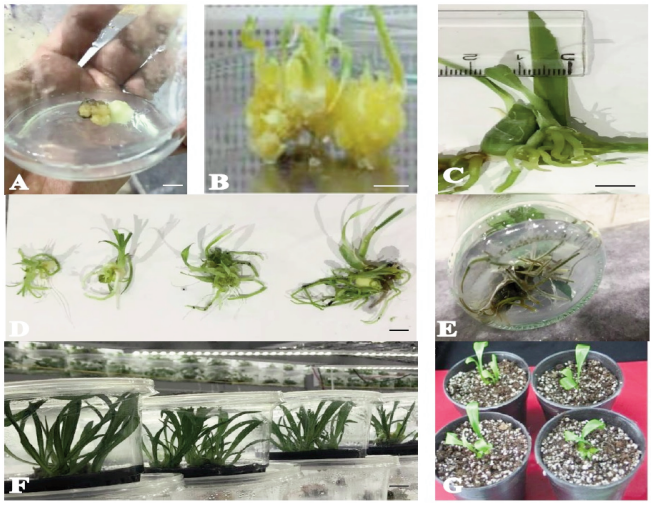

Fig. 3 - Micropropagation process of *Fritillaria imperialis* L. A) Callus production on bulb scales. B) Leaves produced from callus by indirect organogenesis. C) Developed leaves before planting in greenhouse condition. D) Regenerated plantlets from callus on media containing different concentrations of KIN and NAA. E) Rooted plantlets. F) Regenerated leaves in media supplemented with different concentrations of KIN and NAA. G) Acclimatization of plantlets grown into the pots filled with peat moss and perlite (1:1) in an adaptation greenhouse (scale bar = 10 mm).

medium without PGRs (Table 4). The maximum number of 3.16 leaves per explant on twin scale explants was measured on MS medium containing 1.00 mg l^{-1} KIN and 0.50 mg l^{-1} NAA (Figs. 3D, F). Differences of leaf length in samples grown under different concentrations of KIN and KIN in combination with NAA were significant (p≤0.01) (Table 1). No significant difference was seen between NAA and leaf length. There was no any positive effect between increasing leaf length and increasing KIN and NAA concentrations (Tables 2-4). Among all concentrations of KIN used singularly, maximum and minimum leaf length (2.41 and 1.80 cm, respectively) was induced in bulbs treated with 1.00 mg l^{-1} and control (Table 2). On the other hand, among all concentrations of NAA used as singular PGR, maximum and minimum leaf length (2.24 and 1.85 cm, respectively) was induced in bulbs treated with 2.00 and 1.00 mg l^{-1} (Table 3). There was no statistically significant difference among different concentrations of KIN + NAA and leaf diameter (Table 1).

The data clearly show that leaf number is strongly affected by treatments of KIN (p≤0.01), NAA (p≤0.01) and KIN + NAA (p≤0.01) (Table 1). The largest number of leaf (3.80 per explant) was calculated in bulbs grown on medium enriched with 0.50 mg l^{-1} KIN along with 1.00 mg l^{-1} NAA (Table 4; Figs. 3D, F). The smallest number of leaf (1.66 per explant) was obtained in bulbs grown on medium with 2.00 mg l^{-1} KIN along with 1.00 mg l^{-1} NAA. All treatments containing 2.00 mg l^{-1} KIN in combination with all concentrations of NAA produced less than 2.00 leaves per explant (Table 4). Of all concentrations of KIN, the largest and smallest number of leaf (3.33 and 1.85, respectively) was induced in explants grown on medium enriched with 0.50 and 2.00 mg l^{-1} (Table 2). Also, differences in leaf number between all concentrations of NAA were not noticeable.

Root induction and growth

The maximum average of the root length per

Table 1 - Analysis of variance of the effect of different concentrations of KIN and NAA on measured characters of *Fritillaria imperialis* L.

Source of variations	df	Mean of Squares						
		Leaf length	Leaf diameter	Leaf number	Root length	Root number	Callus number	Viability percentage
KIN	3	0.877 **	0.020 NS	4.580 **	4.820 **	4.201 **	7.440 **	413.80 **
NAA	3	0.379 NS	0.041 *	0.690 **	1.023 **	1.397 NS	2.087 *	113.80 NS
KIN × NAA	9	0.686 **	0.030 NS	0.356 **	1.059 **	1.815 **	5.270 **	67.50 NS
Error	32	0.216	0.014	0.099	0.226	0.554	0.636	50.00
cv (%)		22.40	15.80	12.10	12.90	18.03	18.00	8.35

*, ** = Significant at the 0.05 and 0.01 probability level, respectively, NS= Not significant at p=0.05.

Table 2 - Mean comparison of the effect of different concentrations of KIN on measured characters of *Fritillaria imperialis* L.

KIN (mg l^{-1})	Leaf length (cm)	Leaf diameter (mm)	Leaf number	Root length (cm)	Root number	Callus number	Viability (%)
0.00	1.800 b	0.7666 a	2.750 b	2.833 c	3.483 b	3.491 b	78.33 b
0.50	1.933 b	0.8083 a	3.333 a	3.700 b	4.866 a	5.266 a	89.16 a
1.00	2.416 a	0.7083 a	2.450 c	4.375 a	4.341 a	4.858 a	90.00 a
2.00	2.158 ab	0.7666 a	1.850 d	3.766 b	3.616 b	4.116 b	80.83 b

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

Table 3 - Mean comparison of the effect of different concentrations of NAA on measured characters of *Fritillaria imperialis* L.

NAA (mg l^{-1})	Leaf length (cm)	Leaf diameter (mm)	Leaf number	Root length (cm)	Root number	Callus number	Viability (%)
0.00	2.008 a	0.750 ab	2.80 a	3.266 b	3.74 b	4.458 ab	85.00 ab
0.50	2.200 a	0.700 b	2.31 b	3.791 a	4.33 ab	4.333 ab	80.83 b
1.00	1.858 a	0.758 ab	2.79 a	3.950 a	4.48 a	4.975 a	84.16 ab
2.00	2.241 a	0.841 a	2.47 b	3.666 a	3.95 ab	3.966 b	88.33 a

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

explant (5.50 cm) was calculated with treatment of 1.00 mg l^{-1} KIN and 1.00 mg l^{-1} NAA (Table 4). The minimum average of the root length per explant (2.30 cm) was measured with treatment without KIN and NAA (control). Concerning the root length induced by various concentrations of KIN, the maximum and minimum length (4.37 and 2.83 cm) was obtained in media containing 1.00 mg l^{-1} and control, respectively (Table 2). Concerning the root length induced by various concentrations of NAA, the maximum and minimum length (3.95 and 3.26 cm) was obtained in media containing 1.00 mg l^{-1} and control, respectively (Table 3). There was statistically significant difference among different concentrations of KIN, NAA also KIN in combination with NAA and root length ($p \leq 0.01$).

KIN in combination with NAA was superior in induction of root and on the medium fortified with KIN (0.50 and 1.00 mg l^{-1}) + NAA (0.50 and 1.00 mg l^{-1}) optimal of over than 5 roots were produced per explant on MS medium (Table 4). The largest numbers of roots (5.86 per explant) was formed in MS medium supplemented with 0.50 mg l^{-1} KIN plus 1.00 mg l^{-1} NAA (Table 4; Fig. 3E). Bulbs scales cultured on MS medium without any PGRs produced least roots (2.83). The root number (5.40 and 5.00 per explant) in media containing 0.50 mg l^{-1} KIN plus 0.50 mg l^{-1} NAA and 1.00 mg l^{-1} KIN plus 0.50 mg l^{-1} NAA was proper (Table 4). Concerning the root number produced using various levels of KIN and NAA, each one as singularly; the largest and smallest number (4.86 and 3.48) was counted in media fortified with 0.50 mg l^{-1} KIN and control, respectively (Tables 2, 3). Analysis of variance (ANOVA) test demonstrated that concentrations of KIN and KIN in combination with NAA were significant with respect to number of roots produced from scale sections (Table 1).

Callus induction

Current investigation demonstrated variable frequency of callus induction on bulbs scales at various concentrations of KIN and NAA in the culture medium. Callus was emerged after 30 days of culture (Fig. 3A). Callus formation started at the margins of bulbs scales. Callus induction was evident in response to the presence of both KIN and NAA. Maximum (8.16) and minimum (3.00) callus number per explant was observed on explants cultured on medium supplemented with 0.50 mg l^{-1} KIN plus 1.00 mg l^{-1} NAA and medium without PGRs (control), respectively (Table 4).

Viability percentage

Viability percentage of the bulbs scales in the media was changed significantly with the use of different PGRs (Table 4). Viability percentage of the bulbs scales in MS medium supplemented with 1.00 mg l^{-1} KIN without NAA (96.66%) was the maximum. Viability percentage in medium supplemented with 1.00 mg l^{-1} NAA without KIN (73.33%) was the minimum (Table 4). Statistically significant differences were recovered between the means for viability percentage and KIN (Table 1).

Table 4 - Mean comparison of the effect of different concentrations of KIN and NAA on measured characters of *Fritillaria imperialis* L.

KIN (mg l^{-1})	NAA (mg l^{-1})	Leaf length (cm)	Leaf diameter (mm)	Leaf number	Root length (cm)	Root number	Callus number	Viability (%)
0.00	0.00	1.266 ±0.208 d	0.733±0.0289 abcd	2.700±0.000 cd	2.300± 0.200 f	2.836±0.252 h	3.000±0.854 e	76.66±2.887 de
0.00	0.50	1.366±0.208 d	0.533±0.0577 d	2.266±0.058 ef	2.933± 0.115 f	3.233±0.153 e-h	4.000±0.854 cde	80.00±0.000 c-e
0.00	1.00	2.200±0.781 bc	0.900±0.1000 a	3.233±0.252 c	2.766±0.208 f	3.700±0.200 d-h	3.333±0.473 e	73.33±5.774 e
0.00	2.00	2.366±0.513 bc	0.900±0.1000 a	2.500±0.100 e	2.833f ± 0.153	4.733±0.451	3.033±0.462 e	83.33±5.774 b-e
0.50	0.00	1.933±0.493 bcd	0.800±0.1000 abc	3.700±0.000 ab	2.933±0.058 f	4.400±1.015 b-f	3.133±0.666 e	90.00±10.000 abc
0.50	0.50	1.933±0.153 bcd	0.766±0.0577 abc	2.500±0.200 e	4.666±0.058 b	5.400±0.656 ab	5.033±1.514 bc	86.66±15.275 abcd
0.50	1.00	1.700±0.458 cd	0.800±0.1000 abc	3.800±0.100 a	3.366±0.153 ef	5.866±1.422 a	8.166±0.702 a	86.66±5.774 a
0.50	2.00	2.166±0.321 bc	0.866±0.0577 ab	3.333±0.153 bc	3.833±0.289 cde	3.800±0.781 c-h	4.733±1.106 cd	93.33±11.547 ab
1.00	0.00	2.333±0.802 bc	0.666±0.2082 bcd	2.533±0.603 e	3.466±0.208 def	3.333±0.473 f-h	6.233±0.850 b	96.66±5.774 a
1.00	0.50	3.166±0.416 a	0.666±0.1528 bcd	2.566±0.473 de	4.300±0.100 bc	5.000±1.803 abc	3.933±0.153 cde	80.00±5.000 c-e
1.00	1.00	1.700±0.458 cd	0.700±0.1732 abcd	2.466±0.751 e	5.500±0.854 a	4.666±0.777 a-e	4.866±0.603 bcd	90.00±5.000 abc
1.00	2.00	2.466±0.252 abc	0.800±0.2000 abc	2.233±0.451 ef	4.233±0.451 bc	4.366±0.493 b-f	4.400±0.721 cde	93.33±2.887 ab
2.00	0.00	2.500±0.721 ab	0.800±0.1732 abc	1.966±0.058 fg	3.866±0.862 cde	3.766±0.115 d-h	4.86±0.635 bcd	76.66±5.774 de
2.00	0.50	2.333±0.153 bc	0.833±0.1155 abc	1.933±0.115 fg	3.266±0.666 ef	4.100±0.173 c-g	4.366±0.850 cde	76.66±5.774 de
2.00	1.00	1.833±0.351 bcd	0.633±0.0577 cd	1.666±0.231 g	4.166±0.850 bcd	3.700d±0.265 -h	3.533±0.666 de	86.66±5.774 a-d
2.00	2.00	1.966±0.416 a-d	0.800±0.0000 abc	1.833±0.153 fg	3.766±0.702 cde	2.900±0.100 gh	3.700±0.800 cde	83.33±5.774 b-e

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test (Mean ± SD).

Plantlets were successfully acclimatized in an adaptation greenhouse and recorded 95% survival rate after 30 days in pots filled with perlite: peat moss (1:1 v/v) (Fig. 3G). There were no visual morphological abnormalities in the micropropagated plantlets.

4. Discussion and Conclusions

Fritillaria is a rare and critically threatened genus due to the large-scale eradication, irregular grazing and lack of protecting rules. This plant reveals a great commercial potential, therefore, the plants in danger of extinction, like *Fritillaria*, should be protected. *In vitro* propagation is an effective method for conservation and rapid multiplication of the species in danger of extinction like the members of Liliaceae family including *Fritillaria* due to limitations in conventional methods of propagation.

Various factors influence the process of micropropagation from bulb scales and *in vitro* regenerated bulblet explants, especially type, concentration and combination of PGRs particularly auxins and cytokinins. Optimal selection of PGRs is especially important when the amount of the original plant material of rare and endangered plant species is limited (Kulkhanova *et al*., 2015).

For *F. imperialis*, *in vitro* propagation is carried out by bulb scales using mainly NAA, IAA and BA (Witomska and Lukaszewska, 1997; Lukaszewska *et al*., 1998; Witomska *et al*., 1998; Mohammadi-Dehcheshmeh *et al*., 2006; Petrić *et al*., 2013). For most members of the genus *Fritillaria*, micropropagation is done by bulb scales using mainly BA, BAP, TDZ, NAA, IAA and 2,4-D (Petrić *et al*., 2013; Kulkhanova *et al*., 2015). The concentrations of 0.10-4.00 mg l^{-1} of NAA in combination with 0.10-2.00 mg l^{-1} KIN has been applied using bulb scales for *in vitro* propagation of some species of the genus *Fritillaria*, such as *F. anhuiensis* (Xue *et al*., 2008), *F. camtschatcensis* (Okawa and Nishino, 2000), *F. roylei Hook* (Joshi *et al*., 2007), *F. thunbergii* (Seon *et al*., 1999), and *F. ussuriensis* (Sun and Wang, 1991), not for *F. imperialis*. NAA is the most effective auxin in inducing the *in vitro* formation of bulblets from the segments of bulb scales of *F. sonnikovae*, and the maximal regeneration was obtained combining 1.62 µM NAA and 4.65 µM KIN also 5.00 µM BAP and 2.00 µM NAA (Kulkhanova *et al*., 2015). The use of BAP in combination with IAA was effective for micropropagation of *F. unibracteata* (Gao *et al*., 1999). In our study, the use of 0.50 mg l^{-1} KIN and 1.00 mg l^{-1} NAA was useful for shoot and root production through indirect organogenesis.

Cytokinins are generally known to promote the formation of buds in many excised and *in vitro* tissue cultured organs. The medium containing 2.20 µM BA was most effective for shoot formation on bulb scales of *Lilium longiflorum* (Han *et al*., 2004). Similar results were also reported by others (Naik and Nayak, 2005). Bulblets regeneration and shoot multiplication on bulb scale explants were observed in some Liliaceae members, like *F. thunbergii* and *Ornithogalum ulophyllum* (Paek and Murthy, 2002; Ozel *et al*., 2008). Ipek *et al*. (2006) applied different concentrations of BAP, NAA and KIN to obtain bulblets from immature embryos of *Ornithogalum platyphyllum*. Naik and Nayak (2005) induced direct induction of bulblets on the bulb scales grown on the MS media enriched with 1.00 mg l^{-1} NAA and 2.00 mg l^{-1} BA. Naik and Nayak (2005) showed a plant regeneration procedure in *Ornithogalum virens* through direct shoot bud formation and indirect organogenesis using bulb scale as explant cultured on MS medium containing 1.00 mg l^{-1} NAA and 2.00 mg l^{-1} BA. Paek and Murthy (2002) revealed that the maximum bulblet regeneration and leaf production in *F. thunbergii* using bulb scale segments as explants were obtained in MS medium supplemented with a combination of KIN and NAA. Current investigation is consistent with this finding. Our study showed the same importance of KIN and NAA for shoot regeneration. In agreement with us, study of Kukulczanka *et al*. (1989) on *F. meleagris* L. demonstrated that the highest percentage of the regenerating explants, was obtained in case of joint action of cytokinin and auxin. In *F. aurea* Schott, increased TDZ concentrations increased leaf number. The highest regeneration rate was obtained from combination of cytokinin and auxins (Kizil *et al*., 2016). In *F. imperialis*, the number of regenerated shoots was the highest on MS medium supplemented with 0.50 mg l^{-1} TDZ and the number of roots was the highest on MS medium supplemented with 0.20 mg l^{-1} NAA (Rahimi *et al*., 2014).

We observed variable frequency of callus induction at various concentrations of KIN-NAA in the culture medium. Similar findings were reported by others using BAP-NAA (Nayak and Sen, 1995; Naik and Nayak, 2005; Ozel *et al*., 2008). Malabadi and Van Staden (2004) induced shoot bulblets and embryogenic calli in *Ornithogalum longibracteatum* on medium containing KIN. In the study of Çakmak *et al*. (2016) on *F. persica*, all explants including bulb scales showed competency

to regenerate callus and bulblets using 2,4-D and KIN, or TDZ. Bulblet explants responded fairly well to a higher level of TDZ in order to obtain maximum shoot regeneration. Callus induction was evident in response to high TDZ concentration. Current study showed that the maximum root formation was obtained on medium containing both KIN and NAA. Similar to our finding, Ozel *et al*. (2008) successfully rooted bulblets regenerated on 2.00 mg l^{-1} BAP and 0.50 mg l^{-1} NAA. In *F. aurea* Schott, maximum rooting was noted on 0.50 mg l^{-1} IBA (Kizil *et al*., 2016).

In conclusion, *F. imperialis*, bulbous cultivated as ornamental cut flower and garden plant, has important also as medicinal plant and it is at the risk of extinction. Traditional propagation is limited, because production of bulbs is poor and seeds have a low germination rate. Therefore, *in vitro* propagation by direct and indirect organogenesis and embryogenesis is a proper approach. The success of these procedures highly depends on type and concentrations of auxins and cytokinins applied in culture medium, singular or in combination. In our study, 0.50 mg l^{-1} KIN along with 1.00 mg l^{-1} NAA successfully induced leaf and root formation.

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Effect of compost tea and partial root zone drying on tomato productivity and quality

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Abstract: To evaluate the effect of partial root zone drying in combination with compost tea on growth, morpho-physiological traits, yield and quality attributes of tomato (*Lycopersicon esculentum* Mill), a greenhouse experiment was conducted. The ultimate aim of this study was to evaluate the effect of partial root zone drying (PRD) and conventional drip irrigation (CDI) incorporated with compost tea (CT) on tomato productivity and quality. The results of this study indicated positive and significant effect of CT in combination with PRD on fruit size, fruit weight, fruit firmness, cluster per plant, fruit per cluster, fruit lycopene content, pH, TSS and TSS/TA. The PRD treated plant's fruits exhibited better appearance, higher lycopene content, fruit firmness, total soluble solid (TSS), and TSS/titratable acidity (TA) ratio than fruits plants treated with CDI (conventional drip irrigation). Combined treatment with CDI and CT had positive effect on plant height, leaf area, chlorophyll and water content in fruits. But they exhibited the negative effect on fruits blossom end rot, weight loss, chilling injury, and TA content. The results of this study indicated that CT improve more significantly tomato yield and quality under PRD than CDI. Combining PRD and CT led to the maximization of crop water productivity.

1. Introduction

The consumption of fresh fruits and vegetables has been increasing because of their vitamin, mineral, and antioxidant contents. With the increasing consumption of fresh vegetables especially for tomatoes (Consumption Census, 2015) there has been a corresponding rise in concern about the inorganic chemical fertilizer and pesticides residues linked to tomato fruits (Ware, 2017). Besides that, the increasing public concern about negative environmental effects of agricultural practices like conventional chemical fertilization has promoted the evaluation of alternatives like the use of organic fertilizers. Compost is considered as an organic fertilizer. It is made with substance such as organic crop residues, animal wastes, food garbage, organic municipal and industrial wastes. Pane *et al.* (2013) highlighted the beneficial effects of agricultural utilization of

compost, which include improvement on physical, chemical, biochemical and biological properties of the soils. But compost can be heavy and bulky to transport and spread. As an alternative, compost tea (CT) offers the benefits of compost in a lighter-weight package. It is a liquid version of compost, obtained through a liquid-phase of compost and extraction ranging from few hours to two weeks with or without active aeration and the addition of nutrients such as molasses. It is easier to apply to plants and the soil.

The effectiveness of compost teas may vary due to the differences in types of compost, management and procedures used for its preparation (Egwunatum and Lane, 2009; Pant *et al.*, 2012). According to Martin *et al.* (2012), the best results were obtained when aerated compost teas rather than non-aerated teas were used. Most probably dissolved oxygen supports microbial activity (Arancon *et al.*, 2007). Reeve *et al.* (2010) reported that the potential of compost teas for supplementing or substituting other types of fertilizers also seems promising. In a study, Moretti *et al.* (2015) indicated that composted green wastes are advantageous when compared to other organic wastes since they present a lower risk of toxicity due to the presence of heavy metals, pollutants, aromatic hydrocarbons, hormones, pharmaceuticals as well as viruses, fecal coliforms and salmonella (Benito *et al.*, 2005). These materials exhibit excellent biological activity (Ros *et al.*, 2005).

Hatam *et al.* (2015) and Mavity (2016) reported that compost tea is an effective, low-strength, all-natural fertilizer for plants, seedlings, and gardens. They also assert that compost tea reduces salt accumulation in soils that come from commercial fertilizers, improves the ability of soil to hold nutrients, retain water and facilitates the soils pH buffering ability through microbe diversity.

The use of compost tea as a foliar spray or soil drench has been demonstrated to improve plant health, yield and nutritional quality by: enhancing beneficial microbial communities and their effects on agricultural soils and plants. It also enhances mineral nutrient status of plants and induces the production of plant defense compounds that may have beneficial bioactivities in humans (Weltzien, 1991; Hoitink *et al.*, 1997; Diver, 2002; Scheuerell and Mahaffee, 2002; Carpenter-Boggs, 2005; Ingham, 2005). The potential benefits of compost tea are substantial and particularly relevant to crop production in low-input agricultural systems, e.g. minimize water use. Less water input is now one of the globally used modern practice of sustainable agriculture and sustainable food production system.

Indeed, throughout the world, water supplies are limited and water crises are a top global risk. As irrigation of agricultural lands accounts for 70% of water usage worldwide (Khokhar, 2017), even a slight reduction in irrigation water could substantially increase the water available for other purposes. Therefore, there is an urgent need to identify effective irrigation management strategies. Partial root-zone drying irrigation (PRD) is one of the new efficient and productive water-saving irrigation methods that can conserve irrigation water up to 50% in processing tomatoes (Casa and Rouphael, 2015). This technique has the potential to significantly reduce crop water use (El-Sadek, 2014), minimize canopy vigor, but able to maintain crop yields and quality of crops (Sun *et al.*, 2014) when compared to conventional irrigation methods. It is expected that plants under PRD condition will maintain high water saving potentials while adequately watering plants.

In this context, the aim of this study was to check the use of CT in combination with less water use to improve tomato production sustainability. Accordingly, the use of compost tea combined with PRD was checked as a possible management system for promoting tomato growth and decrease need for chemical fertilizer which might lead to sustainable agriculture.

Most of the previous studies were focused on the application of compost as a soil supplement or foliar application for tomato plant or tomato fruit disease control (Vawdrey and Stirling, 1997; Gutierrez-Miceli *et al.*, 2007; Segarra *et al.*, 2009; Souleymane *et al*., 2010). No information is available on the combined effect of compost tea and PRD on morpho-physiological traits, yield and quality attributes of "Vibelco" tomato grown in closed environments. Thus some investigations are required to provide clear information about the effects of compost tea and partial root zone drying in growth promotion, yield and quality among the worldwide consumed vegetables such as tomatoes. Therefore, this work was focused on the effects of compost tea combined with PRD on the growth, yield and main quality parameters of tomatoes grown in closed environments. Based on the initial observations, we hypothesized that the applied compost based tea with deficient water supply may exert a suitable bioactivity on the tomato plants as related to the stimulation of growth, yield and quality parameters of the fruits.

2. Materials and Methods

Experimental conditions

The experiment was conducted in a greenhouse setting at approximately 22°C during daytime temperature and 15°C at night, Relative Humidity (RH) of 67%, 14 hour's photoperiod and ambient light condition at the Chateau Fresno Nursery, 13505 South Fresno, California 93609, USA, from April to September 2017. Seeds of a fresh market tomato (*Lycopersicon esculentum* Mill cv. Vibelco) were sown on March 1, 2017. Thirty three days after seeding, uniform plants were transplanted into 24 wooden boxes (2.53 m length × 0.65 m width × 0.20 m height each). Each box had 4 compartments (0.50 m length × 0.50 m width × 0.20 m height) with one experimental plant per compartment. To avoid lateral water movement, a small piece of plastic (0.50 m length × 0.025 m width × 0.04 m height) was placed centrally on the base of each compartment. The plants were grown in a vermicast and coconut fiber mixture (60:40 v/v). Bees were used for pollination (Fig. 1).

Compost tea preparation

First, a large plastic bin (25 gallons, about 95 l) was filled almost to the brim with water and allowed it to stand overnight to rid it of chlorine, which will otherwise would kill the soil biota in the compost. A close fitting lid was used to exclude mosquitoes and limit odor. Then, to make up a compost "tea-bag", a Hessian sack was used. A measured amount of compost (homemade compost prepared from organic tree leaves) with organic cow manure, that is approximately one-tenth of the volume of water, was placed inside the tea-bag. For every 25 liter bucket, we used about 2.5 liter of compost. The "tea-bag" was tied and securely closed and immersed it in the water for three weeks. The mixture was frequently dunked to speed up the process and obtain better results. After three weeks the mixture was filtered. The whole process was done at ambient temperature. Then the tea was applied to the plant by incorporating it to the irrigation water used for with drip irrigation at the ratio of 1:4 (1 part tea and 4 part water) (Fig. 2).

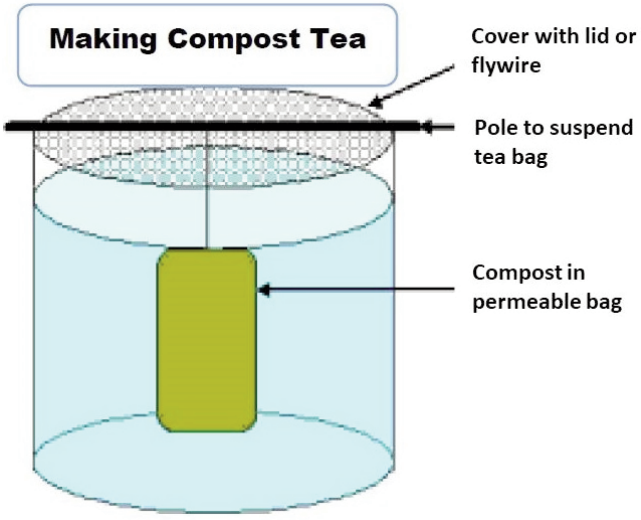

Fig. 1 - Schematic diagram of compost tea production.

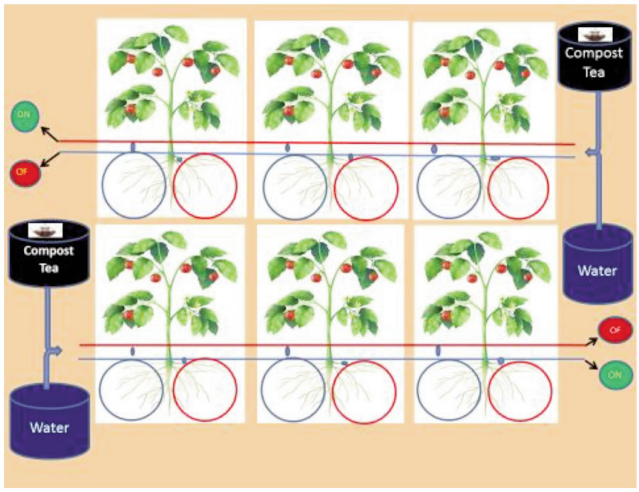

Fig. 2 - Schematic diagram of drip irrigation treatments incorporated with compost tea.

Irrigation treatments incorporated with compost tea

Two weeks after transplanting, irrigation treatments incorporated with compost tea were tested. Treatments were: 1. conventional drip irrigation (CDI) to both sides of the root system, 2) half of irrigation water, in drip irrigation given alternately only to one side of the root system with each irrigation (PRD), 3) CDI plus compost tea, and 4) PRD plus compost tea. In the CDI the irrigation treatment was given 0.10 meter away from the main stem and on both sides of the row. The irrigation (CDI) covered a total area and soil volume of 0.24 m^2 and 0.048 m^3, respectively. The amount of water was given in two equal doses at 10:00 h and at 16:00 h by drip irrigation system with emitters. Two irrigation lines were set up and operated separately for the PRD treatment. Two sprinklers per plant (one on each line) each emitters 4 l/h were placed 0.15 m away from the main stem of each plant. The irrigation in PRD treatment covered a total area and soil volume of 0.018 m^2 and 0.004m^3, but half of irrigated area and soil volume was wetted in PRD treatment. There was some drainage in all treatments, but it was not measured. However, water loss by drainage was minimized by adjusting the amount

of water as the crop developed. The values of the irrigation efficiency presented here might have been underestimated, considering no water loss.

Plant and fruit growth performance

Growth, yield, yield components and blossom-end rot were measured in 12 randomly selected plants/fruits from each treatment. The plant heights were measured with a tape from the base to the tip of the plant. Plant growth and development data were documented in the sampled and tagged plants monthly for three months. Leaf area was measured using a destructive method. Total leaf area (cm^2) was measured by leaf area meter (Model: Delta-T, Cambridge, U.K.). The numbers of clusters were counted per plant from the first to the last cluster during the growing period. The number of fruits was counted when the plants started fruiting. The fruit weights were determined after harvesting the tomato using a weighing balance. After 150 days, one plant per treatment per replication was destroyed and the total vegetative fresh weight was assessed and expressed as kg/plant. Mature green tomato fruit firmness was measured using an Instron Universal Testing Machine with a 0.5 cm^2 plunger. The measurement was taken at the mid-section of the fruit. Water content of fruit was expressed on a dry weight basis. Fruit blossom-end rot incidence was recorded and calculated in percentage of fruits affected per plant.

Fruit quality at harvest or postharvest

For postharvest quality evaluation, 6 replicates of 5 mature green (Cascio, 2017) fruits from each treatment were randomly chosen approximately 125 days after transplanting and were stored in a dark refrigerated room at 4°C with 94% RH. After a storage period of 2 weeks, all fruits were moved to a ventilated room without supplemental light at 24°C with 66% RH and held for 7 days. The following attributes were checked for quality measurements: weight loss, chilling injury, decay, appearance/color change, total soluble solids (TSS), pH, titratable acidity (TA), chlorophyll and lycopene content.

Fruit weight loss was determined prior to and after storage. It was calculated as the percentage of initial fresh weight. Color development was observed visually using a subjective scale with mature green (MG) = 1, breaker (B) = 2, pink (P) = 3, light red (LR) = 5 and red (R) = 6 (USDA, 2005). Chilling injury (surface pitting) was rated visually by estimating the percentage of the injured fruits. Decay (unidentified) was rated visually and calculated as a percentage of fruit affected.

Chlorophyll and lycopene content were determined from three randomly selected fruits from each treatment by grinding pericarp tissue (about 5 g) in 15 ml acetone. The extract was taken for centrifugation at 35,000 rpm for 10 minutes. Before centrifugation, the tubes were covered with aluminum foil to prevent light-induced lycopene oxidation. After centrifugation, the supernatant was decanted and adjusted to 20 ml with acetone. Absorbance of the extracts at 664 nm for chlorophyll and 503 nm for lycopene was measured with a spectrometer (Model 160 A). Total chlorophyll content in milligram per 100 grams of tissue was calculated according to the formula developed by Holden (1976). Lycopene content was calculated using the molecular extension coefficient of 3240 (Davis, 1976) and expressed as micrograms per gram of fresh weight.

Total soluble solid (TSS), pH and titratable acidity (TA) were measured on juice extracted from fruits. The TSS content was determined with a digital refractometer (Atago, Model 1, Tokyo, Japan). The TA was determined by a Metler Auto titrator (Model V 20) and pH was measured with an autocal pH meter (Model PHM 83).

Experimental design and data analysis

A completely randomized design was used with the four treatments replicated six times with four plants per replication for each treatment. Data were analyzed by a complete randomized model using the ANOVA procedure of SAS software version 8.2 (SAS Institute, Cary, NC, USA). Treatment effects on tomato growth, yield and quality components were analyzed using Duncan's Multiple Range Tests to determine significant effects at $p = 0.05$ among four treatment means.

3. Results and Discussion

Figure 3 illustrates the mean plant height over a period of 3 months. Bars represents standard deviations. Plants height increased gradually within 3 months. The different letters in the columns at the same month showed significant differences. The maximum height was obtained from those tomato plants which were irrigated with CDI/full water regime plus compost tea and the difference was significant during the whole experiment. On second and third month, CDI plus CT treated plans exhibited higher height than PRD plus CT treated plans and the difference was significant. On third month, The PRD

treated plants resulted in the significantly lowest height among the four treatments. Similar results were reported by Pal *et al.* (2016), who grew tomato plants under deficit irrigated conditions. Among the four treatments in this study, the growth of CDI treated plants exhibited moderate growth compared to other treated plants.

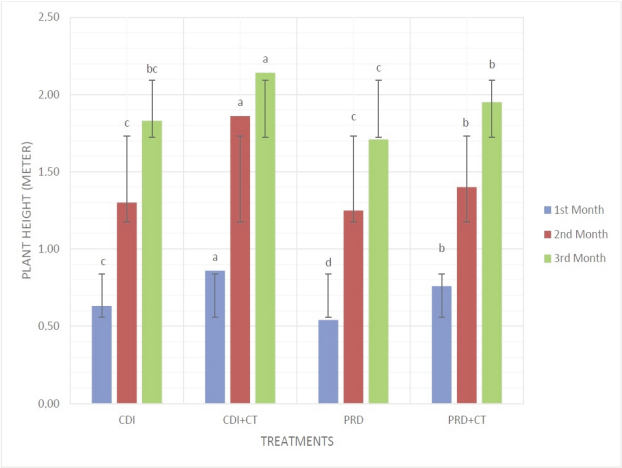

Fig. 3 - Effect of CDI (conventional dripping irrigation) and PRD (partial root-zone irrigation) with compost tea (CT) on plant height over 3 months. Bars represents standard deviations; means followed by different letters at the same month are significantly different at 5% probability level using Duncan's Multiple Range Test (DMRT).

Combined treatment with CDI and CT had positive (+) and significant effect on fruit fresh weight and leaf area of the plant, respectively. The PRD had a negative (-) effect in shoot fresh weight and leaf area of plant. However, PRD had positive (+) effect on the number of fruits per cluster as well as in fruit production in comparison to the CDI treatment (Table 1). Combined treatment with PRD and CT exhibited the positive (+) and significant effect on cluster per plant and number of fruits per cluster. The difference between CDI and PRD treated plants' shoot fresh weight, leaf area, and fruit production were significantly different at 0.05 % level. The CDI treated plants appeared to have excess moisture in the root zone (didn't investigated moisture in root zone in this study), which may have caused root inactivity contributing to lower yield and delayed maturity of the crop as compared to the PRD treated plants. Therefore, this study demonstrates that the deficit of irrigation water with compost tea can induce biological activity of tomato plant which ultimately increased the number of clusters per plant and number of fruits per cluster.

Table 1 illustrates that among the four treatments, partial root zone drying (PRD) plus compost tea (CT) treated plant fruits exhibited the highest percentage blossom end rot. Table 1 also illustrated that the PRD treated plant's fruits showed significantly higher percentage (%) of blossom-end rot as compared to the CDI treated ones. Mathew and Salvadore (2007) reported that the blossom-end rot is a physiological disorder of tomato fruit caused by calcium deficiency or excessive soil moisture fluctuation, which reduce uptake and movement of calcium into the plant. The higher percentage (%) of blossom-end rot in the PRD treated fruits might be due to the reduced movement of calcium to in the PRD treated plants. However, no calcium was analyzed either in the leaves or fruits in this experiment.

Both PRD and CT treatments influenced the fruit size, water content and fresh fruit weight (Table 1). Among the four treatments, fruit size showed significant difference and the largest size of fruits were produced by those plants irrigated with deficit (PRD) water mixed with compost tea. There were some differences in fresh fruit weight and water content in

Table 1 - Effect of CT and PRD on tomato fruit size, total fresh weight, water content, fruit weight, fruit firmness, cluster/plant, fruit/cluster, shoot fresh weight, leaf area, and blossom-end rot

Plant yield parameter	Treatment			
	CDI	PRD	CDI + CT	PRD + CT
Fruit size, diameter (mm)	65.40 d [(X)]	68.30 c	70.27 b	72.03 a
Total fresh weight of fruit (kg/plant)	5.11 d	6.47 c	7.53 b	9.34 a
Fruit water content (%)	95.17 a	96.1 a	92.17 c	94.07 b
Fruit weight (g)	94.4 b	97.33 a	94.57 b	95.5 b
Fruit firmness after harvest (kg/cm^2)	9.97 a	9.13 b	8.30 c	8.17 c
Cluster/plant	8.03 d	9.10 c	10.40 b	11.23 a
Fruit/cluster	5.03 d	5.60 c	6.40 b	6.80 a
Shoot fresh weight (Kg)	10.60 b	11.53 a	8.97 d	9.30 a
Leaf area (cm^2)	453 b	419 d	478 a	432 c
Bloom end rot (%)	5.50 a	6.00 c	6.50 b	8.27 a

[(X)] Means followed by different letters in a row are significantly different at 5% probability level using Duncan's Multiple Range Test (DMRT).

PRD and CDI treated plants' fruits; and the differences were statistically significant (Table 1).

The results of this study indicates that, during conservation, combined CDI and CT treated plant fruits lost more weight than combined PRD and CT treated plant fruits (Table 2). The results of this study also confirm that PRD treated plant fruits lost less water compared to the CDI treated ones. Reduced weight loss in the PRD treated tomatoes during the storage is a positive quality attribute in tomato fruits, especially for distant markets. According to Yadav and Singh (2014) the weight loss of fruits in storage condition is mainly from water loss and from solid constituents. The lower water loss in PRD treated fruit might be due to lesser incidents of micro-cracks in the skin. However, no skin micro-cracks were examined in this investigation.

As demonstrated in this study, chlorophyll and lycopene contents were influenced by irrigation and compost tea treatment (Table 2). Combined treatment with PRD and CT postulated higher lycopene and lower chlorophyll contents compared to the plants treated with a combination of CDI and CT. When compared to the fruits from CDI treated plants, fruits from PRD treated plants exhibited significantly lower chlorophyll and higher lycopene content on the 7th day at 24°C. This was followed by a 2-week storage period at 4°C (Table 2). Klunklin and Savage (2017) also detected significantly higher lycopene content in PRD treated tomato fruits.

Gindi *et al.* conducted a survey in 2016 and concluded that 63 percent of consumers' purchase interest depends on the color of fruits. Fruit colors were affected by irrigation and compost tea treatment. The best color (tone of red) was observed on plants fruit those treated with PRD plus CT. Fruits from PRD treated plants exhibited less visible chilling injury (fruit surface pitting) and decay, but faster color change than the fruits treated with CDI. The less visible chilling injury, decay and faster color changes in PRD treated plants fruit might be due to less water content compared to CDI treated plants fruits.

Tomato flavor is generally determined by the content of soluble solids (TSS) and titratable acidity content (TA).

According to Hong *et al.* (2014), sweetness of the most fruits is closely related to the TSS content. According to Baldwin *et al.* (2015), the sourness in most of the fruits is governed by TA content.

Valero *et al.* (2005), reported that changes in sugar content and organic acid metabolism occur during the ripening process of tomato fruits. Aoun *et al.* (2013) examined that tomato flavor is co-related to the total sugar and acid contents in the fruit. The results of this study demonstrated that the total soluble solid (TSS), TA and pH were affected by the treatment of PRD and CT individually and in combined treatments. Fruits from PRD plus CT treated plant exhibited significantly higher content of TSS, and pH values, but lower TA than fruits from CDI and CT treated plants. The total soluble solid (TSS), TSS/TA and pH increased, while TA decreased in PRD treated fruits than CDI treated ones (Table 2). In an earlier study, Sun *et al.* (2014) also detected higher TSS levels in tomato fruits produced under a PRD condition. The higher TSS and the lower TA in fruits from PRD treated plants were probably due to the less retained water in fruit from PRD treated plant than the fruits from CDI treated plants. The higher pH in fruits from PRD treated plants was compatible with the lower TA

Table 2 - Effect of CT and PRD on tomato fruit weight loss, chilling injury, decay, chlorophyll and lycopene content, fruit color change, pH, TA, TSS content and TSS/TA ratio, 7th day at 24°C followed by a 2-week storage period at 4°C

Phylogical parameter	Treatment			
	CDI	PRD	CDI + CT	PRD + CT
Weight loss (%)	2.18 b [x]	1.88 c	2.93 a	2.03 bc
Chilling injury (%)	14.66 b	9.00 d	17.00 a	10.70 c
Decay (%)	13.67 a	7.50 d	12.30 b	10.20 c
Chlorophyll (mg/100 g fresh weight)	3.80 a	3.30 bc	3.53 b	3.60 b
Lycopene (µg/g fresh weight)	7.00 d	7.80 b	7.43 c	8.10 a
Color change	4.36 c	4.76 b	4.63 bc	5.23 a
pH	4.30 d	4.63 b	4.50 c	4.80 a
TA (% citric scid)	0.49 c	0.45 d	0.53 b	0.56 a
TSS	4.27 d	4.80 b	4.56 c	5.10 a
TSS/TA	8.61 c	10.67 a	8.60 c	9.11 b

[(x)] Means followed by different letters in a row are significantly different at 5% probability level using Duncan's Multiple Range Test (DMRT).

in fruits from PRD treated plants than in the fruits from CDI treated plants.

4. Conclusions

The results of this study demonstrates the significant effect of PRD on biometric variables such as number of cluster per plant, number of fruit per cluster, fruit weight, and fruit size in combination with CT treatments. The results of the study also shows that the plants treated with a combination of PRD, a cost effective water saving method, and CT exhibited slower plant growth, but produced higher yield, and better postharvest quality attributes of fruits.

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Vegetative propagation of *Argania spinosa* (L.) Skeels cuttings: Effects of auxins and genotype

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Abstract: *Argania spinosa* (L.) is an endemic tree species of south-western Morocco; it plays a very important socio-economic and environmental role. However, the vegetative propagation of the argan tree by traditional cuttings is limited by the difficulty of rooting and survival during transplantation in the field. Considering these facts, this study intended to investigate the rooting ability and growth performance of argan tree cuttings, collected from four élite trees rated ASOC1, ASOC2, ASOC3 and ASOC4, and treated with four concentrations (0, 1000, 3000 and 5000 mgL^{-1}) of IBA, NAA and IAA. The results revealed cuttings of ASOC2 and ASOC3 genotypes were relatively less responsive than ASOC1 and ASOC4, this genotype effect was more pronounced in auxin treated cuttings. Treatment of cuttings by IBA was more effective than treatments by either NAA or IAA. Among all the media tested, 3000 mgL^{-1} of IBA with ASOC1 resulted in higher sprouting (81.75%), rooting (60.75%) and survival rates (96.25%). However, with the increase of IBA concentration levels (>3000 mgL^{-1}), adventitious roots and sprouts performances decreased in all the genotypes. *Argania spinosa* could be successfully propagated by cuttings from selected élite trees.

1. Introduction

The argan tree - *Argania spinosa* (L.) Skeels - is a monoecious tree species, evolving in arid and semiarid areas and belonging to the tropical *Sapotaceae* family (M'Hirit *et al.*, 1998). Conventionally, argan plants are propagated by seeds. However, this method is not adequate for argan trees domestication. This species is allogamous and shows extreme variability and heterogeneity (Nouaim *et al.*, 2002; Alouani and Bani-Aameur,

2004; Msanda *et al.*, 2005). In addition, seed propagation does not ensure preserving selected genetic characters for the next generation and could also result in a loss or dilution of favorable mother trees genes (Hartmann *et al.,* 2002). However, vegetative propagation techniques (cuttings, layering, division or separation, budding, grafting and tissue culture) are not used for clonal forestry purposes because the argan tree is a hard-to-root species (Nouaim *et al.*, 2002). Thus, the development of an efficient vegetative propagation technique will provide an opportunity to facilitate domestication, improvement and mass multiplication of élite trees of this species (Leakey *et al.,* 1990; Benbya *et al*., 2018). The most common vegetative propagation methods that have the highest short-term potential for large scale production of woody plants are micropropagation (tissue culture) and macropropagation (rooted cuttings) (Duryea and Dougherty, 1991). The success of cutting techniques depends on several factors that influence rooting efficiency (Hartmann *et al.,* 2002). Adventitious root formation (ARF) is a synchronized developmental process involving various biochemical, physiological and histological events in the induction, initiation, expression and elongation phases of adventitious roots (Nemeth, 1986; Soundy *et al.*, 2008). The large variation in adventitious root formation in *A. spinosa* species is attributed to genotype (Nouaim *et al.*, 2002). In fact, the loss of ability to regenerate roots and shoots by cuttings can be described in terms of ontogenetic stage, physiological and chronological ages, which may eliminate the possibility of successful propagation of selected trees (Rasmussen *et al.,* 2014). The physiological age may also depend on environmental growth conditions and plant responses to stress (Greenwood *et al.,* 2001; Rasmussen *et al.*, 2014). Among external factors, the most important role for adventitious root formation is ascribed to growth regulators, which have been successfully employed in many plant species to improve the rooting ability of cuttings (Singh *et al.*, 2011; Sağlam *et al.*, 2014). The balance of plant hormones in the cutting could affect the development of root primordia, initial root development, root elongation, hardening and further development of the rooted cutting (Jaenicke and Beniest, 2003). In fact, adventitious root induction in cuttings is promoted by high auxin levels and low cytokinin levels in the rooting zone (De Klerk *et al.,* 2001). Auxin, the first identified plant hormone, is involved in various plant growth and development processes, including embryogenesis, organogenesis, vascular tissue patterning (xylem and phloem), flower development, fruit setting, ripening and senescence (Vanneste and Friml, 2009; Davies, 2010). Auxin homeostasis within plant tissues is regulated by the interplay of biosynthesis, conjugation, transport, and signaling pathways (Zhao, 2010). The endogenous auxin, indole-3-acetic acid (IAA), plays a central role in adventitious rooting of cuttings (De Klerk *et al.*, 1999). The difference in rooting ability between easy and hard-to-root cuttings can be attributed to IAA transport and accumulation (Ford *et al.*, 2002). The endogenous auxin increased up to fifteen days to decline thereafter, and exogenous auxin application increased the indole/auxin content (Kochhar *et al*., 2008). Many studies have shown that application of exogenous auxin results in an increased rooting initiation and development. The most common root-promoting compound in the nursery industry is indole-3-butyric acid (IBA) (Hartmann *et al.,* 2002). In addition to enhancing the rate of adventitious root development, exogenous auxin application has been found to reduce rooting development time, to increase the number of roots per plant and root system uniformity (Leakey *et al.,* 1990; Overvoorde *et al.*, 2010). The present study was undertaken to investigate the influence of genotype, auxin type and its concentration, and their combined interaction effect on adventitious rooting capacity and vegetative growth attributes of mature semi-hardwood cuttings of *Argania spinosa.* A low-cost technology of non-mist propagation system was used for all the experiments.

2. Materials and Methods

Experimental site and selection of plant material

This study was conducted under an experimental greenhouse at the biotechnology unit of the Regional Center of Agricultural Research of Rabat, Morocco. Cuttings grew from May 2014 to September 2015. *Argania spinosa* cutting materials were collected from trees of Oued Cherrat forest Arboretum, Benslimane province, Morocco (33°81'96" N ; 7°11'03" W; 45 m altitude), which is located within 2000 m of the Moroccan Atlantic coast and with an average annual rainfall of 460 mm.yr^{-1}. The selection of a healthy mature Candidate Plus Tree (CPT) was based on general growth, phenology (leafing, initiation of flowering, initiation of fruiting, fruiting period, maturation of fruiting, fruit shape and caliber), high biomass (crown diameter) and regeneration ability. The four genotypes (stock plants) were tagged for

identification and were pruned regularly (thrice a year) to encourage production of good shoots and maintain juvenility of the trees. After one-year, semi-hardwood cuttings were collected sequentially from shoots located in the middle-part of the tree crown in the months of April to June. The cuttings were taken early in the morning by using sterile pruning scissors and the collected shoots were kept under shade. After harvesting, these shoots were kept in perforated plastic bags inserted in a cool box to minimize a possible desiccation effect during collection and transportation. At the laboratory, the cuttings were kept in a cold room (4°C) for 48 hours.

Preparation of cuttings and application of auxins

Cuttings were screened by using a calibrated electronic digital Vernier caliper for desired and uniform size of (5±1) mm width, (10±0.5) cm length, with 4-6 leaves and 7-10 nodes per cutting, after removal of the apices. Each cutting was granted by two vertical cuts below the node on the basal end and a slanting cut above the node on the apical portion. The lower thorns and leaves (50% of total leaves) of each cutting were removed, keeping intact leaf buds in each cutting. The base of each cutting was freshly trimmed by 0.5 mm, then it was immersed in a 0.2% fungicide solution (Dithane with active ingredient mancozeb 750 g/kg) for 10 min and washed thoroughly with distilled water. The apical cut ends of the treated cuttings were sealed with tree wound dressings to reduce water loss, prevent diseases and decay. The lower 5 cm portion of the cuttings was dipped for 5 min in a concentrated auxin solution prepared by dissolving the hormone powder into 10 ml of ethanol (95%), and then sterile distilled water was added to a final volume of 1L. The concentrations of auxin solutions were 1000, 3000 and 5000 mgL^{-1} which corresponds to (5.71, 17.12, and 28.54) mM IAA, (5.37, 16.11 and 26.85) mM NAA and (4.92, 14.76 and 24.60) mM IBA respectively. Untreated cuttings (cuttings were dipped in distilled water) were considered as a control set.

Experimental growth conditions

Cuttings were initially raised in a non-mist greenhouse to allow root initiation to take place. The temperature of the greenhouse was 32±2°C, with a 16h/8h photoperiod and 80% humidity. In the greenhouse, the basal cut portion was inserted vertically according to the positive polarity in 1000 cc polystyrene pots (two cuttings per pot). The pots perforated at the bottom were filled with a rooting medium of sterilized and sieved fine river sand. Holes were punched into the rooting medium to allow the insertion of the cuttings without damaging the cambium or removing the rooting hormone. After sticking of the cuttings, the rooting medium was pressed slightly around them. Cuttings were irrigated regularly to field capacity by tap water every two days. The rooting experiment was conducted for several weeks until the cuttings initiated roots. Then, rooted cuttings were removed from pots and substrate was carefully washed away from the root system. Cuttings with roots (≥ 1 mm) were considered as rooted and were included for calculating the rooting ratio. Root shoots (≥ 1 cm) were considered for calculating mean number of roots. The root systems were handled carefully so that no visible damage occurred during transplantation. After data recording, these plantlets were transferred to larger black polyethylene pots (20 cm diameter, 20 cm depth) containing a mixture of sterilized forest soil, peat moss (pH of 6, water retention of 800 ml/l and organic matter content of 20%) and sieved fine river sand rooting medium (1:1:1 v/v). These pots were placed in the greenhouse with full sunlight, at a spacing of 20 cm × 20 cm. A Hoagland nutrient solution (Hoagland and Arnon, 1950) was also used once a week to provide the nutritional needs of plantlets. The cuttings were recorded after 48 weeks, a period that was considered sufficient to measure the survival of rooted cuttings, following a preliminary study in the laboratory.

Experimental design and treatments

The experiment was conducted in greenhouse using a Randomized Complete Block Design (RCBD) with four replications to study the effect of four genotypes (ASOC1, ASOC2, ASOC3 and ASOC4), three auxin types (IBA, IAA and NAA), four concentrations (control, 1000, 3000 and 5000 mgL^{-1}) and their interactions.

Measured parameters

Eight morphological characteristics per cutting, including the number of leaves (LN), leaf size in cm^2 (LS), number of sprouts (SN), sprout length in cm (SL), sprouting rate (SP), number of roots (RN), longest root length in cm (RL), and rooting rate (RP) were measured 12 weeks after planting. The survival rate (SR) was recorded 48 weeks after rooting induction.

Statistical analysis

The data was submitted to tests of analysis of variance (ANOVA) for treatment effects using the general linear model (GLM) procedure of SAS program version 9.1 (SAS Institute, Cary, NC, USA), for all

the evaluated parameters. Comparisons between treatments were performed by using Duncan's Multiple Range Test (DMRT) at P<0.05 level of significance. Values were means ± standard deviation (SE). Data given in percentages was subjected to arcsine $\sqrt{X}$ transformation before statistical analysis.

3. Results

The results have shown that sprouting preceded rooting initiation on the cuttings. Indeed, cuttings developed leaflets after about ten days, sprouts within six weeks and there was no rooting till twelve weeks after planting (Fig. 1), although there were significant differences between genotypes treated with different auxin types and concentrations in the process of adventitious root development and shoot growth of *Argania spinosa* cuttings (Table 1).

Effect of auxin type, concentration and genotype on the number of leaves and leaf size per cutting

The number and size of leaves followed the same pattern; they have a significant response (P<0.05) to auxin type, concentration and for the different genotypes studied. The interaction between auxin type, concentration and genotype was also significant on the mean number of leaves and leaf size (Table 1) The results show that genotypes responded to all treatments including low concentrations (1000 mg L^{-1}). The highest numbers of leaves values (35.75 and

Fig. 1 - Vegetative propagation of *Argania spinosa* through mature semi-hardwood cuttings. (A) Selected plus tree in natural conditions (month of May). (B) Cuttings in polyethylene pots according to a Randomized Complete Block Design (RCBD). (C) Leaf initiation (10 days) and sprouts elongation (6 weeks). (D) Root primordia initiation and adventitious root development (12 weeks). (E) High rate of adventitious roots from semi-hardwood cuttings treated with 3000 mgL-1 IBA (48 weeks). (F) Plant produced from cuttings transplanted in black polyethylene pots under non-mist greenhouse conditions (3 years).

Table 1 - Three-way analysis of variance (ANOVA) for effects of genotype (ASOC1, ASOC2, ASOC3 and ASOC4), auxin type (IAA, NAA and IBA), concentration (0; 1000; 3000; 5000) and their interactions on measured parameters of *A. spinosa* cuttings

Source of variance	Dependent variable	df	F-value	P-value
Genotype				
	No. of leaves	3	99.127	0.000
	Leaf size (cm^2)	3	66.694	0.000
	No. of sprouts	3	56.110	0.001
	Sprout length (cm)	3	10.321	0.000
	No. of roots	3	11.500	0.000
	Root length (cm)	3	12.100	0.000
	Sprouting rate (%)	3	343.16	0.000
	Rooting rate (%)	3	109.75	0.000
	Survival rate (%)	3	57.643	0.000
Auxin				
	No. of leaves	2	15.180	0.000
	Leaf size (cm^2)	2	9.750	0.000
	No. of sprouts	2	1.295	0.274
	Sprout length (cm)	2	3.084	0.046
	No. of roots	2	33.270	0.000
	Root length (cm)	2	38.420	0.000
	Sprouting rate (%)	2	65.200	0.000
	Rooting rate (%)	2	861.00	0.000
	Survival rate (%)	2	1125.5	0.000
Concentration				
	No. of leaves	3	148.10	0.000
	Leaf size (cm^2)	3	165.34	0.000
	No. of sprouts	3	512.55	0.000
	Sprout length (cm)	3	480.47	0.000
	No. of roots	3	281.49	0.000
	Root length (cm)	3	252.28	0.000
	Sprouting rate (%)	3	4638.9	0.000
	Rooting rate (%)	3	2830.6	0.000
	Survival rate (%)	3	8245.3	0.000
Genotype x auxin x concentration				
	No. of leaves	18	6.832	0.000
	Leaf size (cm^2)	18	8.511	0.000
	No. of sprouts	18	00.164	1.000
	Sprout length (cm)	18	8.425	0.000
	No. of roots	18	57.646	0.000
	Root length (cm)	18	14.281	0.000
	Sprouting rate (%)	18	3.681	0.000
	Rooting rate (%)	18	4.715	0.000
	Survival rate (%)	18	1.692	0.047

Df= degrees of freedom; level of significance P<0.05; sprouting rate, rooting rate and survival rate were subjected to arcsine $\sqrt{X}$ transformation before statistical analysis.

34.56) were recorded from ASOC1 treated respectively with 3000 and 5000 mgL^{-1} IBA, while the lowest number of leaves (13.31) was observed for ASOC2 cuttings treated with 1000 mgL^{-1} IAA (Fig. 2). It was noticed that the number of leaves of ASOC1 cuttings, exposed to 3000 mgL^{-1} IBA, is 77% higher compared to the control group.

The number of leaves for ASOC1 cuttings - that received 1000 mgL^{-1} NAA is 73% greater than the control group, while it is 52% higher for the 5000 mgL^{-1} IAA application. The mean number of leaves produced in the control group of ASOC1 is 19.29 leaves per cutting (Fig. 2). According to the results, the highest leaf size mean (32.42 cm^2) was observed in ASOC1 treated by 3000 mgL^{-1} IBA and it is 3 times greater than the control group. Similarly, leaf size (27.29 cm^2) of ASOC1 cuttings receiving 1000 mgL^{-1} NAA is 2.87 times higher than leaf size in the control set. While, the cuttings of ASCO1 that received 5000 mgL^{-1} IAA developed a leaf size 1.52 times bigger than control. Whereas the minimum leaf size (6.82 cm^2) was recorded for the ASOC2 cuttings treated with 1000 mgL^{-1} IAA (Fig. 2).

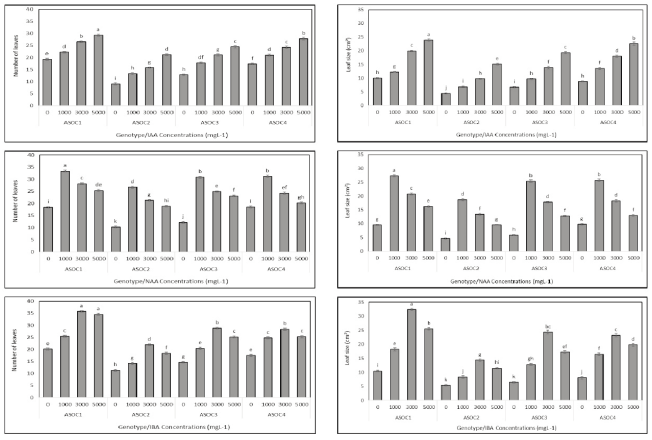

Fig. 2 - Effects of auxin type, concentration and genotype on leaves development (Mean values of leaves number and leaf size cm2) of *A. spinosa* cuttings. Within each treatment, the mean values followed by the same letter are not significantly different (Duncan's Multiple Range Test, P<0.05, mean± SE, n = 32).

Effect of auxin type, concentration and genotype on the number of sprouts and sprout length per cutting

The comparison between various treatments revealed that genotype, auxin type, concentration and their interaction had a significant influence (P < 0.05) on sprout length per cutting. However, the mean number of sprouts is influenced only by genotype and auxin concentration (Table 1). The number of sprouts and sprout length were induced by all the treatments except control (Fig. 3). The pretreatment of ASOC1 with 3000 mgL^{-1} IBA enhanced the number of sprouts (1.81 times) and sprout length by 15.94 cm, in comparison with the control. Thereby, NAA and IAA applications also increased the number and length of sprouted cuttings but were less effective than IBA. Indeed, by using 1000 mgL^{-1} NAA treatment on ASOC1 cuttings, the number of sprouts was 1.63 times greater and sprouts were longer by 12.38 cm than the control set, while by using 5000 mgL^{-1} IAA treatment, the greatest number of sprouts was 1.69 times greater for ASOC4 and the highest sprouts length was 11.19 cm longer for ASOC1 (Fig. 3).

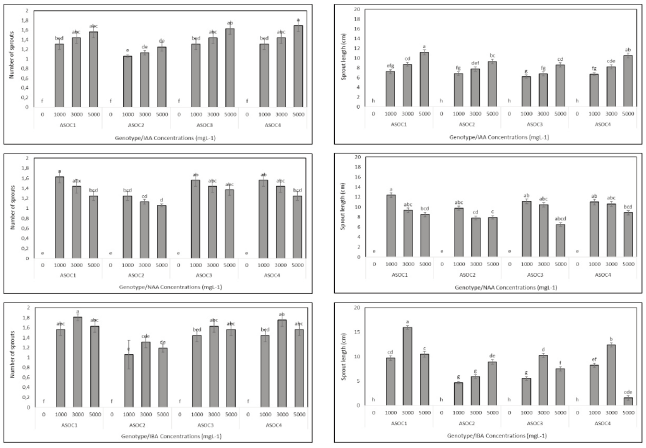

Fig. 3 - Effects of auxin type, concentration and genotype on sprouts growth (Mean values of sprouts number and sprout length) of A. spinosa cuttings. Within each treatment, the mean values followed by the same letter are not significantly different (Duncan's Multiple Range Test, P<0.05, mean± SE, n = 32).

Effect of auxin type, concentration and genotype on the number of roots and longest root length per cutting

The analysis of variance indicated that there were significant differences between auxin type and concentration as well as genotype on the number of roots produced and root length formed on *A. Spinosa* cuttings. The interactions between auxin type and its concentration with genotype influenced (P<0.05) root number and root length (Table 1). It may be inferred from above that there is a synergism between the number of roots and length of roots in all genotypes studied. Indeed, the more there are roots per cuttings and the more there will be longer roots. The adventitious root production increased at lower concentrations of IBA and began to level off between 1000 and 3000 mgL^{-1}, whereas with bigger hormone concentration levels (>3000mgL^{-1}), the number and length of roots were smaller. Similar

rooting response was observed with NAA hormonal treatments (Fig. 4). According to the results of the study, IBA significantly promoted the number and length of roots in comparison with NAA and IAA. Genotypes ASOC2 and ASOC3 were relatively less responsive than genotypes ASOC1 and ASOC4 in term of root number and root length per cutting. The cuttings in the control group and cuttings treated with 1000 mgL^{-1} IAA failed to produce roots, while the cuttings belonging to ASOC1 receiving a 3000 mgL^{-1} IBA application developed a maximum number of roots (41) with an average length of 22.94 cm.

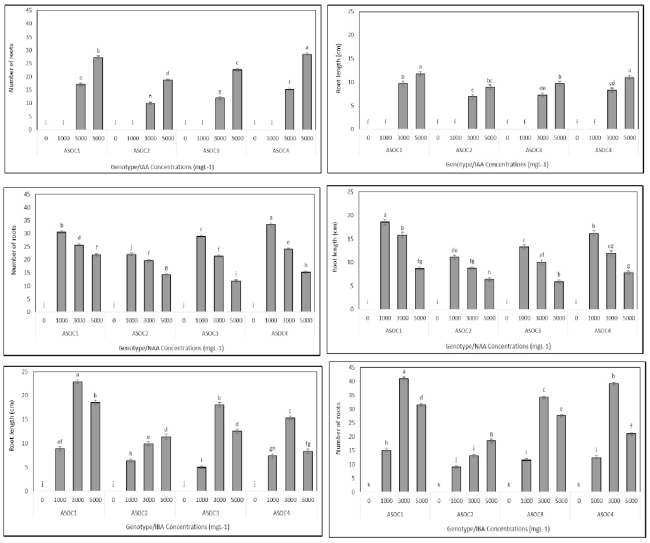

Fig. 4 - Effects of auxin type, concentration and genotype on growing roots (Mean values of roots number and mean longest root length) of *A. spinosa* cuttings. Within each treatment, the mean values followed by the same letter are not significantly different (Duncan's Multiple Range Test, P<0.05, mean ± SE, n = 32).

Effect of auxin type, concentration and genotype on sprouting, rooting and cuttings survival rates

Sprouting, rooting and survival ratios of *A. spinosa* cuttings were significantly influenced (P<0.05) by the genotype from which cuttings were collected, as well as by the type and amount of auxin applied (Table 1). Most cuttings that rooted in the non-mist greenhouse survived and ultimately produced sprouts as a sign of their successful regeneration. The results indicate that an application of IBA, NAA and IAA increased sprouting, rooting and survival rates, and IBA was the most effective auxin. When treated with concentrations >3000 mgL^{-1} sprouting, rooting and survival rates began to decrease rapidly in all cuttings treated with NAA and IBA (Fig. 5). The results indicate that cuttings collected from ASOC1 and treated with 3000 mgL^{-1} IBA had the highest sprouting percentage (81.75%), rooting percentage (60.75%) and survival percentage (96.25 %) per rooted cutting, followed by ASOC4 which had a sprouting ratio of 74%, a rooting ratio of 55.5% and a survival ratio of 94.5%. Among the different concentrations of NAA used, the maximum sprouting percentage (71.5%), rooting percentage (46.5%) and survival percentage (94.5%), were obtained in ASOC1 cuttings treated with 1000 mgL^{-1}. Besides, compared with the control, all IAA treatments enhanced the rooting rate of *A. spinosa* cuttings but were less effective than IBA and NAA. Indeed, the highest sprouting ratio (63.25%), rooting ratio (27.75%) and survival ratio (94.0%) with IAA treatment was obtained with ASOC1 by using 5000 mgL^{-1} IAA.

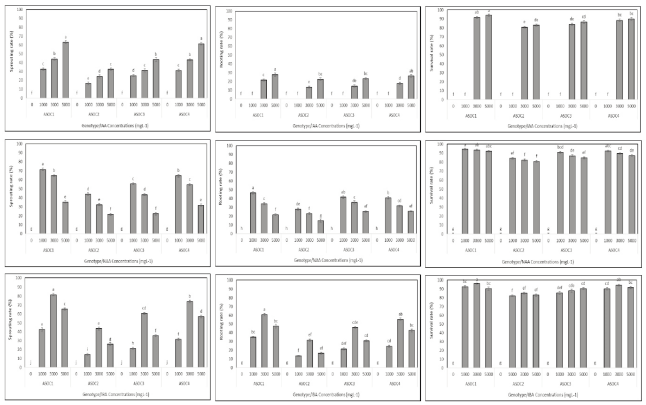

Fig. 5 - Effects of auxin type, concentration and genotype on sprouting, rooting and survival rate of *A. spinosa* cuttings. Within each treatment, values marked by the same letter are not significantly different (Duncan's Multiple Range Test, P<0.05, mean ± SE, n = 32). Mean values of sprouting percentage, rooting percentage, and survival rate were subjected to arcsine √X transformation before statistical analysis.

4. Discussion and Conclusions

The results of the present study indicate that there is a large amount of variability between the genotypes in their sprouting, rooting and survival measurements. These results fit well with those obtained by Nouaim *et al*. (2002), which reported that the rooting capacity of *Argania spinosa* cuttings has proven to be difficult and strongly genotype dependent. The significant variation among genotypes could be due to considerable genetic variation (Prat *et al*., 1998). It could be possible that some of the variation for root response results from the differences in age or in physiological states of initial cutting sources. It has also been reported that cuttings time of collection in different seasons could also

affect the rooting potential in clonal propagation. In fact, the varied effectiveness of auxin concentrations among genotypes and species is likely to be related with differences in the amount of endogenous hormone and associated to root co-promoters in the plant tissues at the time of severance (Hartmann *et al*., 2002). Otherwise, the decreasing in rooting ability could be assigned to low sensitivity of the tissues to auxin or by secondary metabolites accumulation, causing oxidation and inactivation of enzymes and phytohormones which inhibit regenerative potentialities of tissues (Wilson, 1994; Husen and Pal, 2007). Similar observations have been reported by Mabizela *et al*. (2017), who concluded that four studied genotypes of honey bush (*Cyclopia subternata*) had significant differences in rooting potential. Considering the above results, sprouting, rooting and survival ability of *Argania spinosa* cuttings were significantly higher (P> 0.05) in cuttings treated with exogenous auxins. The fact that exogenous auxin treatment exhibited a greater potential for adventitious rooting as well as a rapid growth of cuttings is widely recognized on different plant species (Hunt *et al*., 2011; Gehlot *et al*., 2014). The effect of auxin on adventitious roots growth and development can be explained by its role in wound healing through the inhibition of IAA-oxidase activity and the activation of the enzymatic antioxidant defense system, which helps to restore the redox balance and protect tissues from oxidative damage, particularly during the different steps of adventitious root development (Rout, 2006). While in contact with the basal cell, the auxin pool may have an indirect influence by promoting activity of starch hydrolytic enzymes and enhancing the translocation speed of carbohydrates to the cuttings base (Haissig, 1974; Aminah *et al*., 1995). Consequently, it supplies the cutting with the required energy for hastening cell differentiation of root primordia, growth and development via cell divisions and elongation (Husen, 2008). Auxin could also act through selective proteolysis and cell-wall loosening (Schopfer, 2001). Hence, it regulates the organ growth and development, promotes emergence of shoot buds, sprout length and consequently resulting in a better overall growth of the cuttings (Schroeder and Walker, 1990). In the present study, cuttings treated with distilled water without growth regulators did not show any response on rooting and sprouting. Thus, it has been proved that the IBA application is more effective than NAA and IAA. In most cases, 3000 mgL^{-1} of IBA was the most effective concentration for promoting sprouting, rooting and survival rates of all studied genotypes. Higher efficiency of IBA at inducing adventitious rooting may be explained by higher chemical stability against catabolism and inactivation by conjugation, nontoxic over a wide concentration range, low mobility and available over a longer period of time in the plant tissue (Barrel *et al*., 2001; Ludwig-Muller, 2000; Hartmann *et al*., 2002). Whereas, the higher concentration of IAA stimulates ethylene production in plant cells, which is known to inhibit root induction and elongation (Mulkey *et al*., 1982). Similar to our results, Kesari *et al*. (2009) found a relatively poor rooting yield with IAA treated stem cuttings of *Pongamia pinnata* in comparison to IBA. The result shows that IBA had a stronger effect on sprouting and rooting than NAA, the reason may be that NAA is very stable and more persistent than other auxins and remains present in the tissue in its free form (Dunlap *et al*., 1986).

Thus, NAA decreases the level of nutrients mobilization and translocation to the root primordia and blocks the roots outgrowth (Husen and Pal, 2006). For most genotypes, auxin responses are concentration dependent and tissues react in a distinct manner to varying amounts of exogenous auxins. Thereby, adventitious root production decreased at very low concentrations of IBA (<1000 mgL^{-1}) and increased to levels between 1000 and 3000 mgL^{-1}. However, with the increase in hormone concentration levels (>3000 mgL^{-1}), the number and length of roots decreased. In accord to our results, Akakpo *et al*. (2014) showed a decline in rooting rates of *Vitellaria paradoxa* stem cuttings for IBA concentrations exceeding 3000 mgL^{-1}. This result confirms the findings of Hartmann *et al*. (2002), who demonstrated that too little IBA can decrease rooting and concentrations substantially higher than those normally found in plant tissues may be inhibitory, phytotoxic or even cause cell death. The results of our study revealed that IBA concentrations and genotype strongly influence the sprouting and rooting ability of *A. spinosa*, which confirms previous studies indicating that the optimal IBA concentrations for suitable adventitious root responses vary according to different species. Indeed, Singh and Rawat (2017) achieved a maximum increase in sprouting and rooting on semi-hardwood cuttings of *Zanthoxylum armatum* treated with 3000 mgL^{-1} IBA. Besides, Tsipouridis *et al*. (2003) observed a maximum rooting rate in *Prunus persica* treated with 2000 mgL^{-1} IBA. Husen (2008) reported that cuttings of *Dalbergia sissoo* treated with 2000 mgL^{-1} IBA induced a stronger rooting system. Also, in *Aesculus indica*, the highest rooting rate was recorded in stem

cuttings treated with 4000 mgL^{-1} IBA (Majeed *et al*., 2009). The maximum rooting rate for *Tectona grandis* was obtained with 4000 mgL^{-1} IBA as compared to the other treatments (Husen and Pal, 2007). However, in *Pongamia pinnata*, IBA at 1000 mgL^{-1} was found to be the most effective concentration for rooting ratio and root number (Kesari *et al*., 2009).

The results of this study revealed that adventitious root development and shoot growth of *Argania spinosa* semi-hardwood cuttings were significantly influenced by auxin type, concentration, genotype and their interactions. There was a variation among the different genotypes studied, indeed cuttings taken from ASOC1 and ASOC4 were rooted and sprouted better than those from ASOC2 and ASOC3. It was observed that almost all the treatments, except the control set, were able to induce sprouting and rooting in cuttings, and the IBA application is more effective than NAA and IAA. IBA application at a concentration of 3000 mgL^{-1} to ASOC1 cuttings seemed to be the best treatment in terms of sprouting, rooting and survival rates, and also for the number of leaves, leaf size, number of sprouts, sprout length, number of roots, and root length per cuttings. The present study indicated that *A. spinosa* can be propagated through semi-hardwood cuttings of élite trees and can be usefully applied to promote the role of vegetative propagation of this species through forest restoration and for breeding programs. Thus, further experiments should be conducted to extend the techniques to induce rooting of cuttings from selected élite genotypes used as a source material for argan tree orchards.

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Effect of different nutrient solution and irrigation regimes on growth of Lily (*LA Hybrid 'Fangio'*)

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Abstract: A better understanding of the effects of nutrients element and irrigation levels on production of Lily (*Lilium* LA Hybrid *Fangio*) can lead to optimal uses of nutrients and water. Plant growth is strongly correlated with the amount of irrigation and fertilization. In this regard, a greenhouse experiment was carried out to evaluate the effect of different nutrient solution viz. high concentration of elements (S1), medium concentration of elements (S2), and high concentration of elements (S3) under different irrigation regimes (100, 90, 80 and 70 % of field capacity (FC) in soilless culture. In well-watered treatments (100% FC), S3 enhanced the vase life by 17% compared to S1. The maximum leaf number was observed in the interaction of S3 and 90% FC, whereas its minimum was found in the interaction of S1 and 70% FC. Under 70% FC, S3 increased the leaf length by 6% in comparison with S1. Leaf width was altered by simultaneous use of nutrient solution and irrigation, ranging from S2 and 80% FC (13.3 mm) to S1 and 70% FC (9 mm). In S3, 70% FC decreased the bud length by 9% relative to 100% FC. The days until flowering varied from the interaction of S2 and 70% FC (4.1 days) to S1 and 100% FC (6 days). Under S1 treatments, 70% FC decreased the flower number by 18% compared to 100% FC. The highest weight of daughter bulb was observed in interaction of S2 and 80% FC. In contrast, the lowest weight of daughter bulb was found in S2 and 90% FC.

1. Introduction

Lilies have become economically important, mainly because of beautiful, large, attractive and fascinating form of flowers, long vase life and capacity to rehydrate after long transportation. Bulbs are produced commercially for use in the cut flower and potted plant industries (Aslam *et al*., 2013; Al-Allaq *et al*., 2014). The importance of this genus in the world flower market is due to the existence of a wide variety of hybrids and numerous commercial cultivars (Dhyani *et al*., 2009).

In recent decades, supplying the nutrient solution to plants in order to optimize crop nutrition has been widely used in both soil and soilless cul-

ture under greenhouse conditions (Savvas *et a*l., 2013). Nutrient solution management can provide a sustainable and effective schedule in floriculture by applying different horticultural practices such as water use efficiency. It has been estimated that 100-350 L of water are needed to produce 1 kg of plant dry matter, and this may vary with species and variety, cultivation system and plant growing season (Cassaniti *et al*., 2012). The lack of dependable supplies of good quality water in many regions of the world has become a concern among agricultural, urban, industrial and environmental components (Valdez-Aguilar *et al*., 2009). Optimal irrigation scheduling could lead to higher water use efficiency. It is also very important since it influences the rhizosphere environment, media water potential, and salt accumulation, which in turn affects plant growth, photosynthesis and consequently crop production and quality (Tsirogiannis, 2010). Appropriate use of water supply and nutrients results in better water use efficiency, stressful situations, and control production (Raviv and Blom., 2001). Since in soilless culture systems the water is generally distributed in excess, and consequently the nutrients are removed from the substrate by drainage water and accumulate in the recirculating nutrient solution, which has to be flushed out regularly, representing the negative environmental impacts (Rouphael and Colla., 2009; Wortman, 2015). In bulbous ornamental plants, the flower production and bulb yield are remarkably important in their profitability (De Vroomen, 1993; Maroyi, 2016). An accurate schedule on nutrients and water supplies may decrease production costs and the risk of water pollution (Dufour and Guérin., 2005). Hence, it is essential to have a good knowledge of the plant's mineral requirements in order to avoid nutrient waste. On the other hand, it is necessary to limit mineral imbalance in the medium by assuring a minimal leaching of excess nutrient solution (Chang *et al*., 2010).

Recently, some nutritional solution formulas and irrigation levels have been used to increase the production efficiency, which the suitability of these treatments should be considered with appropriate levels of nutrients and irrigation (Grewal and Maheshwari, 2011; Waraich *et al.*, 2011; Grzebisz *et al.*, 2013; Quaggio *et al.*, 2019). The intent of these studies was to develop the optimal irrigation and nutrient systems that could meet the high productivity of plants with explaining different levels of nutrients and water supply. To our knowledge, there is no published document on the water use and nutrients application on the growth of lily. Therefore, the purpose of present study was to assess the simultaneous application of different irrigation regimes and nutrient solutions on growth of lily (*Lilium* LA sp. cv. *Fangio*).

2. Materials and Methods

Plant material and growing conditions

The experiment was carried out during 2016-2017, under greenhouse conditions at the research unit greenhouse of Islamic Azad University, Gorgan, Iran, for three months. Inside the greenhouse, ventilation was provided automatically when the air temperature exceeded 26˚C. Average day and night temperature were 25˚C and 14˚C, respectively, and average maximum and minimum relative humidity was 70% and 50%, respectively. Uniform sized bulbs of LA Lilium (*Lilium* LA. Hybrid) cv. '*Fangio*' were obtained from a commercial importer (circumference 18-20 cm) and was immersed in a fungicide drench for 10 seconds. The bulbs were cultured in the medium containing cocopeat and perlite (2:1) with EC of 2.95 dS m^{-1}, pH of 5.9, and bulk density of 0.17 g cm^{-3}. The bulbs were cultured in the plastic pots (15 cm height, 17 cm diameter, and 3 liters) on September 2016.

Nutrient solution and irrigation regimes

The study was designed as factorial based on completely randomized design (CRD) with three levels of nutrient solution and four levels of irrigation in six replications. The nutritional solutions contained a low concentration of elements (S1), medium concentration of elements (S2) and high concentration of elements (S3). The nutrient solutions were prepared based on modified Quick nutrient solution (Olfati, 2015). All chemical substrate (solution or elements) used in the study were purchased from Merk (Darmstadt, Germany). Water regimes were applied at 100, 90, 80 and 70% field capacity (FC). It was conducted based on weighing method as 200 ml for well-watered treatment (100% FC) followed by 180, 160, and 140 ml for 90, 80 and 70% FC, respectively. The nutrient solutions used in the experiment are shown in Table 1. The concentrations of ions in the irrigation water were expressed as mgL^{-1} (Table 1). Micronutrients were added to the plants as iron chelate (EDDHA) (0.075 ppm), manganese sulfate 0.001, zinc sulfate 0/01, copper sulfate 0.03, EDTA molybdenum 0.001, and boric acid (H_3BO_4) (0.02 percent. Two weeks after culturing the bulbs (when the

bulbs grew in 10 cm), feeding was carried out manually according to the plant's water requirement.

Growth parameters

At the end of experiment, the bud length, leaf length, leaf width, number of leaves, plant height, number of flowers, time of flowering, number and size of daughter bulb were measured. Flowers were harvested when the first flower in plants was bloomed. Subsequently, vase life was determined. The vase life of individual flowers in an inflorescence was evaluated according to their appearance as the number of days since bud opening till the appearance of deformation because of petal wilting. The vase life of the inflorescence was determined as the number of days from the beginning of the experiment up to the fading of the second flower. The end of vase life was determined by flower wilting, tepal abscission or color change in tepals, while in case of leaves, by color fading of blade, yellowing or dying on 30% of the leaf surface.

Statistical analysis

The data (n=6) were subjected to one-way analysis of variance (ANOVA) and using the SAS software package for windows (SAS, version 9.3, SAS Institute, Cary, NC). When statistical significance (p<0.05) was detected, the mean values subjected to Duncan's multiple range tests.

3. Results

Vase life and plant height

Vase life of lily was affected by nutrient solution (P≤0.05, Table 2). In well-watered treatments (100% FC), S3 enhanced the vase life by 17% compared to S1 (Table 3). Plant height was influenced by the nutrient solution (P≤0.05, Table 2). Plant height was different between treatments, ranging from 18.8 cm in

Table 1 - The concentration of elements used in the nutritional solution

Nutrient solutions (mg L^{-1})	Potassium nitrate	Dipotassium hydrogen phosphate	Potassium dihydrogen phosphate	Calcium nitrate	Ammonium nitrate	Sodium chloride	Magnesium sulfate	Ec (dS m^{-1})	pH
Nutrient Solution 1 (S1)	631.3	43.5	102	512.5	150	14.6	230.6	1.04	5.8
Nutrient Solution 2 (S2)	757.55	52.2	122.4	615	180	17.5	276.7	2.38	5.4
Nutrient Solution 3 (S3)	883.8	60.9	142.8	717.5	210	20.4	322.8	2.74	5.7

Table 2 - Analysis of variance for the studied traits of lily (*Lilium* LA sp. cv. Fangio)

S.O.V	df	Vase life	Plant height	Leaf number	Leaf length	Leaf width	Days until flowering	Flower number	Bulb number	Bulb length	Bulb width
Nutrient solution (NS)	2	22.87 **	117 **	229.4 **	488 **	35.95 **	4.05 NS	0.29 NS	10.68 NS	263 **	0.96 NS
Irrigation regime (IR)	3	1.03 NS	11.2 NS	240.7 **	235 **	16.52 **	57.27 NS	11.12 **	0.12 NS	326 **	9.14 NS
NS×IR	6	2.13 NS	3.24 NS	137.1 **	64.2 **	2.22 *	38.27 NS	0.81 NS	0.4 NS	27.7 NS	8.58 *
Error	55	2.63	8.2	9.05	8.62	0.83	40.8	0.85	0.77	14.6	3.76
CV		10.24	2.4	3.18	2.93	7.81	8.61	10.56	17.32	3.7	17.81

Table 3 - Effect of nutrient solutions and irrigation regimes on post vase life, plant height, leaf number and size of lily (*Lilium* LA sp. cv. Fangio)

Nutrient solutions	Irrigation regime	Vase life (day)	Plant height (cm)	Leaf number	Leaf length (mm)	Leaf width (mm)
Nutrient solution 1 (S1)	100%	14.6±1.8 c	113.0±2.1 c	92.5±2.5 c	101.5±1.9 de	10.6±1.2 fg
	90%	15.1±1.1 a-c	113.5±3.3 c	90.5±2.4 cd	94.8±3.5 f	11.6±1.1 d-f
	80%	15.0±1.4 a-c	114.8±2.5 bc	93.5±3.6 c	96.1±4.1 f	9.8±0.5 gh
	70%	14.0±1.8 ab	112.8±3.7 c	87.5±1.9 d	90.3±4.2 g	9.0±0.8 h
Nutrient solution 2 (S2)	100%	16.5±1.7 ab	115.5±2.1 a-c	96.6±1.9 b	101.5±1.4 de	11.8±0.8 c-e
	90%	16.1±1.4 ab	116.8±1.9 a-c	94.0±3.2 bc	109.5±5.1 a	12.8±0.7 a-c
	80%	17.0±2.1 a	116.5 ±2.3 a-c	105.0±2.6 a	104.1±2.1 b-d	13.3±0.7 a
	70%	15.6±1.2 a-c	116.3.5±4.1 a-c	90.0±1.9 cd	100.2±2.3 e	11.5±0.8 d-f
Nutrient solution 3 (S3)	100%	17.0±1.4 a	115.8±3.1 a-c	96.8±1.5 b	107.5±2.1 ab	12.1±0.6 b-d
	90%	16.1±1.5 ab	118.8±3.3 a	104.6±2.6 a	105.1±2.3 bc	13.7±0.8 a
	80%	15.8±0.5 a-c	118.8±3.2 a	93.5±2.5 bc	102.8±1.8 c-e	13.1±0.6 ab
	70%	16.8±1.2 a	118.0±3.4 ab	90.1±3.8 cd	96.8±2.7 f	10.8±0.8 e-g

plants supplied with the interactions of S3 and 90% FC as well as S3 and 80% FC to 112.8 cm in plants treated with the interaction of S1 and 70% FC (Table 3).

Leaf number and size

The number, length and width of leaves were significantly changed by nutrient solution, irrigation regimes, and their interaction ($P \leq 0.01$, Table 2). The maximum leaf number was observed in the interaction of S3 and 90% FC (104.6 leaf), whereas its minimum was found in the interaction of S1 and 70% FC (Table 3). Under 70% FC, S3 increased the leaf length by 6% in comparison with S1 (Table 3). Leaf width was changed by simultaneous application of nutrient solution and irrigation, ranging from S2 and 80% FC (13.3 mm) to S1 and 70% FC (9 mm).

Bud length, days until flowering, and flower number

Bud length was affected by nutrient solution and irrigation regime ($P \leq 0.01$, Table 4). In plants supplied with S3, 70% FC decreased the bud length by 9% relative to 100% FC (Table 4).The days until flowering was not significantly influenced by nutrient solution and irrigation regimes ($P \geq 0.05$, Table 2). Under the application of both nutrient solution and irrigation regimes, the days until flowering varied from the interaction of S3 and 70% FC (69.1 days) to S1 and 90% FC (77.8 days) (Table 4). Flower number was significantly affected by irrigation regime ($P \leq 0.01$, Table 2). Under S1 treatments, 70% FC decreased the flower number by 18% compared to 100% FC (Table 4).

The number and weight of daughter bulbs

Daughter bulb number was not significantly influenced by nutrient solution and irrigation regimes ($P \geq 0.05$, Table 2). Under the application of both nutrient solution and irrigation regimes, bulb number varied from the interaction of S3 and 100% FC (4.1 bulbs) to S1 and 100% FC (6 bulbs) (Table 4). Daughter bulb weight was affected by the interaction of nutrient solution and irrigation regime ($P \leq 0.01$, Table 2). The highest weight of bulb was observed in interaction of S2 and 80% FC. In contrast, the lowest weight of daughter bulb was found in S2 and 90% FC (Table 4).

4. Discussion and Conclusions

The results showed that high concentration of the elements (S3) improved the flower and leaf attributes of lily. Treder (2005) reported an improvement of bud length and leaf size under the mixture of multicote and liquid fertilization. The results of morphological parameters such as leaf size, number of leaves per plant, and flower number in our study are supported by Khosa *et al.* (2011), as they observed an increase in growth and flowering with the increasing fertilization level of macronutrients. Also, the results of the previous studies have shown that the height of some cultivars might not be affected by fertilization (Treder, 2003). Therefore, it can be concluded that in different cultivars of the lily, the height and some vegetative growth characteristics are influenced by cultivars (Treder, 2003). Our results are also consistent with Devecchi and Remotti (2003), who studied calla (*Zantedeschia aethiopica*) in which different doses of nitrogen and potassium did not significantly affect the length of the floral stem. Moreover, the results of plant height are well-supported by Treder (2005) as the application of multicote at three levels and water-soluble fertilizer for some cultivars of lily at during the vegetation period did not significantly affect the maximum plant height in 'Acapulco', rela-

Table 4 - Effect of nutrient solutions and irrigation regimes on the flower and bulb properties of lily (*Lilium* LA sp. cv. Fangio)

Nutrient Solutions	Irrigation regime	Bud length (mm)	Days until flowering	Flower number	Bulb number	Bulb weight (gr)
Nutrient Solution 1 (S1)	100%	98.9±2.5 c-e	71.5±6.1 ab	9.50±0.9 a	6.0±0.8 a	10.7±2.4 ab
	90%	101.7±2.7 b-d	77.8±6.5 a	8.16±0.8 bc	5.8±0.6 a	10.1±3.1 ab
	80%	98.4±2.9 c-e	74.6±6.9 ab	9.00±1.1 a-c	5.6±0.7 ab	12.1±2.8 ab
	70%	92.7±2.5 f	72.0±3.9 ab	8.00±0.6 bc	5.5±0.7 a-c	10.4±3.4 ab
Nutrient Solution 2 (S2)	100%	109.1±3.2 a	73.6±5.4 ab	9.16±0.8 ab	5.1±0.8 a-d	11.8±2.1 ab
	90%	105.6±2.5 ab	73.3±4.7 ab	8.83±0.9 a-c	5.1±0.4 a-d	8.3±2.6 b
	80%	103.1±3.5 bc	74.0±4.6 ab	9.66±0.7 a	4.6±0.6 b-d	12.5±1.1 a
	70%	99.2±2.8 c-e	74.6±6.6 ab	7.83±0.9 c	5.1±1.1 a-d	11.7±3.2 ab
Nutrient Solution 3 (S3)	100%	103.7±2.4 bc	76.1±6.8 ab	9.66±0.7 a	4.1±0.6 d	10.9±1.9 ab
	90%	108.9±3.3 a	77.5±5.8 ab	7.83±0.8 c	4.3±0.4 d	11.1±2.1 ab
	80%	103.4±2.8 bc	76.8±4.4 ab	9.50±1.0 a	4.5±0.4 cd	10.1±1.9 ab
	70%	95.2±2.7 ef	69.1±5.1 b	7.83±0.6 c	4.6±0.1 b-d	10.7±2.2 ab

tive to other cultivars of lily. Similar to our results, in *Sandersonia aurantiaca*, the number of flowers was not affected by various nutrient treatments (Bernstein *et al.*, 2011). Also, yield, quantified by the mean number of bud per plant, did not differ between the three nutrient solutions or between the four different levels of irrigation tested. The increase of morphological properties of lily fertilization is due to the significant role of essential element presented in the nutrient solution on plant growth. For example (N), Nitrogen is a major constituent of most important plant substances. N compounds comprise 40% to 50% of the dry matter of protoplasm, and it is a constituent of amino acids, the building blocks of proteins. It is also an essential constituent of chlorophyll. N deficiency most often results in stunted growth, slow growth, and chlorosis. Potassium (P) regulates the opening and closing of the stomata by a potassium ion pump, which has a significant role on leaf and flower growth. Additionally the outstanding role of magnesium in plant nutrition is as a constituent of the chlorophyll molecule (Vatansever *et al.*, 2017). Vegetative characteristics of plants are subjective to genetic and environmental factors coupled with optimum fertilizer application and water use method (Lucidos *et al.*, 2013). Marin *et al.* (2011) investigated that the effect of nitrogen, potassium, calcium mixture solutions on Lilium plants, they founded that application of three elements, significantly affected the vegetative growth of *Lilium cv. 'Navona'*expressed as leaf area. Moreover, in this regard, the results of the present study are in accordance with the results of Younis *et al.* (2014) and Asker (2015). The other investigation indicated that the existence of macronutrients enhances the vegetative growth of plants and flowering as N increases (Imran and Gurmani, 2011).

Our results showed that there is no significant change among 100, 90, and 80% FC. However, 70% FC showed a reduction of leaf size, bud number, and days until flowering. Welsh *et al.* (1991) reported that *Photina* × *fraseri* irrigated with 100%, 75%, and 50% represented no different difference in shoot extension and average leaf area. Groves *et al.* (1998) reported similar results with a 40% reduction in irrigation volume resulting in the production of 90% dry weight of C. *dammeri* 'Skogholm'. Also, Rouphael *et al.* (2008) reported that the highest leaf area, and quality index were recorded in irrigation type, using half and full nutrient solution concentration. In another work, the strong correlation of leaf area with high K concentration in the nutrient solution was reported, which is probably due to K role in water relations, turgor maintenance, and cell expansion (Mengel and Kirkby, 2001). Thus, an ample supply of K is recommended for leaf development, which will contribute to plant quality since the foliage is considered an important quality factor to Lilium (McKenzie, 1989). Thus, Lilium can be considered as a species that is compatible with a wide range of nutrient concentrations. Nitrogen is an essential component of chlorophyll, in this connection, the nitrogen content of leaf correlates positively with the leaf chlorophyll content (Loh *et al.*, 2002). Kang *et al.* (2016) showed that an increase in fertilization can reduce the severity of chlorosis in pansy. They pointed out that leaf chlorophyll concentration increased asymptotically with fertilization rate, which finally increase the leaf size and plant height.

In order to reducing over watering, we investigated the water use efficiency and nutrient elements in different nutrient solution and irrigation regimes to ensure adequate water for plants during the growth periods. Outcomes of the present study indicated that Lilium LA Hybrid '*Fangio'* can be properly grown in medium levels of irrigation with no significant change in its morphological properties. 70% FC slightly changed the plant growth particularly leaf size and bud length. Hence, to optimum use of water, we can reduce the water amount to 80% FC even 70% with high use of nutrients concentration.

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Effective pollination period and its influence on fruit characteristics of 'Hayward' kiwifruit

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Key words: anthesis, fruit set, fruit trait, kiwifruit, pollination.

Abstract: Pollination is crucial for producing marketable kiwifruit, and increasing revenue of growers. The objectives of this research were to determine the effective pollination period (EPP) of 'Hayward' *(A. deliciosa* A. Chev. C.F. Liang & A.R. Ferguson) and to determine fruit characteristics in relation to the time of pollination. Hayward kiwifruit showed no significant decrement of fruit set and fruit weight within the 4-day and 2-day period, respectively, however, the mean weight of fruit was ≥ 85 g within 4-day. Fruit set was 100% when pollination was carried out during the first 3 days following anthesis. Fruit set decreased to 20.71% when flowers were pollinated 5 days after anthesis and were practically nil by 6 days after anthesis. Fruit weight and size were the highest on days 1-2 after anthesis and reduced for flowers pollinated 3-4 days after anthesis. The lowest fruit weight and seed weight and number were observed when pollination was done on day 5. Hayward kiwifruit showed no significant drop in fruit set within the 4-day period, and thus, appears to have an EPP equal 4 days after anthesis. Thus, efforts for producing good quality and of marketable size fruit should be concentrated within the first 4 days after anthesis.

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1. Introduction

Hayward kiwifruit (*Actinidia deliciosa*) is the most widely grown *Actinidia* crops (Ferguson, 1990). This cultivar is chosen based on its large fruit production and long storage life. Optimal kiwifruit production is highly dependent on the level of pollination because insufficient pollination is known to lead to unsatisfactory fruit size, shape and uniformity; however, pollination of kiwifruit is impaired by the dioecious nature of the species (Pyke and Alspach, 1986).

Flower receptivity can be evaluated by determining the effective pollination period (EPP). EPP is defined as the number of days following anthesis during which pollination is effective in producing marketable fruit. Various factors may affect the EPP. It was shown that the EPP could be affected by temperature, flower quality, and chemical treatments (Sanzol and Herrero, 2001). Because of dioecious nature of kiwifruit, other factors should be in a favorable condition to determine of EPP in

this species. For example, male vines can be properly distributed, and proper timing of beehive placement can be achieved. In addition, determining the EPP of kiwifruit species/cultivars could allow growers to optimize supplemental pollen applications by applying only during the EPP, and thus, reduce costs.

Kiwifruit flowers are receptive for only a few days following anthesis where pollination can be successful leading to a good marketable fruit set. The EPP may be restricted by limitation in three main events along the reproductive process, stigma receptivity, pollen tube kinetics and ovule longevity (Sanzol and Herrero, 2001).

Estimation reports are available for the different cultivar of kiwifruit. There are variances among the reported duration of the EPP and stigma receptivity. According to Galimberti *et al.* (1987), the EPP of Hayward cultivar reported 3 days in Italy. The EPP for *A. deliciosa* 'Hayward' was determined to be 4 days by Gonzalez *et al.* (1995) in Spain. It was discovered that the duration of stigma receptivity closely fit the EPP, thus it appears that the EPP is limited by stigma receptivity. Sale (1981) reported that the pistillate flowers are receptive for 7-9 days after anthesis (DAA) in New Zealand. Goodwin (2000) also reported this longer period, where the relatively constant and high receptivity was displayed for the first 8 days before dropping. Goodwin *et al.* (2013) found stigma receptivity to be highest during the first 2 DAA in *A. chinensis* 'Hort16A'. They noted that pistillate flowers from *A. chinensis* dehisced their petals after 2 days while *A. deliciosa* typically hold their petals for 5 DAA. The EPP for *A. deliciosa* 'AU Fitzgerald' and *A. chinensis* 'AU Golden Sunshine' was determined to be 4 and ≥5 days respectively by Thompson (2014). In golden kiwifruit cultivars, not only fruit set but also fruit characteristic was affected by DAA pollination. Fruit size, weight, and seed number were reduced on day 5 after anthesis in 'AU Fitzgerald' (Thompson, 2014). Thompson (2014) found that 'AU Golden Sunshine' showed no significant drop in fruit set or size within the 5-day period. However, differences in fruit weight, fruit size index and seed number were found between 1-3 and 4-5 DAA by Brantley (2016).

Fruit set of 'Hayward' cultivar is influence by the time elapsed between anthesis and pollination. It seems that fruit traits in this cultivar may be affected by days after anthesis pollination too. There are some papers reporting contrasting results about the effective pollination period on fruit quantitative and qualitative traits in this cultivar. Therefore, the aims of this research were to evaluate the effective pollination period of 'Hayward' and to determine fruit characteristics in relation to the time of pollination.

2. Materials and Methods

Experimental design

This experiment was conducted using 10-year old vines of 'Hayward'. Kiwifruit vines were grown in an orchard located in Astara, Guilan province, Iran (38°22'N; longitude of 42°50'E), 10 m altitude, trained to a T-bar system with plants spaced of 4×6 m.

Treatment application

Effective pollinated period (EPP) was studied for 3 years. 'Hayward' flower buds were bagged on May 21, 2013; May 18, 2014, and May 23, 2015, using wax paper bags (10.2 × 26.2 cm). Flower buds were bagged 1 day before anthesis; still completely closed but showing some white from petal unfolding, identified as "Stage 57" in BBCH phenology system (Salineroa *et al*., 2009). Anthesis was the day the flower petals opened. The detailed characterization of environmental conditions during the experimental period is shown in Table 1.

Table 1 - Air temperature, relative humidity (RH), sunny hours, precipitation and mean daily transpiration (mm d^{-1}) during three months in 2013-15

Year	Month	Temperature (°C)					Evaporation (mm/d)	Relative humidity (%)	Precipitation (mm)	Sunny hours
		Mean	Max.	Min.	Max. Abs.	Min. Abs.				
2013	April	13.0	16.5	9.5	25.4	4.2	43.1	81	81.8	115.2
	May	17.0	21.7	12.3	27.2	4.8	114.4	76	29.4	236.4
	June	22.5	27.2	17.7	32.8	13.0	175.7	73	58.7	278.3
2014	April	11.1	15.3	6.9	24.8	0.4	68.7	79	95.6	172.7
	May	19.1	23.6	14.5	30.8	10.4	142.8	74	34.7	249.3
	June	23.5	27.9	18.6	32.2	14.8	175	74	15.6	269.4
2015	April	9.6	14.9	12.2	27.2	5.0	59.8	85	44.6	120.6
	May	13.9	19.8	16.9	24.2	7.8	106.9	81	23.5	189.3
	June	20.6	28.1	24.3	33.4	17.0	184.7	70	0.8	236.1

For every year, pollen was collected from staminate vines (Tomuri) one day before anthesis and dried on paper at room temperature. The pollen was then sieved using a fine mesh (0.26 mm) to remove dehisced anthers and other impurities. Bag-isolated pistillate flowers were hands pollinated with the dried pollen before being re-bagged. Twenty four flowers were hand-pollinated each day at 0, 1, 2, 3, 4, 5, 6 and 7 DAA using dried pollen. Uniform and single flower inflorescences were hands pollinated during the study. Flowers were re-bagged using newly labeled bags immediately following hand-pollination to prevent subsequent open pollination.

Data collection

Fruit set of all EPP treatments was determined when bags were removed three weeks later after pollination. For fruit trait analyses, they were harvested on November 7, 2015, when the value of Total Solid Soluble (TSS) detected was =6.2°Brix. Fruit length (L, mm), major width (W1, mm) and minor width (W2, mm), fruit density (g/ml), fresh weight (g), fruit volume (ml), fruit size index [(L+W1+W2) * 3^{-1})] seed number and seed weight (g) as quantity characteristics were measured (Brantley, 2016). To determine the average seed weight of each fruit, weight of 100-seed samples was determined thrice and the average of these values was used to calculate the total seed number for each fruit of each treatment (Goodwin *et al.,* 2013).

Statistical analysis

Combined analysis of three years fruit set data was performed on according to a completely randomized design. Data of fruit traits were analyzed as a completely randomized design. All significant means were separated, using Duncan (P≤0.05). The correlation between fruit weight with seed number and weight were calculated via the software SPSS 22.

3. Results

Fruit set after hand pollination was the highest, averaging 100% during the first 3 days following anthesis (Fig. 1). However, no significant differences were found between the days of treatment 1-4 (P≤0.05). Fruit set dropped down to 20.71% in the flower pollinated at 5 DAA. By 6 DAA, fruit set was practically nil. Thus, the EPP was limited to the first 4 DAA (Fig. 1).

Fruit weight and size (equivalent water volume) were the highest on day 1 (Table 2). A reduction trend was observed in flowers pollinated 2 DAA, however, did not differ from 1 DAA (P≤0.05). Fruit weight and size were reduced for flowers pollinated 3-4 DAA and the lowest value was observed on those pollinated 5 DAA (P≤0.05). Fruit density, fruit length, and fruit size index were the highest in the 1 DAA (Table 2) because the values were reduced on 2-4 DAA and the lowest value observed on day 5. Fruit major and minor width, and seed weight and the

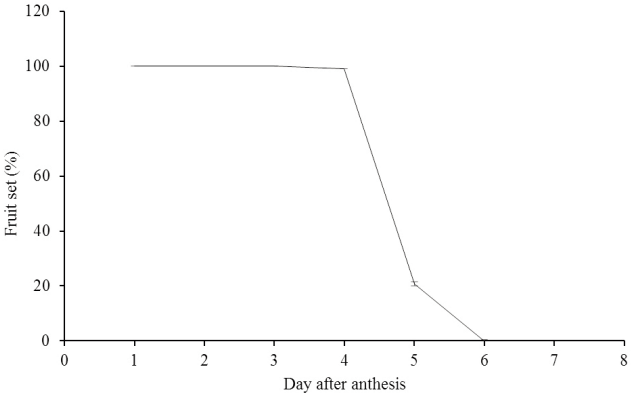

Fig. 1 - Effects of hand pollinating *Actinidia deliciosa* 'Hayward' flowers 1, 2, 3, 4, or 5 days after anthesis (DAA) on fruit set from 2013 to 2015. Values are the mean three years ± SE of three replicates.

Table 2 - Effects of hand pollinating Actinidia deliciosa 'Hayward' flowers 1, 2, 3, 4, or 5 days after anthesis (DAA) on fruit characteristics. Fruit were harvested 7 Nov. 2015

DAA	Weight (g)	Fruit volume (ml)	Fruit gravity (g/ml)	Fruit length (mm)	Major width (mm)	Minor width (mm)	Fruit size index	Seed weight per fruit (g)	Seed number per fruit	Seed weight (mg)
1	105.86 a	103.54 a	1.02 a	70.18 a	53.20 a	47.22 a	56.87 a	1.61 a	1218.97 a	1.33 a
2	95.10 ab	93.54 ab	1.016 b	64.86 b	52.72 a	46.35 a	54.64 b	1.56 a	1326.43 a	1.17 b
3	84.89 b	83.83 b	1.012 bc	63.18 b	50.42 ab	45.11 ab	52.91 b	1.56 a	1239.06 a	1.25 a
4	85.16 b	83.74 b	1.016 b	61.86 b	51.12 ab	45.07 ab	52.68 b	1.56 a	1226.35 a	1.27 a
5	71.73 c	71.10 c	1.008 c	56.44 c	49.37b	43.32 b	49.71 c	0.64 b	498.73 b	1.33 a

Each Values is the mean of three replicates with 15 fruits. Means in each column followed by the same letter are not significantly different according to Duncan's multiple range test (P≤0.01).

number did not show significant reduction 1-4 DAA, however, the value was strongly reduced for flowers pollinated at 5 DAA (P≤0.05).

Correlation analyses showed a positive significant correlation between fruit size with seed number (r = 0.54**), and seed weight (r = 0.58***) (Table 3). The correlation between fruit weight with seed weight was higher than fruit weight with the seed number.

Table 3 - Correlation coefficient between fruit weight with seed number and weight in Actinidia deliciosa 'Hayward'

	Fruit weigh	Seed number	Seed weight
Fruit weight	1	0.54 **	0.58 ***
Seed number	0.54 **	1	0.95 ***
Seed weight	0.58 ***	0.95 ***	1

, * Correlation is significant at the 0.01 and 0.001 levels respectively.

4. Discussion and Conclusions

Effective pollination period is important in kiwifruit because successful pollination results in more seeds per fruit, and seed number directly correlates with fruit size and weight (Hopping, 1976). The results of this study for determination of EPP in 'Hayward' cultivar suggest that flowers should be pollinated within 4 DAA for the successful fruit set. EPP results of this study were similar to that founded by Gonzalez *et al.* (1995) for 'Hayward' in Spain. However, the greatest size, weight and fruit density occurred when flowers were pollinated within 1 DAA (2015). By extending the pollination period from 4 DAA to 5 DAA, a 79.29 % decrease in mean of fruit set was observed along the 3 years of observations.
A similar situation has been recorded under different cultural conditions for 'Hayward' (Gonzalez *et al*., 1995), AU Golden Sunshine' *(Actinidia chinensis*) and 'AU Fitzgerald' (*A. deliciosa*) (Thompson, 2014; Brantley, 2016).

EEP determined by the longevity of the ovules minus the time lag between pollination and fertilization (Sanzol and Herrero, 2001). EEP is affected by the growth rate of the pollen tube. The temperature has a clear effect on pollen tube growth rate (Hedhly *et al.,* 2005), and on flowering period of this study an optimal temperature for pollen tube growth (mean temperature 20.4 to 23.7°C) occurred during 3 years (Manandhar and Lawes, 1980). Due to the viability of ovules for the 7 days following anthesis in kiwifruit (Gonzalez *et al*., 1995), germination of pollen and pollen tube kinetics may be the limiting factor for EPP.

According to results of Gonzalez *et al*. (1995), the viability of ovules is 7 days in kiwifruit and an under optimal temperature, pollen tube reached the ovules 3 days after pollination, so that, there are 4 days between flower anthesis and fertilization. Thus, it appears stigma receptivity may not be the limiting factor for EPP, and under any condition, maximum EPP will be 4 days. However, Hopping and Jerram (1979) observed that the pollen tubes reached the style base and fertilized the ovules inside the ovary by 31 hours and 43 hours respectively. According to this result, there are more than 4 days between flower opening and pollination, and stigma receptivity may be the limiting factor. The difference in the pollen tube kinetics may be the result of pollen origin (Guerrero-Prieto *et al.,* 1985), nutritive stage of the flower (Nyomora *et al.,* 2000) or environmental conditions (Jefferies *et al.,* 1982).

EPP is short for kiwifruit. However, having a genetic component EPP with cultivars, it is affected by the nutritive state of the tree and weather alteration (Gonzalez *et al*., 1995), crop load (Crisosto *et al*., 1988; Buszard and Schwabe, 1995), temperature, flower quality, and chemical treatments (Sanzol and Herrero, 2001) and alternate bearing (Brantley, 2016).

In this study, fruit set averaged 99.8% for 4 DAA and decreased to 20.27% for day 5 in 'Hayward' cultivar along 3 years of studies. In the one-year study of Gonzalez *et al*. (1995) in this cultivar, fruit set averaged 80% after hand pollination during the first 4 DAA before declining to 36% on day 5 and then almost 0% by day 7. They attributed this to the loss of papillar integrity i.e. the unicellular papillae that cover the stigma began to rupture. They considered 80% fruit set to be successful pollination, and based on this, determined the EPP of 'Hayward' to be 4 DAA. They did not publish data for a seed count number or fruit size. Brantley (2016) reported averaging 98% fruit set for the first 4 DAA before declining to 81.5% for day 5 in 'AU Fitzgerald'. It seems plausible that this variance in fruit set was due to the alternate bearing tendencies of the species *A. deliciosa* (Morley-Bunker and Lyford, 1999), flower quality and pollen source (Brantley, 2016).

Presently, no research has been conducted on the effects of alternate bearing on pollination of kiwifruit flowers. However, EPP of *Malus domestica* L. Borkh. cv. Cox's Orange Pippin was influenced by crop loads

of the prior year in a study by Buszard and Schwabe (1995). According to the results of their research, defruited trees in the previous prior year had flowers that were receptive to pollen at opening time while trees that carried a heavy crop load in the previous year had flowers that were not fully receptive to pollen until 3 DAA. We hand-pollinated uniform and single flower inflorescences during the study, meanwhile, Gonzalez *et al*. (1995) did not mention about flower quality. In addition, Gonzalez *et al*. (1995) used the pollen of male C for hand pollination, however, in our study, 'Tomuri' pollen was applied for pollination. The number of seeds is in turn related to the number of viable pollen deposited on the stigma. Even though a successful mating of a pollen tube with an ovule produces a seed, the number of pollen grains needed per seed ranges from 3.0 to 5.2 depending on the male clones (Hopping and Martyn, 1990). This is because some pollen tubes die during the passage through the style.

In this study, no differences in fruit set, seed number and weight were observed between 1-4 DAA. However, differences were observed for fruit weight, size, and density. Fruit weight reduced about 10 and 20 g in 2 and 3-4 DAA respectively. However, fruit weight between 1-4 DAA was ≥85 g and had more than 1200 seed per fruit. Gonzalez *et al.* (1998) observed similar results in *A. deliciosa* 'Hayward'. They detected the bulk of the fruit produced by *A. deliciosa* 'Hayward' with hand pollination were 80-110 g and stated that hand pollination increased the final value of the crop by 10%. Despite no differences in the number of seeds, average fruit weight was reduced from 10 to 20 g between 2-4 DAA. Under the terms of the similar fruit set, fruit weight will not be the same. Similar results were reported in *Actinidia chinensis* 'AU Golden Sunshine' by Thompson (2014).

Gonzalez *et al.* (1998) reported while fruit set is similar to that obtained with hand pollination and mechanical system, a high difference was obtained in fruit quality, where pollination hand still significantly improved mechanical pollination in terms of fruit size and weight. On day 5, not only fruit weight, but also seed number and weight reduced 34 g, 720, and 0.96 g, respectively. Thus, about 60% reduction of seed number on day 5, resulting reduction of fruit weight and size about 31%. Because seed-derived hormones are required to promote fruit growth (Woolley *et al.,* 1988). However, the final fruit size is also affected by the management factors such as crop loadings, nutrients, irrigation, time of anthesis, flower quality, pollination systems, beehives management, type of training systems, the position of the fruit on the vines in any particular training system and leaf to fruit ratio (Sale, 1981; Clinch, 1984; Hopping, 1986; Pyke and Alspach, 1986; Woolley *et al*, 1988; Ferguson, 1990; Gonzalez *et al.,* 1998; Goodwin, 2000).

There are several published relationships between the fruit weight and the number of seed or seed dry weight per fruit (Hopping, 1986; Pyke and Alspach, 1986; Testolin *et al.,* 1991; Goodwin, 2000). According to these researchers, to obtain a size of 70 g and 100 g fruit, 180 to 840 and 620 to 1290 seeds per fruit are required respectively. The seed-to-fruit relationships from these sources display very large scatter and either linear or non-linear positive correlation. Seed number may not be the only factor that plays a role in increasing fruit size. This is thought due to the influence of other factors such as the loading of the vine (Testolin *et al.,* 1991), cultural methods and orchard microclimates (Clinch, 1984), the age of flowers, storage carbohydrates, leaf number, and unknown factors should also be considered. Based on the findings of this study, not only fruit set but also fruit traits of 'Hayward' cultivar were affected by pollination in relation to the time elapsed since anthesis. Therefore, pollination efforts for *A. deliciosa* 'Hayward' may be concentrated within the first 4 DAA for having high fruit set and fruit traits. However, the maximum fruit size and weight obtained within the first DAA in this research.

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Study on relationship between morphological and physiological traits with resistance to rust fungus (*Puccinia allii)* in Iranian garlic clones

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Key words: *Allium sativum* L., garlic rust, combined variance analysis, infection frequency.

Abstract: In the present study we collected 12 clones of garlic from different geographical origin in Iran. The clones were sown in a field trial under natural infection of the rust fungus during two consecutive years. After 210 days, the reactions of the clones to the disease as well as the morphological features of the clones were evaluated. The results of analysis of variance on morphological traits showed a significant difference among the clones in terms of bulb weight, mean clove weight, number of bulb skin, number of cloves in the bulb, leaf temperature and the percentage of clove dry weight, and nutrient uptake for N,P,K, Mn and Zn. The results showed a positive and significant correlation between the leaf temperature, photosynthesis, nitrogen and manganese uptake and percentage of leaf infection at 1% probability level. The results of the infection frequency showed that the clones 'Gilvan1' and 'Lalejin' had the lowest percentage of infection and were identified as resistant clones to the rust disease. The results also showed that garlic clones reacted differently to the rust fungus and are separated into resistant, semi resistant, semi-susceptible and susceptible clones.

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1. Introduction

Garlic *(Allium sativum* L.) is one of the most important vegetable crops in the world. Asian countries such as China, India, Afghanistan and Iran are known as the main growing area of this vegetable. Currently, garlic cultivation and production has been developed with a wide variety from East Asia to South America. Due to the commercial, economic and pharmaceutical-industrial importance of garlic, the interest in increasing pro-

duction, yield and development of new varieties is rapidly growing (Cunha *et al*., 2012). The world average yield of garlic in the last 20 years has been around 10 tons per hectare, while in recent years it increased to 18 tons per hectare (FAOSTATS, 2018). The Iranian plateau is one of the most important centers of vegetation diversity in the world (Mousavi, 1994) and among scientists, it is known as the arc of garlic distribution. Morphological and molecular studies have indicated a wide variety of Iranian garlic clones distributed all over the country (Baghalian *et al*., 2005; Vafaee, 2007). Despite the long history of forced apomixis in the garlic, its cultivars show a great diversity of morphological, physiological and biochemical differences (Etoh and Simon, 2002; Ammarellou *et al*., 2014). Garlic colonies have a wide variety in vegetative traits, taste and flavor, bolting and fertility capacity. The cultivar characteristics differ significantly according to the location of cultivation and climate, and the weather conditions has a significant effect on cloves, flowering and taste of garlic. All garlic colonies are sterile, and therefore genetic variation in this plant may only be due to random mutations or non-sexual variation and to the introduction of new genetic variation using modern molecular techniques. Biochemical and molecular studies indicate that the highest level of heterogenesity occurs within the genetic reserves of central Asia, which may include favorite genes for use in future genetic studies, as well as the improvement of plant programs. Kallo and Bergh stated that cloves' morphological traits, in particular number and size of leaves, height of the flowering stem, number of bulb shells, reaction to temperature, seed dormancy, quality of maintenance, vegetative and maturity period duration, are genetically controlled and these are polygenic traits (Kallo and Bergh, 1993).

One of the most important problems faced by farmers in relation to garlic cultivation is the outbreak of fungal rust disease due to high humidity of cultivated areas. Most herbaceous fungi are destroyed by fungicides, but for many of them there is still no proper and effective fungicide. In addition, the use of fungicides generally causes pollution of the environment, groundwater resources, and products themselves. The cause of garlic rust is the fungus *Puccinia allii* that is an airborne fungus, and its release of spores is very fast (Michael and Sarah, 1995); spores are commonly transported by the wind and causes the disease characterized by a sore or pustules at the leaf level. The teliospores live in non-crop seasons in the soil and plant infected residuals. The disease develops at temperatures below 10 °C and is destroyed by frozen dew. If the garlic is subjected to drought stress or excess water or condensed due to excessive consumption of nitrogen fertilizer, it will become susceptible to the disease (Houshiarfard and Pourabdollah, 2015).

In the world, an overgrowth of rust on garlic has been reported in many cases (Schwartz and Mohan, 1999). For example, garlic rust is one of the major problems in garlic cultivation in the United States. Studies show that the clones do not have complete resistance to garlic rust, they may be tolerant showing (on average) 51% reduction in the product (Coviello, 2007). Selection of resistant clones from the genetic complex of Iranian local clones is one of the prebreeding research priorities of the country in Iran. Identification of resistant crop cultivars has many advantages over the use of chemicals and other control methods. Since the economic importance of garlic rust has been grown up in recent years, few studies have been focused on the identification of morphological traits of garlic related to the yield, but none of the studies addressed on the relationship between morphological, physiological component and the resistance to the rust fungus. This study aimed to investigate the physiological and morphological features in the resistant and the susceptible Iranian garlic clones, with the ultimate scope to achieve resistant colonies to the garlic rust in selected clones.

2. Materials and Methods

Plant materials

Based on geographical distribution of garlic in Iran, we indicated the main cultivation regions with long crop history throughout the country. In each region, we collected one sample which represented the main geographical characteristic of that region to avoid any doubling in sampling and to collect as much as distinct clones. In total we collected 12 superior clones from all over the country (Table 1). The cloves were planted after disinfection with fungicide carbendazim and confidor insecticide in a field trial in Chavarzagh Tarom Zanjan (48 46' 43.34" E - 36 59' 51.33" N) and altitude of 484 meter from sea level and temperate-semi-arid climate in a completely randomized block design with three replications during two consecutive years. Soil texture of cultivation site was clay loam with pH= 7.6 and electrical conductivity of 0.8 dS/m, percentage of neutralizing matter was 6.2%, and available phosphorus and

Table 1 - Origin specifications of Iranian garlic clones

Row	Clone	Country	Province	City/Region	Longitude	Latitude	Altitude
1	Dezfol	Iran	Khozestan	Dezfol	48° 25' 52.57" E	32° 22' 59. 08" N	143
3	Sahneh	Iran	Kermanshah	Sahneh	47° 41' 41.08" E	34° 28' 26. 53" N	1354
4	Lalejin	Iran	Hamedan	Lalejin	48° 28' 34.42" E	34° 58' 25. 22" N	1700
5	Azar shahr	Iran	Azarbayejan sharghy	Azar shahr	45° 58' 59.78" E	37° 44' 1. 64" N	1415
6	Lahijan	Iran	Gilan	Lahijan	50° 0' 12.07" E	37° 12' 25. 46" N	4
7	Khaf	Iran	Khorasan razavi	Khaf	60° 8' 50.48" E	34° 34' 18. 53" N	975
8	Sojas	Iran	Zanjan	Sojas	48° 33' 04.45" E	36° 14' 24. 14" N	1774
9	Hesar	Iran	Zanjan	Hesar	47° 43' 7.24" E	36° 57' 26. 62" N	1158
10	Gilvan 1	Iran	Zanjan	Gilvan 1	49° 4' 48.88" E	36° 48' 10. 53" N	340
11	Gilvan 2	Iran	Zanjan	Gilvan 2	49° 7' 51.54" E	36° 47' 10. 94" N	340
12	Chavarzagh	Iran	Zanjan	Chavarzagh	48° 46' 43.34" E	36° 59' 51. 31" N	484

potassium in the soil were 3.8 and 261 mg/kg, respectively. In the annual cultivation process, the cloves of each clone were grown on 35 cm stacks with cultivation depth of 3 cm and 10 cm spacing. Due to possible genetic variation on resistance to the rust fungus within each clone, the most resistant seedlings of clones were selected for diseases resistance evaluation in the second year. At garlic harvesting stage, the bulbs were harvested at the time of physiological maturity, when the leaves fell and 70% dried (Rubatzky and Yamaguchi, 1997).

Evaluation of morphological traits

The morphological traits were evaluated according to the descriptor recommended by the International Plant Genetic Resources Institute (IPGRI, 2000). The evaluated morphological traits included bulb weight, bulb diameter, number of leaves, mean clove weight, number of bulb sheet, number of cloves in a bulb, color of clove, bulb and leaf, side view of bulb, shape of mature bulb and type of bulb structure. Leaf color and number were evaluated when the plants were fully developed, while the traits of bulb were studied after complete maturation of the plant and bulb harvesting.

Evaluation of physiological traits

At the end of March each year, the leaf chlorophyll content was estimated by measuring 10 leaf samples per replication and estimation of the mean data was done with the help of SPAD device. Other physiological measurements on leaf such as pure photosynthesis, stomatal conductance of H_2O, leaf surface temperature and transpiration sub stomatal CO_2 at the full leaf growth stage were performed by UK manufacturing ADC device (ADC BioScientific LCi Analyser Serial No. 32648). Sampling of green leaves was done at full leaf growth stage to estimate the rate of nutrients absorption and dry matter percentage of the leaf and immediately transferred to laboratory of Zanjan Agricultural Research Center. The percentage of dry weight of cloves and leaves was calculated and recorded by placing a sample of cloves or leaves of each treatment for 24 hours in oven at 70°C and measuring the weight of samples before and after drying. The nutrient uptake was evaluated in Water and Soil laboratory of Zanjan Agriculture Research Center. After sampling the plant leaves, they were immediately transferred to the laboratory and after washing they were dried immediately in a 70° C oven for 48 hours. Then, the samples were digested with acid and the plant extract was prepared. Finally, macro elements such as nitrogen, potassium, phosphorus and micro elements such as zinc, copper and manganese were measured by atomic absorption and calorimetric (Emami, 1996).

Evaluation of resistance to the rust fungus

In order to evaluate the frequency of leaf infection by rust fungus among the clones, the numbers of pustules were recorded at 4 stages (20 April, 5 May, 20 May and 5 June). The evaluation method of infection frequency of the disease was as follows: 10 plants per plot were randomly selected. The average of number of pustules on leaves of 10 plants was determined as the percentage of rust infection in each clone (Clifford and Jones, 1983; Dhingra and Sinclair, 1995).

Methods and tools for analyzing the data

In this study, statistical software SAS and SPSS was used to analyze the statistical data. Analysis of variance of traits and their mean comparison were done using Duncan test at 1% level. The correlation between traits was measured by Pearson's two-

domain test with the SPSS software. At the end of the second year, combined variance analysis was performed.

3. Results

Analysis of variance

The results of two-year combined analysis of variance of morphological traits showed a significant difference between the clones in terms of bulb weight, mean clove weight, number of bulb skin, number of cloves in the bulb at 1% level, while there was no significant difference between the bulb diameter and number of leaves (Table 2).

The results of combined analysis of variance for two years from physiological traits showed a significant difference between the studied clones in leaf surface temperature and clove dry weight percentage at 1% probability level. In terms of stomatal conductance and photosynthesis, the difference was significant at 5% probability level, while there was no significant difference between substomatal CO_2, transpiration, chlorophyll and leaf dry weight percentage among the clones (Table 3).

Combined analysis of variance in terms of nutrient uptake of N, P, K, Mn, and Zn showed that a significant difference was found between the clones at 1% level and in terms of Cu uptake at 5% probability level (Table 4).

The results of combined analysis of variance in terms of the resistance of the clones to the rust fun-

Table 2 - Results of combined analysis of variance for morphological characteristics in some Iranian garlic clones

S.O.V	df	MS					
		Bulb weight	Bulb diameter	Mean clove weight	Number of bulb skin	Number of cloves bulb	Leaf number
Year	1	11295.04 **	5.24 *	2.41 NS	0.12 NS	0.05 NS	0.00 NS
Rep/Year	4	120.94	0.46	1.68	0.06	3.81	0.44
Treat	11	351.53 **	0.59 NS	5.77 **	22.31**	32.77 **	0.46 NS
Treat × Year	11	363.21 **	0.57 NS	0.53 NS	0.42 NS	0.38 NS	0.00 NS
Error	44	103.00	0.57	0.95	0.87	1.84	0.56
CV%		17.48	15.30	25.46	18.73	11.41	10.83

*, **, NS= significant at 5%, 1% and not significant probability levels, respectively.

Table 3 - Results of combined analysis of variance for physiological characteristics in some Iranian garlic clones

S.O.V	df	MS							
		Clove dry weight	Leaf dry weight	Chlorophyl	Sub stomatal CO_2	Stomatal conductance of H_2O	Photosynthesis	Transpiration	Leaf surface temperature
Year	1	590.64 **	0.61 NS	123.76 NS	9531.20 NS	0.0013 NS	233.24 **	160.68 **	894.64 **
Rep/Year	4	0.21	1.84	39.75	2517.86	0.0036	0.37	1.70	7.26
Treat	11	8.55 **	1.89 NS	39.51 NS	455.23 NS	0.0032 *	10.53 *	0.38 NS	3.14 **
Treat × Year	11	13.66 **	1.44 NS	62.30 NS	1061.43 *	0.0032 *	3.74 NS	0.58 NS	1.74 **
Error	44	0.84	2.48	39.16	581.20	0.001	5.21	0.51	0.52
CV%		3.11	12.29	11.18	10.05	17.50	19.38	11.23	1.96

*, **, NS= significant at 5%, 1% and not significant probability levels, respectively.

Table 4 - Results of combined analysis of variance for physiological characteristics in some Iranian garlic clones

S.O.V	df	MS					
		Nitrogen	Phosphorus	Potassium	Manganese	Zinc	Copper
Year	1	26/21 **	0/026 NS	0/0004 NS	445/80 NS	3786/84 **	1/85 NS
Rep/Year	4	0/064	0/009	0/199	25/19	32/23	2.45
Treat	11	0/24 **	0/0071 **	1/326 **	67/36 **	56/87 **	1/28 *
Treat × Year	11	0/098 **	0/0075 NS	0/429 **	22/86 NS	15/60 NS	2/53 **
Error	44	0/017	0/0006	0/09	15/32	9.81	0/61
CV%		2.9	7.31	7.32	10.27	11.08	9.39

*, **, NS= significant at 5%, 1% and not significant probability levels, respectively.

gus at four stages of measurement showed that the difference at the first stage was significant at 1% level. At the next three stages, the difference in resistance between the clones was significant at 5% level (Table 5).

The effect of year was also significant in terms of the leaf surface temperature, photosynthesis, transpiration, percentage of clove dry weight, absorption of nutrients Zn and N and bulb weights at 1% level. In term of resistance to garlic rust, the effect of the year was significant at 4 stages at 1% level.

The effect of year on the treatment showed that the leaf surface temperature, N, P, K and copper nutrient uptake percentage, percentage of dry weight cloves, bulb weight, and percentage of resistance garlic rust were significant at the first and second stages of growth at 1% level and substomatal CO_2, stomatal conductance, and percentage of infection of garlic rust were significant at the 3rd and 4th stages of growth. Other physiological traits including photosynthesis, transpiration, chlorophyll content, dry matter percentage, manganese and zinc intake, and morphological traits including the bulb diameter, mean bulb weight, number of the bulb skin, number of cloves in the bulb and number of leaves did not showed significant differences. The highest coefficient of variation among the physiological traits was related to photosynthesis (19.38). Among the morphological traits, the highest coefficient of variation was related to the mean weight of the clove (25.46). In the study of the percentage of infection of garlic to the rust fungus, the highest coefficient of variation appeared at the second stage of evaluation in the early May (33.60).

Regarding to significance of analysis of variance among the clones, for some traits the mean comparison was done using Duncan's multi-domain test. The results of the two-year mean comparison of morphological traits showed that among the studied clones, the lowest weight was related to the clones ʿChavarzagh' (49.84) and 'Lahijan' (51.47) and the highest was related to the clones 'Sahne' (79.40) and 'Sojas' (61.11). In terms of the mean weight of the clove, the lowest weight belonged to the clone 'Khaf'(1.97) and the highest weight belonged to the clones 'Sahne' (5.14) and 'Gilvan1' (4.98). The mean comparison of the studied clones showed that the lowest number of bulb skin was for the clones 'Chavarzagh' and 'Lahijan' (2.33) and the highest was for the clone 'Azar Shahr' (7.16). In terms of number of cloves in a bulb, the lowest number was related to the clone 'Hesar' (8.66) and the highest was related to the clone 'Khaf (17.33) (Table 6).

Table 5 - Results of combined analysis of variance for resistance to rust in some Iranian garlic clones

S.O.V	df	MS			
		20/04/2019	05/05/2019	20/05/2019	05/06/2019
Year	1	491.42 **	651.86 **	2604.74 **	2947.88 **
Rep. Year	4	0.35	4.65	24.94	11.23
Treat	11	1.38 **	15.19 *	75.02 *	144.83 *
Treat × Year	11	1.38 **	20.63 **	84.50 *	119.35 *
Error	43	0.35	6.29	44.57	63.58
CV%		22.85	33.60	32.58	20.29

*, **, NS= significant at 5%, 1% and not significant probability levels, respectively.

Table 6 - Mean comparison of morphological traits in some Iranian garlic at two years

Treatment	Bulb weight (gr)	Bulb diameter (mm)	Mean clove weight (gr)	Number of bulb skin	Number of cloves per bulb	Leaf number per plant
Gilvan 1	60.13 b	4.96 a	4.98 a	5.83 abc	12.00 cd	6.66 a
Gilvan 2	56.65 b	5.07 a	4.69 a	4.83 c	12.33 bc	6.66 a
Azar shahr	55.08 b	5.05 a	3.15 bcd	7.16 ab	13.16 bc	6.66 a
Hesar	52.77 b	4.77 a	3.85 abc	5.66 bc	8.66 f	7.33 a
Lahijan	51.47 b	4.96 a	3.68 abc	2.33 d	12.00 cd	7.33 a
Khor va biabanak	54.22 b	4.88 a	4.07 ab	2.83 d	11.33 cde	7.33 a
Sojas	61.11 b	5.27 a	2.28 cd	6.66 ab	9.66 def	6.66 a
Dezfol	56.41 b	4.80 a	4.47 ab	2.66 d	14.33 b	7.00 a
Sahneh	79.40 a	5.36 a	5.14 a	6.33 abc	11.50 cde	7.00 a
Khaf	58.45 b	4.10 a	1.97 d	5.83 abc	17.33 a	7.00 a
Chavarzagh	49.84 b	4.93 a	3.89 abc	2.33 d	11.33 cde	7.00 a
Lalejin	60.85 b	5.08 a	3.94 abc	7.33 a	9.33 ef	6.66 a

In each column different letters mean significant differences between samples.

The results of the two-year mean comparison of physiological traits showed that among the studied clones in terms of the leaf surface temperature, the clone 'Gilvan1' had the highest leaf surface temperature (37.98 ℃) while the clone 'Azar shahr' had the lowest one (35.53 ℃). Stomatal conductance was highest in the clone 'Azar shahr' (0.23) while the lowest value was found for the clone 'Gilvan1' (0.15). The clone 'Azar shahr' had the highest photosynthesis rate (13.35) while the clone 'Gilvan1' had the lowest one (8.80). In terms of percentage of the clove dry weight, the highest was related to the clone 'Lahijan' (31.92) and the lowest value was related to the clone 'Azar shahr' (28.11) (Table 7).

The mean comparison of the studied clones in terms of nutrient uptake showed that the highest nitrogen uptake was related to the clones 'Dezful' (4.83) and 'Chavarzagh' (4.77) and the lowest was related to the clones 'Hesar' (4.22) and 'Khaf' (14.32). In terms of phosphorus uptake, the highest uptake was related to the clone 'Lalejin' (0.44) and the lowest was related to the clone 'Sojas' (0.30). In terms of potassium and manganese uptake, the highest uptake was related to the clone 'Dezful' in potassium (4.85) and manganese (42.77), and the lowest was related to the clone 'Lalejin' in potassium (3.62) and manganese (30.89). In terms of zinc and copper uptake, the highest uptake was related to the clone 'Lalejin' in zinc (35.78) and copper (9.21), the lowest zinc uptake was for the clone 'Gilvan1' (24.61) and the lowest copper uptake was for the clone 'Khaf' (7.55) (Table 8).

It is worth noting that all 12 clones were infected with garlic rust, although showing different reaction to the fungus. In two-year mean comparison, it was found that the observation of rust symptoms began from the second half of April, and disease progressed along with the growth of the plants. At the first stage

Table 7 - Mean comparison of physiological traits in some Iranian garlic at two years

Treatment	Leaf surface temperature °C	Sub stomatal CO_2	Stomatal conductance of H_2O	Photo-synthesis	Transpiration	Chlorophyl	Leaf dry weight	Clove dry weight
Gilvan 1	37.98 a	236.33 a	0.15 b	8.80 b	5.96 a	49.45 a	13.33 a	28.83 c
Gilvan 2	36.65 ab	224.75 a	0.20 ab	13.16 a	6.08 a	58.53 a	13.42 a	29.44 bc
Azar shahr	35.53 c	231.83 a	0.23 a	13.35 a	6.37 a	55.76 a	12.17 a	28.11 c
Hesar	36.61 bc	233.50 a	0.22 a	12.85 a	6.46 a	57.26 a	13.05 a	28.66 c
Lahijan	37.03 ab	249.50 a	0.21 ab	10.18 ab	6.31 a	58.70 a	12.38 a	31.92 a
Khor va biabanak	37.30 ab	236.50 a	0.19 ab	11.47 ab	6.29 a	58.96 a	12.55 a	29.39 bc
Sojas	36.98 ab	252 a	0.21 ab	10.78 ab	6.43 a	56.78 a	13.35 a	30.86 ab
Dezfol	35.68 c	247.48 a	0.23 a	12.29 ab	6.28 a	55.45 a	12.32 a	28.76 c
Sahneh	36.60 bc	250 a	0.23 a	12.27 ab	6.61 a	55.26 a	12.60 a	29.62 bc
Khaf	37.65 ab	243.83 a	0.21 ab	11.50 ab	6.74 a	54.23 a	13.91 a	28.75 c
Chavarzagh	37.28 ab	232.25 a	0.22 a	12.36 ab	6.80 a	55.13 a	12.43 a	31.29 a
Lalejin	37.25 ab	238.08 a	0.19 ab	12.35 ab	6.15 a	55.68 a	12.40 a	28.76 c

In each column different letters mean significant differences between samples

Table 8 - Mean comparison of physiological traits in some Iranian garlic at two years

Treatment	Nitrogen	Phosphorus	Potassium	Manganese	Zinc	Copper
Gilvan 1	4.73 ab	0.32 bcd	4.52 ab	42.53 a	24.61 c	8.06 bc
Gilvan 2	4.60 ab	0.35 bc	4.47 abc	37.81 abc	28.69 bc	8.72 ab
Azar shahr	4.67 abc	0.35 bc	4.30 bcd	40.58 ab	31.04 ab	8.52 abc
Hesar	4.22 e	0.35 bc	3.84 ed	36.22 abc	24.80 c	8.71 ab
Lahijan	4.76 ab	0.38 b	4.83 a	35.11 bc	29.03 bc	8.31 abc
Khor va biabanak	4.66 abc	0.35 bc	4.01 cde	40.27 ab	29.48 bc	7.84 abc
Sojas	4.47 cd	0.30 d	3.64 e	36.69 abc	26.63 bc	7.70 bc
Dezfol	4.83 a	0.35 bc	4.85 a	42.77 a	26.98 bc	8.55 abc
Sahneh	4.33 de	0.38 b	3.84 de	37.55 abc	28.53 bc	8.00 bc
Khaf	4.32 de	0.35 bc	3.62 e	36.79 abc	26.71 bc	7.55 c
Chavarzagh	4.77 ab	0.35 bc	4.51 abc	39.83 ab	26.74 bc	8.49 abc
Lalejin	4.48 cd	0.44 a	3.62 e	30.89 c	35.78 a	9.21 a

In each column different letters mean significant differences between samples

of growth, the highest percentage of infection was related to the clone 'Lahijan'. At the second stage, the highest percentage of infection was related to the clone 'Khor va Biabanak and the lowest was related to the clone 'Hesar'. At the third and fourth stages, the clones 'Gilvan1' and 'Lalejin' had the lowest percentage of infection and were identified as resistant to rust. This may indicate that garlic clones behave in different way during seedling and adult plant stages and therefore they have different genetics for resistance to the garlic rust fungus in different growth stages. The most susceptible clone was 'Chavarzagh' showing the highest percentage of infection (Table 9). The first uredospores of garlic rust appeared in the first half of April each year, and the first teliospores appeared two weeks later. The onset and spread of infection completely follow the progressive growth pattern and with the help of ure

Table 10 - Reaction of 12 Iranian garlic clones to rust fungus in a two years field traits under natural infection

Clones	Grouping of resistance			
	Resistant	Semi resistant	Semi susceptible	Susceptible
Gilvan 1	x			
Lalejin	x			
Khaf		x		
Sojas		x		
Sahneh		x		
Hesar		x		
Azar shahr		x		
Gilvan 2			x	
Khor va biabanak			x	
Lahijan			x	
Dezfol				x
Chavarzagh				x

Table 9 - Mean comparison of resistance to rust in some Iranian garlic clones in two year (Difference in days to maturity)

Treatment	Percentage of the disease			
	20/04/2019	05/05/2019	20/05/2019	20/06/2019
Gilvan 1	1.72 b	2.53 bc	3.97 c	5.83 c
Gilvan 2	1.58 b	2.59 bc	4.65 abc	6.42 abc
Azar shahr	1.72 b	2.99 abc	4.66 abc	6.30 bc
Hesar	1.72 b	2.36 c	4.21 abc	6.25 bc
Lahijan	2.01 a	2.86 abc	5.04 a	6.57 abc
Khor va biabanak	1.72 b	3.32 a	4.92 ab	6.52 abc
Sojas	1.72 b	2.45 bc	4.00 bc	5.97 bc
Dezfol	1.72 b	3.00 abc	4.94 a	6.67 ab
Sahneh	1.72 b	2.85 abc	4.29 abc	6.04 bc
Khaf	1.72 b	3.03 ab	4.24 abc	5.95 bc
Chavarzagh	1.72 b	2.88 abc	4.80 abc	7.08 a
Lalejin	1.72 b	2.71 abc	4.55 abc	5.85 c

In each column different letters mean significant differences between samples.

dospores and in the absence of fungicides, the level of plant infection can reach from over 1% to more than 80% within two months.

The results of this study showed that garlic clones respond differently to the rust and are separated into resistant, semi resistant, semi-susceptible and susceptible clones (Table 10). In sum, combined mean comparison of garlic clones in terms of resistance to disease showed that Gilvan1 cultivar had distinct traits compared to other clones. Morphologically, to color pf this clone is violet with code 5, 6-8 skins, side view shape of the compound bulb is broadly ovate, basal plate event, shape of matured bulb is globe and type of bulb structure is regular with two-fan group of cloves. The distinction of this clone with other violet clones is in the number of skins. The next resistant clone, according to the field conditions of the present research, was 'Lalejin' from Hamadan Province, which has white stripes bulb with code 4 and white clove with code 1. The number of skins is 5-8 and side view shape of the compound bulb is broadly ovate, basal plate event, shape of matured bulb is broadly oval, and type of bulb structure is regular multi-shelled.

Traits' correlation

The correlation coefficients between physiological traits and resistance to rust are shown in Table 11. The results of the study showed a positive and significant correlation between the leaf surface temperature, photosynthesis, nitrogen and manganese uptake and percentage of leaf infection at the probability level of 1%. The relationship between the leaf surface temperature and infection frequency indicates that by increasing the leaf temperature, the percentage of infection will be increased. This result is reasonable according to other researchers' reports that the percentage of rust damage has only a direct correlation with the mean temperature. In addition, a positive and significant correlation was observed between percentage of rust infection and photosynthesis and nitrogen, manganese and zinc. Also, a negative and significant correlation was found between percentage of rust infection and transpiration rate at the level of 1%.

The correlation coefficients between morphological traits and resistance to rust are shown in Table 12. A negative and significant correlation was observed between traits such as transpiration rate, bulb weight, bulb diameter, number of bulb skins and type of bulb structure and rust infection percentage at the 1% level. This relationship indicates that garlic rust is effective on the factors affecting the garlic yield, such as the bulb weight, bulb diameter and by increasing the percentage of infection, the value of these traits are reduced.

Results of the infection frequency in four growth stages (Fig. 1) showed that the clones with high infection frequency at the early stages of growth had a higher disease symptomps at the final stages of growth. Therefore, the presence of infection at the early stages reduced the growth, yield and quality of the product. However, those that were resistant to some extent at the early stages of growth had relatively low infection and slow spread of disease, and with better growth of the clones.

Table 11- Correlation coefficients between physiological traits and resistance to rust (*Puccinia allii*) in Iranian garlic clones measured by Pearson's two-domain test

Treat	Percentage of the disease
Percentage of the disease	1
Leaf surface temperature	- 0.602 **
Sub stomatal CO_2	- 0.206
Stomatal conductance of H_2O	0.059
Photosynthesis	0.411 **
Transpiration	- 0.520 **
Chlorophyl	0.217 *
Leaf dry weight	0.059
Clove dry weight	0.160
Nitrogen	0.625 **
Phosphorus	- 0.085
Potassium	0.142
Manganese	0.298 **
Zinc	0.469 **
Copper	0.19

*, **, NS= significant at 5%, 1% and not significant probability levels, respectively.

Table 12 - Correlation coefficients between Morphological traits and resistance to rust (*Puccinia allii*) in Iranian garlic clones measured by Pearson's two-domain test

Treat	Percentage of the disease
Percentage of the disease	1
Bulb weight	-0.462 **
Bulb diameter	-0.285 **
Mean clove weight	0.068
Number of bulb skin	-0.404 **
Number of cloves per bulb	-0.007
Leaf number	0.128
Color bulb	0.183
Color clove	0.009
Color leaf	0.096
Side view of bulb	-0.118
Shape of mature bulb	-0.098
Type of bulb structure	-0.526 **

*, **, NS= significant at 5%, 1% and not significant probability levels, respectively.

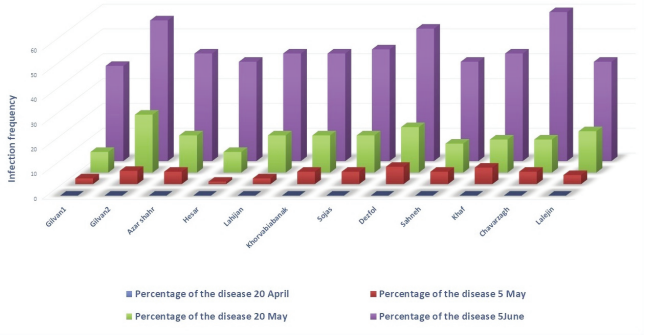

Fig. 1 - Correlation coefficients between Morphological traits and resistance to rust (*Puccinia allii*) in Iranian garlic clones measured by Pearson's two-domain test

4. Discussion and Conclusions

This is the first study on the identification of resistant clones to rust on garlic through the study of morphological and physiological traits collected on

12 Iranian clones from Iran during two crop years. The clones 'Gilvan1' and 'Lalejin' had the lowest number of stomata in the leaf area, because the fungus of the garlic rust through the stomata comes in to the plant, the low number of stomata reduces the possibility of pathogen entry and may be the most important feature of resistance in these clones.

The results of two-year combined analysis of variance of morphological traits are consistent with the research report of Nourbakhshian *et al.* (2007). The results of their research showed that a significant difference was found between the number of cloves in the bulb, clove weight, length and diameter of the clove and amount of dry matter per unit area (Nourbakhshian *et al*., 2007).

Vafaee and colleagues (2009), in a study on the genetic diversity of Iranian garlic clones using morphological traits and AFLP as molecular markers, classified Iranian garlic clones in 6 main groups, while according to morphological traits, the total clones were classified into 4 general groups.

The results of this study showed that garlic clones respond differently to the rust. The results of a study on resistance to garlic rust in Pakistan showed that rust severity of 0.8% was recorded on variety Hazro (Alam *et al*., 2007). A study in the US state of California regarding the susceptibility of the tested clones to garlic rust showed that none of the cultivars including selective later and early clones, and Spanish and Chinese cultivars showed complete resistance and were usually tolerant (Coviello, 2007). Another California breeding program to test the resistance to garlic rust on three genotypes PE493096, PE540315, W12820 showed that all of them were infected with garlic rust and only about 1% of them had less than 26% of the infection (Davis, 2007).

The results of correlation coefficients between morphological traits and resistance to rust are consistent with Nourbakhshian's report on factors affecting garlic yield and bulb weight (Nourbakhshian *et al*., 2008). The results of this study are consistent with the results of other researchers regarding the superior clones' traits (Baghalian *et al*., 2005). Kallo reported that a clove weight, number of cloves and bulb diameter had the most direct effect on garlic yield, and stated that the selection of the clones based on these traits would improve the yield potential of garlic (Kallo, 1988).

The use of commercial clones carrying rust resistance genes is the most efficient, economical and environmental friendly method of rust disease control. Using morphological trait for early screening of the clones for resistance to garlic rust can be very important. The results of the infection frequency showed that the clones 'Gilvan1' and 'Lalejin' had the lowest percentage of infection and were identified as resistant clones to the rust.

We found resistant clones with the introduction of each morphological trait. It should be noted that since the intensity measurements of infection were carried out at four stages, it was found that the earliest symptoms of garlic rust began in early May, and then the growing trend was accompanied by the growth of garlic. Two years of field trails lead us to achieve resistance components and to find resistant clones of garlic to the rust pathogen, however in order to get more in detail on mechanism and genetics of resistance we are following genetics studies on resistance and susceptible clones. By this research we will find the diversity of genes involved in resistance to rust pathogen in garlic and we will explore some more details on mechanisms of resistance in the future.

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Evaluating the salt tolerance of seven fig cultivars (*Ficus carica* L.)

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Key words: abiotic stress, electrolyte leakage, specific leaf area, stem diameter, stem length, stomata conductance, transpiration rate.

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Abstract: The growing demand for both fresh and dry figs worldwide is due to its richness in mineral compounds (i.e. iron and copper) and polyphenols. Considering the position of Iranian cultivars in global fig market, the present study examined the growth and photosynthetic rate of commercial fig cultivars (i.e. 'Sabz', 'Siyah', 'Shah Anjir', 'Atabaki', 'Kashki', 'Mati' and 'Bar Anjir') exposed to six salt treatments corresponding to the following electrical conductivities (EC): 0.5, 2, 4, 6, 8 and 10 dS m^{-1}. The results indicated a decrease trend of stem length, stem diameter and leaf number in salt-exposed plants. The electrolyte leakage and protein content in all cultivars followed an ascending trend. The specific leaf area, relative water content, photosynthetic indices and nitrogen content followed a decreasing trend according with increasing salinity. The 'Siyah' and 'Sabz', as the most salt-tolerant cultivars, had the maximum leaf abscission, the lowest transpiration rate and leaf water content under salt condition, compared to all other tested cultivars. Moreover, they had the most leaf succulence and leaf dry matter content and the lowest specific leaf area, which related to the balance between growth ratio and osmotic regulation under salt conditions. The 'Shah Anjir', as the most salt-sensitive cultivar, could not balance transpiration rate and leaf water content under salt treatment higher than 4 dS m^{-1}.

1. Introduction

The fig (*Ficus carica* L., 2n= 26) of the Moraceae family, is one of the first plants cultivated and consumed by human beings (Duenas *et al*., 2008). According to the FAO, the fig is harvested from 36,535 hectares of cultivated land, with an annual production of over one million tons (FAO, 2017). Iran is the third largest producer of dried figs in the world, as well as the fifth largest fresh fig producer in the world with a cultivated area of 53,101 hectares and a production of 70,730 tons per year of fresh figs (FAO, 2017).

The growing demand for the fig worldwide (Aksoy, 2005) is due to its richness in mineral compounds (i.e. iron and copper) and considerable amounts of vitamins A and C (Flaishman *et al*., 2008). The fig is consumed in fresh, dried, powdered, canned, chocolate-covered forms, and utilized in the preparation of jam, syrup and Muscat-Halvah (De Masi *et al*., 2005).

Significant genetic variation in the fig species, due to obligatory outcrossing, has led to the establishment of new genotypes with desirable properties. According to the latest reports, there are currently more than 600 known fruit-producing cultivars and genotypes, which are distinct in leaf morphology, growth vigor, internal and external color of the fruit, taste and quality index of the fruit, shape and thickness of the fruit, the diameter of the ostiole, and productivity period (Condit, 1955; Toribio and Montes, 1996; García-Ruiz *et al*., 2013).

Sabz or Verde (green), Siyah (black), Shah Anjir (king fig), Atabaki, Kashki and Mati are considered as the most important and marketable cultivars of Iranian figs for local markets or export. The 'Bar Anjir' is the most commonly-used caprifig (Condit, 1955; Pourghayoumi *et al*., 2016).

Several research centers have focused on different aspects of physiology and breeding of fig cultivars and genotypes. For example, some researchers emphasized on genetic diversity of fig using morphological (Khadivi *et al*., 2018) and molecular (Cabrita *et al*., 2001; Khadari *et al*., 2005; Giraldo *et al*., 2008) markers. Some others attempted to study the caprifigs (Dalkılıç *et al*., 2011), while others have considered the variety of the fruit and its qualitative features (Solomon *et al*., 2006; Polat and Caliskan, 2008; Ercisli *et al*., 2012).

In addition, the variation in the behavior of fig cultivars and genotypes to abiotic stress such as chilling (Karami *et al*., 2018), drought (Gholami *et al*., 2012) and salinity (Zarei *et al*., 2016) have attracted the researchers' attention.

Salinity is one of the most important environmental factors which reduces the growth, development and production of plants (Sevengor *et al*., 2011). While some researchers suggested reducing leaf area as the plants' responses to the salinity stress, others enumerate reduced stem length, root length, fresh and dry weights, and relative leaf water content (Yamasaki and Dillenburg, 1999; Bolat *et al*., 2006; Najafian *et al*., 2008; Adish *et al*., 2010; Khayyat *et al*., 2014; Khoshbahkt *et al*., 2014; Soliman and Abd Alhady, 2017). Other important processes that are negatively affected by salinity are protein synthesis (Taylor *et al*., 2004; Murcute *et al*., 2010) and nitrogen metabolism (Owais, 2015; Ashraf *et al*., 2017), whereas in other studies the inhibition of plant growth caused by salinity has been attributed to a decrease in photosynthesis (Garcia-Sanchez *et al*., 2006).

Fig is a moderate salt-tolerant crop (Golombek and Lüdders, 1990). The available studies on the response of fig commercial cultivars to different levels of salinity indicated a significant variation in morphological characteristics, growth parameters, physiological behavior, photosynthetic efficiency, gas exchange ratio, product quality, and productivity (Essam *et al*., 2013; Metwali *et al*., 2014; Alswalmeh *et al*., 2015; Zarei *et al*., 2016, 2017; Soliman and Abd Alhady, 2017).

Considering the position of Iranian cultivars in global fig market, the present study examined the growth and photosynthetic rate of commercial fig cultivars (i.e. 'Sabz', 'Siyah', 'Shah Anjir', 'Atabaki', 'Kashki', 'Mati' and 'Bar Anjir') exposed to six salt treatments corresponding to the following electrical conductivities (EC): 0.5, 2, 4, 6, 8 and 10 dSm^{-1} to identify the most salt-tolerant cultivar.

2. Materials and Methods

The present study was conducted in the plant breeding department, Faculty of Agriculture, University of Shiraz (36° 29' N and 32° 52' E), Iran during 2016-2018.

Plant materials

The plant materials were included six 20-years-old edible fig cultivars (Sabz, Siyah, Shah Anjir, Atabaki, Kashki, Mati), and a caprifig ('Bar Anjir') were located at Estahban fig Research Station (36° 29' N and 32° 52' E) (Table 1). The hard-wood cuttings, 20 cm in length and one cm in diameter, were collected from one-year-old branches on March 25, 2016. The cuttings were treated with a fungicide (Benomyl 2000 ppm) and a rooting hormone (IBA solution 3000 ppm). Then, the upper side of the cutting was covered to prevent the decay, and each cutting was placed in a dark plastic bag (25 x 18 cm^2), which was filled by sand. In the next stage, the bags were located in the shade-house conditions (Temperature: 28±2°C D/18±2°C N, RH=50%, and 50% shade) and were irrigated twice a day. In June 2017, rooted-cuttings were transplanted in pots filled with 500 g of gravel and 20 kg of the media described in Table 2.

Table 1 - The growth and bearing habit of studied fig cultivars (Sabet Sarvestani, 1999; Safai 2002; Jafari *et al.*, 2016)

Parameters	Cultivar						
	'Sabz'	'Siyah'	'Shah Anjir'	'Atabaki'	'Kashki'	'Mati'	'Bar Anjir'
Growth and bearing	Relatively high	High	Moderate	Moderate growth and low bearing	High growth and moderate bearing	Moderate growth and low bearing	Low- moderate growth and high bearing
Bud	Conical terminal buds with curved tip	Curved and pointed terminal bud	Terminal pointed bud	Terminal conical bud	Terminal conical, with tip	Terminal conical bud without tip	Terminal conical bud
Fruit	Medium, yellowish, no neck, thick pulp	Medium, dark purple, no neck, thin skin, flesh uniformly red, low pulp thickness	Large, necked, yellow, pink pulp, fully seed	Large, rounded, reddish-purple fruit, reddish pulp, reddish	Medium, green, , open ostiole necked, white pulp, low seedy	Large, no neck, open ostiole, dark and thick fruit, white, reddish pulp	Medium, with long neck, containing Blastophagous bees
Type of consumption	Dried Fruit	Fresh fruit	Both Fresh and dried fruit	Fresh fruit	Fresh and processed fruit	Fresh fruit	Caprifig
Yield	Very high	High	High	Moderate	Moderate	Moderate	Moderate
Taste	Excellent	Sweet-and-sour	Sweet	Sweet-and-sour	Sweet	Sweet-and-sour	Sweet
Bearing period	Early bearing	Early bearing	Mid-season bearing	Early bearing	Late bearing	Early bearing	Mid-season bearing

Table 2 - Physico-chemical properties of the soil

Soil texture	San (%)	Silt (%)	Clay (%)	EC (ds/m)	CEC (Me/100)	pH	Lime (%)
Sandy clay-loam	58±1.01	26±1	16±0.9	1.45±0.21	10.84±0.81	7.7±0.17	±351.33
Organic C (%)	N (%)	K (ppm)	P (ppm)	Cu (ppm)	Mn(ppm)	Fe (ppm)	Zn (ppm)
1.17±0.05	0.17±0.002	126±1.3	3.2±0.05	0.26±0.002	3.86±0.04	2.85±0.03	0.056±0.001

EC= Electrical conductivity;
CEC= Cation exchange capacity.

The media included the mixture of soil, leaf compost and sand (1:1:1), which was steam-disinfected. A pressure plate extractor (Model ADC, by Santa Barbara, United States) was used to measure the media water capacity. The pots were kept under shade-house condition (Temperature: 30±1°C D/18±0.5°C N, RH=50%, and 50% shade).

Salt treatment

The salt treatments were provided through the irrigation water. Treatments included low salt treatments (EC= 0.5 and 2 dS m^{-1}, A and B, respectively), intermediate salt treatments (EC= 4 and 5 dS m^{-1}, C and D, respectively) and high salt treatments (EC= 8 and 10 dS m^{-1}, E and F, respectively).

In order to avoid osmotic stress, salt treatments were introduced gradually, starting from 1/4 up to the final concentration. Then, irrigation frequency was calculated based on the media filed capacity and water requirement (Essam *et al*., 2013; Zarei *et al*., 2016). Salt treatment was performed within nine weeks from 23/7/2017- to 26/9/2017. In addition, all of the plants were irrigated by distilled water for four months (26/1/2018).

Stem length

The length of the stem was recorded at the beginning and end of the experiment. The difference between the two values was recorded as the difference in stem length (cm).

Stem diameter

The stem diameter was recorded at the beginning and end of the experiment by digital caliper (4 cm above the soil surface). The difference was recorded as the difference in stem diameter (mm).

Number of leaves

The number of expanded leaves was counted at

the beginning and end of the experiment. The difference between the two values was recorded as the difference in the number of leaves.

Specific leaf area (SLA), leaf dry matter content (LDMC) and leaf succulence

The fourth top leaf was harvested after ending the experiment. The leaf area was recorded using a Leaf Area Meter (CI-202 Portable Laser Leaf Area Meter). The leaves were then dried in an oven (75°C, 48 hrs.) and the dry weight was recorded (LDW). The specific leaf area (in cm^2 g^{-1}) was calculated by using the Eq. (1). LDMC and leaf succulence were calculated using Eq. (2) and (3).

$$SLA = LA/LDW \quad (1)$$

where LA is leaf area (cm^2) and LDW is leaf dry weight (g), according to Hunt *et al.*, 2002.

$$LDMC = LDW/LFW \quad (2)$$

where LDW and LFW are leaf dry weight (g) and leaf fresh weight (g) respectively (Garnier *et al*., 2001).

$$Leaf\ succulence = LFW/LA \quad (3)$$

where LFW is leaf fresh weight (g) and LA is leaf area (cm^2), according to Agarie *et al*., 2007.

Relative water content (RWC)

Mature leaves were collected nine weeks after salt application at mid-day, and were transferred immediately to the lab. Then, five similar leaf discs without any vein were separated from each sample and weighted (W_1). Further, the disc samples were placed in distilled water (4 hrs) under laboratory conditions (24 ± 1°C). Subsequently, the samples were surface dried and re-weighed (W_2). Furthermore, the discs were placed in an electric furnace (Model: Memmert, made by Karl Klob factory, Germany) (90°C, 60 min) and reweighted (W_3). Finally, the relative water content was calculated using the Eq. (4) (Barrs and Weatherley, 1962).

$$RWC = \frac{(W1 - W3)}{(W2 - W3)} \times 100 \quad (4)$$

Electrolyte Leakage (EL)

The top expanded leaf was harvested and 5 leaf discs without any vein were prepared. The samples were rinsed three times with distilled water, and incubated in 10 ml of distilled water (at 40°C for 30 min). After cooling, the electrical conductivity was measured using an Electrical Conductivity Meter (H18633 model) (C_1). The samples were then autoclaved (at 120°C for 15 min). After cooling, the electrical conductivity was re-measured (C_2). The Electrolyte Leakage was calculated via Eq. (5) (Sairam *et al*., 1997).

$$EL = (C1/C2) \times 100 \quad (5)$$

Leaf protein content

First, the fresh leaf sample (0.2 g) was powdered with liquid nitrogen. Then, two ml of Potassium Phosphate buffer (38.5 ml NaH_2PO_4, 68.5 ml of Na_2HPO_4, 0.074 g EDTA and 1 g of PVP), pH = 7.15 was added and well-homogenized. The extract was centrifuged at 13000 rpm for 15 min at 4°C, and the supernatant was used to measure protein content. About 20 µl of the extract, 80 µl of potassium phosphate buffer (pH= 7.15) and 5 ml of Coomassie Brilliant Blue (C47H48N3NaO7S2) was stirred for 2 min. In addition, the absorbance was read at 595 nm by using a spectrophotometer (Biowave II model) after incubating 5 min at room temperature. The extraction buffer was used as Blank. The protein content (in mg g^{-1} FW) in the sample was calculated according to the sample absorption using the Bovine albumin serum (C123H193N35O37) standard curve (Bradford, 1976).

Leaf nitrogen content

The Kjeldahl method was used to determine the nitrogen content in fresh leaf samples (Kjeldahl, 1883).

Photosynthetic indexes

The photosynthetic indices were recorded using a compact-portable-photosynthesis-system (LCI, UK). The device was put on attached leaf (third expanded leaf) at midday, then transpiration rate (in mol H_2O m^{-2} s^{-1}) and stomata conductance (in mol CO_2 m^{-2} s^{-1}) were recorded after two min (Evans and Von Caemmerer, 1996).

Experimental design and data analysis

The present experiment was conducted in a Complete Randomized Design. The factors included fig cultivars (seven cultivars) and NaCl treatments (six concentrations), with five replications. SAS Version 9.1.3 (SAS®, 1990) was used for the statistical analysis. Shapiro-Wilks test confirmed the normality of the data. In addition, Leven's test confirmed the variance homogeneity. Further, Tukey test was conducted for mean comparisons ($P<0.01$). Finally, Pearson coefficient was used for analyzing the relationship between the parameters.

3. Results

The results of variance analysis indicated that the studied cultivars had a significantly different behavior

under salt conditions, due to salt concentration and cultivar variation (Table 3).

Stem length

With increasing salinity level, stem length decreased significantly in all cultivars. The greatest effect was observed in 'Bar Anjir' cultivar, which decreased from 78.84 cm (under 0.5 dS m^{-1} salinity) to 28.44 cm (under 10 dS m^{-1} salinity). The lowest effect was observed in the 'Siyah' cultivar, which reduced from 51.3 cm (in 0.5 dS m^{-1} salinity) to 35.8 cm (in 10 dS m^{-1} salinity) (Table 4). The decrease in stem length for all the tested cultivars was between low (0.5 dS m^{-1}) and high (10 dS m^{-1}) NaCl-treated plant. This decrease for most of the cultivars (including 'Sabz', 'Shah Anjir', 'Mati' and 'Bar Anjir') was more than 200%.

Stem diameter

By raising salinity, the stem diameter decreased significantly in all cultivars. The highest reduction in stem diameter was observed in the 'Mati' cultivar, which decreased from 6.35 mm (under 0.5 dSm^{-1} salinity level) to 3.41 mm (under 10 dSm^{-1}). The lowest effect was observed in 'Siyah' cultivar, reducing from 5.37 mm at the lowest salinity level to 3.2 mm at the highest salinity level (Table 4). In addition, the decrease in stem diameter of low and high salt treated plants varied between 157.93 to 219.89% ('Kashki' and 'Bar Anjir' cultivars, respectively).

Number of leaves

With an increase in salinity levels, the number of leaves in all cultivars followed a decreasing trend. The greatest effect of salinity was observed in 'Bar Anjir', which difference in the number of the leaves in the lowest and highest salinity levels was 10.2 leaves. The lowest effect of salinity on leaf number was observed in 'Shah Anjir', and the difference was 3 leaves in the lowest and highest salinity levels in this cultivar. In addition, 'Sabz' and 'Atabaki' cultivars had the lowest number of leaves under intermediate salt conditions (4 and 6 dSm^{-1}) (Table 4). The leaf number of 'Atabaki' cultivar under higher salt condition was significantly lower than 0.5 dS m^{-1} of EC.

Specific leaf area, leaf dry matter content and leaf succulence

The specific leaf area followed a decreasing trend due to an increase in salt concentration in the 'Sabz', 'Siyah', 'Shah Anjir' and 'Kashki' cultivars. In 'Atabaki' cultivar, under EC of 6 and 10 dSm^{-1} the reduction was observed. In 'Bar Anjir' cultivar, there was no significant difference between 2-10 dsm^{-1} salt levels (Table 4). Except for 'Mati' and 'Bar Anjir' cultivars showing 32.21 and 60.55% increase in SLA, the rest of the cultivars had descending trend of SLA during the experiment. Leaf dry matter content of salt-exposed fig cultivars followed a descending trend. The difference in LDMC of seven cultivars under low salt concentration (0.5 and 2 dsm^{-1}) was not remarkable. Under moderate salt condition (4 dsm^{-1}), most of the cultivars had similar values but under higher salt (6 dsm^{-1} and more), 'Shah Anjir' and 'Siyah' cultivars showed an ascending trend. Leaf succulence displayed slight rising trend. 'Bar Anjir' had the highest value under low salt conditions, while 'Siyah' Showed the highest value under moderate and high salt condition. The values of this parameter was unchanged under moderate salt conditions and the decrease started when NaCl reached 8 dsm^{-1} and more (Table 4). Leaf succulence of 'Sabz', 'Shah Anjir' and 'Siyah' under EC of 10 dS m^{-1} were 1.64, 1.64 and 1.61 times higher than their value under EC of 6 dSm^{-1}.

Relative water content of leaf

Salinity had a significant effect on reducing leaf

Table 3 - The interaction of cultivar and salinity on growth and physiological parameters of seven fig (the mean square value is given)

Physiological parameters	Cultivar	Salinity	Cultivar x salinity
Difference in stem length	1273.11 *	5916.23 *	223.68 **
Difference in stem diameter	9.61 **	30.78 *	1.34 **
Difference in leaf number	107.80 **	220.38 *	11.25 **
Specific Leaf Area	174.37 **	27.628 NS	11.23 **
Relative water content	1540.57 **	264.34 **	289.57 **
Electrolyte Leakage	21.31 **	239.41 **	11.23 **
Leaf protein	37007542 **	268172052 **	4586351 **
Leaf nitrogen	0.93 **	5.94 **	0.071 **
Transpiration rate	4.46 **	90.58 **	3.89 **
Stomata conductance	0.099 **	1.025 **	0.074 **

NS, * and **= not significant, significant at 5 and 1% respectively (by Tukey mean comparison test).

Table 4 - The influence of saline water on stem length, stem diameter, leaf number, specific leaf area, LDMC and leaf succulence of fig cultivars

Genotype	Treatments	Stem length (cm)	Stem diameter (mm)	Leaf number	Specific leaf area ($cm^2 g^{-1}$)	LDMC	Leaf succulence
Sabz	A	59.72 b	4.53 c	8.20 c	11.53 b	0.68 b	0.13 i
	B	48.00 c	3.19 d	6.80 d	13.38 a	0.70 b	0.11 i
	C	38.64 c	3.29 d	6.00 d	11.60 b	0.52 d	0.17 h
	D	27.90 d	2.34 e	4.00 e	11.76 b	0.57 c	0.15 h
	E	30.75 d	2.51 e	3.20 f	10.22 c	0.57 c	0.17 h
	F	27.30 d	2.39 e	2.60 f	7.85 e	0.52 d	0.24 g
Siyah	A	51.30 c	5.37 b	5.80 d	4.79 f	0.74 a	0.28 e
	B	38.80 c	4.91 c	4.60 d	3.64 g	0.73 a	0.38 d
	C	30.24 d	3.24 d	2.50 f	3.94 g	0.61 c	0.41 c
	D	36.50 d	3.87 d	3.40 f	3.76 g	0.60 c	0.44 c
	E	28.66 d	3.49 d	1.00 g	3.77 g	0.60 c	0.44 c
	F	35.80 d	3.20 d	2.50 f	2.04 g	0.69 b	0.71 a
Shah anjir	A	49.72 c	4.72 c	4.00 d	11.27 b	0.70 b	0.13 i
	B	52.40 c	5.27 b	6.40 d	11.04 b	0.54 d	0.17 h
	C	29.30 d	3.68 d	5.40 d	10.35 c	0.62 c	0.16 h
	D	32.90 d	3.60 d	2.60 f	9.73 c	0.74 a	0.14 h
	E	33.04 d	3.15 d	1.60 f	9.52 c	0.76 a	0.14 h
	F	23.06 d	2.54 e	1.00 g	6.40 e	0.69 b	0.23 g
Atabaki	A	76.50 a	4.75 c	7.60 c	11.89 b	0.71 b	0.12 i
	B	59.20 b	5.17 b	7.20 c	8.49 d	0.68 b	0.17 h
	C	53.74 c	4.56 c	6.60 d	7.76 e	0.61 c	0.21 g
	D	40.50 c	3.99 d	5.00 e	5.64 f	0.49 d	0.36 e
	E	25.30 d	1.45 f	3.00 f	8.82 d	0.44 e	0.26 g
	F	44.08 c	2.46 e	3.60 f	6.01 e	0.49 e	0.34 e
Kashki	A	78.00 a	5.18 b	12.40 a	9.55 c	0.61 c	0.17 h
	B	53.62 b	5.50 b	7.00 c	9.23 d	0.62 c	0.18 h
	C	50.14 c	3.77 d	8.00 c	9.08 d	0.52 d	0.21 g
	D	38.88 c	2.97 d	4.40 e	7.88 e	0.57 c	0.22 f
	E	46.02 c	3.26 d	3.80 f	6.75 e	0.57 c	0.26 g
	F	45.46 c	3.28 d	6.60 d	6.65 e	0.52 d	0.29 f
Mati	A	68.22 b	6.35 a	9.80 b	5.95 f	0.68 b	0.25 g
	B	64.80 b	5.80 b	9.60 b	8.55 d	0.62 c	0.19 h
	C	52.10 c	4.65 c	5.80 d	5.21 f	0.45 e	0.43 c
	D	38.30 c	4.19 c	3.20 f	4.64 f	0.45 e	0.48 c
	E	33.80 d	4.24 c	5.20 e	4.20 f	0.45 e	0.53 b
	F	30.90 d	3.41 d	2.40 f	7.86 e	0.44 e	0.29 f
Bar anjir	A	78.84 a	3.98 c	13.60 a	5.60 f	0.35 f	0.51 b
	B	58.00 b	4.02 c	11.00 b	8.36 d	0.29 f	0.41 c
	C	48.00 c	3.67 d	8.40 c	8.74 d	0.30 f	0.39 d
	D	43.56 c	3.19 d	7.40 c	8.98 d	0.32 f	0.35 e
	E	32.20 d	2.90 d	4.20 e	8.68 d	0.28 f	0.42 c
	F	28.44 d	1.81 e	3.40 f	8.99 d	0.30 f	0.38 e

Data are average of five replications. In each column means with a common letter have no significant difference at 1% of Tukey test. Treatments A, B, C, D, E and F are 0.5, 2, 4, 6, 8, and 19 dS m^{-1} of EC, respectively.

relative water content in all cultivars. The highest decrease was observed in the 'Siyah' cultivar, which reduced from 90.83% in the lowest salinity level to 53.03% in the highest salinity level. The lowest effect was observed on the 'Sabz' cultivar. The intermediate salt condition of 4 dSm^{-1} did not make a significant difference from 2 dSm^{-1}, except in 'Sabz' (Table 5). The 'Kashki' and 'Bar Anjir' cultivars had the lowest decline of RWC during the experiment (15.15 and 15.33%, respectively), while 'Siyah' showed the stronger decrease in RWC (41.62%).

Electrolyte leakage

Salinity had a significant ascending effect on electrolyte leakage in seven fig cultivars. The highest salinity increased the ionic leakage in 'Siyah' and 'Atabaki'

Table 5 - The influence of saline water on electrolyte leakage, protein, nitrogen, transpiration rate, stomata conductance and RWC of fig cultivars

Genotype	Treatments	RWC (%)	Electrolyte leakage (%)	Protein (mg. g^{-1}. Fw)	Nitrogen content (%)	Transpiration rate (mol H_2O $m^{-2}s^{-1}$)	Stomata conductance (mol CO_2 m^{-2} s^{-1})
Sabz	A	76.20 c	19.53 c	0.90 c	3.73 a	59.72 b	0.46 d
	B	74.80 c	20.75 c	0.93 c	3.55 b	48.00 d	0.13 g
	C	68.20 d	18.76 c	1.21 b	3.28 b	38.64 c	0.20 f
	D	62.55 d	17.80 d	1.79 b	3.13 b	27.90 c	0.20 f
	E	62.22 d	19.20 c	2.20 a	2.47 c	30.75 g	0.06 h
	F	61.88 d	22.01 c	2.45 a	2.31 c	27.30 g	0.05 h
Siyah	A	90.83 a	15.77 e	0.86 c	3.55 b	51.30 b	0.53 c
	B	76.78 c	16.42 d	0.86 c	3.59 b	38.80 c	0.78 a
	C	70.15 c	18.61 c	1.22 b	3.43 b	30.24 e	0.16 g
	D	67.55 d	19.91 c	2.17 a	3.21 b	36.50 e	0.14 g
	E	65.09 d	22.31 b	2.35 a	2.98 c	28.66 g	0.07 h
	F	53.03 e	24.73 a	2.60 a	2.97 b	35.80 g	0.04 h
Shah anjir	A	89.23 b	16.23 d	0.76 c	3.28 b	49.72 b	0.45 d
	B	88.79 b	15.17 e	0.92 c	3.28 b	52.40 b	0.48 d
	C	81.68 b	18.67 c	0.99 c	3.10 b	29.30 d	0.30 d
	D	77.66 c	21.24 b	1.01 b	2.50 c	32.90 d	0.15 g
	E	77.56 c	20.61 c	1.56 b	3.15 b	33.04 f	0.08 h
	F	72.15 c	22.22 c	1.36 b	2.92 c	23.06 e	0.11 g
Atabaki	A	81.16 b	14.37 e	0.91 c	3.13 b	76.50 a	0.67 b
	B	77.22 c	15.39 e	1.01 b	3.34 b	59.20 c	0.36 e
	C	75.33 c	16.56 d	1.15 b	3.16 b	53.74 d	0.38 d
	D	73.47 c	19.29 c	1.62 b	2.97 c	40.50 d	0.40 d
	E	71.09 c	22.75 b	2.04 a	2.43 c	25.30 g	0.06 h
	F	64.65 d	23.05 b	2.31 b	2.29 c	44.08 h	0.03 h
Kashki	A	85.34 b	16.51 d	0.74 c	2.98 c	78.00 b	0.40 a
	B	78.92 c	20.10 c	0.85 c	3.31 b	53.62 c	0.27 f
	C	75.33 c	20.04 c	1.08 b	3.13 b	50.14 e	0.13 g
	D	73.48 c	22.49 b	1.31 b	2.89 c	38.88 e	0.19 g
	E	73.29 c	23.20 b	1.84 b	2.75 c	46.02 e	0.14 g
	F	72.41 c	22.75 c	2.19 a	2.33 c	45.46 e	0.11 g
Mati	A	96.32 a	14.46 e	0.84 c	2.97 c	68.22 a	0.75 a
	B	88.58 b	17.27 d	0.94 c	3.50 b	64.80 b	0.43 d
	C	83.88 b	19.24 c	1.09 b	3.27 b	52.10 e	0.13 g
	D	78.39 c	21.22 b	1.23 b	3.09 b	38.30 e	0.23 f
	E	74.58 c	21.65 c	1.71 b	2.66 c	33.80 f	0.15 g
	F	70.23 c	22.38 c	2.01 a	2.60 c	30.90 g	0.04 h
Bar anjir	A	95.00 a	14.83 e	0.71 c	3.78 a	78.84 d	0.20 f
	B	93.68 a	15.30 e	0.75 c	3.32 b	58.00 d	0.34 e
	C	91.82 a	16.68 d	0.81 c	3.14 b	48.00 b	0.26 f
	D	88.52 b	19.72 c	1.04 b	2.82 c	43.56 b	0.09 h
	E	82.81 b	21.26 b	1.76 b	2.75 c	32.20 f	0.08 h
	F	80.44 b	21.96 c	1.96 b	2.30 c	28.44 g	0.04 h

Data are average of five replications. In each column means with a common letter have no significant difference at 1% of Tukey test. Treatments A, B, C, D, E and F are 0.5, 2, 4, 6, 8, and 19 dS m^{-1} of EC, respectively.

cultivars (Table 5). The electrolyte leakage of 'Atabaki', 'Siyah' and 'Bar Anjir' cultivars showed the highest difference between the moderate and high salt conditions (39.19, 32.89 and 31.65% increases, respectively).

Leaf protein

The results indicated that salinity had a significant effect on leaf protein of studied fig cultivars. Increasing the stress to 4 ds^{-1} of EC increased the

total protein content gradually. The highest amounts were observed in 'Siyah' (2.60 mg g^{-1} FW) and 'Sabz' (2.45 mg g^{-1} FW) cultivars and the lowest in 'Shah Anjir' (1.36 mg g^{-1} FW) and 'Bar Anjir' (1.96 mg g^{-1} FW) cultivars (Table 5). The increase in protein content was expected between moderate (4 ds^{-1}) and high (10 ds^{-1}) NaCl treated plant. This increase for most of the cultivars (including 'Sabz', 'Siyah', 'Atabaki', 'Kashki' and 'Bar Anjir') was more than 200%.

Leaf nitrogen

By increasing salinity levels in all seven fig cultivars, leaf nitrogen content decreased. The highest reduction was observed in 'Bar Anjir' cultivar at EC of 10 ds^{-1} (2.30%) and the lowest reduction was observed in 'Siyah' cultivar, which decreased from 3.55% at the lowest salinity level to 2.99% at the EC of 10 dSm^{-1} (Table 5). The 'Mati', 'Kashki' and 'Atabaki' showed 10.03, 5.04 and 0.88% increase in N content under moderate salt condition (4 dSm^{-1}), while the rest cultivars exhibited a decrease in N content (Table 5).

Photosynthetic indices

The interaction of salinity stress and cultivar on photosynthetic indices (transpiration rate and stomata conductance) was significant. With increasing NaCl levels, the rate of transpiration decreased in seven fig cultivars. The highest reduction was observed in 'Atabaki' cultivar (0.85 mol H_2O m^{-2} s^{-1} at 10 dSm^{-1} of EC). The lowest influence was observed in 'Kashki' (3.41 mol H_2O m^{-2} s^{-1} at 10 dSm^{-1} of EC). For all the studied cultivars this parameter did not differ significantly between 4 and 6 dSm^{-1} (intermediate salt conditions). High salt concentrations (8 and 10 dSm^{-1}) caused a noticeable difference among the genotypes, where 'Atabaki', 'Siyah', 'Mati' and 'Sabz' were fall to 12.18, 19.63, 22.16 and 23.40% of their initial values (Table 5). Stomata conductance decreased by increasing salt concentration in all cultivars, and reached its lowest level at the highest salinity level (10 dSm^{-1}). The lowest stomata conductance at of EC 10 dSm^{-1} was observed in 'Atabaki' (0.03 mol CO_2 m^{-2} s^{-1}) and the lowest in 'Kashki' (0.11 mol CO_2 m^{-2} s^{-1}) (Table 5). The 'Atabaki', 'Mati' and 'Siyah' cultivars displayed the highest decrease of stomata conductance from 05. to 10 dSm-1of EC (3.86, 5.91 and 7.95% decrease of their initial values, respectively)

Correlation analysis

Table 4 indicates the bivariate Pearson correlations among the parameters. The bold faces values indicate high correlated values (higher than 0.5). Difference in the number of leaves had high correlation with relative water content (0.583**), Transpiration rate (0.899**) and stomata conductance (0.915**). Specific leaf area and relative water content were positively correlated (0.680**). In addition, both photosynthetic indices showed high correlation (0.877**) (Table 6).

4. Discussion and Conclusions

The negative effect of salinity leads to the changes in soil structure, competition in nutrients uptake in different parts of the plant and eventually inhibition of nutrients absorption (Gholami et al., 2012). Na+ reduces plant biomass by disrupting the protein synthesis, destroying chlorophyll, and decreasing the activity of the enzymes which are involved in biosyn-

Table 6 - The correlation analysis of physiological parameters of fig cultivars

Physiological parameters	Leaf nitrogen	Leaf protein	Electrolyte Leakage	Difference in stem length	Difference in the number of leaves	Difference in stem diameters	Specific leaf area	Relative water content	Transpiration rate	Stomata conductance
Leaf nitrogen	1									
Leaf protein	-0.170 *	1								
Electrolyte Leakage	-0.225 **	0.017	1							
Difference in stem length	-0.375 **	-0.006	0.068	1						
Difference in the number of leaves	0.331 **	-0.009	-0.055	-0.108	1					
Difference in stem diameters	-0.415 **	0.323 **	0.179 **	0.093	-0.287 **	1				
Specific leaf area	0.393 **	-0.239 **	-0.103	-0.079	0.199 **	-0.480 **	1			
Relative water content	0.351 **	-0.124	-0.122	-0.077	0.583 **	-0.499 **	0.680 **	1		
Transpiration rate	0.362 **	-0.111	-0.098	-0.079	0.899 **	-0.435 **	0.227 **	0.581 **	1	
Stomata conductance	0.195 **	0.025	-0.033	-0.036	0.915 **	-0.149*	-0.020	0.463 **	0.877 **	1

* and **= Correlation is significant at the 5 and 1% levels, respectively.

thesis (phosphoenolpyruvate carboxylase, Ribulose-l,5-bisphosphate carboxylase, pentose phosphate pathway enzymes and glycolysis pathway enzymes) (Demiral, 2005).

The major strategies of plants to overwhelm this stress included: reduction in Cl- and Na+ uptake, leaf loss, decrease in leaf specific area and relative leaf water content, synthesis of osmotic compounds, exclusion of toxic ions into vacuole, change in membrane stability, and increase in the activity of antioxidant enzyme (Mutsushita and Matoch, 1992; Sato *et al*., 2006).

Based on the results of the present study, the stem length in 'Siyah' and 'Sabz' was less affected by salt stress. In addition, under intermediate salt conditions 'Shah anjir', 'Siyah' and 'Sabz' had the lowest stem length. It has already reported that salt-sensitive fig cultivars such as 'Brown Turki' and 'Pius' displayed reduction in stem length under salinity conditions (Alswalmeh *et al.*, 2015; Zarei *et al*., 2016). A similar decrease in stem length under salt conditions was reported in almond (Najafian *et al*., 2008) and pistachio (Adish *et al.*, 2010).

The findings of Soliman and Abd Alhady (2017) and Zarei *et al.* (2016), indicated that the stem diameter in salt-exposed fig cultivars had a linear decrease. Furthermore, the decrease in stem diameter in salt-treated plums (Bolat *et al*., 2006), citrus (Khoshbahkt et al., 2014) and pomegranate (Khayyat *et al*., 2014) showed similar pattern. In the present study, stem diameter of 'Siyah' cultivar was less affected by salt.

Reducing the number of leaves in salt-exposed plants is due to limit leaf production or early leaf aging (Yeo *et al.*, 1991; Munns and Tester, 2008). Based on the results, the highest leaf loss was observed in 'Bar Anjir' cultivar. Similar results are available in almond (Momenpour *et al*., 2018), pistachio (Adish *et al.*, 2010) and fig cultivars (Essam *et al.*, 2013; Alswalmeh *et al*., 2015; Zarei *et al*., 2016; Soliman and Abd Alhady, 2017).

Reduction in the relative leaf water content under salinity stress indicates lower water uptake by plants. Limited access to water due to increase in osmotic potential reduces the cell development and decreases turgor pressure of the cells (Yamasaki and Dillenburg, 1999). In the present study, salinity reduced the relative content of leaf water in fig cultivars, except 'Bar Anjir'. In 'Siyah' cultivar, a dramatic decrease was observed in the relative leaf water content. Under various salt concentrations, the trend of this parameter stayed unchanged.

Under intermediate salt conditions the specific leaf area of 'Siyah' and 'Sabz' had not evident changes. According to Owais (2015), with increasing salt levels, the relative leaf water content in grape genotypes decreased, but this decrease was lower in tolerant cultivars. A similar decrease was observed in the relative leaf water content under salinity stress in fig (Zarei *et al.*, 2016; Soliman and Abd Alhady, 2017) and pomegranate (Khayyat *et al.*, 2014).

The values of SLA and LDMC reveal an important exchange in plant function between high SLA, low LDMC (cultivars with rapid production of biomass) and low SLA, high LDMC (cultivars with an efficient conservation of nutrients) (Poorter and Garnier, 1999). According to our results, 'Siyah' and 'Sabz' cultivars had the lowest SLA and the highest LDMC value, confirmed its production efficiency under various salt concentration. In addition, 'Bar Anjir' and 'Shah Anjir' had the highest SLA and the lowest LSDM under different NaCl concentration, due to their limited production efficiency under salt conditions.

Rising the salinity level, increased leaf succulence. This ability characterizes a balance between growth rate and the necessity of osmotic adjustments (Flowers and Yeo, 1986), which regulate low external water potential encouraged by salinity stress (Flowers and Colmer, 2008). Moreover, it explains the better carbon assimilation capacity per unit area (de Vos et al., 2013). Based on our findings 'Siyah' and 'Sabz' cultivars had the highest leaf succulence, which related to the balance between growth ratio and osmotic regulation under salt conditions.

Peroxidation of lipids is regarded as an extra effect of salinity on plants (Demidehik *et al.*, 2002). The findings of the previous researchers on increasing ion leakage under salinity stress in fig (Abdoli Nejad and Shekhafandeh, 2014; Zarei *et al.*, 2016) and pomegranate (Khayyat *et al.*, 2014) confirms our results.

Proteins biosynthesis is an important biochemical process which is affected by salt stress. Expression of specific genes under NaCl stress assistances the plant to adapt to adverse conditions (Murcute *et al*., 2010). According to our findings, protein content increased under high salt condition, but this increase was greater under intermediate salt for 'Siyah' and 'Sabz' cultivars.

It has reported that sodium chloride treatment reduced leaf protein content in salt-sensitive grape (Alizadeh *et al*., 2010) and figs (Abdoli Nejad and Shekhafandeh, 2014).

In fact, salt inhibits the synthesis of nitrate reduc-

tase, glutamine synthase and glutamate synthase (which are involved in nitrogen metabolism), decreases nitrogen metabolism (Hossain *et al*., 2012), changes active forms of nitrogen, reduces amino acids synthesis, and finally increases the activity of degrading enzymes (De Souza *et al*., 2016). In saline conditions, Cl^- competes with nitrate (Abdelgadir *et al.*, 2005), leading to the decline of nitrogen in different parts of the plant (Yu *et al*., 2016; Hasan and Miyake, 2017). According to the Owais (2015) and Doulati Baneh *et al.* (2014), salinity had a decreasing effect on the leaf nitrogen content in fruit crops. Although the relationship between salinity and nitrogen metabolism is very complex, balanced nitrogen metabolism significantly affects salinity tolerance in plants (Teh *et al.*, 2016). In the present study, the impact of salinity on leaf nitrogen content of different fig cultivars was not the same.

Photosynthesis is another important plant phenomenon, significantly affected by abiotic stress. Reduction of photosynthesis under salinity stress is attributed to stomata factors (reduced CO_2 permeability, stomata closure, decrease in plants transpiration, stomata conductance reduction), and non-stomata factors (cell membrane dehydration, structural changes in the cytoplasm and chlorophyll degradation) (Brugnoli and Lauteri, 1991; Reza *et al.*, 2006; Tabatabaei, 2006). Salinity reduces stomata conductance in hybrids of fig, by decreasing the photo-conductivity of leaf cells (Golombek and Lüdders, 1990; Zarei *et al*., 2016), which is in agreement with the results of the present study. Similar results were reported in pistachio (Adish *et al.*, 2010). According to the results of the present study, 'Atabaki' and 'Kashki' cultivars showed the lowest and the highest stomata conductance under high salt condition, respectively.

Salinity reduces evaporation and transpiration in plants (Bhantana and Lazarovitch, 2010; Dudley *et al.*, 2008). The linear relationship among reduction of plant evapotranspiration, transpiration and increase in salinity levels was reported in pomegranate (Shani and Ben-Gal, 2005; Mohamed Ibrahim and Abd El-Samad, 2018) and date palm (Tripler *et al.*, 2007), which is coordinated with the results of the current study.

In addition, the maximum quantum efficiency of photosystem II, electron transfer, gas exchange, and carbon dioxide assimilation decrease under salt conditions (Joao-Correia *et al.*, 2006). In the present study, Atabaki cultivar had the highest transpiration rate at the low and mild salinity levels, and with increasing salinity, a greater decrease was observed (Table 5). The lowest effect of salinity on transpiration rate was observed in 'Kashki' (Table 5), the cultivar which was able to balance the transpiration level at mild and severe stress levels, probably due to having thicker cuticle.

The most important physiological process affected by this stress is photosynthesis (Sudhir and Murthy, 2004; Acosta-Motos *et al*., 2017). Reduction in photosynthesis efficiency is followed by a series of molecular events including cell membrane dehydration, stomata closure, decreased CO_2 entry, reduction in leaf permeability to CO_2, structural changes in the cytoplasm and subsequent alteration in the activity of enzymes (Tabatabaei, 2006).

On the other hand, nitrogen plays effective role in plant growth and construction of vital plant structures such as amino acids and proteins (Arghavani *et al.*, 2017). Nitrogen is necessary to generate cellular components such as Rubisco, which is responsible for assimilating carbon dioxide. Therefore, by limiting nitrogen uptake, salinity stress affects the photosynthesis efficiency, leading to a decrease in vegetative and reproductive growth (Coruzzi and Bush, 2001; Marschner, 2012; Zarata-Valdez *et al.*, 2015).

In the present research, salinity had a significant effect on growth parameters and photosynthetic indices in seven cultivars of fig. The differences in stem length, stem diameter and leaf number in all cultivars followed a downward trend. The stomata conductance of all fig cultivars was same up to 4 dS m^{-1} NaCl. Moreover, the transpiration rate did not exhibited variation unless in salt concentration higher than 8 dSm^{-1}. The 'Siyah' and 'Sabz' cultivars had the lowest decreases in stem length and diameter, the lowest leaf water content and transpiration rate, and the maximum leaf abscission. Additionally, 'Siyah' and 'Sabz' cultivars had the highest leaf succulence and LDMC and the lowest SLA, which related to the balance between growth ratio and osmotic regulation under salt conditions. The 'Mati', as an intermediate salt-tolerance cultivar, had the lowest leaf abscission under severe salinity levels. The 'Shah Anjir', as the most salt-sensitive cultivar, could not balance transpiration rate and leaf water content under salt treatment higher than 4 dSm^{-1}.

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Optimization of phenolic compounds recovery and *in vitro* antioxidant activity of Algerian eggplant (*Solanum melongena* L.)

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Abstract: The optimum conditions for extraction of total phenolic contents (TPC) and maintaining the highest antioxidant activity from eggplant were determined. Extraction experiments were carried out by investigating the effects of the solvent nature (acetone, ethanol, methanol, or water), solvent concentration (30-90%), extraction temperature (30-100°C), extraction time (30-120 min), solid to solvent ratio (1/25-1/100 g/mL), and number of extractions (1, 2 and 3) on the recovery of phenolic compounds and antioxidant activity of the extracts. The TPC was assessed to determine the polyphenolic component while free radical scavenging activity (FRSA) and ferric-reducing power (FRP) were used to evaluate the antioxidant activity of eggplant extracts. All extraction parameters had significant effects ($p<0.05$) on the TPC extraction and the antioxidant activities. The best conditions were obtained using three extraction steps with aqueous acetone 70% (v/v) at 25°C for 60 min and with 1 g/50 mL solid to solvent ratio. The optimum extraction conditions exhibit the TPC concentrations of 794.94 mg GAE/100 g and antioxidant activities of 737.86 mg TE/g (FRSA) and 28.00 mg TE/g (FRP). The free radical scavenging and ferric-reducing potentials were found to be positively significantly correlated with phenolic content under the influence of all extraction parameters.

1. Introduction

Eggplant (*Solanum melongena* L.) commonly known as brinjal, is an economically important common vegetable grown and consumed

throughout the world. It is a good source of phenolic compounds (Jung *et al.,* 2011; Salerno *et al.,* 2014; Arkoub-Djermoune *et al.,* 2016; Dranca and Oroian, 2017; Sharma *et al.,* 2019), vitamins and minerals, especially iron, compared to other commonly consumed vegetables, and it is nutritionally comparable to tomato (Kalloo, 1993). It is ranked amongst the top ten vegetables in terms of oxygen radical absorbance capacity due to the fruit phenolic constituents (Cao *et al.,* 1996) which are powerful antioxidants (Jung *et al.,* 2011; Piao *et al.,* 2014; Scorsatto *et al.,* 2017; Sharma *et al.,* 2019).

Studies have shown that eggplant extracts suppress the development of blood vessels required for tumor growth and metastasis (Matsubara *et al.,* 2005), and inhibit inflammation that can lead to atherosclerosis (Han *et al.,* 2003). Extracts from eggplant fruit skin have been demonstrated to possess high capacity in scavenging of superoxide free radicals and inhibition of hydroxyl radical generation by chelating ferrous iron (Kaneyuki *et al.,* 1999; Noda *et al.,* 2000; Boulekbache-Makhlouf *et al.,* 2013). Superoxide radicals generated in vivo are usually converted into hydrogen peroxide, and like other free radicals, can damage lipids, proteins, and DNA (Halliwell *et al.,* 1995). From the 120 vegetable species evaluated for antioxidant activity using four different assays (2,20-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid), 2, 2-diphenyl-1-picrylhydrazyl radical, inhibition of lipid peroxidation, and Superoxide scavenging), eggplant ranked among the top 10 species for superoxide scavenging (SOS) activity (Hanson *et al.,* 2006). Nasunin, an anthocyanin isolated from the skin of purple eggplant fruit, is one phenolic compound implicated in both inhibition of hydroxyl radical generation and SOS activity (Kaneyuki *et al.,* 1999; Noda *et al.,* 2000).

Extraction is the first step in isolation of phenolic compounds from plant materials. Considering the compositional diversity of the natural sources of polyphenols, as well as the structure and physicochemical properties of these compounds, specific processes must be designed and optimized for each phenolic source (Santos-Buelga and Williamson, 2003; Pinelo *et al.,* 2005). The extraction protocol must enable complete extraction of phenolics, as well as minimization of oxidation, degradation, and polymerization of desired products (Zuo *et al.,* 2002). Many factors, such as type of solvent (water, methanol, ethanol, ethyl acetate, acetone, and hexane), pH, temperature, time, solid/solvent ratio, and extraction number, can affect the efficiency of the extraction process (Zuo *et al.,* 2002; Mo *et al.,* 2011).

There are several efficient extraction methods for determination of phenolic compounds in solid samples. These include supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), solid-phase microextraction (SPME), ultrasound-assisted extraction (UAE) (Dranca and Oroian, 2017; Ferarsa *et al.,* 2018; Nipornram *et al.,* 2018), and microwave-assisted extraction (MAE) (Mahugo Santana *et al.,* 2009; Dahmoune *et al.,* 2013; Koyu *et al.,* 2018). Solid-liquid extraction (SLE), which was used in this investigation, has been the method of choice of numerous researchers for extraction of phenolics from many sources (Liyana-Pathirana and Shahidi, 2005; Durling *et al.,* 2007; Bachir Bey *et al.,* 2013; Benmeziane *et al.,* 2014; Mokrani and Madani, 2016; Caldas *et al.,* 2018; Mohd Hazli *et al.,* 2019). Effect of extraction process can be generally evaluated based on a one factor one time approach, also known as single experiment, in which only one factor is variable at one time while all others are kept constant. The availability of phenolic compounds in eggplant as an antioxidant source is documented. However, no optimal protocols have been established so far for phenolic extraction from these vegetable. To the best of our knowledge, no data was reported about the effect of extracting parameters on the survey of phenolics from eggplant.

Hence, the objective of this study was to optimize the extraction of total phenolics compounds (TPC) maintaining the highest antioxidant capacity (DPPH free radical-scavenging activity; FRSA and ferric reducing power; FRP) from eggplant (*Solanum melongena* L.) using single factor experiments approach under conditions compatible with food use. The objective in extracting phytochemicals from their plant sources is to liberate these compounds from the vacuolar structures where they are found, either through rupturing plant tissue or through a process of diffusion. The factors that contribute to the efficiency of extraction are, in particular, solvent type (ethanol, methanol, acetone and water), solvent concentration (30%, 50%, 70% and 90%, v/v), temperature (25, 50, 75 and 100°C), time (30, 60, 90 and 120 min), solid to solvent ratio 1/25, 1/50, 1/75 and 1/100 g/mL), and number of extraction (1, 2 and 3). The stability of antioxidant activities (FRSA and FRP) in extracts was also investigated.

2. Materials and Methods

Chemicals

Folin-Ciocalteu reagent was provided from

Biochem, Chemopharma (Montreal, Quebec). Sodium carbonate (Na_2CO_3), acetone, ethanol and methanol were obtained from Prolabo (made in CE). Potassium ferricyanide ($C_6N_6FeK_3$), ferric chloride ($FeCl_3 6H_2O$), trichloroacetic acid, gallic acid and trolox from Biochem-chemopharma (UK). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (Steinheim, Germany).

Plant material

The fresh eggplant (*Solanum melongena L.*) used in this study was purchased from local market of Bejaia city (Algeria) at February 2010. After transferring at the laboratory, the sample was washed with distilled water and wiped then some tests were done on fruits in order to determine their physico-chemical characteristics. Eggplant fruits presented a pH of 4.15, water content of 92.8 g/100 g FW, titratable acidity of 1.24 g/100 g DW, total sugar content of 20.55 g/100 g DW, total soluble solids content (Brix) of 31.48 g/100 g DW and ash content of 0.55 g/100 g DW (Arkoub-Djermoune *et al.,* 2016). The sample was frozen at -20°C before analysis and a quantity of eggplant were mixed and used for each extraction step.

Plant material extraction

Approximately 0.1 g of crushed eggplant was weighed in a glass vial and extracted with 10 mL of the extracting solvent. The mixture was shaken at a constant rate using a water bath shaker, centrifuged for 20 min at 4000 g (nüve NF 200, Ankara, Turkey), and filtered through a Watman filter paper. The extraction process was carried out in duplicate. The obtained filtrates extracts were subsequently used for the determination of total phenolic compounds (TPC) and antioxidant activities: DPPH-free radical scavenging activity (FRSA) and ferric reducing power (FRP) measurements.

Experimental design

In the present study, single factor experiments was used to determine the optimum conditions for extracting phenolic compounds from eggplant. A total of six parameters namely extraction solvent (ethanol, methanol, acetone and water), solvent concentration (30-90%; v/v), extraction temperature (30-100°C), extraction time (30-120 min) solid to solvent ratio (1/25-1/100 g/mL), and number of extractions (1, 2 and 3) were studied in which one parameter was varied at a while the other parameters were fixed. The optimal extracting conditions were selected on the basis of the TPC, FRSA and FRP measurements.

Solvent nature and concentration

By setting extraction time (30 min) and temperature (25°C) and sample/solvent ratio (0.1 g/10 mL), samples were extracted with acetone (30%, 50%, 70%, and 90%; v/v), ethanol (30%, 50%, 70%, and 90%; v/v), methanol (30%, 50%, 70%, and 90%; v/v) and water.

Extraction temperature

Using the best solvent type and solvent concentration, eggplant samples were extracted at temperatures ranging from 25°C to 100°C (25, 50, 75 and 100°C). The best extraction conditions studied were selected according to the value of three responses (TPC, FRSA and FRP).

Extraction time

Eggplant samples were extracted using the best solvent concentration and extraction temperature determined previously. The extracts were prepared by varying the extraction time from 30 min to 120 min (30, 60, 90 and 120 min). The best extraction conditions studied were selected according to the value of three responses (TPC, FRP and FRSA).

Solid to solvent ratio

Eggplant samples were extracted using the best solvent concentration. The extraction procedure was repeated by varying the sample/solvent ratio 1/25, 1/ 50, 1/75, and 1/100 g/mL, while fixing the extraction time and temperature at 60 min and 25°C as determined previously. TPC and antioxidant activity values for consecutive extractions were added for determination of this parameter.

Number of extractions

The final step of this experiment was to determine the effect of the extractions number. In order to determine this effect, the extraction setting with the optimal conditions selected previously: solvent (acetone 70%; v/v), temperature (25°C), time (60 min) and the solid to solvent ratio (1/50 g/mL), the extraction was repeated three times on the solid residue after centrifugation of the mixture at 4000 rpm during 20 min.

Total phenolic compound determination (TPC)

The amount of TPC in eggplant extract was determined using the Folin-Ciocalteu reagent and gallic acid as standard as described by Velioglu *et al.* (1998). In brief, 200 µL of each extract were introduced into test tubes then added with 1.5 mL of Folin-Ciocalteu reagent (previously diluted ten times). After 5 min, 1.5 mL of sodium carbonate (60 g/L) were added. The tubes were mixed and allowed

to stand in darkness at room temperature for 30 min. Absorption at 760 nm against a blank was measured using a Shimadzu UV-Vis spectrophotometer (Kyoto, Japan). The concentration of phenolic compounds extracts is determined by reference to the calibration curve obtained under the same conditions using the Gallic Acid as the standard, expressed as milligram Gallic Acid Equivalent per one hundred gram of the Fresh Weight (mg GAE/100 g FW). All measurements were carried out in triplicate.

Antioxidant activity

Free radical scavenging activity of DPPH· (FRSA). In the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) assay, antioxidants were capable to reduce the stable radical DPPH· to the yellow colored diphenylpicrylhydrazine (DPPH-H). The test is based on the reduction of an alcoholic solution of DPPH· in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H (Gülçin, 2007).

The DPPH· radical-scavenging activity of eggplant extracts was estimated as described by Milardović *et al.* (2006). Briefly, 100 µL of samples extract were mixed with 3 mL of DPPH in methanol (6.10^{-5}M). The mixtures were left for 30 min at room temperature and its absorbance then measured at 517 nm against a blank. All measurements were carried out in triplicate. The percentage scavenging was calculated using the following equation:

$$\text{DPPH-FRSA (\%)} = [(A_{contr} - A_{extr})/A_{contr}] \times 100$$

Where A_{contr} is the absorbance of the control (without extract) after 30 min and A_{extr} is the absorbance of extract. The inhibition percentage were expressed as milligram Trolox Equivalent per gram of the Fresh Weight (mg TE/g FW).

Ferric reducing power (FRP)

The ferric reducing power of eggplant extract was performed by using the potassium ferricyanide-ferric chloride method. Substances, with reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which subsequently reacts with ferric chloride to form ferric ferrous complex who has an absorption maximum at 700 nm (Jayanthi and Lalitha 2011). The reducing power of the extracts was evaluated according to the protocol of Oyaizu (1986). One millitre of different concentrations of the samples was mixed with phosphate buffer (1 mL, 0.2 M, pH = 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 mL, 1 g/100 mL). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (TCA) (1 mL, 10 g/100 mL) was added to the solution which was then centrifuged for 10 min at 3000 g. The supernatant was gathered and mixed with distilled water (1.5 mL) and $FeCl_3$ (150 µL, 0.1 g/100 mL), and the absorbance was measured at 700 nm, increased absorbance of the reaction mixture indicate an increase in reducing power. Results are expressed as milligram Trolox Equivalent per gram of Fresh Weight (mg TE/g FW). The values are presented as the means of triplicate analyses.

Statistical analysis

Results were analyzed using Statistica software (version 5.5.fr; StatSoft, Inc, Tulsa, USA). All values are expressed as mean ± standard deviation (SD) of duplicate extractions and triplicate assays. One-way analysis of variance (ANOVA) with the LSD (Least Significant Difference) test was used to determine significant differences ($p<0.05$) among the means.

3. Results and Discussion

Solvent nature and concentration

The selection of extraction solvent is critical for the complex food samples as it will determine the amount and type of phenolic compounds being extracted. Aqueous alcohols particularly acetone, ethanol and methanol are the most commonly employed in phenolic extraction from botanical materials (Chan *et al.,* 2009).

In this study, diverse solvents, in mixtures with water, were used to extract antioxidant phenolic compounds from eggplant. All solvents used in the present work at different concentrations (30%, 50%, 70%, and 90%) under the same extraction conditions (30 min at 25°C) were capable of extracting phenolic compounds (Table 1). Pure organic solvents were not used because obtained extracts were cloudy. Therefore, dilution with water to 90% was necessary. Solvent type had a significant influence ($p<0.05$) on TPC and antioxidant activities (FRSA and FRP).

The aqueous acetone (70%**;** v/v) extracts showed the highest yield of TPC (1032.16 mg GAE/100 g). Similar results were reported in our previous study (Boulekbache-Makhlouf *et al.,* 2013) that 70% acetone was better for phenolic extraction from eggplant byproduct (peel) than 70% ethanol, and 70% methanol. Kallithraka *et al.* (1995) found that 70% acetone was the best solvent for the extraction of grape seed phenolics especially proanthcynidins.

Similar to the present study, acetone:water mix-

Table 1 - Effect of the solvent type and concentration on the extraction efficiency for TPC and antioxidant activities (FRSA and FRP) of eggplant

Solvent	Concentration	TPC (mg GAE/100 g FM)	FRSA (mg TE/g FM)	FRP (mg TE/g FM)
Acetone	30%	809.06 ± 29.65 c	410.23 ± 19.78 e	18.61 ± 0.59 d
	50%	785.06 ± 31.22 c	200.11 ± 10.01 h	18.78 ± 1.44 d
	70%	1032.16 ± 40.04 a	648.59 ± 14.57 a	26.06 ± 0.42 a
	90%	775.18 ± 26.45 c	365.85 ± 28.37 f	21.33 ± 1.09 c
Ethanol	30%	871.19 ± 32.90 b	360.30 ± 12.01 f	15.44 ± 1.58 ef
	50%	641.04 ± 25.53 de	458.18 ± 34.53 d	13.78 ± 1.44 f
	70%	539.38 ± 37.65 f	298.12 ± 29.32 g	17.23 ± 0.29 de
	90%	512.18 ± 21.32 f	537.80 ± 25.99 bc	24.78 ± 1.13 ab
Methanol	30%	615.62 ± 38.20 e	288.55 ± 18.88 g	16.67 ± 0.44 e
	50%	532.32 ± 47.23 f	305.20 ± 25.32 g	15.50 ± 1.36 ef
	70%	669.28 ± 13.62 de	518.87 ± 19.36 c	22.33 ± 0.50 c
	90%	708.81 ± 33.89 d	638.89 ± 19.85 a	14.00 ± 1.26 f
Water	-	660.81 ± 23.58 de	574.80 ± 17.96 b	23.39 ± 0.48 bc

Values are presented as means ± SD of six measurements. Values with different letters are significantly different ($p<0.05$).
n= 2.

tures have been reported to be one of the most effective solvents for extracting phenolics from different natural sources. Meneses *et al.* (2013) and Mokrani and Madani (2016) demonstrated that 60% acetone was the best solvent for extracting antioxidant phenolic compounds from brewer's spent grains and peach fruit, respectively. Acetone:water mixture is capable to break polyphenol-protein complexes. This fact would explain the high efficiency of this solvent to extract phenolic compounds. Downey and Hanlin (2016) demonstrated that mixtures of acetone ranging from 50% to 70% are more effective in extracting condensed tannins from grape skin.

In the other hand, it has been also demonstrated that acetone is more effective than other organic solvents for extracting phenolics from different raw materials such as berries and apples (Kähkönen *et al.,* 2001), star fruits (Shui and Leong, 2006), onions (Curcic *et al.,* 2012), barley seeds (Liu and Yao, 2007), beach peas (Chavan and Amarowicz, 2013), pistachio byproducts (Mokhtarpour *et al.,* 2014), fenugreek (Mashkor, 2014) and soybean (Lien *et al.,* 2015).

The result also showed that there were no significant difference between aqueous ethanol 50%, methanol (30%, 70%, and 90%), and water extracts. However, the use of water as single solvent provides a cloudy extracts with a high content of impurities (Chirinos *et al.,* 2007).

Generally, acetone is the best solvent for extracting proanthocyanidins and tannins; ethanol efficiently extracts flavonoids and their glycosides, catechols and tannins; whereas phenolic acids and catechin were better extracted with methanol. These facts are in agreement with polarity of the solvent used for the extraction and solubility of phenolics in them since the polarity of acetone, ethanol and methanol is 0.355, 0.654 and 0.762, respectively (Tan *et al.,* 2013). Therefore, there is no single solvent able to extract all of the classes of phenolic compounds from a sample, simultaneously.

Acetone is a more efficient solvent for extracting phenolic compounds with a high molecular weight such as condensed tannins. It is strongly believed that the higher molecular weight of the solvent, the lower the polarity which enable other substances of about the same molecular weight to be easily extracted. This can be associated to ''like dissolves like" or ''polarity versus polarity" principle as both acetone and tannins are of high molecular weight. Acetone has the lowest polarity but contains the highest total phenolic compounds value (Alasalvar *et al.,* 2006; Uma *et al.,* 2010). This would explain why acetone was found to be more efficient for extracting phenolics from eggplant. In addition, the lowest value of TPC was obtained with 70% ethanol, 90% ethanol and 50% methanol. This fact is due to a polarity of the solvent used for the extraction and solubility of phenolic compounds in them because these solvents due to their polarity are more effective for extracting polyphenols linked to polar fibrous matrices (Tabart *et al.,* 2007) and their antioxidant activity depends not only on the concentration of polyphenols but also on their chemical structure (the number and position of hydroxyl groups) (Sroka and Cisowski, 2003). In addition, the effectiveness of phenolic compounds as antioxidants does not only

depend on their composition but also influenced by the degree of polymerization, concentration and interaction of their various chemical structures with colorimetric analysis substances (Moure *et al.,* 2001). However, the acetone:water mixtures are more useful for extracting polyphenol from protein matrices, since they appear to degrade the polyphenol protein complexes (Tabart *et al.,* 2007). According to Grujic *et al.* (2012), mixtures of organic solvent and water have been revealed to be more efficient in extracting phenolic compounds than mono-component solvents. Inevitably, total phenolic content is also influenced by the solubility of phenolic compounds in the solvent used, as their diverse chemical structures might alter their solubility (Chaalal *et al.*, 2012).

The antioxidant activity of the eggplant phenolic extracts was determined by two methods, namely the 1,1-diphenyl-2- picrylhydrazyl free radical scavenging activity (FRSA) and the ferric reducing power (FRP) assays, which have been widely used for the assessment of antioxidant capacity of various plant extracts and natural products.

Determination of scavenging stable DPPH· free radical is a very quick way to evaluate the antioxidant activity of the extracts in a very short time. With this method, it was possible to assess the antiradical ability of an antioxidant by measuring the reduce in the absorbance of DPPH· at 517 nm. As a result of a color change from violet to yellow, the absorbance diminishes when the DPPH· radical is scavenged by an antioxidant through hydrogen donation to form a stable DPPH-H molecule. In the radical form this molecule had an absorbance at 515 nm which disappeared by receiving an electron or hydrogen from an antioxidant to become a stable diamagnetic molecule.

Aqueous acetone 70% was also observed to be the solvent presenting the highest antioxidant activity (Table 1). The percentage of the DPPH· radical-scavenging activity (FRSA) of acetone was 648.59 mg TE/g FM, more three times than acetone 50% (200.11 mg TE/g FM), which represents the lowest value. However, there were no significant difference between 90% acetone and 30% ethanol; 70% ethanol, methanol (30%, 50%) and water extracts with methanol (70%) and ethanol (90%) extract. Our results are in accordance of those reported by Mokrani and Madani (2016), Chaalal *et al.* (2012) and González-Montelongo *et al.* (2010) who shown that aqueous acetone were more efficient for extracting peach, prickly pear seeds and banana peel phenolics, respectively and therefore, produced extracts with higher antioxidant activity. This can be explained by the solubility of phenolic compounds which depends widely on the nature of the solvent (polarity) used, their degree of polymerization, as well as their interaction with other food components and the formation of insoluble complexes (Naczk and Shahidi, 2004). Since acetone was the solvent presenting the highest yield of TPC and antioxidant activity simultaneously, it is believed that phenolics contributing efficiently to the total antioxidant activity of eggplant extract are phenolic compounds with higher molecular weight and lower polarity, according to ''like dissolves like" principle.

Ferric reducing power (FRP) of eggplant extracts was measured by the direct reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$ and was determined by measuring absorbance of the resulting Perl's Prussian blue complex formed after the addition of ferric ions (Fe^{3+}). In this method, the yellow color of the test solution changes to various shades of green and blue depending on the content of reductants (antioxidants) in the sample. These reducing agents reduce the Fe^{3+}/ferricyanide complex to the ferrous form. Thus, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Gülçin *et al.,* 2006; Gülçin, 2011). The same thing as FRSA, 70% acetone extract was observed to have significantly the highest FRP (26.06 mg TE/g FM), two times approxymatively more than 50% ethanol (13.78 mg TE/g FM), which represents the lowest value. Some extract have showed no significant differences at $p<0.05$ (Table 1). A similar result has been reported by Mokrani and Madani (2016) and Chaalal *et al.* (2012) in peach and prickly pear seeds extracts, respectively. The differences observed in the antioxidant activity (FRSA and FRP) of eggplant extracts could be due the variation of the quantity and quality of phenolic compounds present in the different extracts. Thus, by compromising between the yield of TPC and antioxidant activities (FRAS and FRP), 70% acetone was chosen as the best solvent to optimize the following extraction conditions.

Extraction temperature

As depicted in figure 1, extraction temperature demonstrated a significant effect ($p<0.05$) on phenolic content and antioxidant activities of eggplant extracts. The efficiency of TPC extraction and the antioxidant activities were influenced by the temperature. The TPC dropped when the temperature

increased from 25°C to 100°C. With the increase of temperature from 25 to 100°C, TPC, FRSA and FRP decrease from 1032.16 to 886.72 mg GAE/100 g FM (Fig. 1A), from 648.59 to 203.05 mg TE/g FM (Fig. 1B) and from 26.06 to 21.17 mg TE/g FM (Fig. 1C), respectively. This decrease may be attributing to a degradation of phenolic compound by increasing the temperature which has a great effect on the antioxidant activity, in particular the antiradical activity.

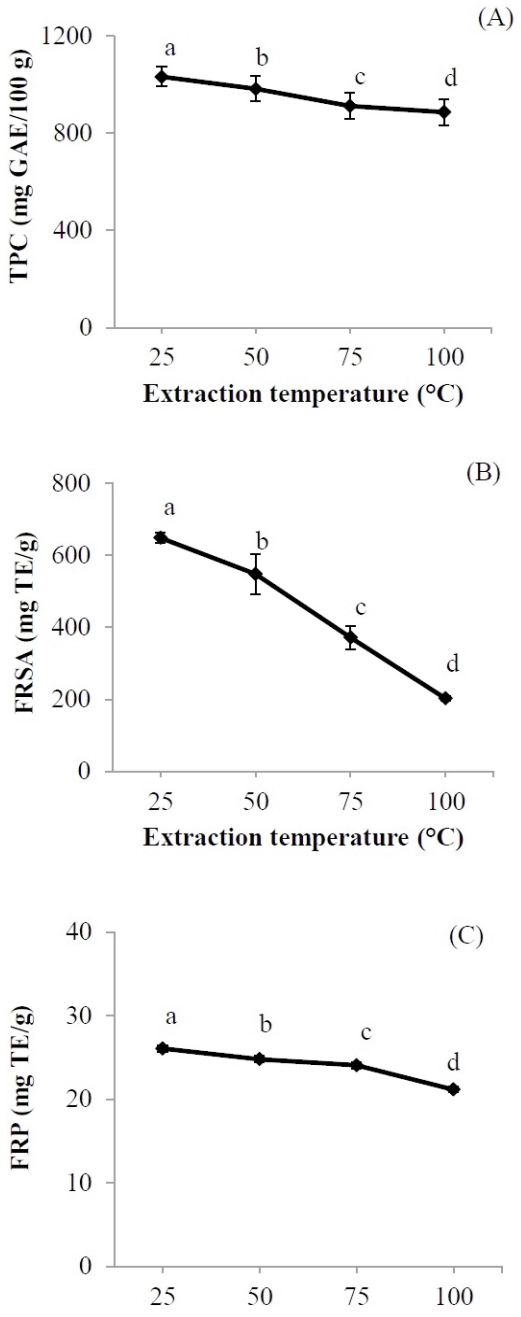

Fig. 1 - Impact of temperature on the extraction efficiency for total phenolic compounds (A), DPPH free radical scanenging activity (B) and ferric reducing power (C) of eggplant (n= 2). Values with different letters are significantly different ($p<0.05$).

In this study, the optimal temperature for antioxidant extraction from eggplant was 25°C. Similar to the result reported by Naczk and Shahidi (2004) and Mokrani and Madani (2016) who found that heating cannot increase the phenolic extraction indefinitely and at temperature above 50°C, the stability of these compounds decreases with dramatic effects with the antioxidant activity. It should be noted that increasing temperature beyond a certain value can lead to decomposition of some phenolic compounds. Rostagno *et al.* (2007) reported a decomposition of isoflavones in soybean during heat treatments. Malonyl isoflavones also degrade when extraction is performed between 75 and 100°C. Extraction between 100-125°C affects acetyl isoflavones and higher temperatures sharply reduced the glucosides concentrations. It is not surprising to find out that the antioxidant activities results showed a similar trend to the total phenolic concentration. This could be due to the fact that each assay measures different kind of phenolics, and each phenolic compound shows different antioxidant properties, which depends on the chemical structure and substitution position (Pokorny, 2003).

According to Liyana-Pathirana and Shahidi (2005) and Hismath *et al.* (2011), heating mobilizes certain antioxidants while promoting concurrent decomposition of antioxidants, which are already mobilized at lower temperatures. When an acetone:water solvent is used for extraction, the evaporation of acetone will change the acetone-water ratio because acetone, with a boiling point of 56.2°C, becomes volatile (Al-Farsi and Lee, 2008). Moreover, Santos-Buelga and Williamson (2003) have reported that some phenolics are thermosensitive, particularly certain flavonoids, such as anthocyanin and flavan-3-ol derivatives which are the most prevalent polyphenols in eggplant. Furthermore, Cacace and Mazza (2003) reported that temperature affected the extraction of anthocyanins and increasing the temperature over 30-35°C resulted in the decomposition of anthocyanins. This could be explained by a higher vulnerability of anthocyanins to high temperature.

Nevertheless, several other studies reported that heat enhanced TPC recovery Ju and Howard (2003), Pinelo *et al.* (2005), Al-Farsi and Lee (2008) and Benmeziane *et al.* (2014). This was probably due to the increased phenolic solubility, faster diffusion rate, better mass transfer, extraction yield, reduced solvent viscosity and surface tension (Richter *et al.,* 1996). According to Wissam *et al.* (2012) an increase

in temperature increases the efficiency of the extraction since heat render the cell permeable, increase solubility and diffusion coefficients of the compounds to be extracted and decreases the viscosity of the solvent, thus facilitating its passage through the solid substrate mass but the use of temperatures higher than 50°C decreases the total polyphenols yield which is probably due to their degradation. The improvement of the antioxidant extraction with temperature was probably due to the increasing diffusivity of the solvent in the solid matrix and the solubility of the phenolic compounds in the solvent, which favour the extraction (Herrero *et al.,* 2005; Juntachote *et al.,* 2006). In fact, the polarity of water (dielectric constant) is decreased when the temperature is increased, due to the breakdown of hydrogen bonds when water is subjected to high temperatures, changing the water properties. Under these conditions, the water becomes less polar and acts like an organic solvent such as methanol or ethanol, increasing the solubility of the organic materials in it (Ballesteros *et al.,* 2017). The effect of extraction temperature is very variable from plant material to another; it depends especially on their composition on phytochemicals and to their resistance to heat. Based on the results obtained with the effect of extraction temperature, we deduced that the phenolic compounds present in eggplant extracts were thermally unstable.

It is well known that the use of water in infusion is very common and easy process used for preparing tea, which extracts the polyphenols from tea with hot water. Which can't be applied in our case to extract phenolics from eggplant because they are very sensitive to heat treatment. While, the use of pure water as single solvent for extraction presents some problems and provides an extract with a high content of impurities (organic acids, sugars, soluble proteins) particularly at high temperatures, that could interfere in the phenolic identification and quantification (Chirinos *et al.,* 2007). When membrane filtration was used, the presence of protein and polysaccharide reduced the filterability. Moreover, the cumulative cost of the concentration operation increases since water is more difficult to remove than acetone and other organic solvents (Bachir Bey *et al.,* 2013). Furthermore, water dissolves many nutrients like sugar and protein. Therefore, aqueous extracts are more susceptible to microorganism invasion during storage. Several studies have reported that acetone:water mixtures are good solvent systems for extraction of polar antioxidants and are more useful for phenolic extraction from protein matrices since phenolic-protein complexes dissolve more easily (Kallithraka *et al.,* 1995; Sun *et al.,* 2002).

Since the highest TPC value was extracted at the temperature of 25°C with an extraction yield of 1032.16 mg GAE/100 g FM, this temperature was chosen as the best temperature for extracting phenolic compounds from eggplant and the extracts provide high phenolic content and maintaining a highest antioxidant activity with health benefits and could be great interest for application in pharmaceutical products.

Extraction time

Extraction time is essential in economizing energy and cost of the extraction process. The extraction time only slightly influenced the TPC and antioxidant activities of eggplant (Fig. 2). The TPC, FRSA and FRP increased when extraction time was increased from 30 to 60 min. After 60 min, further increase of the

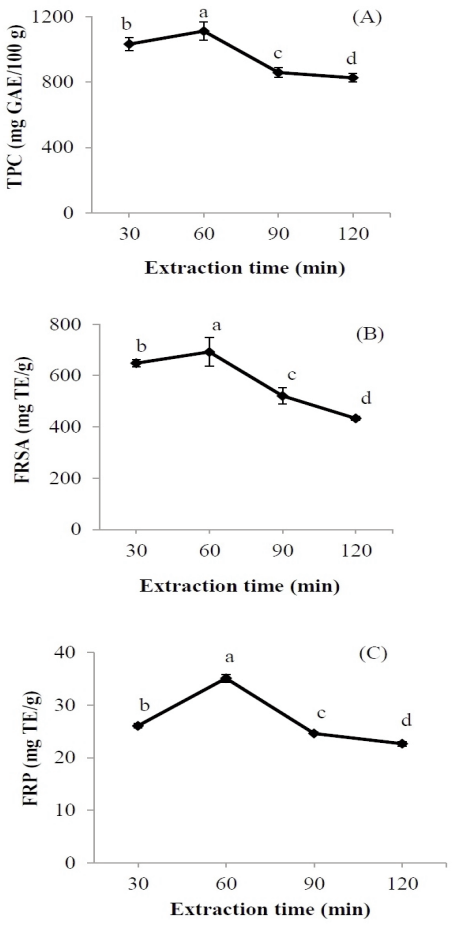

Fig. 2 - Impact of the extraction time on the extraction efficiency for total phenolic compounds (A), DPPH free radical scanenging activity (B) and ferric reducing power (C) of eggplant (n= 2). Values with different letters are significantly different ($p<0.05$).

processing time significantly decreased ($p<0.05$) the rate of phenolics and antioxidant activity. A time of 60 min was selected as the optimal time for extraction. This duration allowed extraction of 1111.23 mg GAE/100 g FM of phenolics, which corresponded to FRSA and FRP of 692.47 and 35.11 mg TE/g FM, respectively.

The optimal time for phenol extraction changes depending upon the material used for extraction but it is an important factor that influence TPC extraction and, hence, antioxidant activity. Moreover, the type of phenolic compounds extracted and the temperature of extraction were also important factors. A similar trend was shown by Liyana-Pathirana and Shahidi (2005) on whole grains, bran of both soft and hard wheat and Al-Farsi and Lee (2008) on date seeds. This observation was well explained by Fick's second law of diffusion, which predicts a final equilibrium between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) after a certain time (Silva *et al.,* 2007).

In addition, Liyana-Pathirana and Shahidi (2005) showed that the total antioxidant activity increased with an increase in extraction time from 15 to 60 min. Beyond 70 min, the total antioxidant activity decreased sharply and reached a minimum at 105 min, probably due to decomposition of the active compounds during the prolonged extraction time. In this investigation, 1 h for TPC extraction was optimal, and prolonged extraction up to 2 h decrease the TPC or the antioxidant activity at 25°C. Our results revealed that an excessive time beyond 60 min is not useful to extract more phenolic compounds from eggplant.

Taking into account these facts, an extraction time of 60 min was selected as the best extraction time for extracting phenolic compounds and antioxidant activities of eggplant.

Solid to solvent ratio

The choice of the solid to solvent ratio was investigated because it influences phenolic recovery and the antioxidant activity. The total phenolic content and antioxidant activity of eggplant extracted by 70% acetone at 25°C for 60 min using four solid/solvent ratios: 1/100, 1/75, 1/50 and 1/25 g/mL are shown in figure 3. Sample/solvent ratio had a significant effect ($p<0.05$) on TPC and the antioxidant activities (FRSA and FRP).

The TPC, FRSA and FRP radicals scavenging capacity increased from 1111.23 to 1258.07 mg GAE/100 g, from 692.47 to 855.50 mg TE/g and from 35.11 to 39.33 mg TE/g, respectively, with the increase of solid/solvent ratio from 1/25 to 1/50 g/mL. The TPC recovery using a ratio of 1/25 was poor. Extraction of antioxidants reached a maximum with a ratio of 1/50, which produced phenolic concentrations of 1258.07 mg GAE/100 g (Fig. 3A). The antioxidant activity changes with the same patherns with phenolic concentrations with a changing ratio from 1/25 to 1/50 g/mL, but with ratio of 1/75 and 1/100 g/mL, the FRSA and the FRP decreased (Fig. 3 B, C). Ćujić *et al.* (2016) have noted that the higher solid to solvent ratio generate a decrease in the consumption of plant material and decrease in the cost of extraction. Additionally, Bucić-Kojić *et al.* (2007) reported a significant difference of the polyphenols recovery yield from grape seeds depending on liquid-to-solid ratio, with the highest polyphenols concentration obtained using a ratio of 40:1. Furthermore, Prasad *et al.* (2012) employed a factorial design approach to identify the significant factors contributing to high extraction yield, antioxidant capacity and phenolic content in the extracts from *Mangifera pajang* peri-

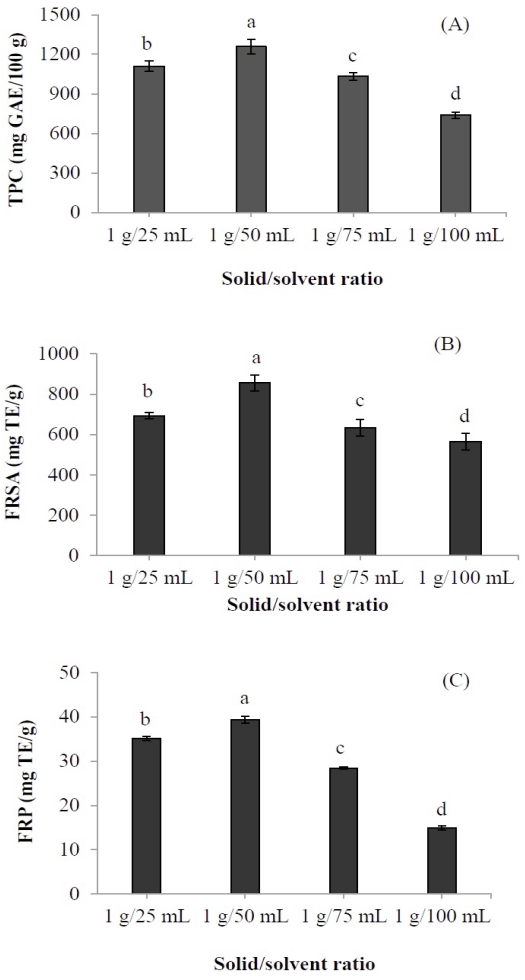

Fig. 3 - Impact of the solid to solvent ratio on the extraction efficiency for total phenolic compounds (A), DPPH free radical scanenging activity (B) and ferric reducing power (C) of eggplant (n= 2). Values with different letters are significantly different ($p<0.05$).

carp. liquid-to-solid ratio was reported as highly significant contributor.

Since the solid to solvent ratio of 1/50 g/mL was selected as the best ratio for phenolics extraction and antioxidant activities of eggplant.

Number of extractions

A series of successive extractions were performed under operating conditions favoring the best extraction: 70% acetone as solvent, a temperature of 25°C, extraction time of 60 minutes and solid/solvent ratio 1/50 g/mL. Three sequential extractions appear sufficient; the first extract contain approximatively 60% of total extractable polyphenols (1258.07 mg GAE/100 g) and exhibits an antioxidant activity of 855.61 mg TE/g (FRSA) and 39.33 mg TE/g (FRP) (Fig. 4). This rate relatively was completed by the second (27%) with a tenor of 558.44 mg GAE/100 g and gives an antioxidant activity of 415.30 mg TE/g (FRSA) and 18.44 mg TE/g (FRP) and third (13%) extraction with a concentration of 264.98 mg GAE/100 g which exhibits an antioxidant activity of 245.95 mg TE/g (FRSA) and 9.33 mg TE/g (FRP). A similar result to that found in the present study was obtained by Benmeziane *et al.* (2014) on the table grape (*Vitis Vinifera* L.) with percentages of 60%, 22%, 13% in the three fractions respectively. According to Bonnaillie *et al.* (2012), depletion of the raw material and the concentration of the extraction medium are conducted mainly in the first three stages, the last stage, rather dilutes the volumes retained in the solid. Consequently, the number of extractions can be limited to three successive contacts with fresh solvent, these authors for their part, found in a series of three successive extractions of dandruff peanut following results: 60%, 35% and 5% respectively.

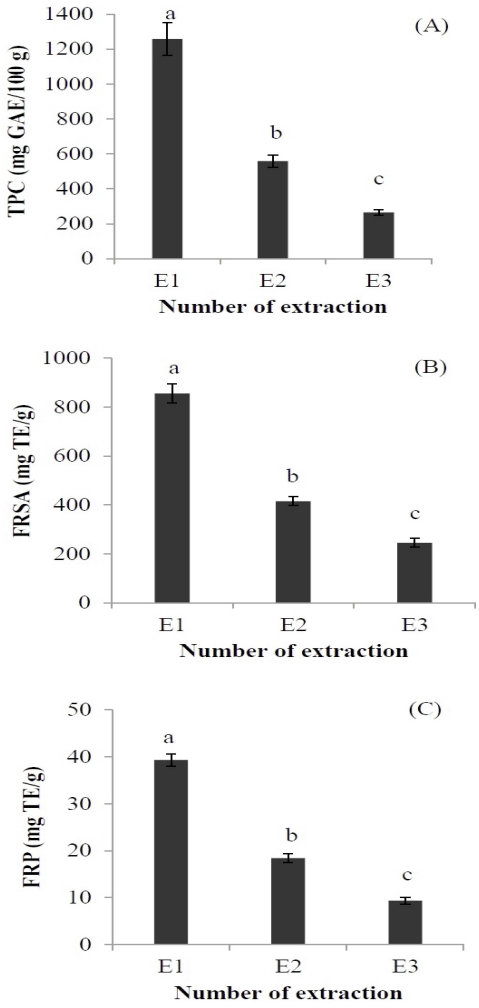

Fig. 4 - Impact of the number of extraction on the extraction efficiency for total phenolic compounds (A), DPPH free radical scanenging activity (B) and ferric reducing power (C) of eggplant (n= 2). Values with different letters are significantly different (p<0.05).

The maximum amount of extractable total phenols in the extract prepared with the mixture of the three successive extractions was assessed to 794.94 mg GAE/100 g FW and maintaining a highest antioxidant activity of 737.86 mg TE/g (FRSA) and 28.00 mg TE/g (FRP) which can be probably attributed to the dilution factor in the three mixture extracts.

Pearson correlation analysis

In order to more appreciate the relationships between antioxidant capacities and phenolic content of eggplant extracts, correlations between assays under different extracting conditions were analyzed.

Under the parameter of solvent type (Table 2), no significant correlations were found between the TPC and the antioxidant activities (FRSA and FRP). This can be explained by a synergism of eggplant phenolics present in the extract which may contribute to the overall observed antioxidant capacity.

Under the influence of extraction temperature (Table 2), TPC was correlated positively with FRSA assay (r= 0.82) at *p<0.05* and FRP assay (r= 0.66) at *p<0.001*.

Concerning the influence of extraction time condition (Table 2), The TPC was observed to be correlated positively with FRSA (r= 0.95) and FRP (r= 0.83) at *p<0.001*. From this correlation, we can believe that eggplant phenolic compounds extracted at different times display antioxidant capacities.

About the parameter solid to solvent ratio, TPC were observed to be positive significantly (*p<0.001*) correlated with FRSA assay with Pearson correlation coefficients of 0.90 and 0.90, respectively (Table 2). Previous studies showed that antioxidant activity of

Table 2 - Pearson correlations coefficient between different assays under influence of extraction conditions

	Total phenolic compounds				
	Solvent type	Extraction temperature	Extraction time	Solid to solvent ratio	Number of extraction
Free radical scavenging activity against DPPH radical	0.23 NS	0.82 ***	0.95 ***	0.90 ***	0.99***
Ferric reducing power	0.26 NS	0.66*	0.83 ***	0.90 ***	0.99***

NS = Not significant.
* = Significant at p<0.05.
*** = Significant at p<0.001.

phenolic compounds depends widely on their structure. Therefore, the solid to solvent ratio affect positively the antioxidant capacity of eggplant phenolics.

Under the influence of number of extraction, correlations between TPC and antioxidant assays (FRSA and FRP) were positively high (0.99, *p<0.001*). We can suggest that the hydrogen electron donating abilities of eggplant extracts were directly proportional to the concentration of total phenolics. This relationship suggested that the phenolic compounds of eggplant extracts might be the major contributors to the analyzed antioxidant activities.

4. Conclusions

In the present study, single factor experiments approach was used to determine the optimization of the extraction process of eggplant phenolics, investigating some variables which such as effect of solvent extraction (solvent type and solvent/water mixture concentration), extraction temperature, extraction time, solid to solvent ratio and number of extractions. To the best of our knowledge, no data was reported about the effect of extraction parameters on the recovery of phenolic compounds from eggplant. The results of the present investigation demonstrated that all the extraction parameters exhibited significant effects (*p<0.05*) on the extraction efficiency of TPC and the antioxidant activity (FRSA and FRP) of eggplant extracts. The optimal extraction conditions, selected by compromising between the rate of total phenolic compounds (TPC) and their antioxidant activities (FRSA and FRP), were extraction with 70% aqueous acetone at 25°C for 60 min using a 1g/50 mL solid to solvent ratio and three successive extractions seem necessary for the depletion of plant material. Therefore the maximum extractions of polyphenols present in eggplant were the optimum conditions for TPC recovery and maintaining the highest antioxidant activity for eggplant vegetable. These conditions allowed recovery of 794.94 mg GAE/100 g FM and produced DPPH free radical scavenging activity (FRSA), ferric reducing power (FRP) of 737.86 mg TE/g FM and 28.00 mg TE/g FM, respectively. A significant Pearson correlation coefficients were found between TPC and FRSA and FRP of eggplant extracts under the influence of extraction parameters. The results obtained in this study indicate that eggplant can be considered as a natural source of phenolics compounds known for their good antioxidant capacity. Since antioxidant compounds provide health benefits, eggplant extracts could be of great interest for application in pharmaceutical products. This study will provide bases for future investigations on the optimization of the extraction of phenolic compounds from eggplant using other models such as response surface methodology. However, it is interesting to test other extraction methods such as microwave and ultrasound extractions, ultrafiltration, supercritical fluid and subcritical water extractions on extracting phenolic compounds from eggplant. These alternative new technologies use less solvent and energy and may increase the safety and the quality of products.

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Morphological and molecular characterization of ancient pomegranate (*Punica granatum* L.) accessions in Northern Italy

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Abstract: The Italian research on *P. granatum* L. is still limited, although the study of local germplasm is extremely important in order to preserve the existing biodiversity and to identify potential useful characters for a renewed industry. The study aimed at characterizing for the first time ancient pomegranates, grown in Emilia Romagna (Italy), through 38 quantitative morphometric descriptors related to leaf, flower, fruit and seed, 42 RAPD and 12 SSR markers. Morphological analyses showed large variation of traits among accessions and the descriptors related to fruit and seed had the highest power of discrimination. The considerable variation found was consistent with ANOVA and PCA results. Among all RAPDs tested, 7 were selected for their polymorphism; whereas among selected SSRs, 8 presented differences in the genetic profiles allowing a good discrimination of the local pomegranate accessions. The genetic relationships among pomegranates were studied by UPGMA cluster analysis and the accessions were clearly regrouped in four different genotypes. The study has highlighted significant differences and interesting pomological characteristics in the local pomegranates, which confirmed the good potential of this germplasm for the pomegranate industry.

1. Introduction

Pomegranate *(Punica granatum* L.) is one of the world most ancient domesticated fruit crops and it is believed to have been first grown in the region between the Caspian Sea and the Caucasus (Zohary and Spiegel-Roy, 1975). Its diffusion occurred across the millennia due to man's activities or gene flow in quite varied environments as concerns climatic conditions, has produced a rich and diversified germplasm. *P. granatum* L. has

a large genetic pool, represented by over 500 described cultivars throughout the world, and by a wide amount of wild plants, and its germplasm is so far only partially explored (Beghè *et al.*, 2016). In spite of the wide genetic diversity, only 50 cultivars were widely cultivated in the main growing areas at the time of the last official survey made by international institutions (IPGRI, 2001). Consequently, the risk of a drastic loss of the existing biodiversity is high.

In the last decade the interest on pomegranate has grown and the world production (estimated to be around 1.5 million tonnes, of which 90% provided by the main producers: India, China, Iran) has rapidly increased (da Silva *et al.,* 2013). The renewed interest in this crop is to be ascribed to socio-economical and cultural factors, which led to a change in food habits in the West, with a growing attention to the nutritional quality of foods (Negri, 2003). Pomegranate, in this respect, is considered one the fruits with the most valuable nutritional properties (Calani *et al.,* 2013).

Pomegranate cultivation is also growing in Italy, and in a ten-year period (since 2008) the surface covered, from a mere 7 ha (2 ha in Calabria and 5 in Sicily), passed to 1142 ha (mainly in Southern Italy, Sicily (364 ha) and Apulia (363 ha) and, with minor productions, also in Venetia (152 ha), Latium (101 ha), Emilia Romagna (44 ha), Tuscany (21 ha) and Lombardy (20 ha). Productions rose from only 69 tons to over 12531 tons (AGRISTAT, 2017).

One of the trends of recent years has been to import from Israel pomegranate cultivars created and patented by Israel breeders, to be utilised in Italian orchards. Although this strategy has led to an increase in production, it hinders any attempt at exploiting Italian cultivars, which have been known for centuries but are not utilised in commercial cultivation.

In Italy, there are several local cultivars which are little known and scarcely diffused in the country. Scarce research, mostly confined to Southern Italy, has been devoted to the characterization of *P. granatum* L. Italian diversity, by means of molecular, morphological and biochemical markers (Adiletta *et al.,* 2018). A research on the whole national territory is unavoidable to select autochthonous genotypes suited to the different environments, and to promote a pomegranate industry able to satisfy the internal demand and to compete with the international product.

The best suited Italian areas for pomegranate growing are the central and southern regions, characterized by a Mediterranean climate, unlike the northern regions, which have a continental climate, with cold and snowy winters. However, in some zones of Emilia-Romagna (44°-45° N and 11°-12° E, Northern Italy) microclimate conditions are such as to allow the cultivation of this species and of other Mediterranean species like olive (Lona *et al.,* 1981; Calani *et al.,* 2013). Ancient pomegranate trees, survived for hundreds of years and adapted to local conditions have been retrieved in these areas. This local germplasm is of particular interest, being the result of a selection process occurred during many centuries in the unfavorable conditions of this territory.

The present research is part of a multidisciplinary project aiming at valorising ancient pomegranate cultivars of Northern Italy. We utilized morphological and molecular markers (Random Amplified Polymorphic DNA (RAPDs) and Simple Sequence Repeat (SSRs)) to characterize ancient pomegranate accessions present in Emilia-Romagna Region. The pomological comparisons also had the purpose of determining the peculiar features and the potential of these plants, for a possible introduction in commercial plantings or for their use in breeding programs.

2. Materials and Methods

Plant materials

The pomegranate germplasm subject of this study was represented by very ancient trees located in a small hill area of Parma province (Emilia-Romagna) (44°69 N, 10°02 E), at an altitude from 150 to 250 m a.s.l. Eight accessions of *P. granatum* L., tagged with an alpha-numerical code (ID): ME1, ME2, ME3, ME4, ME5, ME6, ME7 and ME8 (Table 1), were studied during two seasons, 2014 and 2015. The selected plant material was maintained at pomegranate germplasm collection field in the low hills of the Emilia Appennins, where each accession was replicated 4 times. The pomegranate trees were planted at a spacing of 5 x 5 m and trained to form a bush.

Morphological characterization

The accessions were characterized according to the guidelines proposed by the project EC Project GENRES 29 "Conservation, evaluation, exploitation and collection of minor fruit tree species" (Bellini and Giordani, 1998), integrated by the list of characters proposed by Bellini *et al.* (2007) and by the International Union for the Protection of New Varieties of Plants (UPOV, 2012).

Plant material was randomly sampled from

around the canopy of four plants per each accession, by collecting 40 flowers (20 hermaphrodite also called "long-styled" and 20 male also called "short-styled") at full bloom, on June 1st, 40 adult leaves, from the middle part of the shoot in summer and 12 fruits, at ripening, in the first decade of October. All seeds were extracted from each fruits and 25 of them were randomly selected; arils (the seed fleshy coats, containing edible juice, that represent the seed outer integument or testa) were hand removed to analyze also the tegmen (seed lignified inner integument). The morphological characters evaluated included 38 quantitative traits (Table 2). The linear dimensions were determined with a caliper, and the weight was measured using a semi-analytic electronic scale. From these values other indices have been calculated as indicated in Table 2. Furthermore, some qualitative characters were observed; these traits are reported in Table 1.

DNA extraction and molecular characterization

Total cellular DNA was extracted from young leaves following the CTAB (cetyl trimethylammonium bromide) as reported in Ganino *et al.* (2008).

Forty-two decamer oligonucleotide primers belonging to the AI, AH, OPA, OPC, OPX and OPK series (Table S1 of supplementary data) and twelve couples of SSR primers belonging to the PgAER (Çalişkan *et al.,* 2017), PGKVR (Ravishankar *et al.,* 2015), Pom (Hasnaoui *et al.,* 2012), POM-AGC (Currò *et al.,* 2010) and PG (Ebrahimi *et al.,* 2010) were used for polymorphism detection on the samples. RAPD amplifications were performed as reported in Marieschi *et al.* (2016).

The RAPD profiles obtained with each utilized primer were analyzed by comparison with Gene Ruler 100 pb DNA Ladder plus marker (M-Medical, Milano, IT), with the Kodak digital sciences 1 D Images Analysis Software, calculating the size in base pairs (bp) of each amplicone present in the electrophoretic run of each sample.

SSR amplification reaction was performed as reported in Ganino *et al.* (2008). The amplification condition, for the PGKVR, PgAER, POM-AGC and PG series, were: a first step at 95°C for 5 min followed by 35 cycles of 45 s at 94°C, 45 s at 57°C, 45 s at 72°C, for denaturation, annealing, and primer extension; the last step included 8 min of incubation at 72°C. For the "Pom" serie, the following thermal cycling protocol was used: a first step at 95°C for 3 min followed by 10 touchdown cycles of 30 s at 94°C, 40 s at 65°C (-1°C per cycle), 30 s at 72 and 25 cycles of 30 s at 94°C, 30 s at 55°C, 40 s at 72°C with final extension time of 8 min at 72°C. The amplification products were separated with a CEQ 2000 Genetic Analysis System (Beckman Coulter, Inc.) sequencer on acrylamide gel CEQ Separation Gel LPA-1 (Beckman Coulter, Inc.). A marker CEQ DNA Size Standard kit 400 (Beckman Coulter, Inc.) was used to estimate the molecular weight of the amplified products.

Data analysis

The quantitative morphological characters were evaluated: means, minimum and maximum, standard deviation. The coefficient of variation (CV) was calculated as indicator of variability. All data were subjected to one way analysis of variance (ANOVA) followed by Tukey test to determine the statistically significant differences ($p \leq 0.05$). Correlation analyses between descriptors to reveal possible relationships were car-

Table 1 - *Punica granatum* L. accessions studied, coded (ID) and main qualitative characteristics of their fruit, leaf and flower

ID	Fruit shape	Size	Epicarp colour	Calyx type	Leaf shape	Petiol colour	Mucro	Blade colour	Flower petal colour	Shape short-stiled	Shape long-stiled
ME1	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	yellow	no	yellow	red/orange	medium bell	sinuolate jug
ME2	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME3	oblate/rounded-spheroid	large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME4	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME5	oblate/rounded-spheroid	large/very large	reddish-yellow/red	semi-closed/closed	elliptic	red	no	yellow	red/orange	medium bell	sinuolate jug
ME6	oblate/rounded-spheroid	large	reddish-yellow	semi- closed/open	elliptic	red	no	yellow	red/orange	broad bell	jug with base
ME7	oblate/rounded-spheroid	very small	reddish-yellow/red	open	elliptic	yellow	no	yellow	red/orange	narrow bell	sinuolate jug
ME8	oblate/rounded-spheroid	very small	reddish-yellow/red	open	elliptic	yellow	no	yellow	red/orange	narrow bell	sinuolate jug

Table 2 - Quantitative traits used for characterizing pomegranate accessions and their descriptive statistics analysis using the mean, minimum, maximum, standard deviation (SD) and coefficient of variation (CV)

Trait	Trait code	Mean	Minimum	Maximum	SD	CV (%)
Leaf						
Leaf fresh weight (g)	LFW	0.10	0.070	0.160	0.029	29.13
Leaf length (cm)	LL	5.44	4.450	6.920	0.828	15.21
Leaf width (cm)	LW	1.62	1.260	2.150	0.278	17.21
Leaf shape (length/diameter)	LS	3.50	2.970	3.910	0.341	9.76
Flower						
Flower diameter long-styled (cm)	FDL	1.51	1.220	1.810	0.221	14.57
Flower length long-styled (cm)	FLL	4.75	2.900	5.950	1.193	25.11
Petal number long-styled (cm)	PNL	6.52	5.670	7.500	0.713	10.93
Pistil length long-styled (cm)	PLL	1.75	1.500	2.030	0.231	13.20
Flower diameter short-styled (cm)	FDS	1.48	1.200	1.770	0.163	11.06
Flower length short-styled (cm)	FLS	3.73	2.840	4.490	0.564	15.10
Petal number short-styled	PNS	6.50	6.000	7.400	0.510	7.86
Pistil length short-styled (cm)	PLS	0.43	0.310	0.600	0.105	24.43
Fruit						
Fruit weight (g)	FW	274.02	87.010	388.730	120.303	43.90
Fruit diameter equatorial (cm)	FD	8.22	5.070	9.600	1.799	21.88
Calyx diameter equatorial (cm)	CD	2.14	1.430	2.840	0.545	25.50
Fruit height without calyx (cm)	FL1	6.64	4.400	8.120	1.380	20.80
Total fruit length (cm)	FL2	8.11	5.570	9.530	1.568	19.34
Calyx height (cm)	CL	1.56	1.170	2.220	0.323	20.67
Fruit skin thickness equatorial (mm)	FT	0.42	0.300	0.550	0.096	22.69
Fruit skin and carpellary membranes weight (g)	SCW	129.94	28.980	191.810	66.526	51.20
Number of carpel in equatorial section	NC	6.88	5.330	8.000	0.993	14.43
Fruit shape index (height/diameter)	FSI	0.81	0.730	0.890	0.059	7.21
Calyx shape index (height/diameter)	CSI	0.80	0.610	0.880	0.095	11.93
% Skin and carpellary membranes	SC (%)	45.10	33.300	53.400	7.499	16.63
% Seeds	S (%)	54.92	46.600	66.700	7.491	13.64
Total seeds weight (g)	STW	141.918	58.076	202.556	53.394	37.62
Seed weight (g)	SW	0.27	0.144	0.381	0.080	29.57
Seed length (cm)	SL	0.96	0.786	1.080	0.113	11.73
Seed diameter (cm)	SD	0.69	0.547	0.804	0.090	13.04
Tegmen weight (g)	TW	0.02	0.016	0.029	0.005	19.95
Tegmen length (cm)	TL	0.65	0.544	0.769	0.074	11.35
Tegmen diameter (cm)	TD	0.31	0.253	0.416	0.052	16.88
Woody portion index (tegment weight/aril weight)	WPI	0.09	0.071	0.121	0.021	22.12
Seed shape (length/diameter)	SL/SD	1.39	1.310	1.540	0.083	5.93
Tegment shape (length/diameter)	TL/TD	2.15	1.880	2.410	0.176	8.19
Aril weight (g) [z]	AW	0.25	0.120	0.350	0.080	32.49
Aril weight/tegmen weight	AW/TW	10.04	7.400	12.390	2.162	21.53
% Aril	A (%)	90.35	88.200	92.600	1.653	1.83

[z] Aril weight were calculated by subtracting tegmen fresh weight from whole seed fresh weight.

ried out using a bilateral Pearson correlation. The same characters were also submitted to a principal component analysis (PCA) to evaluate the relationship between pomegranate accessions. The analysis was performed using XLSTAT 2009 software (AddinsoftTM1995-2009).

RAPD bands were treated as binary characters (present = 1 or absent = 0), XLSTAT 2009 software was used to estimate genetic similarities/dissimilarities using Jaccard's similarity coefficient and cluster analysis by using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm.

The size of SSR fragments was determined using a conservative binning approach (Kirby, 1990) through the statistical R software. The information content of the SSR markers under study was evaluated according to number of alleles per locus (N_a), observed (H_o) and expected (H_e) heterozygosity, and polymorphic information content (PIC) (Botstein *et al.,* 1980) using the Cervus 3.0 software (Kalinowski *et al.,* 2007). The level

of similarity/dissimilarity among examined accessions was obtained through the genetic similarity matrix utilizing Manhattan distance and cluster analysis (UPGMA) algorithm, with XLSTAT 2009 software.

Finally, to test the correlations between genetic distance matrices and between the morphological and genetic distance matrices among accessions, Mantel tests were performed (Mantel, 1967). Each matrix distance was obtained by calculating Pearson's index. Mantel tests were performed with 100,000 permutations (p = 0.05). Pearson's r-value was used to measure linear correlation between two matrices.

3. Results and Discussion

Morphological characterization

The data resulting from the 2-year study were grouped and the average values were used for statistical analysis. The accessions showed significant variability in many of the characters analyzed. Descriptive values for each quantitative trait are recorded in Table 2. The coefficient of variation (CV) was used to determine the total variability present in each trait. The CV varied from 1.83% (A%) to 51.20% (SCW%), with seven traits having CV between 15 and 20% and fourteen traits with CV value higher than 20%. According to Audergon (1987), the descriptors with a high CV are more discriminating than the other ones, and can be reliable markers for the characterization of pomegranate accessions. The highest CV values were evident in traits involving fruits and the lowest were in flowers (except FLL and PLS), leaves (except LFW) and seeds (except STW, SW, WPI, AW, AW/TW). These results are consistent with previous studies (Zamani *et al.,* 2007, Mansour *et al.,* 2011).

The mean leaf quantitative values are reported in Table S2 and the traits values presented significant differences between the accessions. Moreover, leaf blade margin color and petiole color next to the shoot was red for accessions ME1, ME7 and ME8, and yellow for the others. All accessions have an elliptic shape and absence of mucro (Table 1).

The flower characteristics are reported in Table 1 and Table S3 and the observed values are comparable with those of Lebanese genotypes studied by Dandachi *et al.* (2017). The flowers of ME7 and ME8 accessions showed a much smaller size than that of the flowers of the other, but presented a similar style length, a feature that favors pistil pollination. Moreover, the long-styled flowers presented a "sinuate jug", except the flowers of ME6 accession that presented "jug with base". ME7 and ME8 presented a "narrow bell" shape in the short-styled flowers whereas ME6 presented "broad bell" shape and other accessions presented flowers with "medium bell" shape.

The mean values of quantitative fruit and seed traits are reported in Tables 3 and S4. Significant variability was observed in total fruit weight (FW), in maximum equatorial diameter (FD) and in fruit length, with calyx (FL2) and without calyx (FL1). In particular, these characters have lower values for ME7 and ME8. All accessions showed fruits with shape "oblate or rounded-spheroid" and "closed or semi-closed calyx", except ME7 and ME8 that have a majority of fruits with "open calyx". The number of locules (NC) was higher in fruits of higher total weight.

Table 3 - Mean values, standard deviation and ANOVA analysis for fruit characteristics

ID	FW [z]	FD	CD	FL1	FL2	CL	FT	SCW	NC	FSI	CSI	SC%	S%
ME1	373.48 ±49.78 A	9.60 ±0.62 A	2.68 ±0.51 A	7.00 ±1.14 A	9.22 ±1.28 A	2.22 ±0.62 A	0.55 ±0.10 A	191.81 ±19.64 A	8.00 ±1.58 A	0.73 ±0.12 A	0.84 ±0.27 A	53.4 ±0.03 A	46.6 ±0.03 D
ME2	303.53 ±93.46 A	8.83 ±1.17 A	2.40 ±0.62 A	7.83 ±0.72 A	9.53 ±1.06 A	1.70 ±0.43 A	0.53 ±0.11 AB	144.42 ±51.87 A	6.67 ±0.58 AB	0.89 ±0.07 A	0.70 ±0.03 A	47.2 ±0.03 AB	52.8 ±0.03 CD
ME3	335.73 ±13.03 A	9.10 ±0.52 A	2.43 ±0.55 A	7.10 ±0.17 A	8.53 ±0.61 AB	1.43 ±0.49 A	0.44 ±0.10 BC	163.25 ±11.59 A	7.67 ±0.58 A	0.78 ±0.06 A	0.61 ±0.23 A	48.6 ±0.02 AB	51.4 ±0.02 CD
ME4	388.73 ±89.49 A	9.40 ±0.84 A	2.15 ±0.78 A	8.12 ±1.03 A	9.00 ±1.27 AB	1.61 ±0.41 A	0.48 ±0.11 ABC	185.73 ±46.34 A	7.60 ±0.55 A	0.86 ±0.06 A	0.82 ±0.31 A	48.3 ±0.03 AB	51.7 ±0.02 CD
ME5	345.68 ±87.85 A	9.50 ±1.14 A	2.84 ±0. 94 A	7.47 ±0.67 A	9.24 ±0.81 A	1.63 ±0.55 A	0.41 ±0.09 BC	181.74 ±47.63 A	7.25 ±0.58 AB	0.80 ±0.05 A	0.85 ±0.27 A	52.5 ±0.01 A	47.5 ±0.01 D
ME6	266.81 ±23.78 AB	8.60 ±0.71 A	1.70 ±0.14 A	6.45 ±0.64 AB	7.95 ±0.78 AB	1.50 ±0.14 A	0.37 ±0.08 BCD	111.03 ±6.31 AB	7.00 ±0.45 AB	0.75 ±0.01 A	0.88 ±0.01 A	41.9 ±0.06 BC	58.2 ±0.06 BC
ME7	87.01 ±21.77 B	5.67 ±0.57 B	1.43 ±0.06 A	4.73 ±0.38 B	5.82 ±0.58 B	1.25 ±0.11 A	0.30 ±0.09 D	28.98 ±7.48 B	5.33 ±0.58 B	0.84 ±0.02 A	0.88 ±0.19 A	33.3 ±0.01 D	66.7 ±0.01 A
ME8	91.21 ±27.45 B	5.07 ±0.11B	1.47 ±0.06 A	4.40 ±0.10 B	5.57 ±0.23 B	1.17 ±0.15 A	0.30 ±0.79 D	32.54 ±10.48 B	5.50 ±0.55 B	0.87 ±0.03 A	0.80 ±0.13 A	35.5 ±0.02 CD	64.5 ±0.02 AB

The same letter show no statistically significant differences (P<0.05).

[z] For explanation of character symbols, see table 2.

According to the list of "pomegranate descriptors" of Bellini *et al.* (2007), the fruits were classified as "large or very large" (ME1, ME2, ME3, ME4, ME5, ME6) and "very small" (ME7, ME8). The first group had a total weight mean values of about 335 g, comparable to those of the fruits of many Italian and Spanish cultivars (Martinez *et al.,* 2006 and Ferrara *et al.,* 2014). Moreover, the epicarp (or "skin") of the local fruits has presented different colors, ranging from reddish-yellow to red. The size of the fruit and the color of the epicarp are two important parameters considered in the international market as concerns the quality of the fresh product (Mansour *et al.,* 2011). Another important parameter for fruit quality was the descriptor "skin thickness". ME7 and ME8 fruits have a thinner "skin thickness" than other accessions, and in field their fruits were more subjected to cracking at the first rainfall in October.

Significant variability was observed in seeds total weight (STW), skin and carpellary membranes weight (SCW) and seeds percentage (S%) (Tables S4 and 3). The mean S% found was of 54.91% and it was similar to that reported in another Italian study (Cristofori *et al.,* 2011). Furthermore, our mean values were lower than those reported on Italian and Iranian cultivars (Ferrara *et al.,* 2014). The seed descriptors showed, for the majority of traits, significant differences between two groups of accessions: ME1, ME2, ME3, ME4, ME5, ME6 and ME7, ME8; the former had SL, SD, SW, TL, TW greater than the latter (Table S4).

Tegmen index (WPI), aril percentage (A%), aril weight (AW) and aril/tegmen ratio (AW/TW) are very important parameters from a qualitative point of view. The WPI is a parameter that refers to the quantity of lignified tissue contained in the seed compared to total seed weight, and consumers greatly appreciate seeds with a limited amount of lignified tissue (Martinez *et al.,* 2006). Accessions ME1, ME2, ME3, ME4, ME5 had a higher quantity of aril (AW) and a lower percentage of the tegmen index (WPI) than ME6, ME7 and ME8. The WPI presented an average value of 7.7% in the first group of accessions, and an average value of 11.9% in the accessions ME6, ME7 and ME8. These values were in agreement with those of other Italian (5.4 to 10%), Spanish (7.4 to 9.7%), Moroccan (6.1 to 10.7%) and Iranian (5.4 to 7.5%) accessions (Martinez *et al.,* 2006; 2012; Sarkhosh *et al.,* 2009). The aril is a tissue valued for the high production of juice; AW and A%, with reference to the individual seed, were high in all accessions, and A% showed an average value of about 90%; a value comparable and higher than that of other Italian and Spanish genotypes (La Malfa *et al.,* 2009).

Correlation among morphometric traits

The correlations found between the quantitative variables, significant at $p < 0.05$, are reported in figure 1. The correlation coefficient can provide infor-

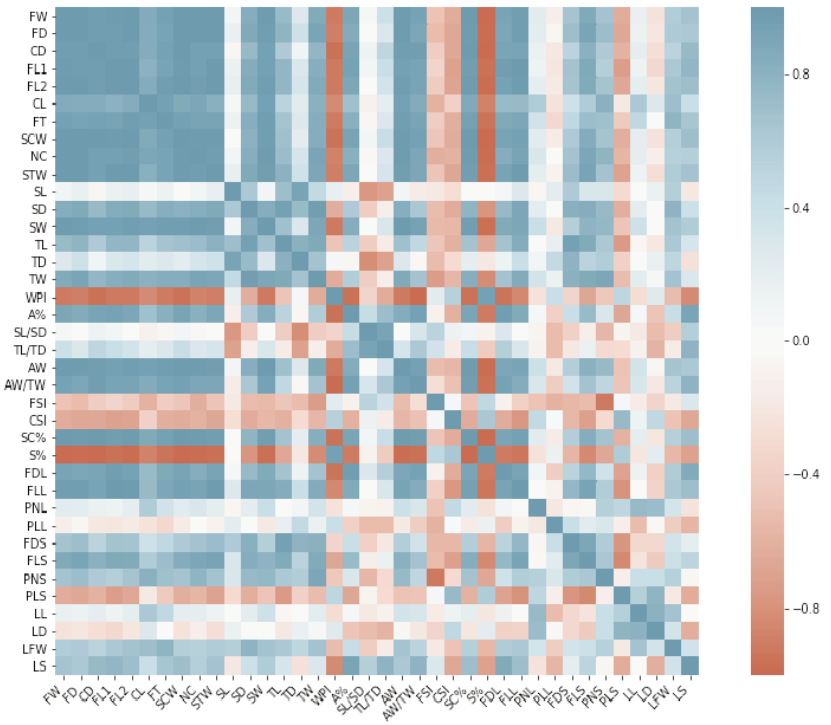

Fig. 1 - Pearson's correlation matrix of the quantitative traits in pomegranate accessions, visualized as a heat map plot. For explanation of character symbols, see table 2.

mation on the traits that are most important in assessing accessions (Norman *et al.,* 2011). Skinner *et al.* (1999) recommended analyzing correlation coefficients close to 0.7: in these conditions, the variance of one trait is strongly dependent on the others. According to this criterion, we estimated two hundred and fifteen valid correlations. Most of the significant correlations among traits coincide with those from the same plant organ, in particular the variables relative to fruits and seeds. A strong correlation value was found for fruit and seed descriptors, and these were also positively correlated with each other (*e.g.* FW was positively correlated with other fruit descriptors, FD, FL1, FL2, CL, SCW, NC, SC%, S% and seed descriptors, STW, SL, SD, SW, TW, A%, AW, AW/TW). The character WPI was negatively correlated with fruit traits relative to dimensions and with SW, SL, SD. The same results were observed in several former works that analyzed accessions from different countries (Martinez *et al.,* 2006; Zarei *et al.,* 2013). Moreover, the trait WPI could be an index of seed hardness, because this feature is strongly dependent on the trait WPI, as reported in Martinez *et al.* 2012 ($r = 0.63$ $p \leq 0.01$). In flowers, positive correlations were found among the variables relative to dimensions: between FLS and FDS and between FLL and FDL. In leaves, a positive correlation was between LD and LL, actually all accessions showed an elliptic leaf shape. Finally, some correlations were observed between seed (SW, A%, AW, TL, TW) and flower characteristics (FDL, FLL, FDS, FLS, PNS); we noted that accessions with small flowers (ME7-ME8) presented fruits and seeds of smaller dimensions. In fact, fruit growth potential is largely determined genetically through the ovary size in several species (Rosati *et al.,* 2009). These correlations between different traits could be due to genetic linkage or to a pleiotropic effect (i.e., when one gene influences two or more seemingly unrelated phenotypic traits) (Iezzoni and Pritts, 1991).

Principal component analysis

The results of PCA revealed the existence of large variability among accessions. The total variance explained by the first three principal components (PCs) in the model was 83.53%. A plot of the percentage of variance explained by seven PCs and eigenvalues associated with the first seven PCs for each quantitative trait are reported in supplementary material (Fig. S1 and Table S5), respectively. The PC1 explained the 54.60 % of total variance and the traits with the greatest weight on this component were related to fruit (FW, FD, CD, FL1, FL2, CL, FT, SCW, NC, SC%), seed (STW, SL, SD, SW, TW, WPI, A%, S%) and some flower traits (FDL, FLL, FLS). The PC2 explained 15.82% of the variability, and showed a strong negative load for TD, LD and PNS, whereas a strong positive load was present for SL/SD ,TL/TD, FSI, and LS. Finally, LD, LL relating to the leaf and PLS relating to the flower showed the highest contribution to PC3 (13.11% of the variability). The comparison of plot scores for PC1, PC2 and PC3 in figure 2 permits to obtain a view of accession dispersion and their clustering based on morphological traits. The accessions, for the first two PCs, were grouped into two main groups highly dissimilar: the first group consists of ME7 and ME8, the second group consists of three sub-groups (sub-group ME6; sub-group ME2, ME3, ME4, ME5 and sub-group ME1). The groups plotted for the PC1 and PC3 were very similar to those on PC1-PC2 plot, though accession ME6 showed less differences with the sub-group ME2, ME3, ME4, ME5. As already reported in the literature (Martinez-Nicolas *et al.,* 2016), fruit characteristics

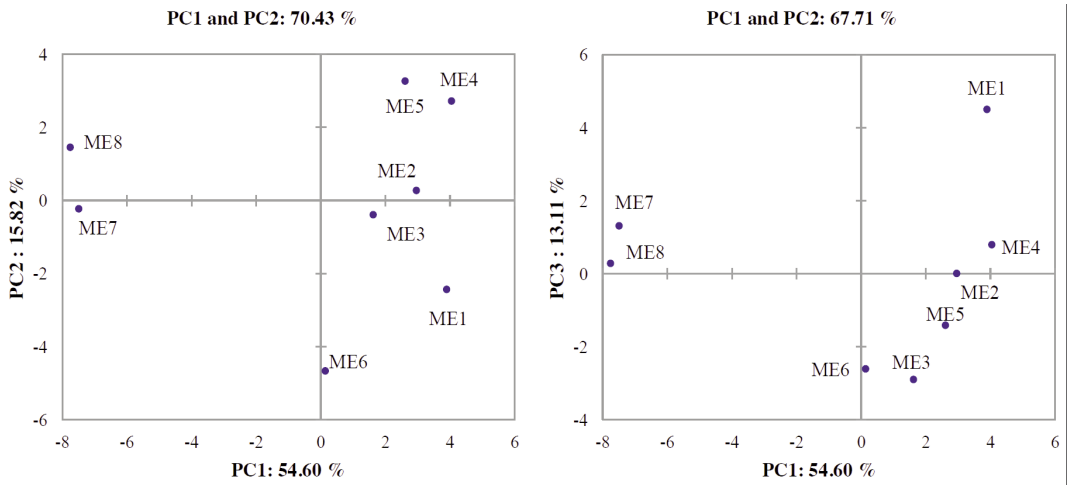

Fig. 2 - Loading plots of the first, second and third Principal Component showing the position of accessions.

had the highest loading values for the first component in principal component analysis. Our results confirmed that the traits related to fruit and seed had the highest power of discrimination, and were, therefore, the most useful for characterization of this local germplasm.

RAPDs and SSRs characterization and genetic relationships

Only 7 RAPD oligonucleotides (AI08, AI12, AI105, AH17, OPA19, OPB08, OPC16) out of 42 belonging to the AI, AH, OPA, OPC, OPX and OPK series, showed polymorphism in two or more accessions, by producing polymorphic and reproducible amplification patterns. The 7 oligonucleotides amplified a total of 84 RAPD fragments, 14 of which were polymorphic, making 16.67% polymorphism (Table 4). The number of polymorphic fragments found per primer was between 1 (OPB8, AI05 and AH17) and 3 (AI12, OPC16, AI08), with a mean of 2 (Table 4) and their size ranged from 250 to 3000 bp. The RAPD markers have been applied in many investigations aimed at the study of polymorphism in pomegranate, for their simplicity and low cost (Kathuria *et al.*, 2017), but these markers have often shown low polymorphism in this species. As reported in literature it is necessary an initial screening by a high number of RAPD primers to detect a discrete number of discriminating markers (Zamani *et al.,* 2010). The level of polymorphism detected in our study is lower than that reported in other works (Sarkhosh *et al.,* 2006; Zamani *et al.,* 2007). However, a percentage of polymorphism similar to ours was detected by Hasnaoui *et al.* (2010). In agreement with these authors we hypothesized that the slightly lower percentage of polymorphism detected could be due to the reduced dimension of the sample collection and to the limited variability in terms of geographical origin. Relationships among accessions were studied by cluster analysis (UPGMA) based on Jaccard's coefficient, and following statistical analysis a dendrogram was produced (Fig. 3A). The genetic distance among the accessions ranged from 0 to 0.8, showing genetic diversity among the pomegranate accessions. In the dendrogram, two main clusters could be identified: the first cluster included only two accessions (ME7 and ME8) at a 0 dissimilarity level (genetic identity). The second cluster comprised all other accessions which presented a level of dissimilarity that varied between 0 and 0.6. This last cluster presented three subgroups: ME1, ME6 and a subgroup that included accessions with genetic identity or with a very little (0.10) dissimilarity's distance (ME2, ME3, ME4, ME5).

Table 4 - Primer sequence of the most informative primers and level of polymorphism found by the RAPD analysis

RAPDs	Sequence	Total n° of bands	N° of polymorphic bands
OPA19	5'-d[CAAACGTCGG]-3'	13	2
OPB08	5'-d[GTCCACACGG]-3'	12	1
OPC16	5'-d[CACACTCCAG]-3'	13	3
AH17	5'-d[CAGTGGGGAG]-3'	13	1
AI08	5'-d[AAGCCCCCCA]-3'	15	3
AI12	5'-d[GACGCGAACC]-3'	15	3
AI105	5'-d[GTCGTAGCGG]-3'	3	1
TOT		84	14
mean		12	2

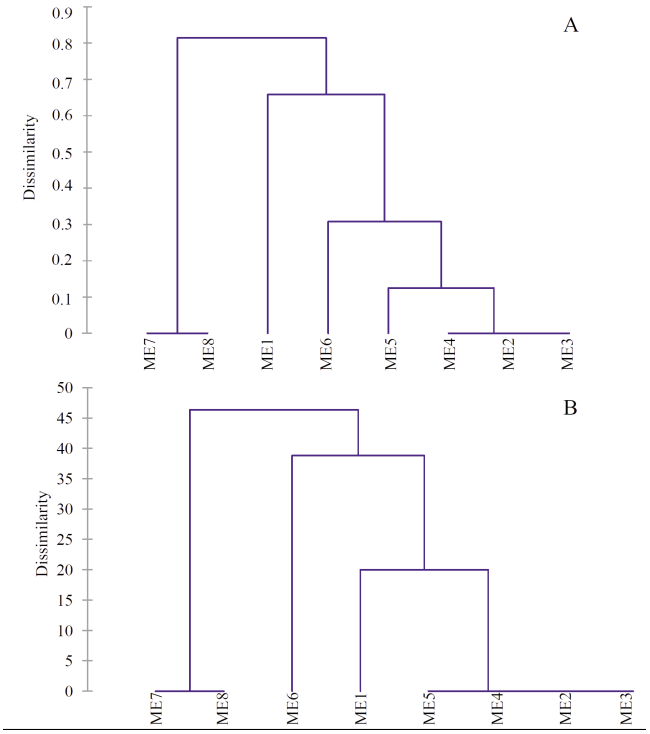

Fig. 3 - UPGMA clusters of 8 pomegranate accessions generated by RAPD markers using the Jaccard similarity coefficient (A) and by SSR markers using Manhattan distance (B).

The SSR molecular technique was utilized as a second molecular method to discriminate the pomegranates and to characterize their genetic profile. Although for *P. granatum* there is not yet a set of the best SSR primers with validity recognized at the international level, these markers have been successfully employed by several researchers to characterize the pomegranate germplasm (Beghè *et al.,* 2016). SSRs utilized in this study have been chosen between the primers which had shown a high discriminating capacity and yielded a total number of 31 reproducible fragments, which allowed a good discrimina-

tion of the local accessions (Table 5). Among 12 selected microsatellites, 10 showed polymorphism and 8 showed differences in the pomegranates genetic profiles. The alleles obtained by amplification of SSRs loci produced four different genetic profiles from eight ancient accessions analyzed (Table S6 of supplementary data). The number of alleles at each locus (N_a) varied between 1, for loci PGKUR114 and PGKUR127, and 5, for loci ssrOeUA-PG6 and Pom021, with an average value of 2.58 and their size ranged from 155 to 319 bp. The values of expected (H_e) and observed (H_o) heterozygosity were always above 0.500, except for the locus PGKVR027, where was not observed heterozigosity, and obviously for the two monomorphic loci (PGKVR114 and PGKVR127). It is important to underline that four primers (PG6, Pom021, Pom045, PgAER154) were highly polymorphic, showing a PIC> 0.5, as defined by Botstein *et al*. (1980) (Table 5). These last markers showed genetic parameters (H_o, H_e, PIC) higher (or similar) to those reported in previous studies where they have been developed (Ebrahimi *et al.,* 2010; Hasnaoui *et al.,* 2012; Caliskan *et al.*, 2017). Instead, the PGKVR-primers presented genetic parameters lower to these reported in literature (Ravishankar *et al.,* 2015); these primers had low polymorphism; of 6 primers 2 resulted monomorphic markers and only 3 (PGKVR027, PGKVR064 and PGKVR165) showed differences in the studied accessions. Similarly, the N_a, H_e, H_o and PIC values in other studies of pomegranate varieties also varied according to the primers tested and the geographic origin of population analyzed (Caliskan *et al.,* 2017). These results confirm the necessity to test different series of SSRs to obtain the best set of markers for each local germplasm.

Table 5 - Number of alleles (Na), Size (bp) expected (He) and observed (Ho) heterozigosity, polymorphic information content (PIC) at 12 loci in pomegranate accessions

SSRs	N_a	Size	H_e	H_o	PIC
PGKVR027	2	236-242	0.429	0	0.305
PGKVR064	2	239-241	0.536	0.750	0.359
PGKVR065	2	202-204	0.571	0	0.375
PGKVR114	1	258	-	-	-
PGKVR127	1	246	-	-	-
PGKVR165	2	307-319	0.571	1.000	0.375
POM-AGC11	2	183-185	0.536	0.250	0.359
PG4	2	198-244	0.571	1.000	0.375
PG6	5	191-199	0.786	0.750	0.653
Pom021	5	203-211	0.857	1.000	0.712
Pom045	3	155-163	0.750	0.750	0.581
PgAER154	4	262-300	0.786	1.000	0.630
TOT	31				
Mean	2.58		0.533	0.542	0.394

The UPGMA cluster based on SSR data divided the set of pomegranate accessions into two main cluster at a dissimilarity level of 45 (Fig. 3B). In the SSRs dendrogram, the first cluster included only two accessions (ME7 and ME8) with genetic identity. The second cluster instead presented all other accessions which a level of dissimilarity that varied between 0 and 38%. This last cluster comprised three genetic subgroups: ME1, ME6 and a subgroup that included accessions with genetic identity (ME2, ME3, ME4, ME5). As confirmed by Mantel's test ($r = 0.399$; $p \leq 0.033$), the SSRs clustering was very similar to that performed by RAPD markers. In fact, it showed the distinction of the same four genetic groups (Fig. 3A and B).

Comparison between morphological and molecular based clusters and potential use of pomegranate genetic resources

It is known that RAPD fragments derived from any region of the genome and that SSR fragments derived only from non-transcribed regions, therefore post-transcriptional modifications and non-nuclear inheritance of some characteristics can't be detected by these markers (Sarkhosh *et al.,* 2006). For these reasons, in literature there are contrasting results about the correlation between these molecular and morphological descriptors (Sarkhosh *et al.,* 2009; Zamani *et al.,* 2010; Basaki *et al.,* 2013). However, researchers agreed that the combination of morphological and molecular techniques are essential for a proper and complete characterization of the germplasm of this species (Beghè *et al.,* 2016).

In this study, the RAPD and SSR analysis reflected the main morphological differences observed among the local accessions studied; the molecular cluster analyses confirmed the same two main clusters detected with PCA analysis using quantitative morphological traits. Moreover, molecular analyses allowed to detect clearly four different genotypes. Analysis of correlation between distance matrices (morphological traits and molecular markers) by Mantel's test confirmed a high statistical significance ($r = 0.412$; $p \leq 0.034$ and $r = 0.583$; $p \leq 0.002$ for SSRs and RAPDs, respectively).

It is important to stress that in populations adapted to difficult ecological conditions, as the germoplasm in study, the polyphenolic content was high (Calani *et al.,* 2013). In this previous study the ME1, ME3, ME5 and ME8 accessions were subjected to phytochemical discrimination fingerprinting in pome-

granate juices. The Emilian pomegranates have presented interesting and peculiar phytochemical profiles. Moreover, the juices were rich in ellagitannins and had high total phenol content and total antioxidant capacity, especially ME8 pomegranate. For these reasons, the local germplasm studied could be considered a source of useful traits (*e.g.* resistance to diseases, frost tolerance, polyphenol synthesis) for genetic improvement of this species. According to pomological descriptors and phytochemical characteristics, we could appreciate peculiar features of these plants. Indeed, the ME7 and ME8 accessions showed some characteristics (*e.g.* small size of fruits, high woody portion in seeds, low pH) that make the fruits unlikely to be used for direct consumption, but have a juice with high antioxidant capacity, and could be successfully employed for the preparation of nutraceutical products or for industrial blending of juices. The other pomegranates (except ME6 accession that presented a high woody portion in seeds) presented good pomological characteristics for which a fresh use of the fruit could also be expected.

4. Conclusions

The present work is a first contribution to the genetic and morphological characterization of the pomegranate germplasm still present in Emilia Romagna region. The morphological traits, in particular those related to fruit and seed, seven RAPDs and eight SSRs have allowed to characterise the genetic diversity of ancient pomegranate accessions. The study, although preliminary and limited to a restricted area, highlighted significant differences and interesting pomological traits in local pomegranates. These results, presented in association with the study of Calani *et al.* (2013), clearly demonstrate a good potential of this germplasm for a commercial exploitation as fresh or processed fruits. The accessions could be used for new pomegranate plantings and could contribute to cross-breeding and the production of new genotypes suited to marginal environments.

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Effect of palm leaf biochar on melon plants (*Cucumis melo* L.) under drought stress conditions

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Abstract: In order to investigate the effect of palm leaf biochar on some characteristics of *Cucumis melo* L. under drought stress, a split plot experiment was conducted in a completely randomized block design with three replications for two consecutive years. The main plot was irrigation level (60, 85, and 100% water requirement) and subplot was biochar in four levels (0, 0.18, 0.24, and 0.36 kg/m^2). Results showed that treatment of 0.24 kg/m^2 biochar and 100% water requirement increased the characteristics of water use efficiency as 88%, shoot fresh weight as 77%, shoot dry weight as 32%, root fresh weight as 100%, root dry weight as 84%, root length as 54%, and average fruit weight 84% compared to treatment without biochar and 60% water requirement. The highest level of leaf N, Mn and K, shoot length, leaf area, leaf number, fruit diameter and fruit flesh thickness in the treatment of 0.36 kg/m^2 biochar and 100% water requirement were higher 58%, 48%, 65%, 18%, 50%, 95%, 43% and 55%, than to of treatment without biochar and 60% water requirement respectively and had no significant difference with the treatment of 0.24 kg/m^2 biochar and 85% water requirement. The highest rates of Fe, Zn and Cu were related to 0.36 kg/m^2 biochar and 60% water requirement as 60, 44 and 66% respectively compared to treatment without biochar and 100% water requirement. The biochar-free treatment with 60% water requirement accounted for the highest amount of proline due to high stress, and the proline content reduced with increasing biochar and decreasing stress in treatments. Generally, the treatments of 0.24 and 0.36 kg/m^2 of biochar increased most of the characteristics, however no significant difference was observed between these treatments. Moreover, in 85% water requirement the drought stress conditions could compensate with the application of biochar. Thus, using 0.24 kg/m^2 of biochar and 85% of water requirement, recommended for the best result.

1. Introduction

Melon (*Cucumis melo* L.) is from cucurbitaceae family that requires

warm weather and high light to grow (Sangeetha *et al.*, 2006). Drought is one of the most important environmental stresses that adversely impact plant growth and crop production. More than 45% of world's agricultural lands are permanently exposed to drought and 38% of the world's population resides in those places (Ashraf and Foolad, 2007). Therefore, the majority of efforts will be focused on producing more crops in water shortage conditions in the future (Sinaki *et al*., 2007). In arid and semi-arid conditions, which consist of the major part of Iran, the lack of sufficient and proper vegetation causes reduction of the return of plant remnants and organic matter to the soil (Tate, 2000). Most of the soils in the arid and semi-arid areas in Iran contain less than 1% organic matter (Asghari, 2011). The organic matter shortage reduces the stability of the soil structure and its flaking, eventually creating a hard and dense soil (Hemmat *et al*., 2010). The use of organic fertilizers such as animal manure is a way of increasing the organic matter content in agricultural soils, however the application of this material cannot meet the needs of these soils (Mesa and Spokas, 2011). Therefore, in order to improving the soil, the use of organic resources such as agricultural waste, compost, urban waste, and sewage sludge is necessary, so that while increasing agricultural products, sustainable development can be achieved in agriculture (Yin Chan and Xu , 2009; Nazmi *et al*., 2012). In recent years, biochar has been used as a soil reformer, an organic carbon source, and somehow a method for carbon sequestration in agricultural soils. Biochar is a char produced from plant biomass and agricultural waste like wheat straw, corn, rice, which is produced during the thermochemical process of pyrolysis; this process is referred to the slow burning of organic matter under low or lack of oxygen condition (Glaser and Birk, 2012). It has been reported in several studies that biochar is a useful reformer to improve the soil physical and chemical characteristics and is effective in preserving soil organic matter, increasing fertilizer efficiency, and enhancing crop production, especially in the soils of subtropical and tropical areas that have long been cultivated (Van Zwieten *et al*., 2010). Biochar enhances the water holding capacity of the soil (Basso *et al*., 2013) and change the particle size distribution and porosity of the soil due to its high specific surface area (SSA) (Sun *et al*., 2014) and also is a direct source for K, Ca, P, Zn, and Cu (Chan *et al*., 2008). In addition, biochar increase the soil nutrient availability due to increasing the cation-exchange capacity (CEC), changing the soil pH. Using a biochar produced from rice plant residues increased the plant fresh and dry weight, root fresh and dry weight, stem length, and leaf number in lettuce and cabbage plants (Carter *et al*., 2013). Addition of biochar increased the soil pH, EC, organic carbon, CEC and N, P, K, Na, Ca, and Mg concentration of the soil and also the P, N, and K contents of the lettuce plants in this soil (Nigussie *et al*., 2012). Depending on the variety and farming conditions during the year, each palm produces about 15-25 dry leaves, each weighting 1.5 to 2.5 kg. The generalization of this amount of plant residues to several million palms in Iran leads to a great deal requiring the management of productivity and optimal use. These wastes can be converted into biochar and then used in soil. In recent years, many areas of Iran have been faced with water shortages and droughts, thus increasing soil water holding capacity by adding organic matter and biochar to soil can increase the potential of land use in these areas. Therefore, the present study was accomplished aiming to exploit the palm leaf biochar in order to increase soil organic matter and diminish the adverse effects of drought stress and investigate its effect on some characteristics of melon plants.

2. Materials and Methods

This experiment was carried out in 2016 and 2017 in an agricultural farm in Zarrindasht region of Fars province, Iran, with a longitude of 54°, 20′ and a latitude of 28°, 20′ with an altitude of 1021 m from the sea level. In this experiment, the Samsouri Varamin early variety melon was used. The remains of palm leaves from Zarrindasht orchards were collected, air dried, and crushed and then packed in aluminum sheets to limit the oxygenation and packs were placed in the oven for four hours at 560 °C to produce biochar (Hall *et al.,* 2008). Table 1 shows some chemical properties of biochar used in the experiment. This experiment was conducted in the split

Table 1 - Some chemical characteristics of biochar used in the experiment

pH (1:7)	EC (dS m^{-1}) (1:7)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)	K (%)	P (%)	N (%)
9	7.5	0.74	0.09	0.83	983.2	32.4	2	1.39

plot form in completely randomized block design with three replications. The main plot was irrigation level in three levels (60, 85, and 100% water requirement) and biochar as subplot in four levels (0, 0.18, 0.24, and 0.36 kg/m^2).

I1B1 = Without biochar with 60% water requirement;
I1B2 = 0.18 kg m^2 biochar with 60% water requirement;
I1B3 = 0.24 kg m^2 biochar with 60% water requirement;
I1B4 = 0.36 kg m^2 biochar with 60% water requirement;
I2B1 = Without biochar with 85% water requirement;
I2B2 = 0.18 kg m^2 biochar with 85% water requirement;
I2B3 = 0.24 kg m^2 biochar with 85% water requirement;
I2B4 = 0.36 kg m^2 biochar with 85% water requirement;
I3B1 = Without biochar with 100% water requirement;
I3B2 = 0.18 kg m^2 biochar with 100% water requirement;
I3B3 = 0.24 kg m^2 biochar with 100% water requirement;
I3B4 = 0.36 kg m^2 biochar with 100% water requirement.

In the year before planting, the farm was fallow plowed well and leveled. Before planting, a soil sample prepared and some its chemical properties were evaluated (Table 2). Biochar was mixed with soil at 10 cm depth and then seeds were planted at an appropriate depth on the rows at distance of 2.5 m and 0.5 m on the row. The drip irrigation was applied, so that, a dripper was placed beside each plant in order to measure the amount of water consumed by the plant. In the 4 to 5 leaf stage, 50 kg/ha of nitrogen, 40 kg/ha of phosphorus and 40 kg/ha of potassium were added to the soil from sources of urea, potassium sulfate and Triple Super Phosphate respectively.

To estimate of the plant water requirement, the meteorological data including minimum and maximum temperature, minimum and maximum humidity, solar radiation, and wind speed were taken from the Zarrindasht Meteorological Office (Table 3). Then, the amount of evapotranspiration of melon plant was measured and the daily water requirement of the plant was obtained using the appropriate formulas for two years. For estimation of potential evapotranspiration parameters (ETOs) and water requirements by the proposed method of FAO using meteorological data and field surveys related to agronomic calendar and different stages of plant growth. It is then calculated by introducing the vegetation coefficient (Kc) according to plant type, stage and duration of growth and its effect on (ETO), evapotranspiration (ETc). Finally, by reducing the effective rainfall, the net requirement of irrigation water (In), which is the soil moisture deficiency, is estimated to be offset by irrigation.

Etc = Eto*Kc, where:
ETc = Actual evapotranspiration of the plant (mm/day)
ETo = Reference evapotranspiration (mm/day)
Kc = Plant coefficient.

Before the melon fruit ripens, parameters of stem length, plant length, leaf area, and number of leaves per plant were measured. At harvest time, the parameters of total yield, average fruit weight, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight determined by scale. Fruit length, fruit diameter and root length determined by a ruler and fruit skin thickness, fruit flesh thickness, determined by a caliper after slicing the fruits. Since it was not possible to separate the leaf at all stages to mea-

Table 2 - Some chemical characteristics of the farm soil

pH (1:7)	EC (dS m-1) (1:7)	N (%)	P (%)	K (%)
7.8	0.54	0.05	0.122	0.014

Table 3 - Some meteorological characteristics during the two years of experiment

Year	Month	Mean minimum temperature (°C)	Mean maximum temperature (°C)	Precipitation (mm)	Potential evapotranspiration (mm)
2016	Mar.	6.4	29.8	30	187.6
	Apr.	12.2	42.8	0.2	314.9
	May.	17.6	44.2	0	397.8
2017	Mar.	9.8	37.2	25.9	185.2
	Apr.	15.2	39.8	3.2	300
	May.	17.8	44.8	0	387.4

sure the leaf area, first several leaves were separated and their length, width, and length by width were calculated. Then, the area of the leaves was measured using the graph paper (mm) and the surface area relation was obtained using the Excel software. The following relation, which has the highest regression coefficient (R^2), was used to calculate the leaf area:

$Y = 1.03 x + 44$

where:

Y (cm^2) = Leaf area

X (cm^2) = Length (cm) * Width (cm)

Leaf proline content were determined by Bates method (Bates *et al*., 1973). Leaf samples washed with distillated water, dried at 65°C for 48 h in an oven and ground. Total N in the leaves was determined by micro-kjeldahl method (Bremner, 1996). The grounded leaf samples were ashed at 550°C and digested with 2 N hydrochloric acid. P concentration in the extracts was determined by the yellow color method and K using flame photometer (Helmke and Sparks, 1996). Concentrations of Fe, Zn, Mn and Cu were determined by an atomic absorption spectrophotometer (PG 990, PG Instrument Ltd. UK) as well. The water use efficiency was calculated as a correlation between plant yield and plant water use during the treatment period (Liu *et al*., 2015).

WUE = Y/V

Where: WUE, Y, and V were water consumption efficiency in kg/m^3, plant yield in kg per plant, and total water consumption in m^3, respectively.

Statistical analysis was performed using the SAS software (Statistical Analysis System) (V9) (SAS Institute Inc. Cary, NC, USA). Differences among the mean values were detected by Least Significant Differences (LSD) test at %5 level.

3. Results

The results revealed that the effects of drought stress and biochar and also the interaction of them were significant on water use efficiency and all physiological characteristics (Table 4). I3B3 and I3B4 treatments increased 88% and 76% in water use efficiency respectively compared to I1B1 treatment, however there was no significant difference compared to treatments of I3B2, I2B3 and I2B4 (Table 5).

Table 4 - Results of analysis of variance (ANOVA) of biochar on some properties of melon plants under drought stress

Source of changes	Degree of freedom	Mean square												
		Water use efficiency	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Shoot length	Root length	Leaf area	Leaf number	Fruit diameter	Fruit flesh diameter	Average fruit weight	Total yield
r (replication)	2	125.59	42833.5 *	3253.9 **	53.3 **	1.0 **	112.5 **	35.5 **	115.1	1626.1 **	10.2	0.07	60257.037 **	1506425.93 **
Stress (a)	2	103.08 **	228545.6 **	6954.6 **	158.6 **	2.9 **	184.2 **	72.8 **	4135.6 **	3603.2 **	351.9 **	2.5 **	597026.20 **	14925655.00 **
r (year)	2	5.69	9183.5	15.3	1.02	0.1	1.2	0.2	76.5	2.2	4.1	0.09	29931.55	748288.89
Biochar (b)	3	37.19 **	229634.7 **	3071.4 **	81.0 **	1.1 **	117.8 **	20.0 **	6129.9 **	3079.3 **	82.0 **	0.6 **	225506.06 **	5637651.49 **
a*b	6	7.44 **	59484.6 **	358.1 **	10.0 *	0.3 **	60.9 **	4.2 *	1087.7 **	618.2 **	25.5 **	0.15 *	38834.35 **	970858.94 **
a*r (year)	8	4.9	11845.3	367.0	4.0	0.1	28.5	2.3	608.7	47.4	6.3	0.2	18493.98	462349.70
Error	36	1.43	13001.4	69.7	4.19	0.0825	13.47	1.777	244.9	117.4	4.78	0.0561	5284.20	132105.19

Table 5 - Effects of biochar and drought stress on some properties of melon plants under drought stress

Treatment	Water use efficiency	Shoot dry weight (g)	Root dry weight (g)	Shoot length (cm)	Root length (cm)	Leaf area (cm^2)	Leaf number per plant	Fruit diameter (cm)	Fruit flesh thickness (cm)	Average fruit weight (g)
I1B1	7.19 c	188.3 e	1.71 d	75 d	10.2 e	167.7 d	76.5 e	36 e	2.15 e	565.2 d
I1B2	7.75 c	193.3 de	2.52 bc	77 cd	12.7 d	186.9 cd	91.5 de	43.3 d	2.76 cd	612.7 cd
I1B3	7.92 c	206 cd	2.34 bc	76.8 cd	13.4 cd	188.3 cd	103.4 bcd	43.3 d	2.64 d	624.6 cd
I1B4	7.88 c	205.2 cd	2.06 cd	77 cd	14.1 bcd	190.2 cd	106.5 bcd	43.4 d	2.84 bcd	637.5 cd
I2B1	7.68 c	200.8 cde	2.5 bc	77 cd	14.7 bcd	193.1 cd	98.5 cd	46.4 cd	2.81 bcd	603.7 cd
I2B2	8.73 bc	216 bc	2.68 ab	81.2 bcd	14.2 bcd	198.8 c	104.2 bcd	46.1 cd	2.83 bcd	687.9 cd
I2B3	11.77 ab	238.7 a	3.14 a	85.5 ab	15.8 abc	212.2 bc	119.7 ab	48.2 bc	3.23 ab	929.3 ab
I2B4	12.01 ab	209.8 bc	2.44 bc	79.6 bcd	15.2 abc	207.5 bc	110 bcd	46.5 cd	3.19 abc	934.7 ab
I3B1	9.02 bc	213.3 bc	2.77 ab	78.1 cd	14.5 bcd	202.4 c	103 bcd	47 c	3.14 abc	723.1 c
I3B2	12.01 ab	222.7 b	2.68 ab	80.8 bcd	15.9 abc	203.1 c	106 bcd	46.5 cd	3.16 abc	922.7 b
I3B3	13.55 a	250 a	3.15 a	83.4 abc	17.5 a	233.9 ab	117.4 abc	50.7 ab	3.3 a	1057 a
I3B4	12.69 a	249.2 a	2.71 ab	88.5 a	16.1 ab	252.7 a	132.5 a	51.7 a	3.35 a	994.7 ab

Interaction of the treatments indicated that the treatment of I3B3 increased shoot fresh weight (Fig. 1), shoot dry weight (Table 5), root fresh weight (Fig. 2), root dry weight and root length (Table 5) by 77, 32, 100, 84, and 71% compared to the treatment of I1B1, respectively. The highest shoot length, leaf area, leaf number per plant, fruit diameter, and fruit flesh thickness (Table 5) were associated with I3B4 treatment, which increased these characteristics 18, 50, 95, 43, and 55% compared to I1B1 treatment respectively and there was no significant difference compared to the I3B3 treatment. Regarding the shoot length, leaf number per plant, and fruit flesh thickness (Table 5), there was no significant difference between I3B4 treatment with the treatment of I2B3. The treatment of I3B3 increased the average fruit weight (Table 5) and total yield (Fig. 3) by 84% compared to the treatment of I1B1, however there was no significant difference compared to the treatments of I3B4 and I2B3 with increase rates of 73, 63, and 62%, respectively. The biochar-free treatment with 60% water requirement also accounted for the lowest rates in all characteristics. The results indicated that the effects of drought stress and biochar as well as the interaction of them on leaf proline content and all chemical characteristics except for the interaction of P were significant (Table 6). The treatment of I3B4 increased the N (Fig. 4), K (Fig. 5), and Mn (Table 7) as 58, 65, and 48%, respectively, compared to the treatment of I1B1. Regarding N ele-

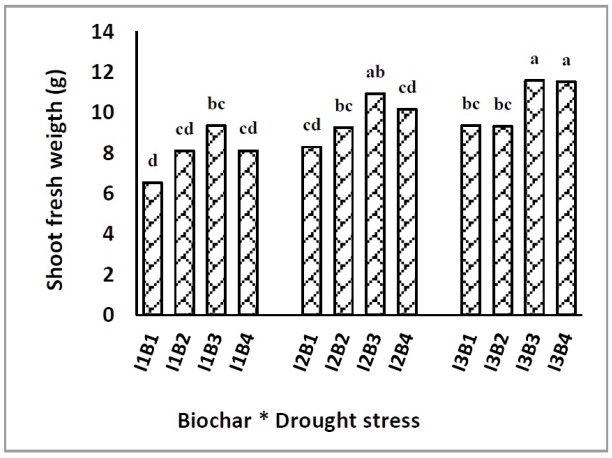

Fig. 1 - Effects of the interaction of biochar and drought stress on shoot fresh weight of melon.

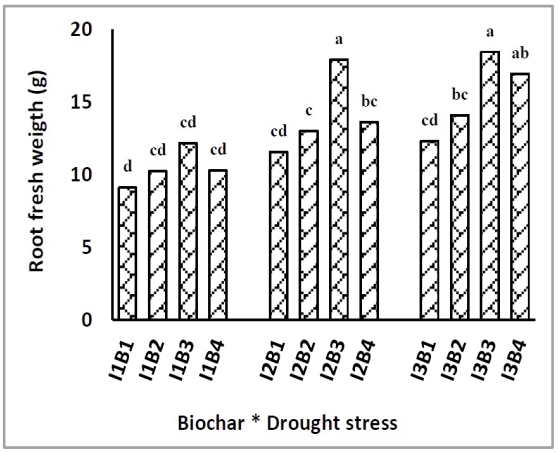

Fig. 2 - Effects of the interaction of biochar and drought stress on root fresh weight of melon.

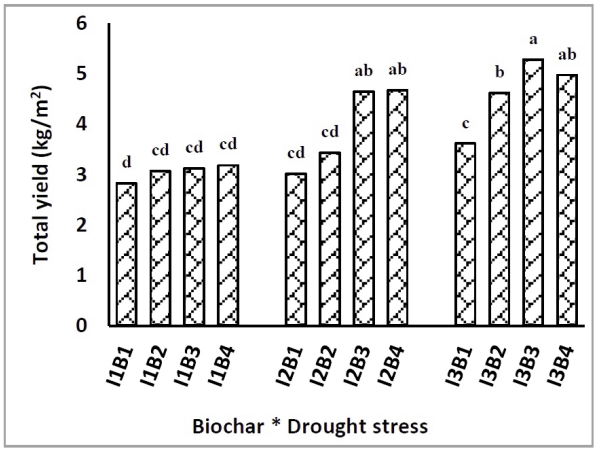

Fig. 3 - Effects of the interaction of biochar and drought stress on yield of melon.

Table 6 - Results of analysis of variance (ANOVA) of chemical characteristics of melon leaf

Source of variation	Degree of freedom	Mean square							
		N	P	K	Fe	Zn	Cu	Mn	Proline
r (replication)	2	1.0 **	0.004 *	0.2 *	3505.7 **	144.7 *	4	68.2 *	1.5 *
Drought stress (a)	2	3.1 **	0.06 **	2.1 **	14716.9 **	1321.4 **	24.9 **	970.7 **	166.2 **
r (year)	2	2.3	0.02	1.2	20.0	39.9	0.2	5.8	0.04
Biochar (b)	3	1.2 **	0.01 **	1.0 **	34202.0 **	836.3 **	63 **	363.5 **	13.7 **
a*b	6	0.3 **	0.008 NS	0.2 **	9413.6 **	184.6 *	11.8 **	200.9 **	2.2 **
a*r (year)	8	0.08	0.001	0.07	1356.1	46	0.66	53.2	2.5
Error	36	0.0734	0.00123	0.0446	623.32	76.479	3.135	15.31	0.528

* and ** indicate significant difference in 1 and 5% level respectively; NS= not significant.

ment, the treatments of 0.36, 0.24 and 0.19 Kg/m^2 and 100% water requirement was not significantly different in comparison to treatments of I2B3 and I2B4 (Fig. 4). In terms of K, there was no significant difference between the treatments of I3B3 and I3B4 (Fig. 5). In addition, the Mn content in the treatments of I3B3 and I3B4 was not significantly different in compared to I2B3 and I2B4 treatments and the lowest rate was also related to the biochar-free treatment with 60% water requirement (Table 7). The treatment of 0.36 Kg/m^2 increased the P level by 20% compared to the treatment without biochar and accounted for the highest rate, although it was not significant compared to the treatment of 0.24 Kg/m^2 (15%) (Figs. 6 and 7). Mean comparison of drought stress treatments suggested that 100% and 85% water requirement increased the leaf P content 36% and 10% compared to the treatment of 60% water requirement respectively and were significantly different compared to each other (Figs. 6 and 7). The interaction of drought stress and biochar treatments showed that the treatment of 0.36 Kg/m^{2}and 60% water requirement increased leaf Fe (Fig. 8), Zn, and Cu (Table 7) by 60, 44, and 66%, respectively, compared to treatment without biochar with 60% water requirement, with the lowest rate being associated

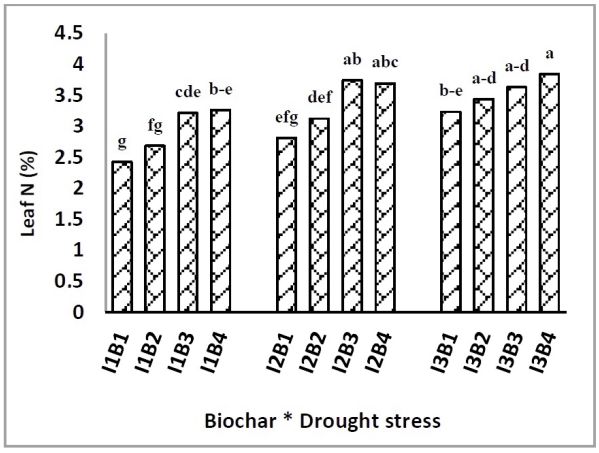

Fig. 4 - Effects of the interaction of biochar and drought stress on melon leaf N content.

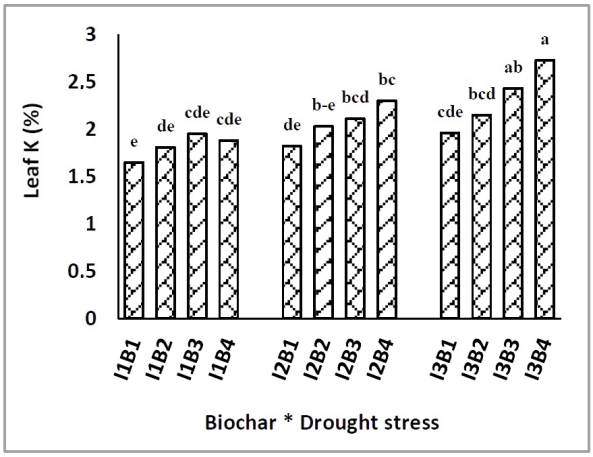

Fig. 5 - Effects of the interaction of biochar and drought stress on melon leaf K content.

Table 7 - Mean comparison interaction of biochar and drought stress on some characteristics of melon leaf

Treatment	Zn (ppm)	Cu (ppm)	Mn (ppm)
I1B1	44.07 bcd	9.51 d	44 d
I1B2	46.87 bcd	10.18 d	47.17 d
I1B3	54.97 ab	14.38 ab	51.51 bcd
I1B4	63. 7 a	15.75 a	51.98 bcd
I2B1	38.83 cde	9.98 d	49.68 cd
I2B2	35.2 de	10.55 d	50.9 bcd
I2B3	51.3 abc	11.92 bcd	56.88 abc
I2B4	45.5 bcd	13.98 ab	59.1 ab
I3B1	25.27 e	8.41 d	50.9 bcd
I3B2	42.37 bcd	11.35 bcd	52.73 bcd
I3B3	40.03 bcd	11.01 bcd	59.37 ab
I3B4	43.53 bcd	10.85 cd	65.34 a

In each column, mean values with the same letters do not have a significant difference in 1% probability level of the Duncan's test.

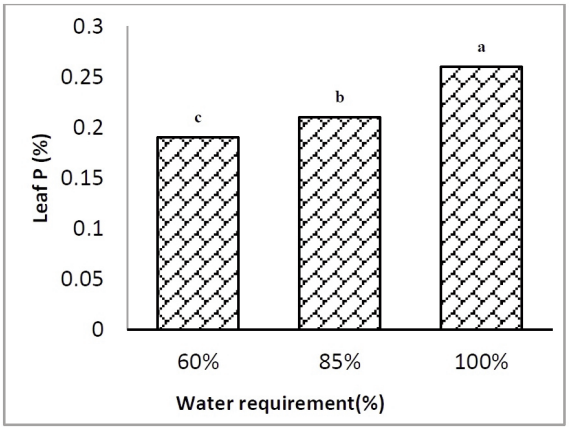

Fig. 6 - Effects of drought stress on melon leaf P.

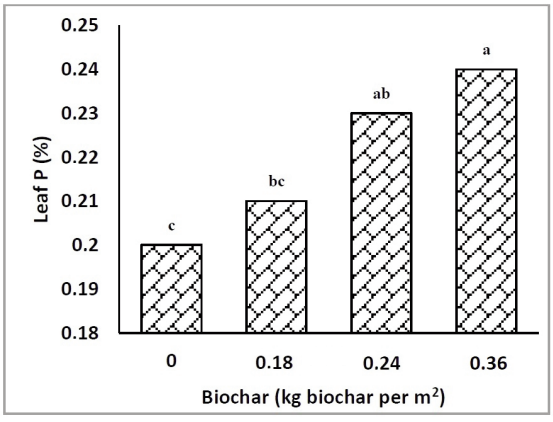

Fig. 7 - Effects of biochar on melon leaf P.

with the biochar-free treatment with 100% water requirement. For Fe, the treatments of I1B2, I1B3 and I1B3 had not significantly difference to each other and with compared to I2B3 and I2B4 (Fig. 8). The leaf Zn content of the treatments of I1B3 and I1B4 and I2B3 was not significantly different (Table 7). In the case of Cu, the treatments of I1B3 and I1B4 were not significantly different from I2B4 (Table 7). The lowest level of proline was related to I3B3, and it was not significantly different from the treatment of I3B4. Moreover, the highest rate of proline was related to the biochar-free treatment and 60% drought stress and was significantly different from other treatments (Fig. 9).

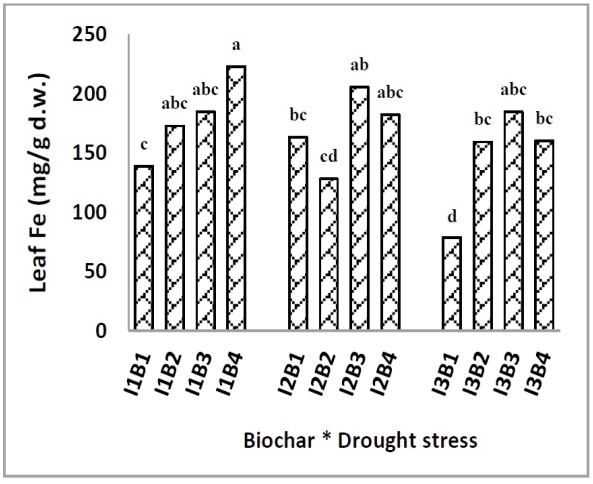

Fig. 8 - Effects of the interaction of biochar and drought stress on melon leaf Fe.

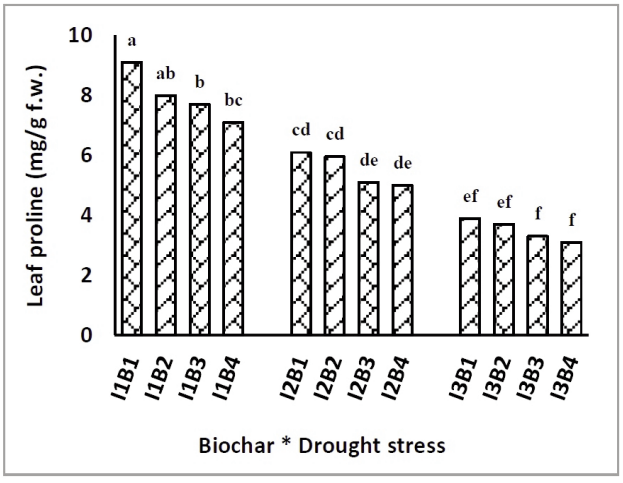

Fig. 9 - Effects of the interaction of biochar and drought stress on leaf proline content of melon plants.

4. Discussion and Conclusions

Reduction in water resources affects the physiological processes of the plant and hence reducing the growth and yield. In this experiment, the water shortage of root media was compensated with the application of biochar and hence increasing in water holding capacity, and the water use efficiency of plant improved without decreasing growth and by increasing the nutrient supply and hence total yield. The treatments of I3B3 and I3B4 increased water use efficiency by 88 and 76% compared to the treatment of I1B1 respectively, but there was no significant difference compared to the treatments of I3B2, I2B3 and I2B4. In this experiment, no significant difference was observed between 85% and 100% water requirement, particularly in 0.24 and 0.36 Kg/m^2, indicating the fact that biochar application in 85% water requirement significantly reduced plant water use and hence, a significant effect on the water use efficiency of the plant. Akhtar *et al.* (2014) reported that the use of biochar obtained from rice bran and flaxseed increased water use efficiency in all irrigation treatments compared to the biochar-free conditions. Uzoma *et al.* (2011) indicated that the application of 10, 15, and 20 tons cow manure biochar per hectare significantly increased the water use efficiency of corn plants in a sandy soil. I3B3 treatment increased the shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and root length by 77, 32, 100, 84, and 71%, respectively compared to the treatment of I1B1. The treatment of I3B3 had no significant difference in comparison to the treatments of I3B4 and I2B3.

Roots grew well in biochar beds, which can be due to the improvement of the physical and chemical conditions of the soil and therefore, reducce the soil resistance to the root growth (Chan *et al*., 2008). Biochar can improve water permeability of the soil and facilitate root infiltration and increase root weight and length.

The highest shoot length, leaf area, and leaf number per plant were related to I3B4 in compared to I1B1 treatment, however treatment of I3B4 was not significantly different from the treatment of I3B3. In this experiment, decrease in irrigation and increase in plant stress caused decrease in the shoot length, leaf area, and leaf number per plant, and with increased irrigation and biochar application, and hence decreasing stress, these values increased. In the stress conditions, the plant size reduces due to reduced transpiration, hence reducing its leaf cells

and leaf size. With increasing water use efficiency and thus decreasing stress, the biochar leads to an increase in the leaf area and leaf number per plant (Olympios, 1992). The shoot length increases because of the effect of biochar in increasing available P that causes increase in root growth and absorption of nutrients (Hossain *et al*., 2010). Moreover, Sang and Gio (2012) showed that the biochar with increasing chlorophyll content, leads to the improvement of photosynthesis, carbohydrate synthesis and biomass production; the result of which include the increase in the leaf area, leaf number per plant, hence increase in weight and length of the root and shoot of the plant. The treatment of I3B4 increased fruit diameter and fruit flesh diameter by the rate of 43 and 55 in compared to the treatment I1B1, although there was no significant difference between the treatments of I3B3. In this experiment, fruit diameter, fruit flesh diameter and hence the yield increased with the application of biochar. This is due to the nutritional elements available in the palm leaves (direct) and also the improvement of soil physical, chemical and biological characteristics (indirect) by biochar (Major *et al*., 2010). Biochar significantly leads to increase in the organic carbon and soil fertility (Kumar *et al*., 2013), increased growth and crop yield (Spokas *et al*., 2010), and increased plant dry matter (Van Zwietn *et al*., 2007). The treatment of I3B3 increased the average fruit weight and plant yield by 84% compared to the I1B1 treatment, however it was not significantly different compared to the treatment of I3B4, I2B4, and I2B3. Other researchers (Zhang *et al*., 2010) also attributed the increase in corn growth and yield in biochar treatments to increased availability of the nutritional elements and improved physical properties of the soil, such as decreasing the apparent density. Furthermore, biochar improves soil chemical properties including functional groups and CEC (Kharea *et al*., 2013), in addition to increased plant access to nutrients and improved plant growth (Lehmann and Joseph, 2009). Uzoma *et al*. (2011) indicated that biochar application increased the growth and yield of corn compared to control, and had a significant effect on shoot length and number of leaves in different stages of corn growth in sandy soil. In this experiment, addition of 0.19, 0.24, and 0.36 Kg/m^2, especially the treatments of 0.24 and 0.36 Kg/m^{2}and without stress, increased other vegetative characteristics. Moreover, the application of biochar with 85% water requirement did not significantly change these characteristics, but increased stress (60% water requirement) decreased plant vegetative properties. N is considered as a mobile element, so N level reduces in conditions of water shortage. Accordingly, it can be conclusively claimed that the addition of biochar to the soil, by increasing water retention, decreases the nitrate leaching from the soil and increases the availability of N in the soil, and this effect is stable for at least five months (Clough *et al*., 2013). Generally, there are varying reports on the effect of drought stress on nutrient content in plant species. The decreased rate of N in water shortage conditions (Muni Ram and Singh, 1995; Alam, 1999) and its strengthening under drought stress have been reported (Abdel Rahman *et al.*, 1971). In this experiment, the treatments of I1B4, I1B3 and I1B2 increased N content of the plant as 34, 32 and 10%, the treatments of I2B4, I2B3 and I2B2 increased N content of the plant as 52, 54, and 28%, and finally the treatments of I3B4, I3B3 and I3B2 increased N content of the plant by 58, 50 and 41% compared to treatment I1B1. It is concluded that the plant N content increases with application and increasing the biochar level and decreasing drought stress.

Results showed that biochar utilization increased leaf P content under stress and non-stress conditions. The effects of organic matter on increasing P availability in the soils depend on their phosphorus content. Due to the low amount of absorbable phosphorus in palm leaf biochar, this increase can be attributed to acids released from organic matter. These acids reduce the P stabilization in the soil and transform it into an absorbable form. The absorption of nutrients and available water by plant roots are closely related to each other. Water relations affect all physiological processes related to the solubility and availability of nutrients (Alam, 1999). In this experiment, application of 0.36, 0.24, and 0.19 Kg/m^2 increased 20, 15, and 5% of plant P, respectively, in comparison to the control (without biochar). Moreover, the treatments of 100% and 85% of water requirement increased the plant's P rate as respectively 36% and 10% in comparison to the treatment of 60% of water requirement. It can be concluded that the leaf P content increased with increasing biochar level and decreasing drought stress. Biochar application increased K under stress and non-stress conditions. Increasing the soluble K due to the application of biochar depends on their composition, especially their K content, the rate of K release, and the effect of organic molecules on the release of K from soil minerals (Jalali, 2011; Najafi-Ghiri, 2015). At the presence of higher water rate, univalent ions

such as K in the soil solution increase relatively more than bivalent ions such as Ca and Mg, however as the soil becomes dry gradually, clay colloids absorb K (univalent ions) more strongly to their surface and prevent the separation of these ions (Kafi *et al*., 2009). In addition, since the overall growth of the plant, including the absorption activity of roots reduces due to stress, they will not be able to absorb K from the surface of clay colloids and, hence, the rate of absorption of these elements decreases (Radin and Eidenbock, 1984). In the present experiment, treatments of I1B4, I1B3 and I1B2 increased the K rate as 13, 18, and 9%, also, treatments of I2B4, I2B3 and I2B2 increased the K rate as 39, 27, and 23%, and finally treatments of I3B4, I3B3 and I3B2 increased the K content by 65, 47, and 30%, respectively. This results leads to the conclusion that the addition of biochar reduces stress and, as a result, increases the K content of the plant. Biochar application increased Fe, Zn, and Cu under stress and Mn under non-stress conditions. The researchers have suggested that drought stress stops the activity of older roots and only the tip of the roots absorb nutrients, hence the bivalent cations such as iron are absorbed more than the univalent ones and adsorption of the anions is limited (Martins *et al*., 2003). In the case of Zn and Cu elements, maybe in conditions of drought stress, continuous wetting and drying in the soil leads to the release of these elements from the clay layers and their concentration increases in the soil, hence increasing the adsorption phenomenon (Logan *et al.*, 1997). Mn and Fe have an inverse relationship with each other in terms of absorption by the plant, that is, increasing the Mn absorption decreases the Fe absorption (Martins *et al*., 2003). Changes in the availability of micro elements in the soil are affected by the characteristics of organic matter and soil. The nutrients of organic matters are released through its decomposition. Although various mechanisms are responsible for increase or decrease of retaining nutrients in the soil (Sposito, 1984), studies have shown that adding biochar to the soil is effective on the capability of use of ions due to affecting ion exchangable capacity and microbial activity (Atkinson *et al*., 2010). In an experiment, drought stress increased soil Zn and Cu and reduced Mn (Alizadeh *et al*., 2008). Drought stress increased Zn, Fe, and Cu content in the sage plant (Sodaeizadeh and Mansouri, 2014). In an experiment, biochar application increased Fe and Mn elements in amaranth plant (Habibi *et al*., 2017). In this experiment, 60 and 85% water requirement increased the Fe content (76 and 107%), Zn (74 and 13%), and Cu (13 and 18%) compared to the 100% water requirement (without stress) and the treatments of 0.24 Kg/m^2 in 60 and 85% water requirement and 0.36 Kg/m^{2}in 60 and 85% stress, increased the Fe rate as 33%, 48%, 60%, and 32%, the Zn as 24, 16, 44, and 3%, and Cu as 51, 25, 65, and 47% respectively, compared to the treatment of I1B1. Moreover, in the present experiment, the Mn level increased with the use of biochar and decrease in the drought stress, so that treatments of I1B4, I1B3 and I1B2 increased the Mn by 18, 17, and 7%, the treatments of I2B4, I2B3 and I2B2 increased the Mn by 34, 29, and 15%, and eventually, the treatments of I3B4, I3B3 and I3B2 increased the Mn by 38, 44, and 19%, respectively.

The use of biochar reduced proline content under stress and non-stress conditions. This finding suggests that biochar decrease the water evaporation and keeping moisture in the root media, because of its large pores on its surface or improving the soil texture, and can improve root growth and hence reduce stress. Under drought stress conditions, the water potential of the leaf decreases substantially, which, solutions such as proline accumulation in the leaf in order to adapt to the osmotic conditions. Proline decreases in leaves under stress due to decreased synthesis and increased oxidation. It was observed that drought stress caused reduction in leaf water capacity of grape and, thus, increased proline and proline rate was reduced through the use of biochar in cultural media (Rasouli and Golmohammadi, 2009). In this experiment, the proline content decreased with increasing biochar treatments from 0.19 to 0.36 Kg/m^2 and the increase of water requirement from 60 to 100%, with the lowest amount of proline being related to 0.24 and 0.36 Kg/m^{2}and 100% water requirement.

The results of this study revealed that adding palm leaf biochar to the soil especially in drought stress conditions reduces the water consumption rate and improve plant growth and yield. Treatments of 0.24 Kg/m^{2}and 100% water requirement increased the shoot fresh weight, root fresh weight and plant yield compared to without biochar and 60% water requirement. In general, the most effective treatments were 0.24 and 0.36 Kg/m^2 and there was no significant difference between these treatments in most of the characteristics. Using biochar, especially 0.24 and 0.36 Kg/m^2, could compensate the drought stress effects and improve plant growth and yield.

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Short note

AHS
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Influence of different ornamental shrubs on the removal of heavy metals in a stormwater bioretention system

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Key words: blue-green infrastructure, rain gardens, urban stormwater runoff, water sensitive urban design.

Abstract: Several laboratory studies have shown the ability of bioretention systems to remove pollutants from stormwater. However, to our knowledge, no existing research has addressed the use of ornamental shrubs for improving water quality in bioretention systems in Italian cities. In this short note, we evaluated the potential of three ornamental shrub species (*Lonicera pileata* Oliver, *Cotoneaster horizontalis* Decne., *Hypericum hidcoteense* 'Hidcote') for the removal of heavy metals in a stormwater bioretention system. Pot experiments in "pot prototypes" using an alternative bioretention system filter media have been carried out under controlled conditions. The ornamental shrubs were irrigated with semisynthetic stormwater with known heavy-metal concentrations. Experimental results indicate that the removal of heavy metals by the system is very efficient. However, there was not a significant effect of the plant on the system's retention efficiency. The removal of lead and cadmium by the system was over 87%. In order to provide accurate information for bioretention design, future research should comparatively assess plant species in a laboratory-scale filter column and *in situ*.

1. Introduction

Urban stormwater runoff contains pollutants which can impact the quality of surface, seepage, and ground water (Eckley and Branfireun 2009; Göbel *et al.*, 2007). Stormwater carries different pollutants, both organic and inorganic (Barbosa *et al.*, 2012), including copper, zinc, lead, cadmium, sediments, polycyclic aromatic hydrocarbons, and de-icing salts (Muthanna *et al.*, 2007) so that its quality management is of crucial

importance to urban development and water resource planning (Zgheib *et al.,* 2012). In particular, cadmium has become an increasing problem because of its toxic effects on biological systems (Mishra and Tripathi, 2008). Additionally, contaminated soils and waters represent an environmental and human health problem, which may be partially solved by the phytoremediation technology (Mojiri 2012; Dadea *et al.,* 2017).

New approaches to improve water quality as well as water cycle in urban areas have been proposed, for example with Best Management Practices (BMP), Low Impact Design (LID), Sustainable Urban Drainage System (SUDS), Water Sensitive Urban Drainage Systems (WSUD) and sponge cities ((Pompêo 1999; Raja Segaran *et al.,* 2014, Fletcher *et al.*, 2015; Griffiths 2017). These systems have been implemented around the world because they provide important environmental, economic and health benefits such as improving water quality, reducing flood risk, increasing amenity and increasing biodiversity in cities (Griffiths, 2017). Retention and degradation of stormwater pollutants using the above systems are becoming an important ecosystem service in urban environments (Kabir *et al.,* 2014). According to Kabir *et al.* (2014), more than 75% of metals, such as Pb, Zn, Cu, and Cd is retained by blue-green infrastructure.

In particular, bioretention systems, also known as biofilters or rain gardens, have been used to remove a wide range of pollutants, such as suspended solids, nutrients, metals, hydrocarbons, and microorganisms from stormwater runoff (Muthanna *et al.,* 2007; Sun and Davis 2007; Hatt *et al.,* 2009; Blecken *et al.,* 2010; Megharaj *et al.,* 2011; Trowsdale and Simcock 2011; Weerasundara *et al.,* 2016). Well-designed bioretention systems can remove several pollutants from the urban runoff via physical, chemical, and biological processes, including plant uptake, sedimentation, filtration, and sorption on mulch and soil layers, and biodegradation by soil microorganisms (Weerasundara *et al.,* 2016). A bioretention system consists of several layers of filter media, normally a soil/sand/organic media matrix (approximately 0.7 - 1 m deep), a mulch layer and both woody and herbaceous plants (Sun and Davis 2007; Davis *et al.,* 2009; Liu *et al.,* 2014).

Plants not only assimilate pollutants directly from wastewater and rooting media into their tissues, but also act as catalysts for purification reactions by increasing the environmental diversity in the rhizosphere and promoting a variety of chemical and biological reactions that enhance pollutant removal (Zhang *et al.,* 2011). The benefits of bioretention by vegetation have not been well quantified (Davis *et al.,* 2009) and the majority of studies have focused on herbaceous plants in bioretention systems (Sun and Davis 2007; Read *et al.,* 2008; Feng *et al.,* 2012; Barrett *et al.,* 2013; Payne *et al.,* 2014). Woody shrubs may also provide low maintenance and might be an attractive cover for stormwater systems (Environmental Services Division, 2009).

Feng *et al.* (2012), conducted a large-scale stormwater biofilter column study and found that vegetation and the type of filter are significant factors for the treatment of metals. While most studies evaluated individual plant performance for metal uptake, some plant species have been shown to improve the performance of stormwater biofiltration systems (Read *et al.,* 2008; Houdeshel *et al.,* 2012). Therefore, the assemblage of different species may be suitable for increasing biofilter efficiency and maximizing the spectrum of removed pollutants, but this topic remains largely unexplored.

Species mixes might also be preferred for aesthetic and ecological reasons (Read *et al.,* 2008). However, higher concentration of heavy metals can cause damage to plants by reducing growth and the rates of photosynthesis and respiration, so that further understanding on species' tolerance to pollution is needed (Hossain *et al.,* 2012; Ovečka and Takáč 2014). Plant species suitable for the use in bioretention systems are provided by North American and Australian bioretention design guidelines (Environmental Services Division, 2009; Houdeshel *et al.,* 2012). However, this information is not based on data from replicated experiments (Dylewski *et al.,* 2011) and little is known about the most suitable type of plant for bioretention systems in terms of survival and performance for Italian cities. Therefore, the objectives of our study were: i) to evaluate an alternative bioretention filter media; and ii) to test the hypothesis that species association may increase heavy-metal retention by the system constituted by different plant combinations and substrates; and iii) to understand the heavy-metal effect on chlorophyll and root/shoot ratios.

2. Materials and Methods

Experimental setup and planting material

Three species potentially suitable for planting in bioretention systems were chosen across a range of

evergreen ornamental shrubs commonly grown in urban areas in Central-Northern Italy. 70 plastic pot prototypes (Fig. 1) with a truncated pyramid shape (418 x 310 mm, 347 x 245 mm base, and 575 mm height) with lateral taps at the bottom, were put in a greenhouse facility at the University of Florence in Sesto Fiorentino, Italy, in October 2013 (Fig. 2). The pots consisted of four layers: (1) The drainage layer at the bottom of the pot was filled with 150 mm of perlite (AGRILIT 2, Perlite Italiana) and (2) a filter sheet (DRENALIT F130, Perlite Italiana) was placed to separate the 300 mm substrate layer (3) (AgriTERRAM TV, Perlite Italiana) from the drainage layer, followed by a 50 mm mulch layer (4) (GEOBARK Pine Bark) to cover the soil and improve pollutant retention (Muthanna *et al.,* 2007). The substrate basic properties were pH 6-7, EC <40 mS/m, cation-exchange capacity (CEC) 55-60 meq/100 g, total organic content <20-25%, bulk density 400 kg/m^3 ± 5%, and vertical permeability >13 mm/min. The system consisting of AGRILIT 2 and AgriTERRAM TV (Perlite Italiana), known as PERLIROUND™, is used for the greening of roundabouts and traffic islands (Perlite Italiana, 2011). Three-year-old plants of *Lonicera pileata* Oliver, *Cotoneaster horizontalis* Decne., and *Hypericum hidcoteense* 'Hidcote' were potted in the containers. Each pot contained 2 plants of the same species, namely *Lonicera pileata* (Lp), *Cotoneaster horizontalis* (Ch), and *Hypericum hidcoteense* 'Hidcote' (Hh), or plants of two species, in all possible combinations (Lp + Ch, Lp + Hh, and Ch + Hh). 5 additional pots were prepared as previously described but left unplanted. The experiment was carried out from October 2013 until June 2014. Plants were grown at 28/18°C day/night temperatures and exposed to natural daylight, and the light transmission was of 90%. Relative humidity was always above 60%.

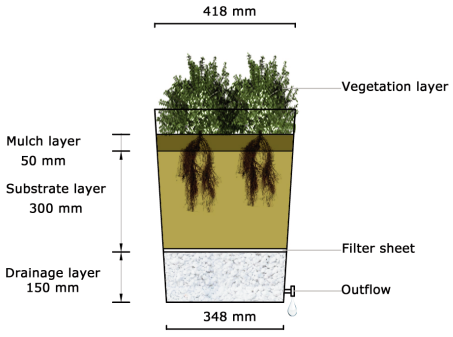

Fig. 1 - Schematic drawing of the bioretention pot prototype. Not to scale.

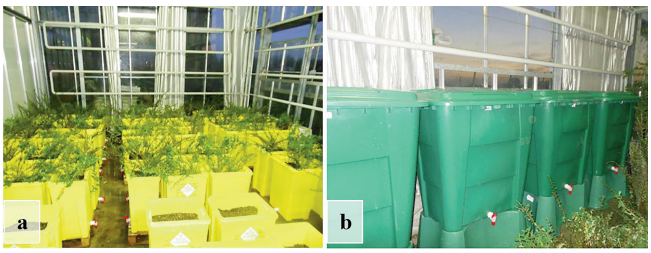

Fig. 2 - Photo of the greenhouse experiment at the University of Florence, Italy: (a) bioretention pot prototypes, (b) 200-L plastic water storage tanks.

Measurement of pollutants and plant growth

Synthetic stormwater runoff was prepared using tap water that was left to stand at room temperature in 200-L plastic water storage tank for 24 h to dechlorinate and thermally equilibrate (Fig. 2) (Sun and Davis, 2007). The first irrigation with synthetic stormwater started on April, 3rd 2014 after approximately 6 months of plant growth in the pots. Plants were irrigated with synthetic stormwater with heavy metal concentrations (Pb and Cd) once per week for 3 weeks. The total volume of runoff applied to each pot was 5 L, this amount was based on rainfall precipitation in Florence (Vijaya Kumar *et al.,* 2013). The concentrations (mg L^{-1}) of pollutants in our synthetic stormwater were 2.02 (mg L^{-1}) in the first irrigation and 1.97 in the successive irrigations for Pb and 0.37 (mg L^{-1}) in the first irrigation and 0.39 mg L^{-1} in the successive irrigations for Cd. These values are the highest concentrations of highway runoff reported in the literature (Kayhanian *et al.,* 2012). To determine the effect of plants on pollutant removal from stormwater, the water that drained from the tap (outflow) was collected during the first and second irrigations. We collected 60 samples from the "stormwater plants'' and 10 from the unplanted containers "stormwater soil". We also collected stormwater (inflow) in order to assess its quality, before each irrigation. Furthermore, pH was measured immediately after each sampling using a pH Electrode LE407. Samples were filtered through 0.45 µm membrane filter (Swinnex Filter Holder) and acidified with 1% of Nitric Acid. The samples were sent to an accredited analytical chemistry laboratory (Research Centre for Agriculture and Forestry, Laimburg, Italy) and analyzed according to standard methods for Pb and Cd using ICP. The removal efficiency was calculated as percentage of inflow concentrations.

A Minolta SPAD-502 leaf chlorophyll meter was

used for non-destructive data collection. The instrument is able to provide a rapid and reasonably accurate estimate of leaf Chl. Measurements were made before the first irrigation and after the second irrigation. SPAD readings were recorded for 3 positions on each leaf and for 3 different leaves on a single shrub (Table 1). At the end of the experiment, dry weight (DW) of roots, stems and leaves was determined in 36 treated plants and in 36 control plants. The total plant DW and shoot/root ratio were calculated.

Experimental design and statistics

The experiment was a randomized complete block with five blocks (Rao, 2007). The outflow data were checked for normality using Kolmogorov-Smirnov and Ryan-Joiner tests using Minitab 17. The data did not fit a normal distribution and we used a non-parametric Kruskal-Wallis test to analyse statistical differences among treatments. In order to determine whether there was a statistically significant effect between treatments on the plant-growth parameters, including stem, roots and leaves, a post- hoc comparison on means was conducted by Duncan's test (SPSS Statistics) with $p<0.05$.

3. Results and Discussion

Mean outflow concentrations and reduction are shown in Table 2. Outflow Pb concentrations ranged in the first irrigation from 4.13 µg/L in *Lonicera* + *Cotoneaster* to 9.37 µg/L in *Lonicera pileata* + *Hypericum hidcoteense* 'Hidcote'. Cd concentrations ranged in the first irrigation from 1.57 µg/L in *Lonicera* and *Cotoneaster* to 3.23 µg/L in *Cotoneaster* + *Hypericum*. However, Pb concentrations ranged in the second irrigation from 5.88 µg/L in soil to 237.80 µg/L in *Lonicera* + *Lonicera*. Cd concentrations ranged in the second irrigation from 1.44 µg/L in soil to 8.34 µg/L in *Cotoneaster* as single species.

We found that the different shrub species did not affect the reduction and there was no significant difference in metal concentration between the effluent from soil-only controls and shrubs or mix of species. Based on the results above, heavy metals are mainly retained by physical processes (i.e., sedimentation and chelation) within the PERLIROUND substrate and we were unable to determine removal by vegetation uptake. However, previous studies have highlighted the limited role of plant uptake in the removal of metals from storm water in bioretention systems (Read *et al.,* 2008; LeFevre *et al.,* 2015). Several factors could interact with the Cd uptake, for example the interaction of soil composition, pH, organic matter, and available mineral elements may decrease or increase the plant availability of Cd (Chizzola and Lukas, 2006). Furthermore, effective vegetation metal removal performance in bioretention has been attributed to species (i.e. hyperaccumulating plants), root architecture, plant age, and leaf area and the species chosen may not be metal accumulators or alter the soil chemistry/ecology to enhance metal retention (Muerdter *et al.,* 2018). Based on the average effluent concentrations, reduction efficiency for Pb and Cd was more than 87%. Removal was very high in non-vegetated bioretention containers >99.4%, this is due to the absence of roots and soil compaction (Rycewicz-Borecki *et al.,* 2016). Similarly,

Table 1 - Effects of Cd and Pb on the SPAD clorophyll in three ornamental shrubs

Treatments		A		B		C		D		E		F	
		Lp	Lp	Hh	Hh	Ch	Ch	Lp	Hh	Lp	Ch	Ch	Hh
Control - without heavy metals	Mean	69.03	68.10	38.90	38.23	49.70	53.80	42.67	36.93	58.60	67.17	41.53	47.43
		(6.55)	(3.73)	(1.54)	(0.29)	(1.39)	(1.41)	(7.61)	(4.83)	(0.10)	(3.10)	(0.58)	(3.47)
	Mean	44.37	56.17	43.50	44.60	56.13	60.70	49.40	38.67	54.50	61.43	63.43	47.03
		(4.08)	(2.76)	(8.44)	(3.75)	(4.22)	(5.47)	(4.76)	(4.36)	(5.60)	(5.75)	(8.13)	(5.55)
	Mean	51.27	52.80	42.83	42.17	62.93	61.67	54.43	38.83	53.70	66.30	66.20	45.50
Treatment with heavy metals		(1.58)	(1.57)	(1.81)	(1.29)	(6.37)	(3.21)	(1.66)	(3.97)	(4.47)	(2.41)	(4.22)	(3.74)
	Mean	75.40	66.53	38.57	41.70	60.57	69.70	66.30	43.57	77.03	65.53	62.70	44.50
		(8.59)	(9.37)	(4.40)	(2.17)	(4.30)	(4.25)	(8.83)	(2.61)	(4.74)	(6.33)	(2.98)	(2.85)
	Mean	70.90	63.73	43.27	42.33	61.57	61.53	52.97	43.80	69.20	60.23	60.93	40.33
		(10.62)	(14.17)	(5.43)	(2.81)	(7.09)	(5.89)	(6.75)	(5.16)	(12.33)	(2.97)	(4.34)	(4.44)
	Mean	60.87	70.57	41.23	46.30	44.77	44.40	55.20	41.43	65.77	65.00	65.63	40.37
		(13.55)	(9.64)	(3.09)	(3.64)	(3.49)	(1.75)	(2.67)	(2.61)	(7.16)	(2.31)	(1.91)	(1.75)

Lp= *Lonicera pileata*, Hh= *Hypericum* 'Hidcote', Ch= *Cotoneaster horizontalis*.

Standard deviation in brackets. SPAD readings were recorded for 3 positions on each leaf and for 3 different leaves on a single shrub. Treatments were at 2 plants per pot, each pot contained 2 plants of the same species (column A, B and C) and plant mix (2 species, column D, E and F).

Rycewicz-Borecki *et al.* (2016), found that compacted soil conditions of unplanted controls retained significantly more Cu, Pb, and Zn than *Carex praegracilis*, and *Carex microptera* treatments.

The outflow concentrations changed over time and the removal efficiency was lower in the second irrigation for the majority of planted pots and not for the unplanted ones. This may be due to soil compaction. The lower removal rate could be attributed to leaching of Pb and Cd from the bioretention media as the concentration of heavy metals in the bottom layer increases (Muthanna *et al.,* 2007).

Reduction rates in this study agree with the rates observed in previous experiments carried out on bioretention systems in laboratory (Davis *et al.,* 2003; Kabir *et al.,* 2014; Wang *et al.,* 2017; Muerdter *et al.,* 2018).

The results suggested that plant growth was not influenced by heavy-metal treatments for the majority of species. It is likely that the heavy metal concentrations were below the tolerance limits of these species or the length of exposure time was not long enough.

However, we found statistically significant differences (Duncan multiple range test; $p<0.05$) in root/shoot weight ratios for *Hypericum* sp. The addition of heavy metals appeared to increase the root/shoot ratio (Table 3). This observation may be due to the fact that low and moderate doses of Cd could stimulate multiplication, rooting, and biomass

Table 2 - Outflow concentrations and reduction efficiencies for Pb and Cd

	Soil (Unplanted pots)	*Lonicera* sp. & *Lonicera* sp.	*Hypericum* sp. & *Hypericum* sp.	*Cotoneaster* sp. & *Cotoneaster* sp.	*Lonicera* sp. & *Hypericum* sp.	*Lonicera* sp. & *Cotoneaster* sp.	*Cotoneaster* sp. & *Hypericum* sp.
Outflow concentration (Pb) (µg/L) 1st irrigation	7.36 (8.97)	8.88 (8.54)	4.17 (1.84)	7.03 (11.02)	9.37 (5.67)	4.13 (1.58)	7.07 (12.17)
p value (Kruskal-Wallis test)	NS	NS	NS	NS	NS	NS	NS
Reduction % (Pb)	99.6	99.6	99.8	99.7	99.5	99.8	99.7
Outflow concentration (Pb) (µg/L) 2nd irrigation	5.88 (1.87)	237.80 (313.60)	53.04 (79.04)	49.42 (31.49)	20.52 (4.69)	13.94 (6.52)	80.32 (107.64)
p value (Kruskal-Wallis test)	NS	NS	NS	NS	NS	NS	NS
Reduction % (Pb)	99.7	87.9	97.3	97.5	99.0	99.3	95.9
Outflow concentration (Cd) (µg/L) 1st irrigation	2.08 (2.32)	1.45 (0.88)	1.77 (1.05)	2.38 (4.28)	2.68 (3.41)	1.57 (1.82)	3.23 (3.78)
p value (Kruskal-Wallis test)	NS	NS	NS	NS	NS	NS	NS
Reduction % (Cd)	99.4	99.6	99.5	99.4	99.3	99.6	99.1
Outflow concentration (Cd) (µg/L) 2nd irrigation	1.44 (0.93)	3.78 (2.28)	2.54 (1.52)	8.34 (6.97)	2.22 (1.69)	2.04 (1.72)	7.34 (10.42)
p value (Kruskal-Wallis test)	NS	NS	NS	NS	NS	NS	NS
Reduction % (Cd)	99.6	99.0	99.3	97.9	99.4	99.5	98.1

Standard deviation in brackets. Kruskal-Wallis test; significant at $p<0.05$.
NS= not significant.

Table 3 - Effect of heavy metals on stem dry weight (SDW), root dry weight (RDW) leaf dry weight (LDW), total dry weight (TDW) and root/shoot

Treatments	SDW (g)	RDW (g)	LDW (g)	TDW (g)	Root/Shoot (g)
Lonicera sp. & *Lonicera* sp. without heavy metals	22.93 (4.07)	10.37 (3.73)	19.53 (3.71)	52.83 (7.93)	0.63 (0.26)
Lonicera sp. & *Lonicera* sp. with heavy metals	27.97 (4.04)	11.85 (1.64)	24.13 (5.11)	63.95 (10.28)	0.60 (0.06)
P value (Duncan multiple range test)	NS	NS	NS	NS	NS
Hypericum sp. & *Hypericum* sp. without heavy metals	19.92 (8.43)	9.32 (4.51)	13.38 (7.41)	42.62 (19.70)	0.46 (0.21)
Hypericum sp. & *Hypericum* sp. with heavy metals	22.40 (5.70)	6.75 (4.14)	20.48 (7.61)	49.63 (16.22)	0.71 (0.14)
p value (Duncan multiple range test)	NS	NS	NS	NS	<0.01
Cotoneaster sp. & *Cotoneaster* sp. without heavy metals	55.03 (11.37)	11.45 (7.62)	16.73 (7.01)	83.22 (24.79)	0.24 (0.06)
Cotoneaster sp. & *Cotoneaster* sp. with heavy metals	65.87 (6.79)	16.35 (3.19)	18.00 (2.92)	100.22 (10.72)	0.22 (0.03)
p value (Duncan multiple range test)	NS	NS	NS	NS	NS
Lonicera sp. & *Hypericum* sp. without heavy metals	28.28 (7.68)	10.73 (4.87)	20.50 (6.45)	59.52 (15.85)	0.54 (0.14)
Lonicera sp. & *Hypericum* sp. with heavy metals	26.65 (4.89)	8.92 (3.01)	16.50 (2.75)	52.07 (9.38)	0.47 (0.07)
p value (Duncan multiple range test)	NS	NS	NS	NS	NS
Cotoneaster sp. & *Hypericum* sp. without heavy metals	43.07 (18.61)	11.62 (6.98)	17.28 (4.23)	71.97 (23.38)	0.37 (0.21)
Cotoneaster sp. & *Hypericum* sp. with heavy metals	45.90 (24.22)	10.23 (5.04)	18.75 (6.51)	74.88 (33.52)	0.38 (0.18)
p value (Duncan multiple range test)	NS	NS	NS	NS	NS
Lonicera sp. & *Cotoneaster* sp. without heavy metals	45.38 (21.95)	13.35 (4.47)	19.07 (6.50)	77.80 (28.10)	0.35 (0.11)
Lonicera sp. & *Cotoneaster* sp. with heavy metals	49.18 (17.28)	16.23 (5.78)	18.95 (7.81)	84.37 (16.94)	0.31 (0.15)
p value (Duncan multiple range test)	NS	NS	NS	NS	NS

Standard deviation in brackets. Duncan multiple range test; significant at $p<0.05$.
NS=not significant.

production in heavy metal-tolerant shrubs (Wiszniewska *et al.,* 2017). Furthermore, the genus *Hypericum* L. has been described as a cadmium hyperaccumulator (Gardea-Torresdey *et al.,* 2005).

SPAD readings ranged from 36.93 *Hypericum* sp. to 77.03 in Lonicera. Differences in chlorophyll content (Table 1) were statistically significant (One-Way ANOVA Test; $p<0.05$) in mono-specific pots between *Hypericum*, *Lonicera* and *Cotoneaster* (Table 1, columns A,B,C) as well as in mixed pots containing, respectively, *Hypericum* and *Lonicera,* and *Lonicera* and *Cotoneaster* plants (Table 1, columns D and F). This result agrees with previous studies that found that mixed heavy metals decrease the chlorophyll content in various plants (Chandra and Kang, 2016). The concentration of non-essential metals like Pb and Cd may be the cause of low chlorophyll content and could also have several negative impacts via oxidative stress (Nadgórska-Socha *et al.,* 2013).

Recent studies have suggested that laboratory-scale filter columns do not satisfactorily replicate field-scale conditions leading to calls for in situ evaluation of bioretention systems (Trowsdale and Simcock, 2011; Liu *et al.,* 2014). Furthermore, previous studies conducted in greenhouses in which plants were grown in pots have shown that pot size can have a limiting effect on plant growth, nutrient efficiency and photosynthesis rates (Ray and Sinclair, 1998). Future research should comparatively assess plant species in a laboratory-scale filter column and in situ.

4. Conclusions

This study tested an alternative bioretention system filter media and species design. The reduction of Cd and Pb concentrations was over 87% similar to other studies, however there were no differences between replicates with plants and the soil-only control. Therefore, the presence of vegetation did not significantly affect heavy metal removal. Some species appeared Cd and Pb tolerant suggesting they would be appropriate in selections for bioretention systems in Mediterranean cities. The long-term effects of these, and other, metal contaminants is however advisable for future studies. Plant selection for bioretention systems has received considerably more research attention in recent years than previously, but important research gaps still remain, e.g. the impact of bioretention vegetation on emerging contaminants (Muerdter *et al.,* 2018). Our alternative bioretention system filter media can be used to assess other plant species and different pollutants (e.g. nutrients, metals and emerging contaminants). More in depth study is recommended to help landscape architects and horticulturalists in the selection of suitable species or species mixes for bioretention systems.

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