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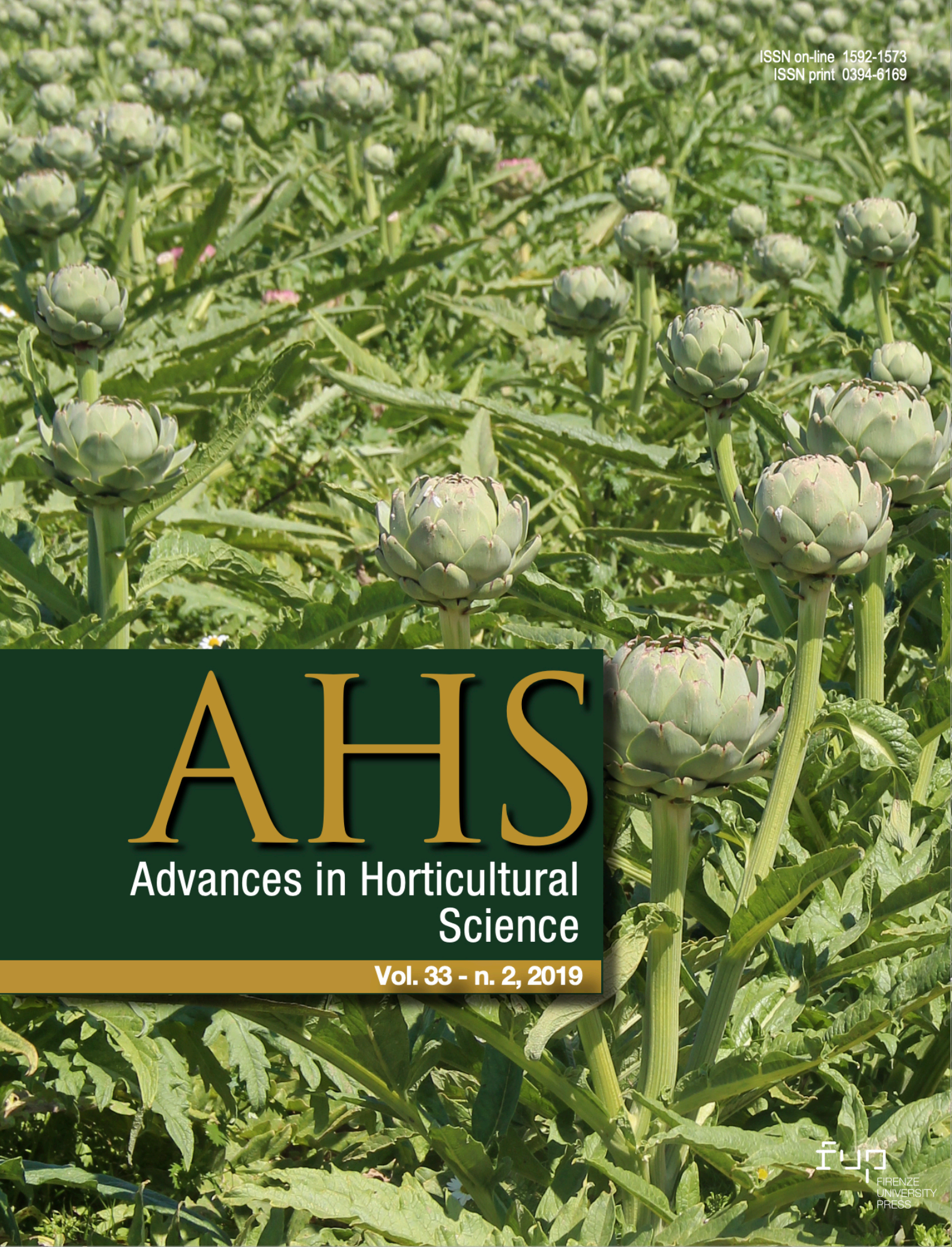

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Diversity of morpho-physicochemical traits in Iranian sour cherry genotypes using multivariate analysis

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Key words: fruit quality traits, genetic diversity, *Prunus cerasus* L.

Abstract: In this study, morpho-physicochemical characterization of sour cherry genotypes from Iran was investigated. Thirty-four morphological and eight physicochemical traits were recorded. Sour cherry genotypes had a high variability in traits related to fruit characters such as fruit weight, stone volume, total anthocyanin content and total soluble solid. As a result, sour cherry genotypes exhibit total phenolic content and antioxidant activity higher than "Ciganymeggy" and "Erdi botermo" cultivars. Principal component analysis (PCA) suggested that leaf dimensions, fruit weight, stone weight, and stone volume could be sufficient for identification of genotypes. Hierarchical cluster analysis classified sour cherry genotypes and "Ciganymeggy" and "Erdi botermo" cultivars into two main clusters. The first cluster was characterized by a upright tree vigour, depressed fruit pistil end, reniform shape of fruit, high sweetness, dark red juice, flower high length and diameter, fruit and stone weight and length and diameter, total soluble solid, low total phenolic content, high total flavonoid content and high total anthocyanin content.

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1. Introduction

Sour cherry, *Prunus cerasus* L., is known as tetraploid (2n =4x = 32), originated through natural hybridization of the large statured, cold sensitive sweet cherry (*P. avium* L., 2n = 2x = 16), and the low growing, cold tolerant ground cherry (*P. fruticosa* Pall., 2n = 4x = 32) (Olden and Nybom, 1968). This species originated around the Black and Caspian Seas and were cultivated in temperate and cold regions. Sour cherry spread slowly from its origin to other regions due to human and animal migrations (Pérez-Sánchez *et al.,* 2008). Sour cherry fruit is mostly used for industrial preserves (jams, purees, juices and concentrates), while only a small portion is assigned to fresh consumption. Sour cherry is also used as a sweet cherry rootstock. This rootstock is more resistant to soil wetness and cold

climate than wild sweet cherry and mahaleb forms.

In 2014, the total world production of sour cherry reached 1.1 million tons, being Turkey, the Russian Federation, Poland, Ukraine, Iran and Serbia the most important producing countries (Faostat, 2014). The main sour cherry producing areas in Iran are the Ardebil, Azerbaijan, Khorasan and Alborz provinces.

From the viewpoint of fruit quality, several studies for characterization of fruit traits have been accomplished recently. Sour cherry is a valuable source of vitamins (A, B1, B2, C, E, K, and Niacin), carotenoids like beta-carotene, minerals, fiber, various sugar like fructose, glucose, maltose, antioxidant agents such as caffeic acids, cyaniding-3-O-glucosylrutinoside and flavoids (Mulabagal *et al*., 2009; Ferretti *et al*., 2010). This products has positive effects on human health (Ataie-Jafari *et al*., 2008; Saric *et al.*, 2009; Kuehl *et al*., 2010). Analysis of flavonoids from *P. cerasus* identified kaempferol, quercetin, quercetin 3-O-glucoside, and isorhamnetin 3-O-rutinoside (Piccolella *et al.*, 2008). The main anthocyanins found in cherry are cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, cyanidin-3-O-glucosylrutinoside, cyanidin-3-O-sophoroside, pelargonidin-3-O-glucoside, peonidin-3-O-rutinoside and cyanidin-3-O-arabinosylrutinoside (Chaovanalikit and Wrolstad, 2004).

Much of the genetic diversity is available in the wild types and natives from the center of origin. Wild species are probable gene resources for the breeding objectives such as resistance to pests and diseases, more appropriate cultivars for table and industry, extending cherry season and developing new resistant and dwarfing rootstocks. Therefore, it is necessary to characterize and preserve these species (Demirsoy and Demirsoy, 2004; Aliyoun Nazari *et al*., 2012).

Description of the morphological characteristics is the usual methodology accepted from a legal point of view for patenting and registration of varieties. Several quantitative and qualitative evaluations showed a clear difference between sour cherry, with a more marked variability within the sour cherries group, probably due to the more intense domestication processes that have taken place. Morphological characterization continues to be the first step for germplasm description and classification, and the statistical method of factor analysis is a useful tool for screening the accessions of a collection (Badenes *et al*., 2000; Hajilou and Fakhimrezaei, 2011). Several morphological characterization studies have been carried on the sour cherry (Krahl *et al*., 1991; Rodrigues *et al*., 2008; Rakonjac *et al*., 2010; Najafzadeh *et al*., 2014).

As an origin of the subgenus *Cerasus*, Iran has a rich cherry germplasm. The area of this study is part of a large growing area in North West of Iran. Sour cherry has been cultivated in this area for many years. However, the conservation and characterization of local cultivars is important to avoid the loss of genetic variability and as a potential source of genetic variation for future sweet and sour cherry breeding programs. These genotypes show distinctive agronomic characters such as low susceptibility to fruit cracking, high levels of soluble solids and early fruit maturity. The objective of this study was to survey, identify and characterize sour cherry genotypes existing in the province of East Azerbaijan - Shabestar (Iran) for their later introduction into a germplasm bank.

2. Materials and Methods

Plant materials

The plant material was located on the Shabestar town in west side of the East Azerbaijan province, in north-west of Iran. A total of 15 sour cherry genotypes and two cultivars, “Ciganymeggy” and “Erdi botermo”, were used in this study.

Evaluation of morphological and physicochemical traits

Characterization of vegetal material and fruits was based on sour cherry descriptors developed by the International Union for the Protection of New Varieties of Plants - UPOV (UPOV, 2006). Thirty four morphological (16 qualitative and 18 quantitative) and eight physicochemical traits were recorded as described in Table 1 and 2. In this study, a total of 17 sour cherry genotypes, including 15 local sour cherry genotypes and two cultivars, “Ciganymeggy” and “Erdi botermo”, with three replicates for each genotype were evaluated. The evaluation for morphological characters was based on 30 measurements of each trait.

For the analysis of physicochemical traits, fruits were picked at the commercial maturity stage. All fruits were collected from a single plant, randomly from all cardinally oriented branches with different directions around the canopy. All samples were stored in a freezer at -20°C. The frozen fruit material (5 g) was homogenized with a polytron (2 min on ice) with 10 mL of extraction solution, consisting of 0.5 N HCl in methanol/Milli-Q water (80% v/v). The mixture

Table 1 - Sixteen qualitative traits and their states and codes studied of sour cherry genotypes

No.	Trait	1	2	3	4	5	6	7	9	11
1	Tree vigour	Very weak		weak		medium		strong	Very strong	
2	Tree habit	upright	Semi-upright	spreading	drooping					
3	Tree branching			weak		medium		strong		
4	Tree bud distribution	along entire branch	only on middle distal part of branch	only on distal part of branch						
5	Flower arrengment of petal	free	intermediate	overlapping						
6	Flower shape of petal	circular	medium obovate	broad obovate						
7	Flower arrangement	solitary	double	In clusters	irregular					
8	Starting bloom from April	9-11 day		11-13 day		13-15 day		15-17 day	17-19 day	›19 day
9	Fruit ripening time from June	5-10 day		10-15 day		15-20 day		20-25 day	25-30 day	› 30 day
10	Fruit pistil end	pointed	flat	depressed						
11	Stone shape	narrow elliptic	broad elliptic	circular						
12	Fruit shape	reniform	oblate	circular	elliptic					
13	Fruit color of skin	Orange red	Light red	Medium red	Dark red	Brown red	blackish			
14	Fruit color of flesh	yellowish	pink	Medium red	Dark red					
15	Fruit sweetness			low		medium		high		
16	Color of juice	Colorless	Light yellow	pink	Medium red	Dark red				

Table 2 - The range of 26 quantity variability in Sour cherry genotypes traits, mean and coefficient of variations (CV %)

No.	Trait	Unit	Min	Max	Mean	CV (%)
1	Flower diametr	Mm	23.5	39.5	28.1	12.4
2	Petal length	Mm	10.8	13.7	11.9	8.1
3	Petal width	Mm	9.6	14.0	11.6	11.9
4	Pestil length	Mm	11.4	13.6	12.4	5.1
5	Number of stamens	-	31.9	37.0	34.5	4.1
6	Fruit length	Mm	13.5	19.1	15.2	8.8
7	Fruit diameter	Mm	13.1	22.6	17.6	10.9
8	Fruit length/ diameter	-mm	0.8	1.0	0.9	6.1
9	Length of stalk	Mm	41.0	54.0	46.8	7.3
10	Fruit weight	Gr	12.4	54.3	21.4	55.7
11	Stone length	Mm	5.6	9.5	7.1	11.7
12	Stone diameter	Mm	5.5	9.0	7.4	13.2
13	Stone volume	cm^3	0.1	0.4	0.2	36.0
14	Stone weight	Gr	2.2	3.5	3.0	13.5
15	Leaf blade length	Mm	67.0	96.8	78.9	10.2
16	Leaf blade width	Mm	36.8	53.4	43.4	9.8
17	Leaf blade length/ blade width	-	1.7	2.0	1.8	4.0
18	Petiole length	mm	13.6	18.4	16.2	8.2
19	pH	-	2.0	3.6	3.3	13.9
20	Total soluble solid	%	12.1	23.3	16.8	20.9
21	Vitamin C	mg/100g FW	10.5	13.2	11.7	7.7
22	Titratable acidity	%	1.9	2.7	2.3	9.0
23	Total phenolic content	mg GAE/100 g FW	228.0	289.0	241.8	6.0
24	Total anthocyanin content	mg cyanidin 3-glucoside /100 g FW	60.1	130.3	98.0	21.5
25	Antioxidant activity	µg TE/ 100g FW	52.3	67.7	60.5	7.5
26	Total flavonoids content	mg QE/ 100 g FW	141.8	155.8	145.8	2.5

was incubated overnight at 4°C and then centrifuged for 20 min at 4°C and 20000 g. Supernatant was recovered and the volume measured. This hydroalcoholic extract was used for total phenolics, anthocyanins, flavonoids, and antioxidant capacity assays (Cantin *et al.,* 2009). The content of phenolic compounds in methanol extracts was determined according to the Folin-Ciocalteu method (Waterhouse, 2001). Absorbance was measured at 725 nm using a spectrophotometer (UV-2100 SPECTROPHOTOMETER). Total anthocyanin content (TAC) was determined using the pH differential method (Giusti and

Wrolstad, 2001). The absorbances of the extracts at 510 and 700 nm were measured against a blank. TAC was calculated and expressed as mg cyanidin 3-glucoside equivalent/100 g of FW. Total flavonoid content of each extract was determined following colorimetric method (Chang *et al*., 2002). The antioxidant capacity was measured using the DPPH method adapted from Brand-Williams *et al.* (1995). Titratable acidity was established by titration with 0.1 N NaOH and sugar content was measured as total soluble solids (TSS) using digital refractometer (Atago PR 100, Japan). Vitamin C content was estimated according to the titration with 2, 6-Dichlorophenolindophenol method (AOAC, 2000).

Statistical analysis

Statistical analysis were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). To obtain basic statistics for the entire plant material studied, maximum and minimum values, mean, and coefficients of variation (CV %) were calculated for each trait. Relationships among the species were investigated by principal component analysis (PCA). PCA was performed using SPSS statistics software. Scatter plot of the first two PCs and the cluster analysis were created by PAST statistics software (Hammer *et al*., 2001).

3. Results and Discussion

Characteristics of cultivars

Several researchers have reported the morphological variation between some *Prunus* subgenus *Cerasus* genotypes such as for sweet cherry (*P. avium*), sour cherry (*P. cerasus*), mahaleb (*P. mahaleb),* marmareh (*P. incana*) and tomentosa cherry (*P. tomentosa*) (Ganji-Moghadam and Khalighi 2007; Khadivi-Khub *et al*., 2008; Perez-Sanchez *et al.*, 2008; Zhang *et al*., 2008; Rakonjac *et al*., 2010; Aliyoun Nazari *et al*., 2012).

Morphological characteristics of the studied genotypes are resumed in the Table 1 and 2. Results showed that high variation among studied genotypes was found for fruit weight (CV=55.7%) and stone volume traits (CV=36%). This result is compatible with Zhang *et al.* (2008) report. They observed high morphological variation among populations, where the highest variations were in fruit weight, fruit width, and leaf width. Tree habits of the studied genotypes are different. Most of the genotypes have drooping, three have spreading and one has upright tree habit ("Erdi botermo").

TPC values ranged between 228 and 289 mg GAE/100 g FW of sour cherry genotypes, which is in good agreement with previously published results (Dragovic-Uzelac *et al.,* 2007; Khoo *et al.,* 2011; Alrgei *et al*., 2015). "Erdi botermo" had the lowest TPC among the studied genotypes and N02 had the highest, that is consistent with the results of Papp *et al.* (2010) that reported the "Erdi botermo" have lowest TPC among all tested genotypes. Behrangi *et al.* (2015) reported that TPC is versatile on the basis of fruit type, stage of growth, farm of landing, extraction method, component of TPC experiment and other factors. Therefore TPC decreased by transforming fruit from first stages of growth to fully ripe form that is compatible with our results.

As shown in Table 2, total antioxidant capacity (AC) of sour cherries was between 52.3 and 67.7 µg TE/100 g FW. The total AC of different sour cherry cultivars showed significant difference (Blando *et al.,* 2004; Bonerz *et al.,* 2007; Khoo *et al.,* 2011). The lowest TAC was found in N02 genotype (60.1 mg cyanidin 3-glucoside/100 g FW), while "Erdi botermo" had the highest TAC (130.3 mg cyanidin 3-glucoside/ 100 g FW). These differences in TAC showed that the plant growth region and the harvest period might have an impact on plant growth and metabolite concentration (Premier, 2002). Sour cherry is one of the richest source of flavonoid (Marinova *et al.,* 2005), that is consistent with our results.

Principals component analysis

Eighty percent of the variability observed was explained by seven components (Table 3). For each trait, a factor loading of more than 0.51 was considered as being significant. PC1 represents mainly fruit pistil end, fruit color of skin, fruit length, fruit diameter, fruit weight, stone length, stone diameter, stone volume, leaf blade length, leaf blade width and total flavonoids content with significant positive effects, also tree habit, flower shape of petal, fruit ripening time with negative effects and account for 29.65% of the variance. The second principals component with 13.4% of total variance included traits of the tree branching, vitamin C, titratable acidity with negative impacts and the trait of flower diameter, petal length, petal width and stone weight with positive impacts. High absolute values of the correlations between variables related to the growth, fruit and leaf size, and PC1 or PC2 were also established by Krahl *et al.* (1991) and Rakonjac *et al.* (2010) in sour cherry, by Lacis *et al.* (2010) and Rakonjac *et al.* (2014) in sweet cherry, Aliyoun Nazari *et al.* (2012) in

marmareh (*P. incana*) and by Khadivi-khub *et al.* (2012) in *Prunus* subgen. *Cerasus*. PC3 was correlated with starting bloom, stone shape, pistil length, fruit length/diameter, pH and length of stalk. The remaining components explain less variability.

Grouping of cultivars

Hierarchical cluster analysis classified native sour cherry genotypes and "Ciganymeggy" and "Erdi botermo" cultivars in two main clusters (Fig. 1). The first major cluster is divided into two subgroups; subgroups I consisted of Erdi botermo cultivar and and subgroup II contained Ciganymeggy and some of the genotypes, indicating that these sour cherry genotype had high similarity to Ciganymeggy cultivar. The second cluster included the native genotypes. The

Table 3 - Eigen values and cumulative variance for seven major factors obtained from principal component analysis (PCA) and traits within each factor for sour cherry genotypes

Trait	Factors						
	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Tree vigour	-0.10	0.32	0.12	-0.62**	0.02	0.30	-0.52
Tree habit	-0.87**	0.05	0.19	0.14	0.14	-0.15	0.00
Tree branching	0.14	-0.74**	0.21	0.21	0.02	-0.24	-0.08
Tree bud distribution	0.32	0.02	0.15	-0.60**	-0.04	-0.29	-0.39
Flower arrangement of petal	0.32	-0.34	0.35	-0.02	-0.14	0.39	0.32
Flower shape of petal	-0.90**	0.10	0.19	-0.03	-0.19	-0.20	-0.04
Flower arranement	-0.36	-0.39	0.18	-0.22	0.13	0.64**	-0.22
Starting bloom from April	0.02	-0.15	0.61**	-0.24	-0.48	0.14	0.27
Fruit ripening time from June	-0.87**	0.08	-0.10	0.15	0.20	0.02	-0.02
Fruit pistil end	0.81**	-0.13	-0.02	-0.29	-0.18	-0.13	-0.04
Stone shape	0.18	0.34	0.62**	0.50	0.02	0.26	0.07
Fruit shape	-0.83**	0.22	0.26	0.25	0.07	-0.05	-0.06
Fruit color of skin	0.80**	-0.12	-0.27	0.10	0.34	0.11	0.09
Fruit color of flesh	0.16	-0.50	0.14	0.26	-0.55**	0.26	-0.06
Fruit sweetness	0.63**	0.02	-0.51	-0.08	0.28	0.15	0.16
Color of juice	0.90**	-0.10	-0.19	0.03	0.19	0.20	0.04
Flower diameter	-0.28	0.71**	-0.15	0.17	0.05	0.15	0.39
Petal length	-0.10	0.77**	-0.14	0.32	-0.32	-0.03	0.15
Petal width	-0.10	0.70**	-0.12	0.14	-0.48	0.29	0.06
Pestil length	-0.18	0.08	0.70**	0.06	-0.36	0.02	-0.38
Number of stamens	-0.21	0.11	-0.46	0.46	0.40	-0.02	-0.19
Fruit length	0.90**	0.33	0.09	0.03	0.04	-0.11	-0.07
Fruit diameter	0.81**	0.23	-0.19	0.22	-0.13	-0.18	-0.15
Fruit length/diameter	-0.15	0.01	0.52**	-0.38	0.33	0.24	0.28
Length of stalk	0.01	-0.02	0.59**	-0.13	0.40	0.07	0.11
Fruit weight	0.86**	0.16	0.21	-0.14	0.28	-0.19	0.07
Stone length	0.89**	0.11	0.25	0.14	-0.10	-0.03	0.09
Stone diameter	0.60**	-0.20	0.47	-0.02	-0.23	-0.34	0.05
Stone volume	0.88**	-0.05	0.31	0.01	-0.17	-0.16	0.09
Stone weight	0.35	0.77**	0.26	-0.03	0.13	0.08	-0.05
Leaf blade length	0.64**	0.50	0.11	-0.17	0.01	0.17	-0.28
Leaf blade width	0.64**	0.41	0.03	-0.09	0.07	0.34	-0.49
Leaf blade length/leaf blade widht	0.05	0.49	0.21	-0.23	-0.13	-0.39	0.43
Petiol length	0.38	0.14	-0.39	-0.13	-0.12	0.18	0.54**
Total soluble solid	0.47	-0.17	0.31	0.45	0.45	-0.16	-0.13
pH	0.12	0.31	0.67**	0.59	0.09	0.25	0.01
Vitam C	-0.05	-0.56**	-0.13	-0.09	-0.15	0.07	0.19
Titratable acidity	0.21	-0.51**	0.47	0.04	0.39	0.21	0.31
Total phenol content	-0.10	0.14	0.40	0.12	0.27	-0.67**	-0.07
Total anthocyanin content	0.51	-0.20	-0.24	0.20	-0.59**	-0.12	0.00
Antioxidant activity	0.12	0.36	0.01	-0.42	0.34	-0.08	0.28
Total flavonoids content	0.61**	-0.32	-0.24	0.50	0.08	0.09	-0.25
Eigen value	12.45	5.63	4.69	3.32	2.42	1.43	1.18
Cumulative Variance (%)	29.65	43.06	54.24	62.16	69.30	75.11	80.54

first cluster were characterized by a upright tree vigour, depressed fruit pistil end, reniform shape of fruit, high sweetness, dark red juice, flower high length and diameter, fruit and stone weight and length and diameter, total soluble solid, low total phenolic content, high total flavonoid content and high total anthocyanin content. Perez-Sanchez *et al.* (2008) suggested that dendrogram gained from morphological characteristics clearly showed the relationships among the cultivars of sweet, sour and duke cherries. In addition, Khadivi-khub *et al.* (2012) reported that dendrogram obtained from morphological characteristics clearly separated some *Cerasus* genotypes. The second cluster were characterized by small fruit and stone, drooping or spreading tree habit.

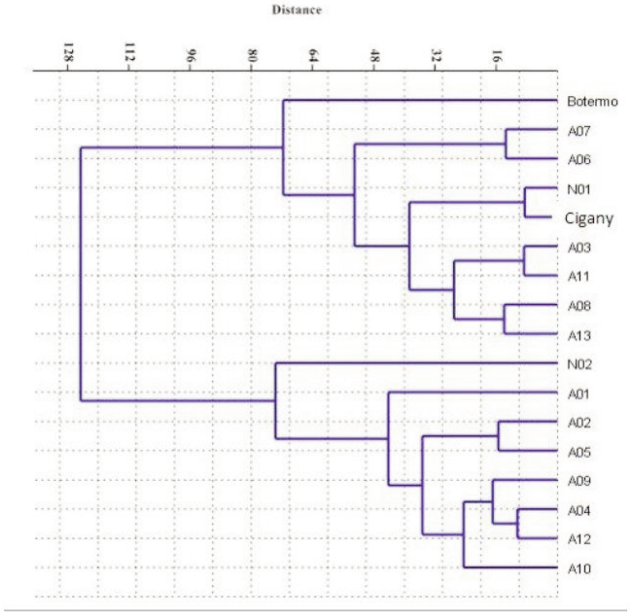

Fig. 1 - Dendrogram of 17 sour cherry genotypes based on morphological traits by PAST software.

Scatter plot was prepared according to the PC1 and PC2 by PAST software (Fig. 2). Starting from the positive to the negative values of PC1, these genotypes indicated a gradual decrease in fruit pistil end, fruit color of skin, fruit length, fruit diameter, fruit weight, stone length, stone diameter, stone volume, leaf blade length, leaf blade width and total flavonoids and an increase in tree habit, flower shape of petal, fruit ripening time. Starting from the negative towards the positive values of PC2, the genotypes indicated a gradual increase tree branching, vitamin C, titratable acidity and a decrease flower diameter, petal length, petal width and stone weight.

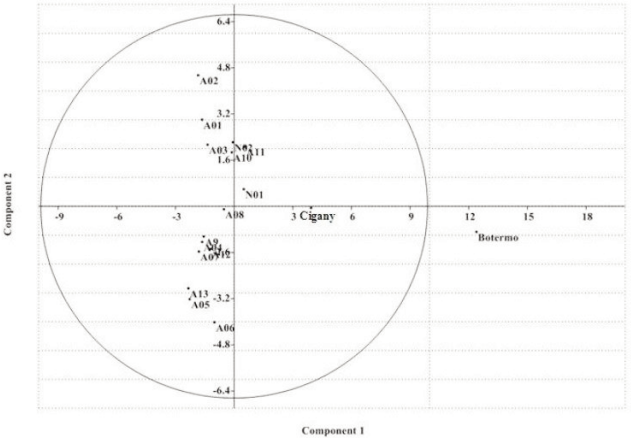

Fig. 2 - Factor scores for the first two principle components (PCs) for Sour cherry genotypes.

4. Conclusions

Morphological characterization continues to be the first step for the description and classification of germplasm and statistical methods like principal components analysis (PCA) are useful tools for screening the accessions of a collection (Cantini *et al.,* 1999; Badenes *et al.,* 2000). PCA is used for data reduction that transforms the original variables into a limited number of uncorrelated new variables. This technique, producing a smaller set of composite variables, account for much of the variance among the set of original variables and allows visualization of the differences among the individuals, identification of possible groups and finding relationships among individuals and variables (Martinez-Calvo *et al.,* 2008). High correlations were found between some traits and principal components, which could reduce the number of traits to be studied in sour cherry germplasm. For instance, measuring the traits of PC1 (such as FW, SL, SD. SV, FPE, and FCS) is suggested for future studied in sour cherry genotypes. Dependent on the trait, a certain number of genotypes were observed that showed lower or higher values than the commercially grown cultivars involved in this study. Especially in reference to the fruit weight, a high portion of genotypes were characterized by smaller fruits than that of “Ciganymeggy” and “Erdi botermo”. In addition, native genotypes showed higher values of total phenolic content and antioxidant activity traits than the commercial cultivars.

We conclude that this is the first study of sour cherry native genotypes, which deals with the morphological and physicochemical variation basis of genetic diversity. Although these accessions does not

represent the whole sour cherry germplasm in Iran, considerable genetic diversity observed in both morphological and physicochemical characteristics indicate rich and valuable plant material for sour cherry improvement.

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The responses of enzymatic and non-enzymatic antioxidant systems of scion on different rootstocks under water stress deficit

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Abstract: Selecting of the specific type of rootstock is an appropriate and short-term method for increasing drought tolerance by improving the antioxidant systems in plants. In this research, the responses of the antioxidant systems of fig scion 'Sabz' were investigated on various rootstocks at different irrigation levels. Graft combinations were 'Sabz' on 'Sabz' (Sa/Sa), 'Siah' (Sa/Si) and 'Torch' (Sa/T) rootstocks, plus 'Sabz', 'Siah' and 'Torch' cultivars with no grafting, for a total of six groups. The plants were irrigated with 4 levels of 25, 50, 75 and 100% of water requirement (WR) for a duration of 12 weeks. The experiment was performed in a randomized complete design with 5 replications per treatment. The results showed that the 'Torch' rootstock induced the greatest amount of anthocyanin, glutathione and ascorbic acid in 'Sabz' (Sa/T) at 25% WR. Superoxide dismutase and catalase activities of 'Sabz' grafted on 'Siah' were more evidence compared to 'Sabz' grafted on 'Torch' rootstock at 25% of WR. The cv. Sabz grafted on both 'Siah' and 'Torch' rootstocks indicated higher chlorophyll content, chlorophyll stability index and shoot growth than cv. Sabz with no grafting. As a result, both rootstocks (T and Si) with the activation of enzymatic and non-enzymatic antioxidant systems caused the scion protects its integrity and be able to tolerate more water stress.

1. Introduction

Water scarcity is one of the most important environmental stresses, with its greatest impact on agriculture worldwide, especially in arid and semiarid regions (Knapp *et al.*, 2001; Alizadeh *et al.*, 2011). In recent years, the issue of climate change, combined with global warming, has been a major contributor to the increased water scarcity and plant losses in many parts of the world (Kramer and Boyer, 1995). Since most of a plant's processes are directly or indirectly affected by water, it is clear that most plants are affected by moderate to long-lasting drought throughout their life cycles (Bhattacharjee and Saha, 2014). The effects of drought stress on plants depend on genotype, rate and severity of the stress, age and stage of plants growth and development (Rostami and

Rahemi, 2013). Plant tolerance to water stress depends on their morphological, physiological and biochemical mechanisms that determine the responses of the plant under stress conditions (Penella *et al.*, 2014).

The fig (*Ficus carica* L.) belongs to the Moraceae family, a perennial fruit tree, which generally has a high tolerance to water deficit (Faghih and Sabet Sarvestani, 2001). This tree is managed in two ways, irrigated or rain-fed, and is cultivated in many parts of the world with diverse climatic conditions such as Iran, Turkey and other Mediterranean countries. The total area in the world cultivated with figs is reported to be about 388368 hectares with a production of 918813 tons per year (El-Shazly *et al.,* 2014). Iran, with an annual production of more than 76414 tons of fig, ranks fourth in the world (FAO, 2012). Most fig trees in Iran are cultivated in Estahban, a semi-arid region in the southeast of Fars province (Shirbani *et al.,* 2013).

Drought stress stimulates the accumulation of active oxygen species in plants. These reactive oxygen species are active forms of the oxygen molecule that are produced by the excitation of an oxygen molecule, or the transfer of one, two or three electrons to oxygen molecules, which ultimately lead to the formation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (Gill and Tuteja, 2003) These reactive oxygen species may be the first step in the degradation of oxidative processes, such as lipid peroxidation, chlorophyll breakdown, protein oxidation, and nucleic acid damage (Anjum *et al.,* 2011; Gu *et al.,* 2013). Plants have specific defense mechanisms that provide protection on the molecular level. These mechanisms comprise non-enzymatic antioxidants, such as ascorbic acid (ASA), glutathione (GSH) and anthocyanin and enzymatic antioxidants include catalase (CAT) and superoxide dismutase (SOD) (Guerfel *et al.,* 2009; Liu *et al.,* 2012 a).

Superoxide dismutase is a major scavenger that counters superoxide damage. Catalase catalyzes the decomposition of the excess of hydrogen peroxide to water and oxygen and protects cells from oxidative damage. These two enzymes play an important role in the degradation of reactive oxygen species (Mittler, 2002). With regard to the climate change models that predict an increase in drought and excessive groundwater depletion in the future, water scarcity appears to be the main limiting factor in agriculture (Alizadeh, 2005). In this regard, the use of drought tolerant rootstocks can play an important role in managing water absorption, water use efficiency, survival potential, growth capacity and the success of grafting in dry conditions.

Different rootstocks show different levels of tolerance in response to drought, and their ability to have high tolerance depend on improving the vegetative growth of the scion, the horizontal and vertical expansion of the roots, the ability to absorb water and minerals and antioxidant system activities for scavenging of active oxygen species (Ballesta *et al.,* 2010; Corso and Bonghi, 2014).

How the rootstock genotypes may be effective in response to water deficit stress has been investigated in many studies on fruit trees, such as apples and grapes (Alizadeh *et al.,* 2011; Liu *et al*., 2012 b; Corso and Bonghi, 2014). Most of these researches focus on the effects of rootstock on vegetative growth, fruit yield and quality, nutrition and hormones, and the scion's water content in response to drought. However, there are limited investigations on how rootstock affects the antioxidant systems of the scion in response to water deficit. The aim of this study was to investigate the effect of different fig rootstocks on the enzymatic and non-enzymatic antioxidant systems of the 'Sabz' scion in response to different levels of irrigation.

2. Materials and Methods

In early November, offshoots semi-hard woodcuttings of different cultivars ('Sabz', 'Siah' and 'Torsh') were disinfected with a commercial bleach (Clorax) solution containing 5% NaOCl and 2% benomyl for 20 min. Then, they were wrapped in a wet cotton cloth and placed at 4°C for 15 days to ensure that their chilling requirement was satisfied. After that, the bottoms of the cuttings treated with IBA solution (1000 mg/L) and cultured in cartonplast boxes containing perlite. They were put in a greenhouse with natural sunlight, relative humidity of 64% and an average temperature of 38/16±2°C day and night respectively.

After 2 weeks, rooted cuttings were transferred to 1 L plastic pots containing a mixture of soil: peat: sand (1:1:1, v/v/v). After 8 weeks, when the length of the new shoots reached 20 cm, cultivar Sabz as scion were grafted (cleft grafting) onto the three rootstocks ('Sabz', 'Siah' and 'Torsh'). The place of grafting was firmly enclosed with cellophane. To maintain moisture, grafted plants were covered with plastic bags to ensure grafting success. After 15 days, the plastic bags were removed and the cellophanes were

opened. Subsequently, all grafted and non-grafted plants (controls) were transferred to 10 L pots without drainage that contained the aforementioned ratio of the soil mixture. The field capacity of the soil used for potting was determined according to the protocol described by Richards (1949). The pots were irrigated daily at field capacity by the help of a balance. After complete deployment, the experiment was carried out by selecting 40 plants of each grafted combination ('Sabz'/ 'Sabz', 'Sabz'/ 'Siah', 'Sabz'/ 'Torsh') and 40 plants from cultivars with no grafting ('Sabz', 'Siah' and 'Torsh') in a randomized complete design with 4 levels of irrigation 100%, 75%, 50% and 25% of water requirement (WR). Three months after the start of treatments, various indices were measured as follows.

Anthocyanin

Extraction solution containing methanol, water and concentrated chloric acid (HCl) was prepared by a ratio of 80: 20: 1. Leaf samples were kept in the extract solution at 4°C in the dark for 48 h. Then, the extract passed through a Whatman filter paper (No. 1) and was read at 530 and 657 nm. Anthocyanin content was measured using the microplate reader spectrophotometer according to the method described by Alexieva *et al.* (2001).

The amount of anthocyanin was calculated as µg g^{-1} fresh weight.

Glutathione

The method used by Moron *et al.* (1979) was followed to measure glutathione concentration. An amount of 200 mg of fresh leaves was homogenized with 2 ml of cooled Trichloroacetic Acid (TCA) (5%), and they were centrifuged for 30 min at 15,000 rpm at 4°C. From supernatant extract, 75 µl was transferred to a vial containing 300 µl sodium phosphate buffer (0.2 M, pH 8) and 750 µl DTNB (5, 5-di-tiobis-2-nitrobenzoic acid) 0.6 mM. The extract was read at 412 nm by a spectrophotometer microplate reader. Glutathione concentration was calculated using standard curve.

Ascorbic acid

To determine the concentration of ascorbic acid, 1 g of fresh leaf tissue was thoroughly crushed in 5 ml of TCA (10% cold), and then centrifuged at 3500 rpm for 20 min. The supernatant was isolated and diluted to reach 10 ml. One ml of aqueous extract was mixed with 0.2 ml of DTC reagent (2, 4-di-nitrohydrazine, thiourea, copper sulfate) and was incubated at 37°C for 3 h. Then, 1.5 ml of sulfuric acid (65%) was added and mixed thoroughly. The extract was allowed to stand at room temperature for 20 min. The absorbance was read at 520 nm using a spectrophotometer. The ascorbic acid was calculated in µg g^{-1} F.W. (Elavarthi and Martin, 2010).

Antioxidant enzymes

To prepare leaf extract containing enzymatic antioxidant, 200 mg of fresh leaf tissue was ground with liquid nitrogen in a mortar. Then, 1.2 ml of K_2PO_4 buffer 0.2 M (pH 7.8) containing 0.1 mM EDTA (Ethylenediaminetetraacetic acid, $C_{10}H_{16}N_2O_8$) was added to the samples and homogenized. The samples were centrifuged at 15,000 *g* for 20 min at 4°C. The supernatants were separated and then the same operation was repeated on residual. The resulting extract was used for determining the activity of antioxidant enzymes (Elavarthi and Martin, 2010).

Catalase activity

Catalase activity was determined by the method of Aebi (1984) as described by Elavarthi and Martin (2010) by measuring the amount of hydrogen peroxide (H_2O_2) degradation via reducing the absorbance at 240 nm with a spectrophotometer microplate reader. To this purpose, 3 ml of the reaction mixture was made up of 2 ml of leaf extract, diluted with 50 mM K_2SO_4 buffer at pH=7 and H_2O_2 10 mM, which reached 3 ml by distilled water, the reaction started and the absorbance of the samples were recorded for 1 min.

Superoxide dismutase

Superoxide dismutase activity (SOD) was evaluated using a modified nitro-blue tetrazolium (NBT) method (Elavarthi and Martin, 2010). Accordingly, 2 ml of the reaction mixture including 50 mM phosphate buffer (pH= 7.8), 2 mM EDTA, 9.9 mM methionine, 55 µM NBT (Nitro blue tetrazolium chloride, $C_{10}H3_{30}Cl_2N_{10}O_6$), 0.025% triton X-100 to was added to 40 µl of diluted sample (×2) and then, added 20 µl of Riboflavin (1 mM). The reaction began by exposing the samples under a fluorescent tube (15 Watts) for 10 min. The blank received the same chemical mixture but without leaf samples throughout the steps. The absorbance of the specimens was read at 560 nm in a microplate reader spectrophotometer and one unit of enzyme activity was taken as the quantity of enzyme, which reduced the absorbance reading of the sample to 50% in comparison to control. Finally, SOD was calculated as U g^{-1} fresh weight.

Chlorophyll stability index

Chlorophyll stability index (CSI) was measured

according to the method used by Murty and Majumber (1962). The fresh leaf samples were emerged in 20 ml of distilled water and placed in a warm water bath of 56±1°C for 30 min, and the chlorophyll contents of the samples were determined. The chlorophyll stability index was obtained from the following formula.

CSI (%)= 1-(chlorophyll content without heating/chlorophyll content after heating) × 100.

Chlorophyll content

Total chlorophyll, chlorophyll a and chlorophyll b content of leaves were measured using the Dimethyl sulfoxide (DMSO) method introduced by Hiscox and Israelstam (1979). Leaf samples (100 mg fresh leaf) were submerged in 7 ml of dimethyl sulfoxide solution and put in the dark for 17 h. After, they were incubated in an oven at 60°C. By adding 3 ml of dimethyl sulfoxide to the samples, the volume was adjusted to 10 ml. Then, 200 µl of the samples extract were transferred to a plate and read by microplate reader spectrophotometry at 633 and 645 nm wavelengths. The following formulas were used in order to determine the total chlorophyll concentration as mg g^{-1} fresh weight (FW).

$$Ch_a \text{ (mg g}^{-1}\text{ FW)} = \frac{12.7(A663) - 2.69(A645) \times \text{volume made}}{\text{w.t of the sample} \times 10}$$

$$Ch_b \text{ (mg g}^{-1}\text{ FW)} = \frac{22.9(A645) - 4.68(A663) \times \text{volume made}}{\text{w.t of the sample} \times 10}$$

$$TCh \text{ (mg g}^{-1}\text{ FW)} = \frac{20.2(A645) + 8.02(A663) \times \text{volume made}}{\text{w.t of the sample} \times 10}$$

In these formulas, Ch_a represents chlorophyll a, Ch_b = chlorophyll b, TCh = total chlorophyll; A = absorbance in the wavelength (nm).

Cell membrane injury

The cell membrane damage index, measured by electrolyte leakage, is an indicator of estimating the tolerance of cellular protoplasts and the ability of the membrane to maintain integrity under conditions of water scarcity (Bajji *et al.*, 2002). This index was measured using the method followed by Kocheva and Georgiev (2003). A punching machine was used in order to prepare leaf samples measuring 1 cm in diameter. The leaf sample discs of 1 cm in diameter were washed three times with distilled water to remove surface contamination. Then they were transferred to vials containing 20 ml of deionized water and kept at 10°C for 24 h. After measuring their electrical conductivity, they were placed in an autoclave of 120°C for 15 min, after cooling at 25°C, the electrical conductivity was re-measured. Cell membrane damage (CMI) was obtained from the following formula.

$$CMI\ (\%) = [1-(T1/T2)] / [1-(C1/C2)] \times 100$$

T and C are the stressed and control samples, respectively, while 1 and 2 are the primary and secondary EC measurements.

The experiment had a factorial layout, based on 4 irrigation levels and 6 combinations of grafted and non-grafted plants in a randomized complete design with 5 replications and 2 plants per replicate. Data analysis was performed using SAS software version 9.3 and Duncan's Multiple Range Test at 5% of probability was used for the means comparison.

3. Results

Analysis of variance showed that the interaction between cultivars and different levels of irrigation were significant on all measured traits except anthocyanin (Table 1).

Anthocyanin

The results showed that increasing drought stress from 100% to 25% of WR the anthocyanin level increased in all grafted and non-grafted plants (Fig.

Table 1 - The variance analysis of some traits of fig cultivars under water stress condition

Source of variation	df	Mean square				
		SOD	CAT	Ant	GSH	ASC
Cultivar (A)	5	0.0259 **	10.9 **	0.008 **	14.8 **	68.3 **
Water stress (B)	3	0.4569 **	5.5 **	0.014 **	3.8 **	42.2 **
A × B	15	0.0293 **	2.3 **	0.0001 ns	3.3 **	15.8 **
Error	96	0.0028	0.044	0.0001	0.55	1.29
C.V.	-	3.63	19.33	14.07	22.41	10.64

SOD= Superoxide dismutase; CAT= catalase; GSH= glutathione; ASC= ascorbic acid; Ant= Anthocyanin.
**significant at 1% probability, NS; non-significant.

1A). A comparison of cultivars with no grafting ('Sabz', 'Siah', and 'Torsh') showed that 'Torsh' and 'Siah' had anthocyanin levels higher than the amount found in the 'Sabz'. However, 'Torsh' rootstock increased the amount of the anthocyanins in the scion ('Sabz') compared to the non-grafted 'Sabz' cultivar (Fig. 1B).

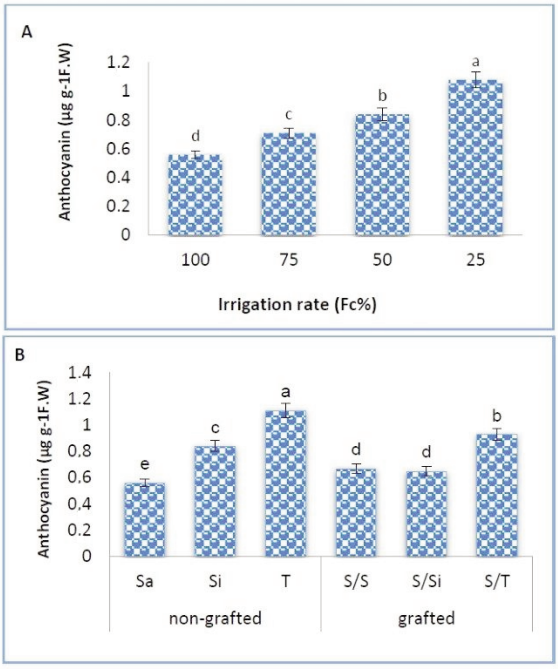

Fig. 1 - Main effects of irrigation levels (A) and grafted and non-grafted cultivars (B) on leaf anthocyanin rate. Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Glutathione

The amount of glutathione in the leaves of non-grafted 'Torsh' increased with increasing the stress intensity from 100% to 25% of WR, while the 'Siah' cultivar reduced the glutathione significantly. The decrease in glutathione concentration in the leaf of the 'Sabz' non-grafted and the 'Sabz'/'Sabz' combination was not significant with increasing drought stress (Fig. 2A). Furthermore, both 'Siah' and 'Torsh' rootstocks significantly increased the glutathione level of the 'Sabz' scion at 75% of WR. However, there was no a significant difference in glutathione rate between 75%, 50% and 25% of WR in Sa/To combination.

Ascorbic acid

The results showed with increasing stress from 100% to 25% of WR, no increase in ascorbic acid content was observed in non-grafted 'Siah' and 'Sabz' cultivars. The non-grafted 'Torsh' showed an increase of 47.63% in the ascorbic acid content in the severe water stress compared with control. The 'Sabz' and 'Siah' rootstocks reduced the ascorbic acid content of the 'Sabz' scion at 50% and 25% of WR, compared to the non-grafted 'Sabz'. Nonetheless, 'Torsh' rootstock also reduced the ascorbic acid content of the 'Sabz' scion when 100% of WR was supplied, but this rootstock caused a significant increase in the ascorbic acid content (15.55%) of the 'Sabz' scion in the 25% of WR (Fig. 2B).

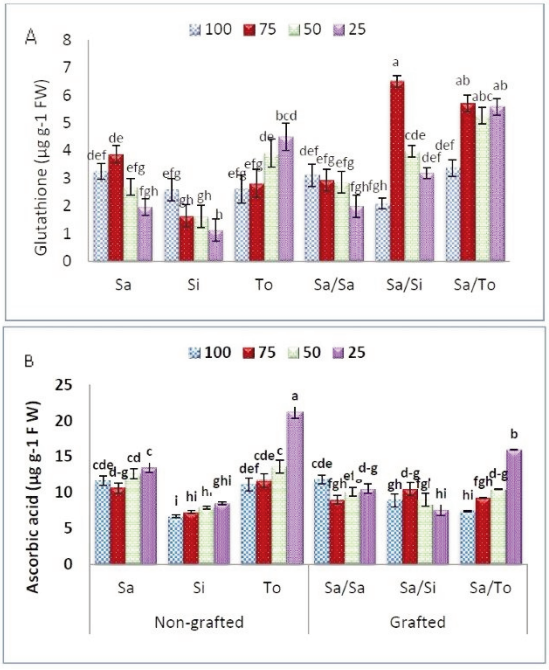

Fig. 2 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50, 25% WR) on: Glutathione (A) and Ascorbic acid (B). Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Superoxide dismutase

Superoxide dismutase activity increased with increasing drought stress in all non-grafted and grafted cultivars (Fig. 3A). Comparison of cultivars with no grafting showed that 'Siah' at 50% of WR had the highest enzyme activity compared with 'Torsh' and 'Sabz' cultivars. At 25% of WR, the SOD activity in the 'Sabz' rootstock was 1.78 times higher than the 'Siah' rootstock, and in the 'Siah' rootstock it was 1.63 times higher than in the 'Torsh' rootstock. Each of the three rootstocks reduced the activity of the enzyme compared to the non-grafted 'Sabz' cultivar when supplied with 50% and 25% of WR, although the differences in these values were not statistically significant (Fig. 3A).

Catalase activity

The activity of catalase enzyme increased significantly in all grafted and non-grafted cultivars in

response to intense drought stress (Fig. 3B). Comparison of non-grafted cultivars showed that catalase enzyme activity in 'Torsh' at 75%, 50% and 25% of WR was significantly higher than the two other cultivars ('Sabz' and 'Siah'), which did not differ significantly. However, only 'Siah' rootstock increased the activity of catalase enzymes (by 31.13%) in the 'Sabz' scion when 25% of WR was applied compared to the non-grafted 'Sabz' cultivar.

Analysis of variance showed that the interaction between cultivars and different levels of irrigation were significant on all measured traits (Table 2).

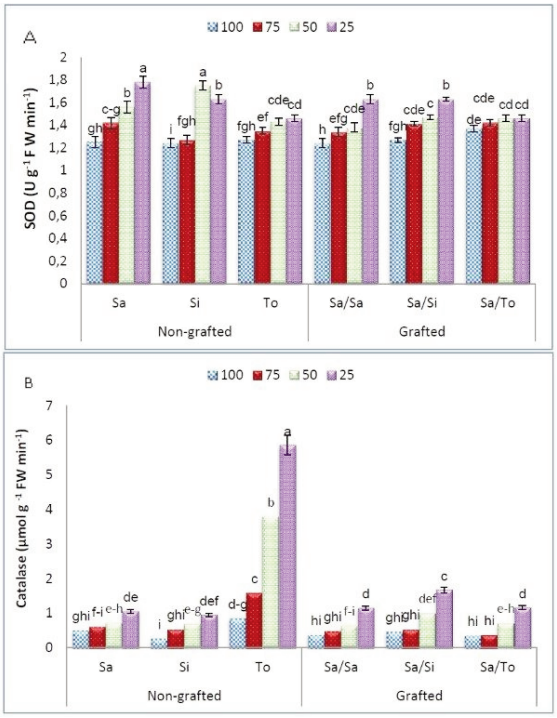

Fig. 3 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50, 25% water requirement: WR) on Superoxide dismutase (SOD) activity (A) and Catalase activity (B). Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Total leaf chlorophyll

The results showed that in non-grafted cultivars, 'Torsh' at 100%, 75% and 50% of WR showed higher chlorophyll contents than the other two cultivars. In grafted rootstocks, 'Siah' and 'Torsh' significantly increased the chlorophyll content in the leaves of the scion 'Sabz' in 100% and 75% of WR. 'Siah' rootstock also increased the chlorophyll content of the 'Sabz' scion by 42.52% in 50% of WR compared with non-grated 'Sabz' (Fig. 4A).

Chlorophyll stability index

The results showed that the chlorophyll stability index decreased as the drought stress intensified

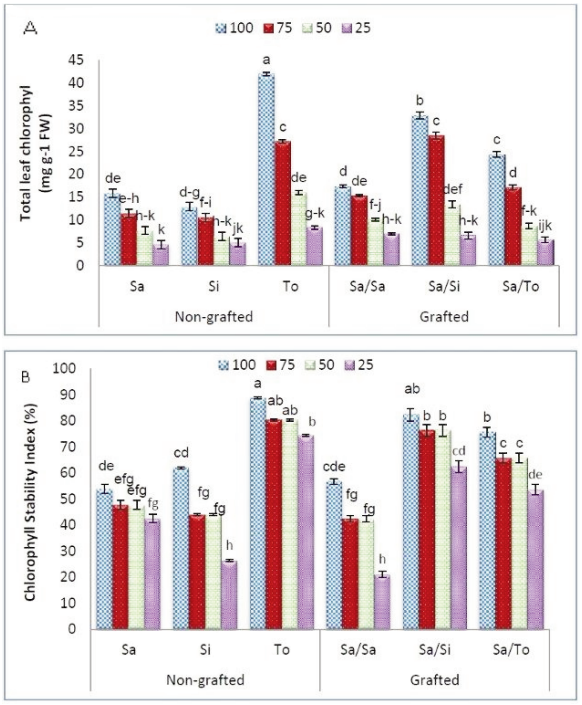

Fig. 4 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50 and 25% WR) on leaf chlorophyll content (A) and chlorophyll stability index (B). Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Table 2 - The variance analysis of total Chl, CSI, CMI, shoot FW and Shoot DW of fig cultivars under water stress condition

Source of variation	df	Mean Square				
		Total Chl	CSI	CMI	Shoot FW	Shoot DW
Cultivar (A)	5	685.6 **	5449.9 **	469.7 **	13355.4 **	1895.5 **
Water stress (B)	3	1963.6 **	8539.5 **	11707.8 **	22405.1 **	2749.35 **
A × B	15	108.2 **	134.7 **	180.4 **	691.71 **	83.24 **
Error	96	12.45	41.75	26.47	119.36	19.38
C.V.	-	23.83	12.45	5.59	13.03	12.77

Chl= chlorophyll; CSI= chlorophyll stability index; CMI= cell membrane injure; FW= fresh weight; DW= dry weight. **significant at 1% probability

(Fig. 4B). Among the non-grafted cultivars, 'Torsh' showed more stability in the leaf chlorophyll content than the other two cultivars at different levels of WR. 'Sabz' scion on the 'Siah' and 'Torsh' rootstocks showed the higher chlorophyll stability than non-grafted 'Sabz' and 'Sabz'/'Sabz' graft combination at all levels of WR (Fig. 4B).

Cell membrane injury

The results showed that high levels of water stress increased leaf cell membrane damage in all grafted and non-grafted plants (Fig. 5). The differences in the damage to the cell membranes of the 'Siah', 'Torsh' and 'Sabz' were not significant at 100% of WR, but the 75% and 50% of WR induced significantly greater damages to the leaf cell membrane of 'Siah' than the other two cultivars. At 25% of WR, the non-grafted 'Sabz' cultivar showed lower levels of membrane damage in its leaf cells, compared to 'Siah' and 'Torsh'. The results also showed that the effect of the 'Sabz', 'Torsh', and 'Siah' rootstocks on the 'Sabz' scion was not significant at all levels of water requirement, compared to the non-grafted 'Sabz' cultivar (Fig. 5).

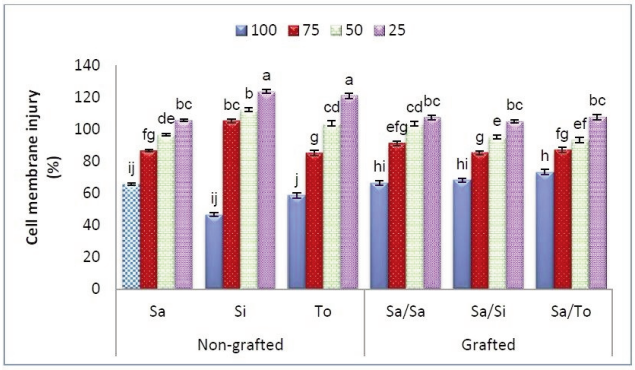

Fig. 5 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50 and 25% of WR) on membrane injury index. Sa= Sabz; Si= Siah; To= Torsh. Values are means ± SE (n=5).

Shoot fresh and dry weight

The results showed that with increasing water stress deficit the shoot growth decreased in all non-grafted and grafted combinations of plants. However, 'Siah' and 'Torsh' rootstocks are associated with higher scion shoot fresh and dry weight compared to non-grafted (Sa) and self-grafted (Sa/Sa) Sabz cultivar (Fig 6. A and B).

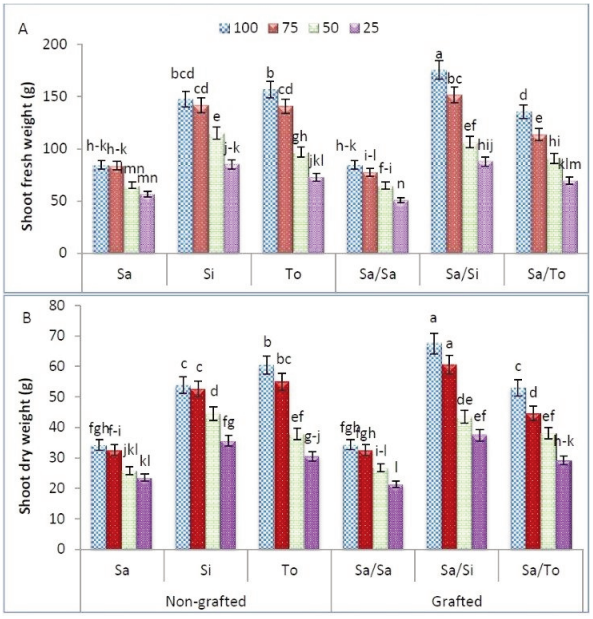

Fig. 6 - Interaction of fig grafted and non-grafted combinations and different levels of irrigation (100, 75, 50 and 25% WR) on shoot fresh weight (A) and shoot dry weight (B). Sa= Sabz; Si= Siah; To= Torsh.

4. Discussion and Conclusions

The increase in anthocyanin content in stressed leaves in this study confirms the protective role of anthocyanin against sunlight and active oxygen species in stressed plants (Gholami *et al.,* 2012 a). Stimulation of anthocyanin production has been proven by osmotic pressure and it is believed (in most cases) that tissues that contain more anthocyanins are more tolerant to drought (Chalker-Scott, 1999). Anthocyanins are able to protect cells against environmental damage through protection of cell membranes, organelles and nucleic acids (Neil *et al.,* 2002).

In this study, 'Torsh' rootstock increased the leaf anthocyanin in the 'Sabz' scion. Accordingly, the type of rootstock can increase the production of anthocyanins and thus counteract the reactive species of oxygen, increasing the ability of the plant to tolerate conditions of water stress.

In the green tissues of plants, ascorbic acid is a major antioxidant soluble in water. Leaf ascorbic acid changes seasonally, but as the leaf ages it remains at a constant level. However, exposure to stress can significantly change this situation and increase it (Sircelj and Batic, 2007). Ascorbic acid, like carotenoids, plays an important role in protecting the photosynthetic system against the harmful effects of reactive

oxygen species (ROS). In this research, the amount of glutathione and ascorbic acid increased in 'Torsh' rootstock, and this effect was transmitted to the 'Sabz' scion, which may be due to the rapid reaction of this rootstock to severe drought stress and the need for glutathione and ascorbic acid, which can confront ROS more efficiently. Even though the changes in glutathione and ascorbic acid were not significant at different levels of irrigation in the non-grafted 'Sabz' cultivar, by grafting the 'Sabz' scion on the 'Siah' rootstock, the glutathione level in the 'Sabz' scion increased significantly in 75% of water requirement and did not show any changes in ascorbic acid. In this regard, Tausz *et al*., (2004) also reported that drought stress leads to a decrease in glutathione concentration, and the oxidation and reduction of compounds ultimately leads to the system's degradation. An increase in the amount of glutathione in conditions of water shortage may be necessary to adjust the level of ascorbic acid in the plant (Gholami *et al*., 2012 b).

It seems that increased levels of glutathione in non-grafted 'Torsh' cultivar and 'Sabz' grafted on 'Torsh' rootstock have been effective in regulating ascorbic acid levels at mild and severe stress levels. Sircelj *et al.* (2005) also reported an increase in the level of glutathione and ascorbic acid at moderate levels, indicating compatibility with oxidative stress in apple trees, and stated that the decrease in glutathione levels at severe stress levels indicates severe oxidative stress.

Many researchers have shown that drought stress leads to oxidative stress in the plant (Mittler, 2002; Gill and Tuteja, 2010; Gholami *et al*., 2012 b; Shirbani *et al*., 2013). Excessive forms of stress can damage the plant by producing reactive oxygen species. The antioxidant defense system and the breakdown of active oxygen species are known to be under dry conditions (Zarafshar *et al.,* 2014). Under mild and moderate water deficit conditions, a number of compatible plant species increase the activity of enzymatic antioxidants such as superoxide dismutase and catalase, although severe drought stress may cause damage to cells by stronger stimulations or impairments via reactive oxygen species that suppress enzymatic antioxidant activity (Guerfel *et al.*, 2008).

Superoxide dismutase and catalase are active enzymes for the elimination and degradation of harmful oxygen species in plants and maintain oxidative equilibrium during oxidative stress (Gill and Tuteja, 2010). Superoxide dismutase enzymes have been reported to play an important role in the antioxidant metabolism of plants under environmental stress conditions, such as water deficit, via regulating their gene expression or activities (Xu *et al.*, 2010). High SOD activity in 'Siah' and 'Sabz' non-grafted cultivars were achieved in 50% and 25% of WR respectively, and the activity of catalase in the non-grafted 'Torsh' cultivar and in the 'Sabz'/'Siah' graft combination (at 25% of WR) were also comparatively high. These results depend on the plant's better protection against oxidative damage caused by water stress. It seems that the scion of the 'Sabz' cultivar on the 'Siah' rootstock led to greater improvements in the antioxidant system.

Chlorophyll content has a positive correlation with the rate of photosynthesis. Therefore, the decrease in chlorophyll content under drought stress condition is a common symptom of oxidative stress, which may be due to photo-oxidation of pigments and chlorophyll degradation (Anjum *et al.,* 2011; Shirbani *et al*., 2013). The reduction of chlorophyll content was observed in all cultivars under stress in this study. According to Guerfel *et al.* (2009) the reduction of chlorophyll content can be attributed to its susceptibility to environmental stresses, especially drought. Chlorophyll levels tend to decrease or otherwise remain unchanged during the period of drought stress in many species. This, however, depends on the duration and severity of the drought (Anjum *et al.*, 2011).

In this study, chlorophyll contents at 75% and 50% of WR decreased less sharply in 'Sabz'/Siah graft combination. This indicates that the light dissipation and antioxidant systems may prevent the degradation of chlorophyll molecules (Niu *et al*., 2008). Sircelj *et al.* (2005) also reported no reduction in the chlorophyll content of apple leaves 'Elastar' under water stress is due to a strong antioxidant system and an efficient light dissipation system. Anjum *et al.* (2011) attributed the decrease in the content of chlorophyll to the chloroplast membrane damage, swelling, lamella distortion and the occurrence of small droplets of lipids under drought stress conditions.

The chlorophyll stability index is an indicator of measuring the membrane's integrity and the pigments stability under stress conditions (Ananthi *et al.*, 2013). Surendar *et al.* (2013) reported that a decrease in chlorophyll content under stress was due to the destruction of the chloroplast membrane with increasing phosphatase activity, which is located on the membrane. In this study, intense water stress (25% of WR) reduced the chlorophyll stability in all grafted and non-grafted rootstocks compared to

100% of WR. This could be due to the degradation of chlorophyll when proteolytic enzymes, such as the chlorophyllase enzymes, are produced. The values of the high chlorophyll stability index in the non-grafted 'Torsh' cultivar and the 'Sabz'/'Siah' or 'Sabz'/'Torsh' graft combinations indicate a higher chlorophyll content in the leaves, which leads to an increase in the rate of photosynthesis and the production of more dry weight which helps the plant with stand dehydration (Babu *et al.,* 2008; Ananthi *et al.,* 2013; Surendar *et al.,* 2013).

Under conditions of water scarcity, the cell membrane of the leaves are subject to changes such as increased permeability and reduced selectivity, which can be observed through an increase in electrolyte leakage (Zarafshar *et al.,* 2014). In this study, the overall increase for leakage under stressful conditions was observed in all grafted and non-grafted rootstocks as compared to fully irrigated plants.

The non-grafted 'Siah' cultivar experienced a greater damage to the cell membrane of its leaf, compared to the non-grafted 'Sabz' and 'Torsh' cultivars. This indicates that the rootstock's ability to maintain the integrity of its cell membrane in severe stress conditions is considered an important factor in determining tolerance to drought (Bolat *et al.,* 2014). Undesirable performance of the cell's metabolism during periods of drought stress leads to the stimulation of reactive oxygen species or the disruption of systems that prevent or reduce the activity of reactive oxygen species, which would damage the cell membrane and increase electrolyte leakage (Guerfel *et al.,* 2008; Karimi *et al.,* 2013). Therefore, the fact that damage to the cell membranes of the non-grafted 'Sabz' cultivar occurred less than in the non-grafted 'Siah' rootstock may indicate a more tolerance to drought in the 'Sabz' cultivar. However, Shahidi-Rad *et al.* (2015) reported that leaf abscission occurred more rapidly in 'Siah' than 'Sabz' cultivar in 16 days of water deficit stress, but after rewatering, 'Siah' recovered more efficiently and more rapidly than 'Sabz'.

The growth of grafted plants (Sa/Si and Sa/T) showed that by activating their enzymatic and non-enzymatic antioxidant systems, they could maintain the growth of the scion much higher than control (Sa).

In this study, both rootstocks affected the scion (Sabz cultivar) antioxidant systems and increased SOD and catalase (enzymatic), ascorbic acid and glutathione (non-enzymatic) in high water deficit. Both combinations of Sa/T and Sa/Si indicated higher chlorophyll content, chlorophyll stability index and shoot fresh and dry weight than non-grafted 'Sabz' cultivar. Consequently, they tolerated higher water deficit.

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Relationship between chlorosis, photosynthesis and the nutrient content of plane trees in the presence of chemical and organic fertilizers

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Key words: mycorrhizal fungi, nutrient acquisition, organic matter, symbiosis, urban trees.

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

Competing Interests:
The authors declare no competing interests.

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Abstract: Chlorosis disorder is a major problem affecting the growth and physiological processes of many trees including plane trees (*Platanus orientalis* L.). This experiment was conducted to study the relationship between leaf chlorosis disorder and the nutritional status and some important physiological characteristics of plane trees. The experiment was arranged in a randomized complete block design with six replications and four treatments including control, manure (M), manure + fertilizer (20-5-10) (MF), and manure + fertilizer + mycorrhizal fungi (MFA) (*Glomus intraradices* + *G. mosseae*). The results showed that although all treatments significantly improved the nutrients content, soluble carbohydrates content, photosynthesis rate and chlorophyll content in the leaves, they mostly reached their peak in the mycorrhizal inoculated plants. Nitrogen (N), phosphorus (P) and zinc (Zn) were increased in the AMF amended trees compared to the control plants. The photosynthesis rate was enhanced by all the mixtures at least by 60% compared to the control. The most Chlorosis (17.5%) to the leaves recorded on the control plants, while leaf damage dropped to less than 2.9% at mycorrhizal treatment leading to the improved nutritional balance in the plane trees. The results proved the effectiveness of including mycorrhizal inoculation to the common fertilization practices to prevent leaf chlorosis in the plane trees.

1. Introduction

Plane tree *(Platanus orientalis* L.) is among the most common ornamental and street trees planted in the urban landscape in Iran and some Mediterranean countries (Anselmi *et al.*, 1994; Khorsandi *et al.*, 2016). They are known for their longevity and wide distribution in the temperate zones.

However, the chlorosis as an important physiological disorder in the plane trees has affected a majority of them in Iran in recent years (Khorsandi *et al.*, 2016). The problem is a common physiological disorder affecting many plants around the globe. It is especially a major problem in

the calcareous soils and soils with high pH (Wallace, 1982).

The chemical properties of the soil and the adequate supply of nutrients are major factors affecting natural plant growth and extension (Cekstere and Osvalde, 2013), therefore important factor in nutrient uptake is the availability of the nutrients in the soil. Most trees cultivated in the alkaline and calcareous soils are exposed to the incidence of chlorosis which is reported to be basically due to Fe deficiency (Mortvedt, 1986). Several factors can contribute to leaf chlorosis including nutritional disorders and a disorder in the chlorophyll biosynthesis. Indeed, the lack of some nutrient elements such as nitrogen (N), zinc (Zn) and expecially iron (Fe), lead to the chlorosis in plants (Godde and Dannehl, 1994). Moreoever, following the lack of sufficient chlorophyll, the affected plant will not be able to operate photosynthesis process, resulting in stunted growth (Miller *et al.*, 1984).

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that colonize the roots of the most land plants and increase host nutrient acquisition (Desiro *et al.*, 2014) and it is claimed that virtually all trees acquire nutrients through symbiotic mycorrhizal fungi (Brundrett, 2009). Mycorrhizal inoculation is documented as a method to the improve nutrient uptake in many plants (Lehmann *et al.*, 2014; Varga, 2015; Young *et al.,* 2015). AMF are effective symbionts for plants, and their symbiotic relationship can increase plant growth (Vafadar *et al.,* 2014). Moreover, there is a lack of information on symbiosis relationship between the plane tree and AMF fungi.

In the present study, we added AMF to the common fertilization program of the plane trees in the urban landscape to study the following items. Firstly study the effect of mycorrhizal association on the trees response and then, observe how nutrient content and different physiological processes are associated with the leaf chlorosis disorder. To the best of knowledge, this is the first report attempting to discover the correlation between different plane tree physiological processes and leaf chlorosis disorder under AMF inoculation.

2. Materials and Methods

Experimental site and treatments

The experiment was conducted during 2013-2014, on the campus of the Isfahan University of Technology in Isfahan (32°39’ N, 51°40’ E; 1600 m), Iran. The site is characterized as having an arid climate with cold winters, 122.8 mm average annual rainfall and 23.4°C average annual temperature. Twenty-four uniform 15-year-old plane trees (*P. orientalis* L.) were selected.

The experiment was a randomized complete block design (RCBD) with four treatments. Treatments included control, manure (M), manure + fertilizer (water soluble 20-5-10 N-P-K compound fertilizer with 12.8% sulfur, 1.3% magnesium oxide, NovaTec Solub, Compo, Germany) (MF), and manure + fertilizer + mycorrhizal fungi (MFA). Six replications were prepared for each treatment. The plants were inoculated with two AMF inoculations including *Glomus intraradices* and *G. mosseae* (both of them have been transferred to new genera, so Index Fungorum considers them now as *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) and *Funneliformis mosseae* (Nicolson & Gerd.) (Schüßler and Walker, 2010). The AM fungi were provided by the Institute of Soil and Water Research, Tehran, Iran. Inoculum was comprised of a mixture of spores (80 spores g^{-1} for *G. intraradices* and 80 spores g^{-1} for *G. mosseae*). The mixtures of filling materials were placed into 0.5 × 0.5 m holes, depending on the treatment in early spring. This technique provides a nutrients in a zone in and around each hole. With the first wetting, the nutrients are released from the fertilizer into the soil and the manure slowly lower the pH of the soil surrounding the hole. Over a period of time, a zone of soil around each hole is modified to be lower in pH and rich in micronutrients in approximately the correct proportions. Two identical holes were drilled around each tree about one meter away from the tree trunk and filled up with the corresponding mixture. During the process, we avoided drilling into large buttress roots. In M treatment, trees received 5 kg of manure per hole mixed with the soil of the drilled hole. In MF treatment, 100 g of fertilizer per hole was added to the manure. Trees of MFA treatment received the AMF inoculums by adding 250 g of mycorrhizal inoculums into each hole mixed with manure and fertilizer (500 grams of inoculum per each tree in total). The control group did not receive any treatment (two identical holes were drilled). The trees were irrigated once a week. Some chemical and physical properties of the soil and cow manure are presented in Table 1.

Measurements

Various morphological and physiological parameters were measured 5 months after treatment. The

Table 1 - Some chemical and physical properties of the soil and cow manure used in research

Factors	Texture	pH	EC (dS m^{-1})	Organic matter (%)	N (%)	P-available (mg kg^{-1})	K-exchangeable (mg kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})
Soil	Clay	7.9	1.53	1.15	0.15	140	235	1400	21
Manure	-	8.02	15.23	20.4	3.07	791	2030	12300	194

mineral contents of the plant leaves were determined in the second year of the experiment. Plant samples were oven-dried at 65°C for 48 h and then were ground to determine their mineral composition. The determination of the total N in the leaf samples was based on the Kjeldahl method (Baker and Thompson, 1992). The extraction of P, K, Fe, and Zn from the plant tissue material was performed by using 2 M hydrochloric acid (HCl) after dry ashing at 550°C for 5.5 h. The concentrations of Fe and Zn were determined by atomic absorption spectrophotometer (670 Shimadzu, Kyoto, Japan) (AOAC, 2006). P concentration was determined by vanado molybdate phosphoric acid method with a spectrophotometer (UV-160A UV-Visible Recording Spectrophotometer, Shimadzu, Tokyo, Japan) (Cottenie, 1980). LCI Portable photosynthesis and transpiration rate analyzer (Li - 6400; LICOR, Lincoln, NE, USA) was used to measure the net photosynthesis rate (A) between 09.30-11.30 h on 10 fully expanded current-season leaves situated at the mid-canopy height. The soluble sugars were measured according to the phenol-sulfuric acid method (Dubois *et al.,* 1956). The extraction of the leaf chlorophyll pigments was carried out using 100% acetone according to Lichtenthaler (1987).

For evaluation of the leaf chlorosis extent, 100 leaves from each tree were selected randomly and both leaf surfaces were scanned by a scanner (Canon i-SENSYS MF4010, Canon Inc., Korea). The leaf chlorosis was determined by digital image processing using MATLAB software. A range of color was defined for the leaf chlorosis in the program and total leaf area was examined pixel by pixel by the software and the percentage of pixels which was defined as chlorotic areas were calculated by the software (Rathod *et al.*, 2013).

Data analysis

Data were assessed for normality and log-transformed used to make data conform to normality when necessary prior to analysis. Non-homogeneity data were observed in leaf chlorosis, being the data transformed with the formula

$$arcsin\ \sqrt{(\text{leaf chlorosis}/100)}$$

to obtain homogeneity. The experimental data were statistically analyzed by the analysis of variance (ANOVA). The significance of the differences between treatments was estimated using the least significant difference (LSD) test at $P \leq 0.05$, and graphs were drawn using Excel 2010. Statistical correlation was calculated by Pearson's correlation coefficient (r). This test was used to measure the strength of a linear association between the leaf chlorosis and other variables including nutrients content, photosynthesis rate and chlorophyll and soluble sugar contents. The value r = 1 means a perfect positive correlation and the value r = -1 means a perfect negative correlation. The experimental data were statistically analyzed with Statistical Analysis Systems (SAS) software, version 9.1 and Statistics, version 8.0.

3. Results

AMF inoculation increased all nutrients content including P, N, Fe and Zn in the leaves of the treated trees (Table 2). N, P and Zn reached their peak value only when the fertilizer mix amended by mycorrhizal inoculums. P and Zn increased by 424% and 425%

Table 2 - Influence of arbuscular mycorrhizal (AM) fungi and other treatments on nutrient uptake of plane tree (*Platanus orientalis* L.)

Treatment	Nutrient			
	N (g kg^{-1})	P (g kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})
Control	17.47 d	1.85 c	54.93 b	4.82 d
Manure	19.14 c	5.25 b	148.99 a	15.09 c
Manure + Fertilizer	19.65 b	7.31 b	146.71 a	17.32 b
Manure + Fertilizer + AMF	20.39 a	9.71 a	170.14 a	25.31 a

Means in the same column followed by the same letters are not statistically different at $P \leq 0.05$ by the Least Significant Difference test (LSD).

respectively, compared to the control plants. All treatments (regardless of the composition of the mixture) successfully enhanced Fe and N contents in the leaves compared to the control plants (Table 2).

All treatments significantly improved the soluble carbohydrates content in the leaves; however, it reached the peak in the mycorrhizal inoculated plants. The soluble carbohydrates content increased by 35.44% and 13.82% compared to the control of non-fertilized treatment and non-inoculated plants, respectively (Fig. 1).

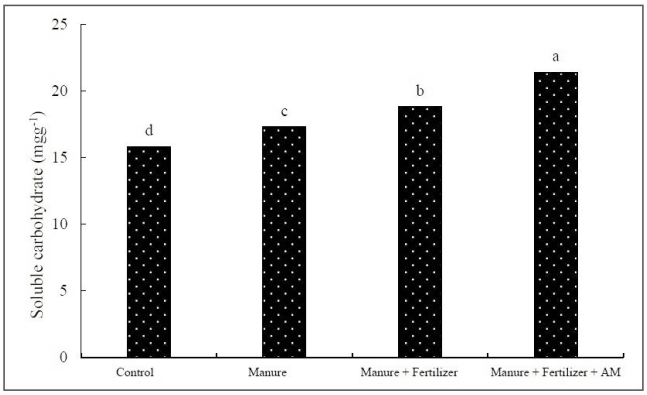

Fig. 1 - Influence of arbuscular mycorrhizal (AM) fungi and other treatments on soluble carbohydrate content in plane tree (*Platanus orientalis* L.). Means are separated by LSD test at P≤0.05.

All treatments increased the photosynthesis rate at least by 60% compared to the control, although no significant difference was observed between the treatments (Fig. 2). The same trend was observed in the case of chlorophyll content (Fig. 3), where it increased at least by 32% compared to the control.

Leaf chlorosis was influenced dramatically by the treatments (Fig. 4). The most leaf chlorosis was recorded in control non-fertilized treatments. Leaf chlorosis reduced to less than 2.9% on inoculated trees by AMF, while 17.5% of leaves tissue were affected by chlorosis in the control plants. The AMF inoculated plants showed an increase of 13.44% compared to the plants that received manure + fertilizer.

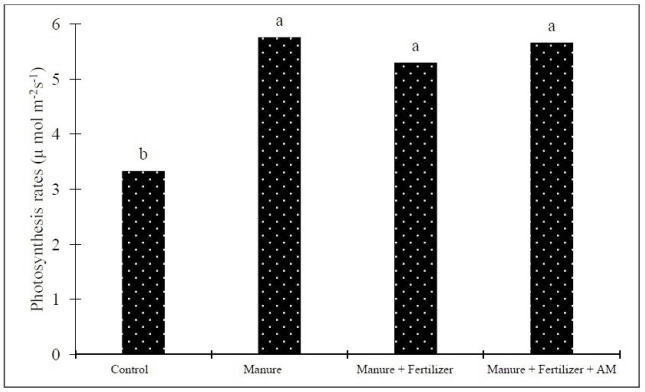

Fig. 2 - Influence of arbuscular mycorrhizal (AM) fungi and other treatments on photosynthesis rates in plane tree (*Platanus orientalis* L.). Means are separated by LSD test at P≤0.05.

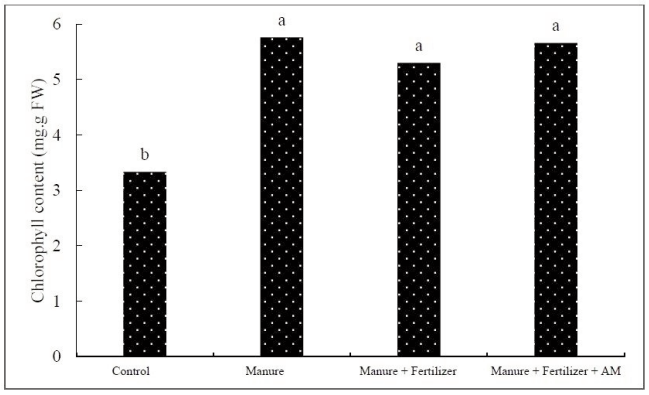

Fig. 3 - Influence of arbuscular mycorrhizal (AM) fungi and other treatments on chlorophyll content in plane tree (*Platanus orientalis* L.). Means are separated by LSD test at P≤0.05.

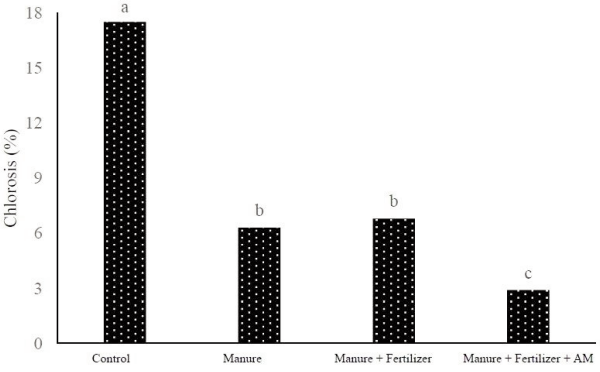

Fig. 4 - Influence of arbuscular mycorrhizal (AM) fungi and other treatments on chlorosis damage in plane tree (*Platanus orientalis* L.). Means are separated by LSD test at P≤0.05.

A strong relationship was found between leaf chlorosis and Fe, N and Zn contents (Fig. 5).

A significant and linear relationship was also found between chlorosis and the chlorophyll content and net photosynthesis in the plane tree leaves (Fig. 6). Leaf chlorosis in the plane trees resulted in a dramatic and linear decline in the soluble carbohydrate in the leaves (Fig. 6).

4. Discussion and Conclusions

Two explanations can be presented for increasing Fe content under any treatments (except for the con-

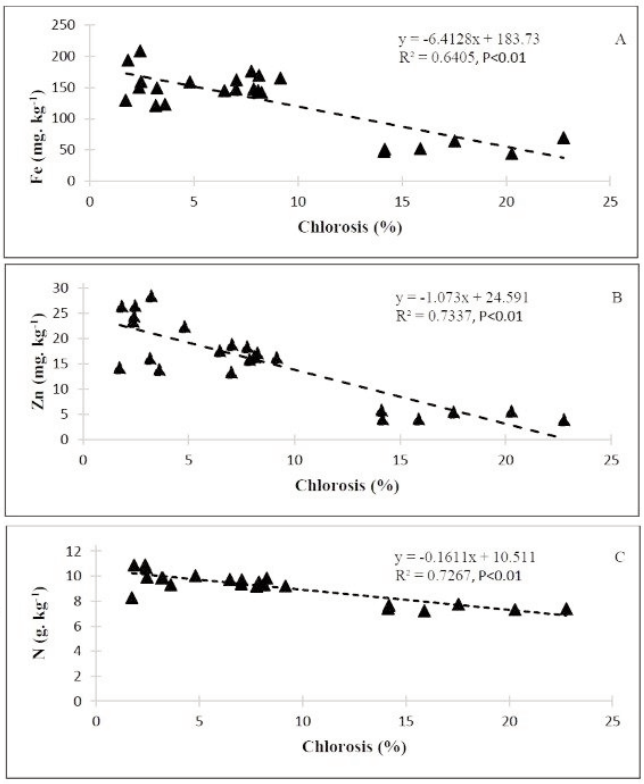

Fig. 5 - Relationship between chlorosis with Fe (A), Zn (B) and N concentration (C) in plane tree (*Platanus orientalis* L.).

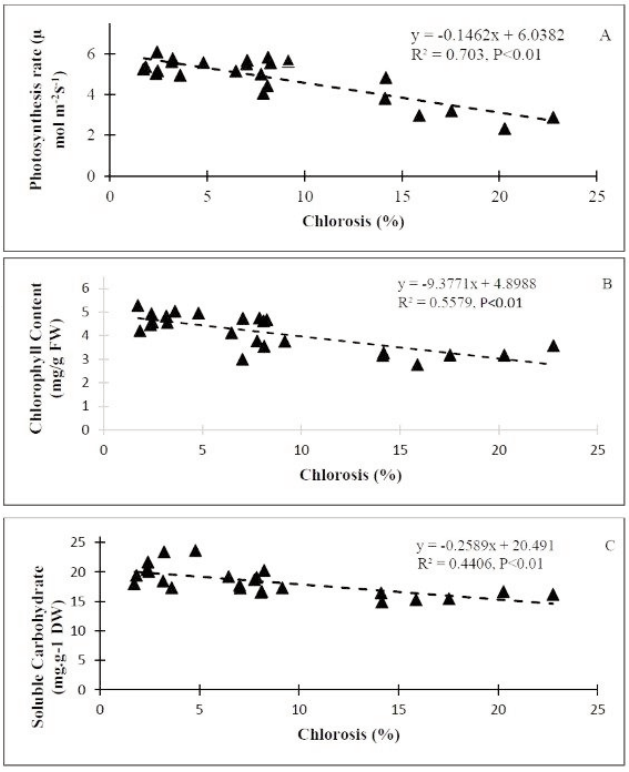

Fig. 6 - Relationship between chlorosis with Photosynthesis rate (A), Chlorophyll Content (B) and Soluble Carbohydrate (C) in plane tree (*Platanus orientalis* L.).

trol) in this experiment (Fig. 1). Firstly, as the soil of the site was a calcareous and compacted soil, any treatment increasing the air flow in the soil could improve Fe content in the plant (Lucena, 2003). Secondly, using the manure itself can increase Fe content in the plants (Mortvedt, 1986). It is documented that the manure provides micronutrients including Fe and improves the structure of the soil (Lucena, 2003). Our results at least partly confirm the findings that combining Fe fertilizers with the organic matter is more favorable in terms of Fe uptake than Fe sources applied alone (Mortvedt, 1986). It has been reported that the application of $FeSO_4$ (4-8 kg $tree^{-1}$) mixed with manure, cotton seed cake, or other organic substances in 8 to 10 holes in the soil around the crowns of apple trees (*Malus sylvestris* Mill.) resulted in marked correction of Fe chlorosis (Zheng-Qing and Cang-Zhen, 1982). It is also well established that as a result of the decomposition of organic matters in the soil, compounds such as humic acid (HA) and fulvic acid (FA) are produced in the soil (Nardi *et al.,* 2002). These acids are well known as naturally-occurring chelating agents (Mortvedt, 1986; Nardi *et al.,* 2002). There are many reports showing enhanced micronutrients uptake by the plants receiving HA, FA (Nikbakht *et al.,* 2008; 2014) or organic matter (Atiyeh *et al.,* 2002). It is shown that inoculation turfgrass (*Lolium prenne* L.) with AMF receiving HA not only improved plant growth but also showed more elevated nutrients content in the leaves than in non-inoculated (control) plants or plants receiving only HA (Nikbakht *et al.,* 2014).

Researchers believed that the role of AMF in NO^{-3} transport to the root surface is significant (Subramanian and Charest, 1999; Javaid, 2009). They especially insist that the role of AMF is of value and importance in nitrate uptake in Mediterranean and (semi-) desert ecosystems which are characterized by calcareous soils.

N, P and Zn uptake reached their peak value when the fertilizer mixture amended by AMF (Table 2). These results confirm the well-documented effect of AMF inoculation on nutrients uptake (Brundrett, 2009; Varga, 2015; Young *et al.,* 2015). A strong relationship between leaf chlorosis and Fe, N and Zn contents implies that the chlorosis is not only because of Fe deficiency in the plant, but also other nutrients including N and Zn play they own role (Fig. 5). It indicates that leaf chlorosis in the plane trees was not simply due to Fe deficiency. It is well documented that Fe is an essential element for many vital processes in a plant including photosynthesis, respiration, N

fixation, chlorophyll and hormone synthesis; Fe is also a constituent of heme proteins (cytochromes, catalase, and peroxidase) (Briat and Lobreaux, 1997). As a result, affected plants by Fe deficiency suffer severe metabolic and structural disorders (Javaid, 2009). There are also some reports indicating that the major cause of Fe deficiency is the very low solubility of Fe oxides in the soil (Mortvedt, 1986). It shows the importance of the fact that the role and priority of each element in the plane tree chlorosis remain to be investigated further.

Fe deficiency depresses the synthesis of chlorophyll, which results in the decrease of photosynthetic products, which in turn affect plant growth (Wang *et al.,* 2008). As a result of carbohydrates synthesis reduction in chlorotic leaves, which slows the movement of K^+ from the leaf to the phloem vessels, a decline in the production of biomass is reported (Maldonado-Torres *et al.,* 2006). These explain why we found a relationship between chlorosis and chlorophyll content, net photosynthesis and soluble carbohydrate content of the leaves (Fig. 6). Moreover, increased photosynthetic capacity by AMF is in agreement with the results of the previous study by Birhane *et al*. (2012). It seems this process has improved nutrition, leading to higher photosynthetic rates (Vafadar *et al.*, 2014). To the best of our knowledge, no similar information has yet been provided for interaction effect of fertilizers and AMF inoculation on plane trees and its relationship with the leaf chlorosis disorder.

This study demonstrated that AMF inoculation added to the common fertilizer program served successfully as a biological and environmental-friendly method to overcome chlorosis disorder of the plane trees. In addition, the findings of this study suggest that in calcareous soils drill hole nutrition should be considered as a standard method to prevent nutritional disorders in the urban landscape. The results also revealed that Fe is not the only nutrient participating in the leaf chlorosis of plane trees. It suggests further investigations to study the weight and importance of each nutritional element in chlorosis disorder of the plane trees.

In this study we mainly focused on the effect of improved media around the plane trees, rather than specific effect of AMF. Indeed, this study was one of the primary trail in a series of experiments we want done later. In the later, we specifically will studied the AMF effect on the plane trees. Our experiment creates a paradigm for future studies of relationship between plane trees and microorganisms.

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AHS
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Embryogenesis in Valerian (*Valeriana officinalis* L.) using leaf segments

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Key words: glutamic acid, *in vitro* culture, MS medium, somatic embryogenesis, *Valeriana officinalis* L.

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

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The authors declare no competing interests.

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Abstract: A protocol for direct embryogenesis induction without an intervening callus production was developed in valerian *(Valeriana officinalis* L.) using the leaf segments. Direct somatic embryogenesis was induced using by half-strength MS medium supplemented with 2,4-D (0.5 mg·l^{-1}), glutamic acid (100 mg·l^{-1}), 4% sucrose and 8 g·l^{-1} agar. The embryo germination and plantlet formation were enhanced on MS medium supplemented with NAA (0.1 mg·l^{-1}) and Kin (2 mg·l^{-1}). Regenerated plants with well-developed root and shoot systems were successfully (72%) transferred to the greenhouse.

1. Introduction

Valerian (*Valeriana officinalis* L.) is an herb belongs to Valerianaceae family which grows in several geographic areas of the world including Iran. The genus *Valeriana* encompasses nearly 250 species, found mainly in temperate zone regions. Some species of this genus have high economic demand in Iran and also, around the world. Plants of this genus have sedative, antispasmodic and anxiolytic properties mainly due to the production of iriod esters, known as valepotriates, in the plant rhizomes (Hiller and Zetler, 1996). The conventional methods of propagation of valerian are from seed and rhizome. Considering the importance of the genus *Valeriana* in the medicinal world (O'Hara *et al.,* 1998) concentrated efforts are being made to improve the propagation using biotechnological approaches. Plant regeneration has been described from shoot tip, axillary bud explants, via callus and embryo-like structures derived through suspension cultures of *V. wallichii* (Mathur *et al.*, 1988; Mathur and Ahuia, 1991; Mathur, 1992). Also, plant regeneration from adventitious shoots, seedlings, suspension cultures of *V. edulis* (Enciso-Rodriguez, 1997; Castillo-España *et al.,* 2000), shoot regeneration from leaf segments of *V. officinalis* (Abdi and Khosh-khui, 2007), shoot organogenesis and somatic embryogenesis from leaf explants of *V. jatamansi* (Rong *et al.*, 2014) and from shoot buds of *V. jatamansi* (Kaur *et al.,* 1999) and shoot

organogenesis and somatic embryogenesis from leaf explants of *Valeriana jatamansi* Jones have been described (Chen *et al.*, 2014). Somatic embryogenesis is an efficient and high volume propagation system for the large number of plants within a short period. Successful genetic transformation attempts have mostly employed embryogenic callus or cell cultures as the target tissue in several medicinal plants (Leena and Jaindra, 2003). However, a major limitation of this callus system is the repeated subculture to select embryogenic callus portions among highly proliferating non-embryogenic tissue. This process is not highly producible and furthermore increases the chance of somaclonal variation. As these limitations have become unavoidable, strategies to improve plant regeneration must necessarily include manipulation of the medium to embark upon new morphogenetic pathways (Pedroso and Pais, 1995). Direct somatic embryogenesis offers several advantages in medicinal plant improvement, as cost effective and large-scale clonal propagation is possible using bioreactors, ultimately leading to automation of somatic seed production and development of artificial seeds. Besides, such a system could provide a new source for use in genetic transformations. The plant derived from direct somatic embryogenesis usually is unicellular in origin and hence genetically uniform. The leaf segments of valerian are an excellent source for the induction of indirect embryogenesis and the factors affecting this process had been studied (Castillo-España *et al.,* 2000). Direct somatic embryogenesis and factors controlling it have been studied in many plant species (Pedroso and Pais, 1995; Chen *et al.,* 1999; Desai *et al.,* 2004; Kuo *et al.,* 2005; Quiroz-Figueroa *et al.,* 2006; Thengane *et al.*, 2006; Jayanthi *et al.,* 2011). Based on our knowledge, there is no report about direct somatic embryogenesis on *Valeriana officinalis.* The aim of this study was to establish a method for asexual multiplication of *Valeriana officinalis* through direct somatic embryogenesis.

2. Materials and Methods

Plant material and culture methods

Fresh leaves of valerian were collected from 4-month old greenhouse-grown plants (Fig. 1A). They were washed with tap water and a few drops of Rica (a commercial detergent). They were surface sterilized by 70% Ethyle Alcohol for 1 min. and rinsed twice with sterile distilled water. The leaves were immersed in a solution of 1.5% sodium hypochlorite for 10 min and rinsed four times with sterile distilled water. The leaves were cut into 7-8 mm^2 segments and transferred to 150 ml glass jars with 25 ml of half-strength MS medium (Murashige and Skoog, 1962). The pH of media was adjusted to 5.8 by 0.1 N HC1 before autoclaving for 15 min at 121°C and 1.5 $kg \cdot cm^{-2}$ pressure. Cultures were placed initially under the dark condition for 2 weeks and thereafter they were maintained at 25±3°C under 16 h photoperiod provided by cool white fluorescent lamps (45 $\mu mol \cdot m^{-2} \cdot S^{-1}$) with relative humidity of 75-85%.

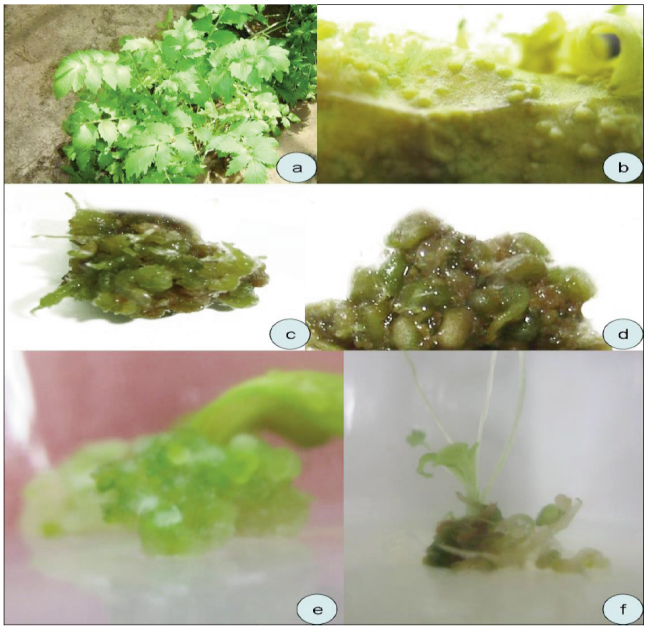

Fig. 1 - Effects of different treatments on *in vitro* culture of valerian, (A) 4-month old greenhouse-grown plant of valerian, (B) Initiation of small embryo-like structures in the 10 to 13 day after culture in induction media, (C) and (D) Embryo formation in top and cut end of valerian leaf explant after 4 weeks. (E) Callus initiated in M2 medium with great potential for regeneration. (F) Germination of embryo mass in medium supplemented with 2 mg l^{-1} Kin and 0.1 mg l^{-1} NAA.

Embryo induction and germination

For embryo induction, after preliminary experiments, MS media were supplemented with 0.5 $mg \cdot l^{-1}$ 2,4-D, 0.5 $mg \cdot l^{-1}$ Naphthalene acetic acid (NAA), 100 $mg \cdot l^{-1}$ glutamic acid (Glu) or different concentrations of sucrose (3, 4, and 5%) (Table 1). For embryo germination, two separate experiments were conducted. In the first experiment, the explants that formed embryo were divided to 4 segments (each fragment was about 0.2±0.03 g) and transferred to MS medium containing NAA (0.5, 1, 2 and 5 $mg \cdot l^{-1}$), gibberellic acid (GA_3) (0.5, 1 and 2 $mg \cdot l^{-1}$), or MS added to different combinations of NAA (0.1 and 0.2 $mg \cdot l^{-1}$) and

Table 1 - Different media used for induction of direct somatic embryogenesis in *Valeriana officinalis* L.

PGR	Additives (mg·l^{-1})												
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}	M_{12}	M_{13}
NAA	-	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
2,4-D	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-
Glutamic acid	-	-	-	100	100	-	-	100	100	-	-	100	100
Sucrose (%)	3	3	3	3	3	4	4	4	4	5	5	5	5
Response (%)	-	-	-	12.4±0.3	11.21±1.1	5.28±0.1	3.4±0.03	57.21±2.7	43.31±2.33	9.8±0.15	8.8±0.1	25.28±1.24	13.39±2.28

kinetin (1.5 and 2 mg·l^{-1}). In the second experiment, embryos were transferred individually to MS medium supplemented with NAA (0.1 and 0.2 mg·l^{-1}) and kinetin (1.5 and 2 mg·l^{-1}) to stimulate the germination and roots and shoots development.

Acclimatization

The 4 cm plantlets were transferred to small pots containing 1/3 vermiculite, 1/3 perlite and 1/3 sand (v/v). The pots were placed in transparency box and maintained under 25±5°C temperatures and 70% relative humidity for 4 weeks and then transferred to greenhouse.

Data analysis

The experiment was conducted as a completely randomized design in a factorial arrangement with 4 replications and each replicate with 12 explants. The means were compared with Duncan's new multiple range test (DNMRT) at 5% probability level. To determine the efficiency of embryo induction medium, responsive leaves that formed embryo was recorded after 4 weeks. For embryo germination experiments the percent of germinated embryo was recorded after 3 weeks and the number of plantlets was recorded 6 weeks after culture. Plantlets were recognized when they developed roots and shoots.

3. Results

Embryo induction

The leaf segments showed swelling and initiation of small embryo-like structures in the 10-13 days after culture (Fig. 1B). In the following weeks, embryogenic clumps were visible at the cut end and surface of the explants (Fig. 1C and D). Embryoid formation in cut edges of leaf explants was higher than other parts of leaves. Well-developed embryos were observed all over the cultured explants within four weeks of culture. Different medium showed different embryogenesis response. When Glu and sucrose (4%) were added to medium, embryogenesis response increased. Maximum embryogenic response of leaf explants (57.21±2.7%) was observed on M_8 medium supplemented with 2, 4-D, 4% sucrose, and 100 mg·l^{-1} glutamic acid. NAA addition instead of 2, 4-D decreased percentage of explants response in M_9 (43.31±2.33%) medium. Increasing sucrose concentration more than 4% reduced embryogenic response on M_{12} (25.28±1.24%), and M_{13} (13.39±2.28%) (Table 1).

The explants on M_2 and M_3 media did not exhibit any embryogenic response. However, explants on these media initially showed slight swelling and subsequently resulted to callus production. M_2 showed higher percentage of callus proliferation compared to M_3 (data not shown). Callus initiated in M_2 medium had a great potential for regeneration (Fig. 1E). The explants on M_1 medium did not exhibit any response.

Embryo germination

For embryo germination experiment, two kinds of explants were used. In the first experiment, the explants that formed embryo were divided to 4 segments (each fragment was about 0.2±0.03 g) and used as mass embryo. In the second experiment the embryos were transferred individually to MS medium supplemented with various supplements. Somatic embryo germination response varied greatly in various hormone supplements (Table 2). MS without growth regulators showed low response to embryo germination. In this treatment, germination percentage per number of embryoid pieces and also number of plantlet per pieces was not considerable (Table 2). MS+NAA (0.5, 1, and 2 mg·l^{-1}) led to rooting of somatic embryos (Fig. 2A). The inclusion of GA_{33} (0.5, 1, and 2 mg·l^{-1}) in the germination medium increased the germination percentage and consequently plantlets with both well-developed shoots and roots. The combination of Kin-NAA enhanced somatic embryo germination percentage more than other treatments. Among Kin-NAA treatments, 2 mg·l^{-1} Kin and 0.1 mg·l^{-1} NAA showed high frequency of embryos and also plantlet formation (Table 3). The somatic embryos were easily isolated with a pair of forceps, each developing into a single plant. The highest recovery

Table 2 - Effects of various supplements on germination of *Valeriana officinalis* L. embryos

Media	Response
MS + without growth regulators	Germination and plantlet production was low
MS + NAA	Rhizogenesis
MS + Kin and NAA	Plantlets with both well-developed shoots and roots
MS +GA_3	Plantlets with both well-developed shoots and roots with vigorous growth of plantlets

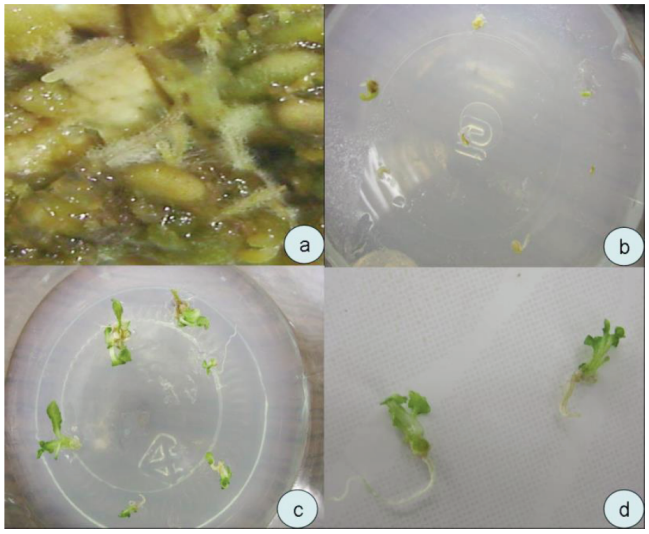

Fig. 2 - Effects of different treatments on *in vitro* culture of valerian (A) Rhizogenesis response of embryo in medium containing NAA. (B) Individual embryos in medium containing 2 mg l^{-1} Kin and 0.1 mg l^{-1} NAA. (C) Well germinated embryos in medium containing 2 mg l^{-1} Kin and 0.1 mg l^{-1} NAA. (D) Plantlets with developed shoots and roots obtained from embryo germination.

of somatic embryos (78+3.21%) was possible on MS medium supplemented with NAA (0.1 mg·l^{-1}) and Kin (2 mg·l^{-1}), this condition showed considerable germination percentage and plantlet formation while the fewest somatic embryos germination and developing into a single plant (41+1.73%) was observed in MS medium supplemented with a low concentration of NAA (0.1 mg·l^{-1}) and Kin (1.5 mg·l^{-1}) (Table 4).

Table 3 - Effects of various supplements on frequency and plantlet/embryo mass in *Valeriana officinalis* L.

Plant growth regulator	Explants response (%)	Plantlet per each embryo mass
PGR free	13.71 g	1.27+0.1 e
GA3 (mg·l^{-1})		
0.5	23.24 f	2.71+0.01 d
1.0	38.00 d	3.00+0.10 c
2.0	31.70 e	2.71+0.11 d
Kin+NAA (mg·l^{-1})		
1.5+0.1	43.37 d	3.21+0.12 c
1.5+0.2	37.75 d	4.00+0.02 b
2.0+0.1	61.32 a	5.21+0.12 a
2.0+0.2	51.37 b	4.27+0.13 b

Table 4 - Effects of growth regulators on percentage of embryo germination and number of plantlets

PGR (mg l^{-1})	Explant response (%)	Plantlet per each embryo mass
Kin + NAA		
1.5 + 0.1	49+1.2 c	23 bc
1.5 + 0.2	41+1.73 d	19 c
2.0 + 0.1	78+3.21 a	37 a
2.0 + 0.2	58+2.41 b	25.6 b

4. Discussion and Conclusions

Using different plant tissue culture technique and plantlet regeneration have been reported in different species of Valeriana genus, such as *V. officinalis* (Abdi and Khosh-Khui, 2007; Abdi *et al.*, 2008; Reza *et al.*, 2009), *Valeriana wallichii* (Mathur *et al.*, 1988), *Valeriana edulis ssp. procera* (Enciso-Rodriguez, 1997), *V. jatamansi* (Kaur *et al.*, 1999; Das *et al.*, 2013), *Valeriana glechomifolia* (Salles *et al.*, 2002; Bello de Carvalho *et al.*, 2004), *Valeriana glechomifolia* (Bello de Carvalho *et al.*, 2004). However, direct somatic embryogenesis have never been reported from any explant of the species. In this study, the effects of different plant growth regulators (PGRs) and different supplement on the induction of direct somatic embryogenesis were investigated. We established an efficient embryo induction, somatic embryogenesis and plant regeneration system from leaf explants using various supplement and different types and concentrations of PGRs. The induction and development of *in vitro* somatic embryos comprise complex processes including cell division, differentiation, growth, and pattern formation (Capron *et al.*, 2009)., the composition of the basal culture medium, the type and levels of plant growth regulators (PGR), the level of carbon sources and the balance of organic and inorganic nitrogen sources are key factors for *in vitro* embryogenesis system. Primary nitrogen sources, including Glu, is very important in plant tissue culture in order to stimulate the cellular growth

and the connection between cells and tissues (Young *et al.,* 1999). The productions of direct somatic embryos on leaf explants of *V. officinalis* are possible by the addition of the Glu to the culture medium and increasing the sucrose level to 4%. Absence of any callus formation indicated that the process of embryo development was direct. Glutamate occupied a central role in amino acid metabolism in plant. It can form by action of the glutamate synthase utilizing glutamine (Bohinski, 1991). Glutamine as a nitrogen source for purins and pirimidins biosynthesis, was significantly stimulated the direct embryogenesis in *Valeriana officinalis* in this study. This finding is in parallel with the results of Chowdhry *et al.* (1993), Gex *et al.* (2006) and Shahsavari (2011). This could explain the enhanced rate of induction and development of somatic embryos in the present investigation. Additionally, during the metabolism and protein synthesis the nitrogen originated from amino acids is quickly assimilated into carbonic skeletons (Lea, 1993). This stimulation suggested that organic nitrogen was a growth-limiting factor in Valerian cultures and the inclusion of glutamine decreased the culture lag phase, which indicated that glutamine was much more readily assailable than inorganic nitrogen. The somatic embryos in this study were formed more in the cut edges of leaf explants. Increasing in embryogenic competence of wounded tissue probably was related to the endogenous growth regulators changes in leaf tissue (Ivanova *et al.,* 1994). This study also demonstrated that 2, 4-D is more efficient than NAA to induce somatic embryos formation in valerian. Shoot organogenesis and somatic embryogenesis were also reported in cucumber in the presence of 2,4-D and NAA (Kuijpers *et al.*, 1996) and the same results were reported in Cassava leaf explants by Sofiari *et al.* (1997). Plant tissue studies on *V. edulis* revealed that 2,4-D induced somatic embryos while NAA induced shoots (Castillo-España *et al.,* 2000). Increasing the level of the sucrose from 3% to 4% showed a positive effect in embryo induction, while the induction response decreased at the presence of 5% sucrose in the culture medium. Similar results have been also reported for the other plant species (Luo *et al.,* 1996; Biahoua and Bonneau, 1999; Nakagawa *et al.,* 2001) Sucrose as common carbohydrate in the phloem sap of many plants have affects in plant tissue culture and the formation of somatic embryos in culture medium (Luo *et al.,* 1996; Nakagawa *et al.,* 2001) for this reason adding high concentration of sucrose can enhance the somatic embryos induction (Luo *et al.*, 1996; Nakagawa *et al.,* 2001). The positive effect of sucrose may related to the vital function of the sucrose such as controlling the several developmental processes in the cells (Gibson, 2000; Smeekens, 2000), nutritional function (serve as a carbon source during somatic embryogenesis) and osmotic regulator or cell osmolarity (Biahoua and Bonneau, 1999). The positive role of sucrose in the present study may be interpreted as both nutritional and osmotic regulatory functions of this carbohydrate. The conversion of somatic embryos to plantlets is a multi-step process. In many embryogenetic systems the transfer of somatic embryos into PGR free culture medium enhances the development of the somatic embryos and their conversion to plantlets. One of the determinative factors for the low rates of somatic embryo conversion to plantlets is associated with the residual effects of 2, 4-D. Prolonged expositions to this PGR normally reduces the conversion and increases the number of abnormal somatic embryos (Cruz *et al.,* 1990). When somatic embryos of *V. officinalis* were culture in medium supplemented with Kin-NAA and GA_3, the number of plantlets increased. The positive role of cytokinins may be related to reversion of negative effects caused by 2, 4-D to the cultures (Parrot *et al.,* 1988). The role of the GA_3 in promoting the germination of somatic embryos is well documented in other embryogenic systems (Deng and Cornu, 1992). Our results are in agreement with the findings of Castillo-España *et al.* (2000) who used combination of Kin-NAA for embryo germination in *Valeriana edulis* ssp. Procera. In conclusion, the induction of direct somatic embryogenesis in valerian using leaf segments as described in this study could be useful in rapid propagation of the elite plant, which has best characters for medicinal purposes. Furthermore, direct embryogenesis can be beneficial for gene transformation via particle bombardment or *Agrobacterium* in short time, avoiding somaclonal variation. In addition, the identified protocol does not seem induce a proliferation of callus before the differentiation of somatic embryos. Regenerated plants with well-developed root and shoot systems were successfully (72%) transferred to greenhouse.

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The roles of sodium nitroprusside, salicylic acid, and methyl jasmonate as hold solutions on vase life of *Gerbera jamesonii* 'Sun Spot'

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Abstract: The present study investigates the roles of nitroprusside (SNP), salicylic acid (SA), methyl jasmonate (MJ), and their interaction with 8-hydroxyquinoline sulfate (8-HQS) in regulating the peroxidase activity (POX), water uptake, the relative water content (RWC), the contents of malondialdehyde (MDA), soluble sugar, proline, and protein content in the petals and the stem bending of *Gerbera jamesonii* 'Sun Spot' cut flowers. Cut flowers were treated with various concentrations (50, 100, and 200 µM) of hold-solutions containing 8-HQS, SA, MJ, and SNP. Hold solutions were used alone or in combination with 100 µM 8-HQS for 24 h. Distilled water was used as control and sucrose (4%, w/v) was added to all solutions. The findings showed that 50 µM SA+ 100 µM 8-HQS markedly improved the RWC, the contents of proline, anthocyanin, carotenoid, protein, and soluble sugar, and activities of POX in the petals and markedly reduced water loss and the contents of MDA in the petals, compared with other treatments, especially the control. Meanwhile, the combination of plant growth regulators (PGR) with 8-HQS markedly improved positive indexes than use alone PGR. This phenomenon seemed to be due to more absorption of PGR. Among different concentrations of PGR, 50 µM is the most effective treatment for the improvement of the vase life of *Gerbera jamesonii* cut flowers. The results also demonstrated that SA+8-HQS improves the vase life of gerbera cut flowers by enhancing the membrane stability and water retaining capacity as well as increasing proline, antioxidant activity, and pigment contents.

1. Introduction

Vase life and quality are key factors that contribute to the aesthetic and benefits of cut flowers (Mansouri, 2012). The short vase life of most cut flowers is mainly due to the water loss, which is an important physiological process that affects the main quality characteristics of cut flowers, such as appearance (Salehi Salmi *et al*., 2018).

Gerbera jamesonii is a commercially popular cut flower that ranks 10 in the globe auctions. This plant is a member of the family asteraceae that

originates from Africa and Madagascar and extends to China (Parthasarathy and Nagaraju, 1999; Hind, 2007). Sensitivity to microbial contamination at the stem base is a major postharvest problem of this plant (Balestra *et al*., 2005). Microorganisms cause stem end blockage in cut flower (He *et al*., 2006; Liu *et al*., 2009) and also secretion of toxic compounds, and thereby accelerated wilting (Williamson *et al*., 2002). Vase life of gerbera has been studied extensively with different treatments (Nair *et al*., 2003; Solgi *et al*., 2009; Shabanian *et al*., 2018). Witte *et al.* (2014) reported that treating cultivars of gerbera stems by sucrose in combination with an antimicrobial compound (HQC, Chlorine) resulted in less bending than the same concentration of the antimicrobial compounds alone. Perik *et al.* (2014) noted that other factors might also be involved in bending and showed that a mixture of chemicals delayed the time to bending in six tested cultivars of gerbera.

Sodium nitroprusside (SNP), the inorganic nitrous compound (nitroferricyanide) with the formula $Na_2[Fe(CN)_5NO].2H_2O$, is an important signaling molecule. This molecule has diverse physiological functions for plants such as inducing tolerance to adverse environmental factors (Shi *et al*., 2016; Kumar Rai *et al*., 2018). Application of SNP in vase solution resulted in extending the vase life of gerbera cut flowers (Shabanian *et al*., 2018); however, there is very limited information regarding the positive effects of exogenously applied SNP in extending the vase life.

Ortho-hydroxybenzoic acid or salicylic acid is an endogenous plant growth regulator. Exogenous application of salicylic acid (SA) can affect the antioxidant capacity of plant cells and prolong vase life of cut flowers, such as rose (Alaey *et al*., 2011) and anthurium (Promyou *et al*., 2012). However, little work has been reported on the role of SA on cut flower vase-life improvement and physio-chemical attributes related to senescence.

Methyl Jasmonate (MJ) has been found as a naturally occurring substance in higher plants. In this context, the application of MJ induced antioxidant system activity can suppress fungal infection and enhance stress resistance (Kanani and Nazarideljou, 2017). To our knowledge, MJ effects on specific physiological and biochemical processes in gerbera cut flower have not been studied yet.

As a derivative of 8-hydroxyquinolines, 8-hydroxyquinoline sulfate is widely used as antibacterial since the beginning of the 1950s. This compound is active against gram-negative bacteria of the Enterobacteriaceae family, fungi of the Candida genus, and mycoplasma (Chupakhina *et al*., 2012). In previous studies, 8-hydroxyquinoline sulfate (8-HQS) remarkably increased vase life of rose cut flowers (Ichimura *et al*., 1999).

In the present study, the role of three plant growth regulators (i.e., SNP, MJ, and SA) in regulating the activities of the antioxidant enzyme, relative water content (RWC), water uptake, the contents of malondialdehyde (MDA), protein, pigments and soluble sugar in the petals, and time of stem bending of gerbera cut flowers were investigated. The objective of the present study is to provide a theoretical basis for the application and optimization dosage of plant growth regulators in combination with an antimicrobial compound, i.e., 8-HQS, in improving the vase life of gerbera cut flowers during the vase-holding period.

2. Materials and Methods

Plant material and treatment

'Sun Spot' cut-gerberas (*Gerbera jamesonii*), harvested at normal harvest maturity, were obtained from a commercial grower (Dezfol, Khuzestan, Iran). The length of the stem varied between 65 and 70 cm. The harvested flowers were packed into parchment paper and transported to the laboratory within 1-2 h. Then, stems were re-cut to a uniform length of 55, under distilled water to avoid air embolism. Each six-flower sample was placed randomly in 250 mL of various concentrations (50, 100, and 200 µM) of hold-solutions containing 8-HQS, SA, MJ, and SNP. Hold solutions were used alone or in combination with 100 µM 8-HQS for 24 h. Distilled water was used as control and sucrose (4%, w/v) was added to all solutions. To maintain the proper concentrations of hold-solutions, the mouths of the vases were covered with plastic wrap (around the stem) to minimize evaporation and to prevent contamination. Then, flowers were individually sited in glass bottles of 25 cm height, approximately 150 ml of distilled water in each bottle, under laboratory condition. The laboratory was maintained at 22°C, 60±5% relative humidity, and 16 mmol m^{-2} s^{-1} photons irradiance using cool fluorescent lamps for a 12 h photoperiod (07:00-19:00 h).

Measurements

- Time of stem bending was determined as described by Perik *et al*. (2012).
- Relative fresh weight (RFW) was calculated by the

following formula:

$$RFW\ (\%) = [(FW_{t=10} - FW_{t=0}) / FW_{t=0}] \times 100$$

where $FW_{t=10}$ is the fresh weight of flower (g) at 10th day and $FW_{t=0}$ is the fresh weight of the same flower (g) at first day (He *et al*., 2006).

- Water uptake (mL) was calculated by subtracting in the weight of the remaining water at the end of the experiment from the initial weight.
- Soluble carbohydrate content in petals was measured by the anthrone colorimetric method according to the method of Xue (1985).
- Total anthocyanin content of petal was measured by the pH differential method of Yang *et al*. (2009).
- Total carotenoid content of gerbera petal tissue was estimated using the method of Wellburn (1994).
- Flavonoid petal tissue was measured according to the method of Markham (1982).
- Protein content in petal was estimated by the method of Bradford (1976) using bovine serum albumin as the protein standard.
- Malondialdehyde (MDA) content in petals was measured by the thiobarbituric acid reaction following the procedure of Hodges *et al*. (1999).
- Peroxidase (POX) activity was evaluated by oxidation of guaiacol, as a substrate, according to Chance and Maehly (1955).

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in SAS software. Means were compared by one-way ANOVA and Duncan's multiple range test at the 5% level of significance.

3. Results and Discussion

Vase life

As shown in figure 1, hold-solutions affected senescence of *Gerbera jamesonii* in a dose-dependent manner. Compared with control, different concentrations of SA, with or without 8-HQS, all prolonged the senescence of gerbera as shown by the tighter stem and more showy flowers. Compared with other concentrations of SA, 50 µM SA+ 100 µM 8-HQS markedly prolonged the longevity of the cut flowers. As shown in figure 1, different concentrations of SNP, with or without 8-HQS, all prolonged the length of vase life of gerbera cut flower, compared with control. Compared with control, 50 µM, 100 µM, and 200 µM SNP noticeably increased the length of vase life of *Gerbera jamesonii* cut flower by 65%, 78%, and 43%, respectively. However, there was no significant difference in the stem bending of flowers among different concentrations of SNP. Among various concentrations of 8-HQS, stem bending of cut flowers treated by 100 µM 8-HQS was significantly lower than those treated by other concentrations. Compared with control, 50, 100, and 200 µM 8-HQS increased the vase life by 69%, 100%, and 78%, respectively. Different concentrations of MJ all significantly increased vase life, compared with the control (Fig. 1). However, MJ treatments were less effective on vase life compared with 8-HQS, SA, and SNP treatments.

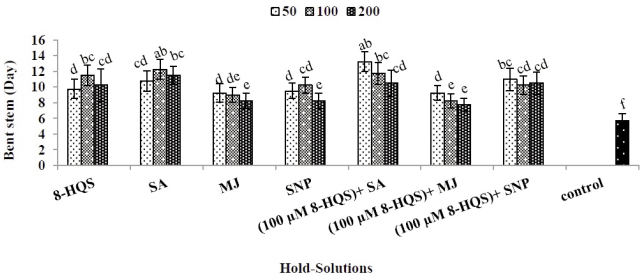

Fig. 1 - Effect of the different preservative solutions (µM) on time of stem bending in gerbera cut flowers. Columns followed by different letters are significantly different at P=0.05.

Shabanian *et al*. (2018) showed that SNP extended the vase life of gerbera cut flowers as compared with their respective control treated with water alone. In agreement with this finding, treatments with SNP extended the vase life of other cut flowers, e.g., carnation (Zeng *et al*., 2011), chrysanthemums (Mansouri, 2012), gladiolus (Dwivedi *et al*., 2016), and rose (Liao *et al*., 2013). The mechanism of SA action, as a hold solution, in vase life of cut flowers has not been clarified; however, published data suggest some association with ethylene production. Zhang *et al*. (2003) showed that application of SA resulted in suppression ACC synthase and ACC oxidase activities and biosynthesis of ethylene in kiwifruit. In Gladiolus, the maximum vase-life was obtained once flowers treated with a solution containing 100-ppm 5-sulfosalicylic acid + 4% sucrose (Ezhilmathi *et al*., 2007). 8-HQS is a subclass of quinolones with a wide variety of biological effects. The 8-hydroxyquinoline derivatives emerged as a hold-solutions being widely explored for several biological functions such as antifungal effects (Oliveri and Vecchio, 2016) and antimicrobial (Abouelhassan *et al*., 2017). According to van Doorn (1997), the bending of gerbera cut flowers was caused by low turgescence of the flower scape when facing water uptake problems. In addition, he notified that bacte-

ria in the vase water were the most common cause of xylem blockage affecting water uptake. Accordingly, antimicrobial compounds such as 8-hydroxyquinoline citrate (Elhindi, 2012) and essential oils (Salehi Salmi *et al*., 2018) were applied to improve vase-life of cut flowers. The effect of the application of MJ on vase life of cut flowers varies widely among species and cultivars. These reports indicated that the ethylene production rate might change with the kind of genes, which were stimulated by MJ (Salimi *et al*., 2016). The results of the present study indicated that the decline in the vase life was significantly less in cut flowers in MJ-treated, compared with other treatments.

Water loss and water uptake

Comparing the results of the different hold-solutions revealed statistically significant differences such that the maximum and minimum amounts of water loss, in the 10th day, occurred on cut gerbera hold in 100 µM 8-HQS+ 200 µM MJ and 100 µM 8-HQS+ 100 µM SNP, respectively (Fig. 2). From figure 2 the maximum water loss occurred also on cut gerbera hold in MJ 200 µM alone (together with 100 µM 8-HQS+ 200 µM MJ). Also, data showed that cut flowers treated with 50 and 100 µM 8-HQS; 200 µM SA; 50 and 100 µM SNP; 50, 100, and 200 SA+ 100 µM 8-HQS; 50 µM MJ+ 100 µM 8-HQS; 50, 100, and 200 µM SNP + 100 µM 8-HQS hold solutions lost lower water the control (Fig. 2).

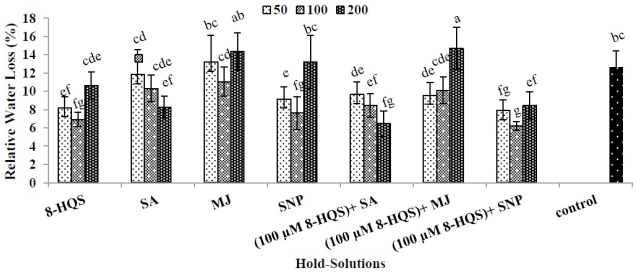

Fig. 2 - Relative water loss of cut flower of gerbera in various hold solutions with different concentrations (µM). The indicator was determined on 10st day. Columns followed by different letters are significantly different at P=0.05.

The lowest amount of water uptake appeared on 10th day in the cut flowers treated with 100 µM 8-HQS+ 200 µM MJ (Fig. 3). However, there was no significant difference between this treatment and 50 µM MJ, 200 µM MJ, and 200 µM SNP control treatments. In other treatments, water uptake was increased over 10 days of postharvest life in comparison with control. However, water uptake amounts showed significant differences among hold-solutions such that the maximum amount of it was observed in a cut flower treated with 100 µM 8-HQS+ 100 µM SNP (Fig. 3). Cut flower senescence is closely associated with water uptake stem and RWC of petals, whereas, these characteristics are closely related with the contents of osmoregulation substances such as soluble sugars and soluble proteins (Hou *et al*., 2018).

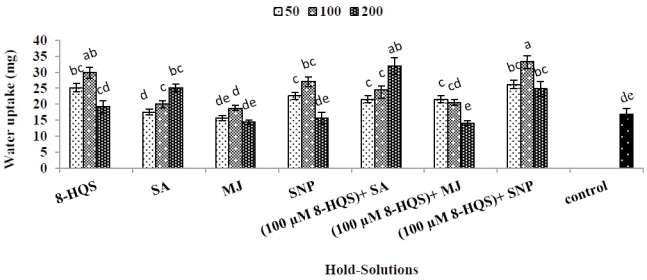

Fig. 3 - Effects of different concentrations (µM) of various hold solutions on the water uptake. The indicator was determined during 10 days. Columns followed by different letters are significantly different at P=0.05.

Soluble carbohydrates of petal

Changes of sugars content of gerbera petals are shown in figure 4. Maintenance of elevated total soluble carbohydrates content exhibited by the flowers under hold-solutions treatments can be correlated with the delay in senescence and the increase in vase life of Gerbera flowers. The results indicate that treatment with hold-solutions, except the high concentration of MJ, with or without 8-HQS, caused a significant decrease in reducing sugars compared with the control. Reducing carbohydrate starvation or its symptoms led to unwanted color changes and eventually increased susceptibility to microorganisms. Postharvest treatments can reduce carbohydrate starvation during the vase life phase. Postharvest treatments like sugar feeding often have

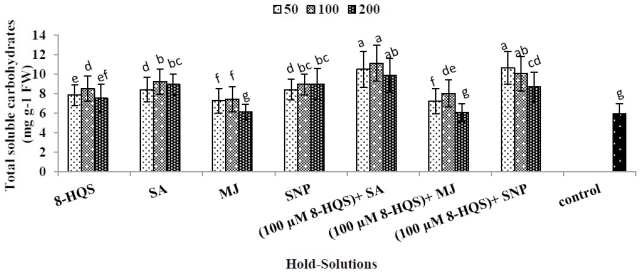

Fig. 4 - Total soluble carbohydrates in petals of hold-solutions-treated and untreated gerbera cut flowers stored at 22°C for 10 days.

a positive effect on vase life in general. It seems that 8-HQS, by preventing vascular blockage, caused sugars directly to reach flowers in the transpiration stream via xylem. Increased sugar caused by exogenous sucrose is well known from earlier studies (Ichimura *et al*., 1999; Promyou *et al*., 2012). Han *et al*. (2018) have illustrated that SNP treatment inhibited significantly the degradation of sucrose of peach fruit at the end of storage. They suggested sugars were significantly affected by SNP treatment probably due to the activities of sucrose metabolism enzymes. Yu *et al*. (2016) found that MJ treatment could increase the encoding level and enzyme activity of sucrose phosphate synthase, which resulted in the enhancement of sucrose content.

Petal pigments

The highest concentration of anthocyanin in gerbera florets was in the SA+ 8-HQS treatments, followed by the 8-HQS and SA treatments. The MJ, with or without 8-HQS, treatments did not result in a significant increase in anthocyanin concentration compared with that of the control (Fig. 5). Carotenoid content of petals were increased under treatment with all concentrations of SA with or without 8-HQS, all concentrations of SNP with or without 8-HQS, high concentrations of 8-HQS, and all concentrations of MJ with 8-HQS (Fig. 6) while the control had the lowest amount of carotenoid content among all treatments at the end of the experiment.

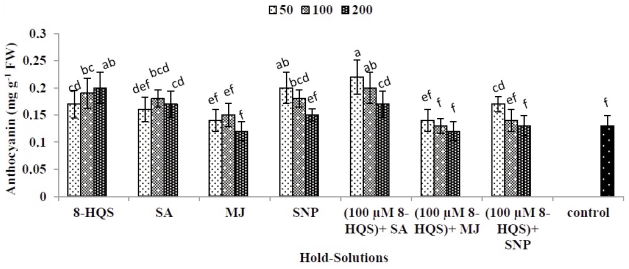

Fig. 5 - Effects of different concentrations (µM) of various hold solutions on anthocyanin content. The indicator was determined during 10 days. Columns followed by different letters are significantly different at P=0.05.

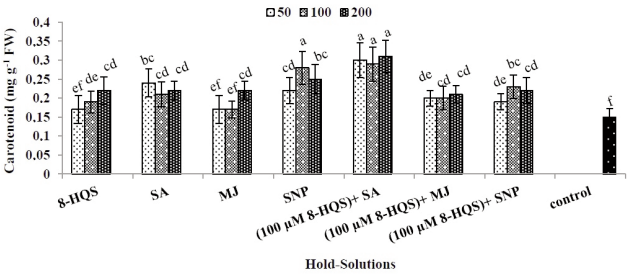

Fig. 6 - Effects of different concentrations (µM) of various hold solutions on amount of carotenoid. The indicator was determined during 10 days. Columns followed by different letters are significantly different at P=0.05.

Figure 7 depicts the contents (expressed as mg/g fresh weight) of flavonoid obtained from petals of gerbera cut flowers untreated and treated with hold solution at different concentrations. By comparing untreated-control samples, hold solutions differences could be clearly established (Fig. 7). In particular, the concentrations of petal flavonoid were significantly ($p<0.05$) higher in treated cut flowers with SNP, SA or MJ; except 50 µM SA, 200 µM MJ, 50 µM SNP treatments; than Control. As can be seen, cut flowers treated with 100 and 200 µM SNP+ 100 µM 8-HQS exhibit higher concentrations of flavonoid than the other treatments.

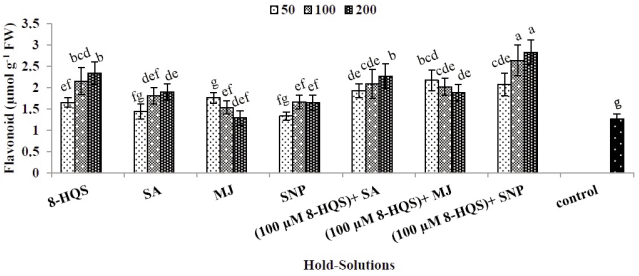

Fig. 7 - Effects of different concentrations (µM) of various hold solutions on flavonoid. The indicator was determined during 10 days. Columns followed by different letters are significantly different at P=0.05.

Despite the results of this study, most investigations on vase life of cut flowers do not present data on the changes in pigmentation and those that use subjective color grades for evaluation. Browning and discoloration are important factors in determining display quality of cut flowers and in many cases are the major reason for the termination of vase life (Elhindi, 2012; Khalaj *et al*., 2017; Salehi Salmi *et al*., 2018). Petal coloration is caused by the accumulation of pigment, including carotenoids, flavonoids, and betacyanins, within epidermal cells. Anthocyanins are synthesized via the phenylpropanoid and flavonoid pathways (Tanaka *et al*., 2008). Carbon metabolite levels, directly and indirectly, affect almost every metabolic process in a plant life. Anthocyanin and Carotenoid biosynthesis occur concomitantly with sugar accumulation in plant tissue (Hara *et al*., 2003; Zhang *et al*., 2015). Similarly, in our study, some vase solutions promoted soluble carbohydrates contents in the petals of gerbera cut flowers (Fig. 4), accompanying higher anthocyanin contents and presenting better ornamental quality of petal color (Figs. 5, 6, and 7). It is reported that application of MJ enhanced

accumulation of flavonoids in *Daucus carota* (Sircar *et al*., 2012).

POX activity

The POX activity in the petal of gerbera flowers that were treated with different concentrations of all hold-solutions, except 100 µM MJ+ 100 µM 8-HQS, slightly increased during vase life (Fig. 8). The highest activity of POX was observed in cut flowers hold in 50 µM 8-HQS, 100 µM SA, and 200 µM 8-SNP solutions. The enzymatic antioxidant system can work against the accumulation of reactive oxygen species (ROS). Regulation of the antioxidant status and ROS production by SNP in plant cells subjected to either biotic or abiotic stressors is well established (Vidal *et al*., 2018). Previously, it has been shown that SNP provides protection in broccoli florets against rapid yellowing after harvest (Shi *et al*., 2016). This study was carried out to provide evidence for the ability of SNP to regulate flower senescence through regulation of the antioxidant status of gerbera petal cells. Salicylic acid can also act as a protector against several stressful impacts, scavenge free oxygen radicals, and counteract oxidative damage by regulating cellular redox balance and accelerating the transformation of superoxide anion and enhancing the activities of antioxidant enzymes (Zhang *et al*., 2003). SA treatment reduced chilling injury in anthurium via improving the activities of SOD, CAT, and POX (Promyou *et al*., 2012). Kumar Rai *et al*. (2018) reported that SA and SNP enhanced tolerance to heat stress in *Lablab purpureus*, by elevating antioxidant enzyme activity of POX, SOD, and CAT and thus alleviating heat-induced oxidative damage. The present study, for the first time, indicated that SA and SNP treatment delayed the senescence of gerbera flowers via improving the activity of antioxidant enzymes of POX. MJ was reported to stimulate POX activity in banana plants and reduce the level of O_2 and H_2O_2 (Sun *et al*., 2013). In this regard, Fan *et al*. (2016) reported inducing resistance responses in eggplant fruit by increasing the expression of POX genes.

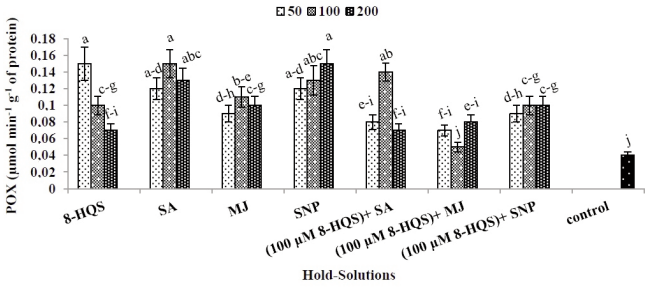

Fig. 8 - Effects of different concentrations (µM) of various hold solutions on activity of POX. The indicator was determined during 10 days. Columns followed by different letters are significantly different at P=0.05.

Protein

There was a significant difference among the type of hold-solution treatments on protein in the 10th day, although the protein content showed no significant differences among concentrations of a hold-solution. Protein content for SA treatments, with or without 8-HQS, increased compared with the control; however, it was not significantly different from other treatments to control (Fig. 9). The increase observed in the protein content, through treatment with SA, was likely the result of less protein degradation (Alaey *et al*., 2011) or an increase of protein synthesis (Ezhilmathi *et al*., 2007). Under flower senescence, the stimulation of protein synthesis leads to protein accumulation that may involve in the enhanced activity of enzymes as a defense mechanism (Promyou *et al*., 2012). To support the accumulation of proteins

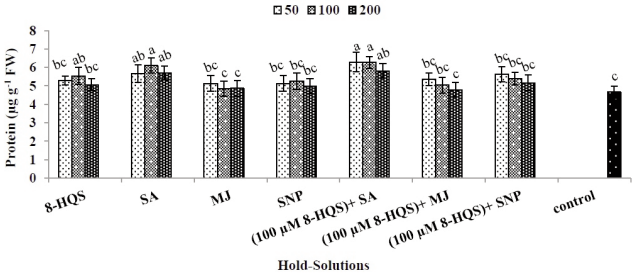

Fig. 9 - Petal proteins of gerbera cut flower in various hold solutions with different concentrations (µM). The indicator was determined on day 10. Columns followed by different letters are significantly different at P=0.05.

due to SA treatment, it was reported that SA results in a pronounced increase in total protein content and the formation of new proteins in roses (Alaey *et al*., 2011). It seems that MJ by increasing antioxidant defense enzymes leads to maintaining carbohydrate at high levels, as antioxidant inhibits the oxidation of cell biomolecules like proteins and carbohydrates (Kanani and Nazarideljou, 2017). In the present study, different concentrations of SNP and SA, with or without 8-HQS, could markedly increase the contents of soluble sugars and soluble proteins, which increased the RWC of petals and water uptake. These increases were helpful in increasing the water retaining capacity and also played an important role in increasing vase life of gerbera cut flowers. In addition, Schouten *et al*. (2018) suggested that SNP enhances flow through xylem vessels by increasing the ionic strength of the vase water.

MDA

In the present experiment, a noticeable decrease in the MDA in control treated cut flowers compared with the other vase-solutions (Fig. 10). Accumulation of elevated amounts MDA in control treated cut flower was recorded. This accumulation indicates the presence of oxidative stress in gerbera petals. Zhang *et al*. (2015) reported over-reduction of the electron transport chain in mitochondria as the main source of O_2 production under specific stress conditions. Production of H_2O_2 can occur during lipid catabolism as a side-product of fatty acid oxidation. ROSs are also involved in the detoxifying reactions catalyzed by cytochromes in both the cytoplasm and the endoplasmic reticulum (Kumar Rai *et al*., 2018). Obviously, the observed high activity of POX in SA- and SNP-treated cut flowers are as a protective mechanism against senescence. It has been revealed that MJ mitigates the ROS effects in maize seedlings subjected to oxidative stress (Ahmadi *et al*., 2018).

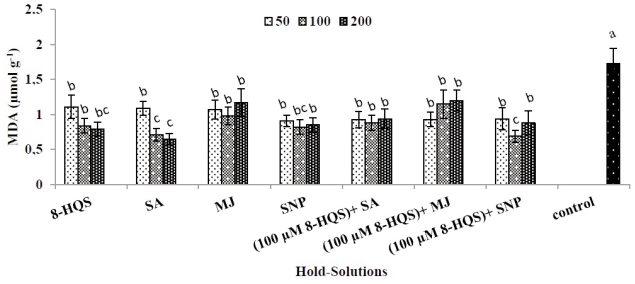

Fig. 10 - Effects of different concentrations (µM) hold solutions treatment on MDA of petal. The indicator was determined on day 10. Columns followed by different letters are significantly different at P=0.05.

4. Conclusions

Treatment with SA+8-HQS extends the vase life of gerbera cut flowers at relatively low SA concentrations and leads to generating the maximum cost-effectiveness. In conclusion, our results demonstrated that 8-HQS improves the vase life through increasing water uptake and consequently increases total soluble carbohydrates. Also, this effect may be exerted by improving the membrane stability and increasing proline, antioxidant activity, and pigment contents in the presence of SA.

Acknowledgements

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Water retention of substrates potentiates the quality of lettuce seedlings

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Key words: chemical properties, *Lactuca sativa* L., physical properties, root system morphology, shoot morphology.

Abstract: A difficulty in the production of lettuce seedlings in containers is to assure the production of shoot biomass with limited portion of roots, restricted to a small volume of substrate. Therefore, we investigate if substrates associated to lettuce cultivars interfere in the seedling quality. The treatments, outlined in a two-factorial scheme, were two cultivars of lettuce and four substrates, arranged in a randomized complete block design, with three replications. The results showed that seedlings produced in the substrate with higher water retention capacity had higher performance in relation to shoot morphology and root system morphology. In conclusion, the data show that the seedlings quality of lettuce cultivars associate with the types of substrates studied and that seedlings produced in substrate with higher water retention have better quality.

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1. Introduction

In horticultural crops the supply of quality seedlings to the producers is important to obtain high production after the establishment of the plants in their growth medium. Such quality is related to the plants resistance to biotic and abiotic stresses (Zhao *et al.,* 2016). Among vegetables, lettuce (*Lactuca sativa* L.) is one of the most cultivated (Kim *et al.,* 2016), with a world production of approximately 25 million tons (FAO, 2014). In order to maintain this production in an upward manner, quality seedlings must be provided to producers. Thus, the production of seedlings is one of the most important stages in lettuce cultivation, because this process reflects on the productive performance of the plants (Auler *et al.,* 2015). However, a difficulty in the production of seedlings in containers is to ensure the production of shoot biomass with limited portion of root (Lemaire, 1995), restricted to a small volume of substrate, in response to the species/cultivars used.

As the substrates have a wide variation in their physicochemical pro-

perties (Fermino and Kämpf, 2012), choosing a suitable material is essential to the development of the plants (Mondragón-Valero *et al.,* 2017). The substrates used must be low cost and easy to handle (Noya *et al.,* 2017), to have porosity around 85% (Kämpf *et al.,* 2009) and water retention capacity (Graceson *et al.,* 2013). For lettuce, it is necessary to choose material that ensures these physical characteristics, since it is a species with high water demand (Nunes *et al.,* 2017). Moreover, the choice of the cultivar is also important, because the genotypes can interact with biotic and abiotic factors, influencing the quality of the seedlings produced (Martins *et al.,* 2017).

Knowing that substrates used in seedlings production are essential to plants germination and establishment (Auler *et al.,* 2015) and that crop productivity is linked to this input (Smiderle *et al.,* 2001), this question arises: how substrates associated to lettuce cultivars affect the seedlings quality?

Therefore, based on the hypothesis that the quality of lettuce cultivars is dependent of the water retention capacity of the substrate, the objective of the present study was to evaluate if substrates associated to lettuce cultivars interfere in the seedlings quality. This study provides a view of the development of lettuce seedlings using different substrates to improve the seedlings quality (e.g., increase the growth of shoot biomass and root system) grown in greenhouses.

2. Materials and Methods

Plant material, treatments description and experiment site

The seeds of lettuce used in the work were of the cultivars Mimosa Roxa Salad Bowl (Purple), of bright greenish purple color, and Mônica SF 31 (Green), of medium green color, both of the group crisphead.

The materials used as substrates were carbonized rice husk (CRH), Horta 2® (HOR), TN Gold® (TNG) and a mixture (MIX) composed of 40% CRH, 40% HOR and 20% TNG. The composition of HOR consists of pine bark, vermiculite, acid correction and fertilizers (nitrogen, phosphorus and potassium) in quantities not supplied by the manufacturer. The composition of TNG consists of sphagnum peat, expanded vermiculite, dolomitic limestone, agricultural gypsum and fertilizers (nitrogen, phosphorus and potassium) in quantities not supplied by the manufacturer. No fertilizer was added to the substrates. The rice husk used in the work was carbonized (Kämpf *et al.,* 2006).

The experiment was developed in the Brazilian subtropics, in the city of Passo Fundo/RS (28° 15' 46" S, 52° 24' 24" W), from April to May (Fall) of 2017. The trial took place on trays kept on metal benches, 1.2 m above the soil surface, in a agricultural greenhouse of 90 m^2, with semicircular roof, installed in the northeast-southeast direction. The galvanized steel frame was covered with low density polyethylene film, with anti-ultraviolet additive and with a thickness of 150 microns, and the sides were covered with anti-aphid screen.

The irrigation used was with sprinklers, in the mechanized system, with a flow rate of 2 l min^{-1} per unit. The irrigation regime consisted of four sprinklers per day, with total wetting of seven minutes. The water blade supplied to the seedlings was 4.35 mm day^{-1}. During the execution of the experiment, the photosynthetically active radiation (PAR) and the mean air temperature inside the greenhouse were monitored, with mean values of 110.5 µmol m^{-2} s^{-1} and 17.4°C, respectively.

Experimental design

The treatments, outlined in a two-factorial scheme, consisted of two lettuce cultivars (Purple and Green) and four substrates (CRH, HOR, TNG and MIX). The production of the seedlings was carried out in trays of expanded polystyrene, with dimensions of 0.34 m of width and 0.68 m of length. Each tray had 128 cells, with a volume of 35 cm^3. The experimental design was randomized blocks with three replicates (n= 3; one replicate per tray, i.e. three trays were used in the experiment).

On April 20, the trays were filled with the substrates CRH, HOR, TNG and MIX, and after that, five seeds of the lettuce cultivars were sown in each cell. In each tray each treatment was composed of 16 seedlings, that is, a total of 48 seedlings per treatment (16 seedlings/treatment x 3 replicates). Considering that we used 8 treatments, our experiment consisted of a total of 384 seedlings (16 seedlings/treatment x 3 replicates x 8 treatments).

Determination of physicochemical properties of substrates

A sample of 1 l of each substrate was collected and analyzed to obtain physicochemical attributes of the materials.

The physical attributes determined in the substrates were: density (D), total porosity (TP), aeration space (AS), readily available water (RAW) and buffer water (BW).

The chemical attributes determined in the subs-

trates were nitrogen (N), phosphorus pentoxide (P_2O_5), potassium oxide (K_2O), organic carbon (OC), hydrogenionic potential (pH), electrical conductivity (EC) and cation exchange capacity (CEC) (MAPA, 2014).

Regarding the seedlings, the evaluations began one week after sowing. Morphological attributes of the shoot and the root system of the seedlings were evaluated.

Determination of shoot morphology

In relation to the shoot morphology, forty-eight seedlings per treatment were evaluated. Four days after sowing the percentage of seed germination was evaluated by means of the equation:

$$\text{Germination (\%)} = (\text{seed germinated/total number of seeds}) \times 100 \quad (1)$$

After the germination, thinning was performed, leaving one plant per cell in each tray. In addition, the date of emergence of the cotyledons and the issuance of the first, second and third leaves were noted. Thirty-three days after sowing, the stem base diameter (SBD) and the shoot height (SH) of the seedlings were measured with a digital caliper. The fresh (SFW) and dry (SDW) weight of the shoot was also evaluated. In order to obtain the dry weight, the plants were kept in a drying oven with forced air circulation, at 65°C for 48 hours, until constant weight, and weighed in an electronic analytical balance.

Determination of root system morphology

Regarding to the root system morphology, forty-eight seedlings per treatment were evaluated. The roots were collected and washed in water to eliminate the substrate fragments. Thus, the roots were scanned and then the images obtained were analyzed by WinRHIZO® software. The attributes evaluated were the total root length (TL, cm), root surface area (SA, cm^2) and root volume (RV, cm^3). The roots were grouped by software in different diameter classes in relation to their total length (Böhm, 1979): very thin roots (VTR, Ø <0.5 mm), fine roots (FR, Ø 0.5 to 2 mm) and thick roots (TR, Ø > 2 mm). The fresh (RFW) and dry (RDW) weight of the root system was also evaluated, following the methodology described previously.

Determination of seedlings quality

The seedlings quality was obtained by models of plant development. These development models are mathematical models that consider plant growth variables (Cournède *et al.,* 2013), such as shoot morphology and root system morphology. Thus, the seedling vigor index (SVI) was determined according to Abdul-Baki and Anderson (1973), by the equation:

$$\text{SVI} = \text{germination (\%)} \times (\text{shoot length} + \text{root length}) \quad (2)$$

It was determined, also, the Dickson quality index (DQI), proposed by Dickson *et al.* (1960), by the equation:

$$\text{DQI} = (\text{TDW}) / (\text{H/SBD} + \text{SDW/RDW}) \quad (3)$$

where TDW = total dry weight (g); H = shoot height (cm); SBD = stem base diameter (cm); SDW = shoot dry weight (g); RDW = root dry weight (g).

In addition, dry matter accumulation (DMA) of the shoot and of the root system was determined, according to Atif *et al.* (2016), by the equation:

$$\text{DMA} = (\text{DW/FW}) \times 100 \quad (4)$$

where DW = dry weight (g); FW = fresh weight (g).

Statistical analysis

The data were submitted to analysis of variance and the means of the treatments were compared by Tukey test, at 5% probability of error, with the aid of the Assistat® program (Silva and Azevedo, 2016).

3. Results

Physicochemical properties of substrates

The results of the physical characterization of the substrates used in this experiment (Table 1) showed

Table 1 - Physical properties of the substrates used in the study

Substrates	Density (kg m^{-3})	Total porosity (m^3 m^{-3})	Aeration space (m^3 m^{-3})	Readily available water (m^3 m^{-3})	Buffer water (m^3 m^{-3})
Carbonized rice husk	170±12.33 c	0.879±0.11 b	0.365±0.02 b	0.395±0.10 a	0.009±0.003
Horta 2®	241±05.26 a	0.837±0.10 d	0.303±0.01 d	0.149±0.02 d	0.020±0.001
TN Gold®	088±10.98 d	0.916±0.13 a	0.519±0.11 a	0.202±0.08 c	0.007±0.002
MIX	183±07.47 b	0.869±0.14 c	0.325±0.08 c	0.259±0.09 b	0.030±0.001
Mean	170.50	0.87	0.378	0.251	0.016
Coefficient of variation (%)	13.58	14.11	15.26	14.45	12.25

MIX= mixture composed of 40% Caborized rice husk, 40% Horta 2® and 20% TN Gold®. Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (P≤0.05, n= 3).

that, considering the density of the materials, the TNG substrate is the lightest. With the values of TP, AS, RAW and BW of Table 1 we elaborated a graph to visualize the relation between air and water in each substrate (Fig. 1). We observed that TNG material showed an unbalanced air-water relation (Fig. 1). In addition, we observed a better balance between air-water in the MIX substrate, that is, when the other materials were combined (40% CRH, 40% HOR and 20% TNG).

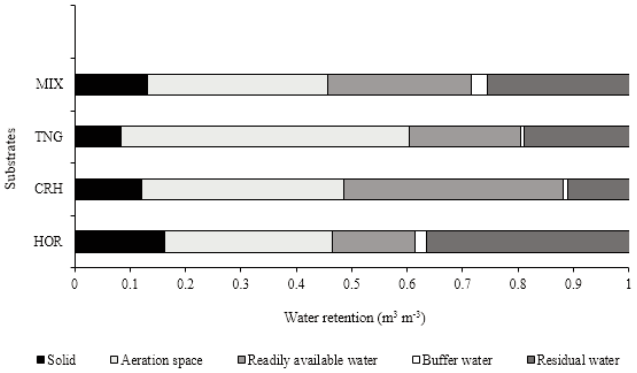

Fig. 1 - Physical characterization of the substrates used in the study; n= 3 CRH= Carbonized rice hull; HOR= Horta 2®; TNG= TN Gold®; MIX= mixture.

In addition, also with the values of TP, AS, RAW and BW of Table 1 we elaborated a graph to visualize the water retention curve of each substrate (Fig. 2), according to De Boodt and Verdonck (1972). The HOR and MIX substrates presented higher water retention, requiring volumes of 0.385 m^3 m^{-3} and 0.285 m^3 m^{-3}, respectively, to remain in the range of water easily available to plants (10-50 -cm H_2O) (Fig. 2). On the other hand, the CRH substrate had greater drainage of water (Fig. 2).

The four materials showed availability of nutrients, except for K_2O. Among the substrates, CRH presented 23% more pH than TNG. The opposite was obtained for EC and CEC, with CRH being 58% lower

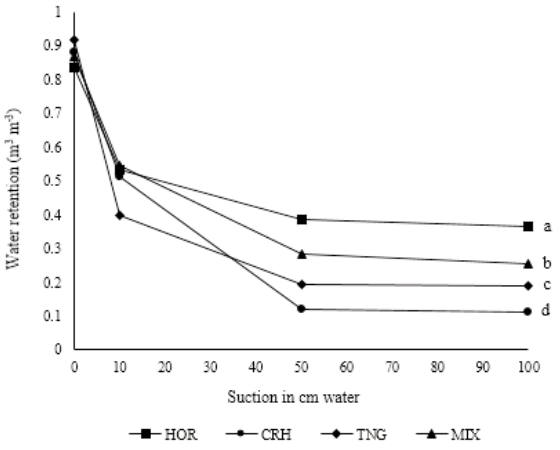

Fig. 2 - Water retention curve of the substrates. CRH: Carbonized rice hull; HOR: Horta 2®; TNG: TN Gold®; MIX: mixture. Different letters indicate significant differences by the Tukey test (P≤0.05, n = 3).

than the HOR for EC and 85% lower than TNG for CEC (Table 2).

Shoot morphology

In all treatments there was 100% germination of the seeds. Up to the first true leaf, the two cultivars took the same period (11 days) to differentiate. The differences began to be identified from the second true leaf. For this attribute, the two cultivars produced in the CRH and the Green cultivar produced in the TNG took longer to emit the third true leaf (Fig. 3). As a consequence, at the time of transplantation the third leaf was poorly expanded.

We did not observe effects of the cultivars on the shoot morphology of the seedlings. This means that the seedlings of both cultivars can be produced on any of the substrates. However, we observed only effect in relation to the substrates for the SH, SFW and SDW.

The seedlings produced on the HOR substrate had higher SH (5.86 cm ± 1.82) than those produced on the CRH substrate (3.24 cm ± 0.77), but did not differ

Table 2 - Chemical properties of four substrates

Substrates	N % (m/m)	P_2O_5 % (m/m)	K_2O % (m/m)	OC % (m/m)	pH	EC (mS cm^{-1})	CEC (mmolc kg^{-1})
Carbonized rice husk	0.69±0.01 a	1.71±0.10 a	0	07.21±03.03 d	7.2±1.0 a	0.19±0.01 c	134.60±12.2 d
Horta 2®	0.36±0.09 c	0.39±0.06 d	0	12.60±06.22 b	6.1±2.2 c	0.45±0.01 a	278.60±15.4 b
TN Gold®	0.65±0.01 b	1.37±0.11 c	0	31.16±11.02 a	5.6±1.3 d	0.36±.0.2 b	892.98±13.7 a
MIX	0.35±0.02 c	1.48±0.03 b	0	10.38±04.33 c	6.4±3.1 b	0.37±0.03 b	230.05±11.2 c
Mean	0.51	1.23	0	15.33	6.32	0.34	384.05
Cofficient of variation (%)	12.25	15.90	0	16.30	11.51	17.91	14.15

MIX= mixture composed of 40% Caborized rice husk, 40% Horta 2® and 20% TN Gold®. Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (P≤0.05, n= 3).

statistically from the seedlings produced in TNG and MIX (Fig. 4 A). In addition, the seedlings produced on the HOR substrate showed higher SFW (0.27 g ± 0.17) and SDW (0.012 g ± 0.0033) than those produced on other substrates (Fig. 4 B and C, respectively). In general, seedlings obtained on the HOR substrate produced 71% more shoot biomass than those grown in CRH (Fig. 4).

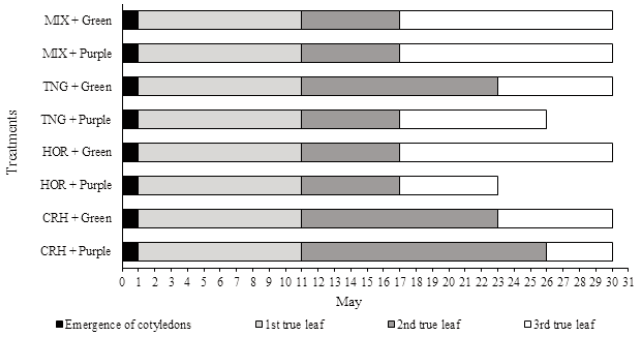

Fig. 3 - Combination of two lettuce cultivars produced in four substrates in relation to the period of emergence and expansion of the first true leaves. CRH= Carbonized rice hull; HOR= Horta 2®; TNG= TN Gold®; MIX= mixture; Purple=Mimosa Roxa Salad Bowl cultivar; Green= Mônica SF 31 cultivar.

Root system morphology

Regarding the root system morphology, we observed significant differences for the interaction between substrates and cultivars in relation to RV and TR (Table 3). For the interaction between the factors, the best combination corresponded to the seedlings of the Green cultivar produced on the HOR substrate, both for root volume and for thick roots (Table 3).

We also observed significant differences for substrates only for RFW, RDW, SA and FR (Table 4). In this respect, we observed that the seedlings produced in HOR presented a larger SA, but did not differ statistically from the seedlings produced in MIX (Table 4). The seedlings developed in CRH had smaller amount of FR in relation to those obtained in the other substrates (Table 4). Regarding the RFW, seedlings produced in HOR were superior to those obtained in CRH, but did not differ from the seedlings produced in TNG and MIX (Table 4). In addition, the seedlings obtained from HOR and MIX had a higher RDW in comparison to those developed in CRH and TNG (Table 4).

In relation to lettuce cultivars the significant differences occurred for RFW, RDW, TL, SA and FR (Table 4). Thus, we observed that the seedlings of the Green cultivar presented superior performance of the root

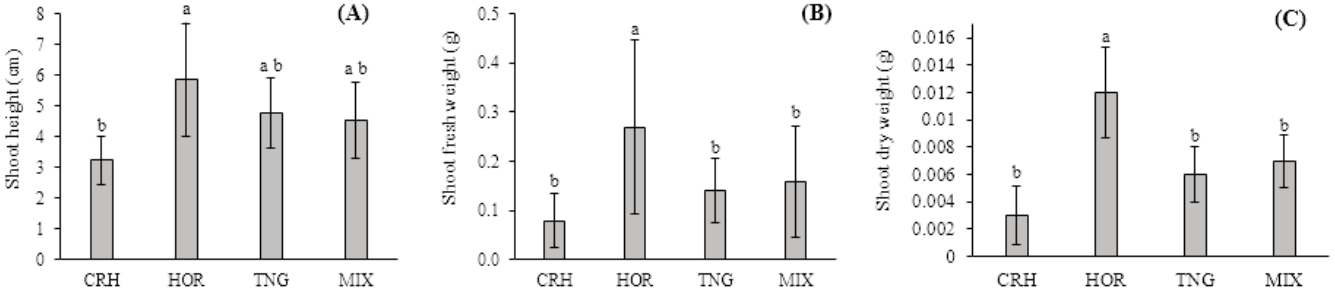

Fig. 4 - Shoot morphology of lettuce seedlings produced in substrates. (A) Shoot height (SH, cm); (B) Shoot fresh weight (SFW, g); (C) Shoot dry weight (SDW, g). Values are means ± standard deviation. Different letters above columns indicate significant differences by the Tukey test (P≤0.05, n = 3). CRH= Carbonized rice hull; HOR= Horta 2®; TNG= TN Gold®; MIX= mixture.

Table 3 - Root system morphology of two lettuce cultivars (*Lactuca sativa* L.) produced in four substrates

Substrates	Root volume (cm³)		Thick root (cm)	
	Purple cv.	Green cv.	Purple cv.	Green cv.
Carbonized rice husk	0.019 ±0.004 A b	0.043±0.017 A c	0.090±0.15 A a	0.078±0.10 A c
Horta 2®	0.074 ±0.029 B a	0.185±0.023 A a	0.501±0.41 B a	3.021±0.51 A a
TN Gold®	0.053 ±0.014 A ab	0.079±0.027 A bc	0.209±0.17 A a	0.660±0.61 A bc
MIX	0.066 ±0.007 B a	0.111±0.019 A b	0.420±0.22 B a	1.440±0.78 A b
Mean	0.07		0.80	
Cofficient of variation (%)	21.77		53.88	

MIX= mixture composed of 40% Caborized rice husk, 40% Horta 2® and 20% TN Gold®. Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (P≤0.05, n= 3).

Table 4 - Root system morphology of two lettuce cultivars (*Lactuca sativa* L.) produced in four substrates

	Total root length (cm)	Root surface area (cm²)	Fine root (cm)	Root fresh weight (g)	Root dry weight (g)
Substrates					
Carbonized rice husk	21.18±05.89	2.81±1.13 c	5.01±2.52 b	0.030±0.01 b	0.0017±0.0008 b
Horta 2®	28.42±10.55	6.54±2.48 a	9.87±2.30 a	0.087±0.04 a	0.0036±0.0010 a
TN Gold®	24.44±13.36	4.39±1.78 bc	8.61±2.58 a	0.060±0.04 ab	0.0023±0.0006 ab
MIX	28.73±09.55	5.54±1.53 ab	9.09±1.93 a	0.061±0.02 ab	0.0032±0.0006 ab
Cultivars					
Purple	21.92±08.13 b	3.70±1.54 b	7.20±3.07 b	0.038±0.01 b	0.0021±0.0008 b
Green	29.46±10.54 a	5.94±2.23 a	9.55±2.52 a	0.080±0.04 a	0.0033±0.0010 a
Mean	25.69	04.82	08.37	00.05	0.0027
CV (%)	26.28	21.32	20.07	46.77	35.41

MIX= mixture composed of 40% Caborized rice husk, 40% Horta 2® and 20% TN Gold®. Data presented as mean ± standard deviation. Means followed by the same letter in the column did not differ significantly by the Tukey test (P≤0.05, n= 3).

system in relation to the seedlings of the Purple cultivar (Table 4).

Seedlings quality

Regarding the seedlings quality, we observed statistical diferences for the substrates and for the cultivars. Analyzing only the substrates, there were significant differences for the DQI. As for the cultivars, the significant differences were observed regarding the SVI and DQI.

Seedlings produced on the HOR substrate presented higher DQI (0.000249 ± 0.000089) than those produced in CHR (0.00011 ± 0.000071), but did not differ statistically from seedlings produced in MIX (Fig. 5). This higher quality of the seedlings produced on HOR increased by 56% in relation to those produced on CHR material (Fig. 5).

The Green cultivar, regardless of the substrate, presented superiority regarding the SVI (Fig. 6 A) and DQI (Fig. 6 B) in relation to the Purple cultivar.

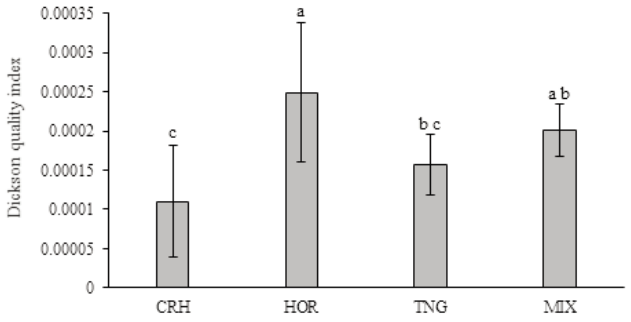

Fig. 5 - Quality of the development of lettuce seedlings produced in four substrates. Values are means ± standard deviation. Different letters above columns indicate significant differences by the Tukey test (P≤0.05, n= 3). CRH= Carbonized rice hull; HOR= Horta 2®; TNG= TN Gold®; MIX= mixture.

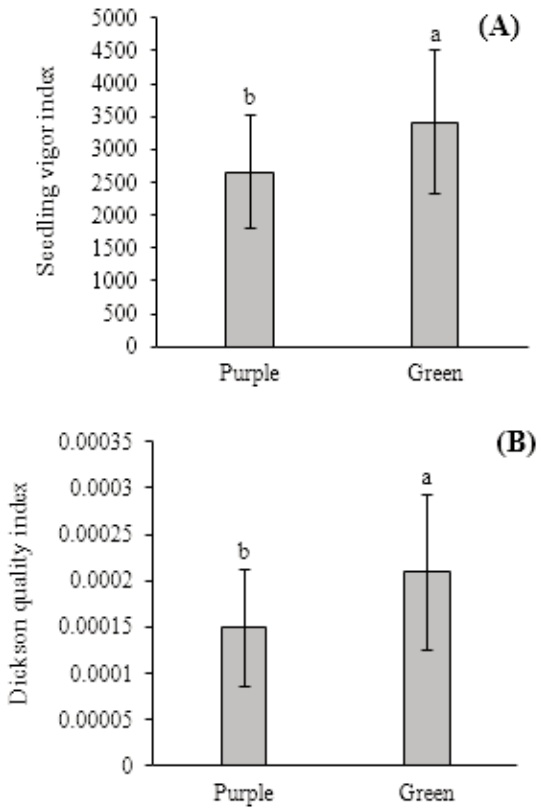

Fig. 6 - Development of seedlings of two lettuce cultivars produced in four substrates. (A) Seedling vigor index (SVI); (B) Dickson quality index (DQI). Values are means ± standard deviation. Different letters above columns indicate significant differences by the Tukey test (P≤0.05, n = 3). Purple: Mimosa Roxa Salad Bowl cultivar; Green: Mônica SF 31 cultivar.

4. Discussion and Conclusions

In general, the research showed that the quality of lettuce cultivars was associated with the types of substrates studied. However, our study showed that

substrates with higher water retention promoted greater development of seedlings, through models of plant development. In this way, the physical characterization of the substrates used in the production of seedlings allows to select materials with greater water availability, in order to increase the seedlings quality.

The higher quality of the seedlings produced in the HOR substrate was attribute to the higher availability of water of this material, because the water retention capacity of the substrates influences the growth and development of the seedlings (Graceson *et al.,* 2013), covering the shoot morphology (Prevedello and Armindo, 2015) and the root system morphology (Ferraz *et al.,* 2005). In practice, the data referring to the root system morphology indicated that seedlings of the Green cultivar produced in the HOR substrate have a more structured lump, which improves seedling sustainability after transplanting and increases plant survival.

As in CRH there is predominance of large particle sizes, this impairs water retention by the material (Zorzeto *et al.,* 2014), which explained the lower quality of the seedlings produced in this substrate. In practice, the expansion data of the first true leaves showed that seedlings produced in the CRH would not be suitable for transplant because, in addition to lower water retention, this substrate has pH above the ideal range (5.0 to 6.5) (Bunt, 1988), which reduces the availability of nutrients to plants (Lemaire, 1995).

The higher the seedlings quality delivered to the producers the better the development of the plants in their growth medium and the lower their susceptibility to stresses after transplantation. Through the DQI, we verified that more robust seedlings were produced in the HOR substrate. During the production of the seedlings we observed that the PAR mean (110.5 µmol m^{-2} s^{-1}) was below ideal for the lettuce, corresponding to 196.7 µmol m^{-2} s^{-1} (Ferentinos *et al.,* 2000). This may have reduced the photosynthetic rate of the plants, reducing accumulations of biomass and, therefore, DQI values.

As in our study, other studies showed higher growth and development of seedlings produced in substrates with higher water retention (Smiderle *et al.,* 2001; Costa *et al.,* 2007), as verified in the HOR, and lower performance for seedlings produced in substrates with low water availability (Freitas *et al.,* 2013), as observed in the CRH.

Considering the physicochemical characterization of the substrates, the chemical properties are less relevant than the physical properties (Belda *et al.,* 2016). This is because fertirrigation is provided to the plants, according to the need of the cultivated species. However, the physical quality of the substrates must still be weighed in the choice of materials. The density of the substrates, for example, is linked to plant stability (Noya *et al.,* 2017; Wisdom *et al.,* 2017). Very light substrates (< 100 kg m^{-3}) do not sustain plants and very dense substrates (>300 kg m^{-3}) impair the root growth of seedlings due to mechanical impediment (De Boodt and Verdonck, 1972; Fermino and Kämpf, 2012). In addition, a common problem in substrates is insufficient aeration (Nemati *et al.,* 2002) and, therefore, the nurseryman should choose materials with higher aeration levels to improve root growth and increase the acquisition of water and nutrients by seedlings (Jones and Dolan, 2012).

Thus, in order to maximize the quality of the seedlings produced, nurserymen must obtain the physical characterization of the substrates, selecting materials with greater water retention capacity. In addition, seedling development models, such as SVI and DQI, can be used as indicators of the quality of seedlings produced, as we have verified in our study.

In conclusion, the data show that the seedlings quality of lettuce cultivars associate with the types of substrates studied. In addition, we proves that substrates with greater water retention promote greater development of the seedlings. We emphasize that the use of development models can be an alternative to analyze the seedlings quality provided to producers in order to increase lettuce production. We suggest to substrate producers to sell materials with a clear label informing the physicochemical characteristics of the substrate so that nurserymen and producers can establish an adequate management to potentiate the lettuce production chain.

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Yield and physiological response of Perilla (*Perilla frutescens*) under different soil fertility treatments

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Abstract: Medicinal plants are one of the main natural resources of Iran from ancient times. Perilla is one of the most important medicinal plants of the mint family *Lamiaceae*, since there is no study about adaptability of Perilla in Iran climate conditions and different fertilizer systems, this experiment was conducted in two experimental sites. The experiment was conducted as split-plot factorial based on a randomized complete block design with three replications at two experimental regions. The main factor was three chemical fertilizer levels (control, 50, 100, 200 kg/ha) and subplots were different kinds of organic fertilizer (control, humic acid, and compost application) and inoculation with *Piriform osporaindica* (inoculation and without). Among levels of chemical treatments, 50 and 100 kg/ha lead to a better result. Also, humic acid allows to achieve the highest amount of measured traits between different treatments of organic fertilizer. The highest plant yield (147.2 g/m^2) and rosmarinic acid yield per area (3.432 g/m^2) was achieved in 100 kg/ha at chemical fertilizer with humic acid and biological fertilizer application and the lowest plant yield (89.86 g/m^2) and rosmarinic acid yield per area (1.253 g/m^2) was observed in control. Also, the highest stomatal conductance was obtained with application of compost fertilizer (67.67 mmol H_2O m^{-2}s^{-1}). Integrated application of the studied fertilizers showed the more positive effect on yield and quality of Perilla than individual application of those fertilizers.

1. Introduction

The Perilla is a medicinal plant belonging to the *Labiatae* family and is widely cultivated in Southeast Asian countries (Igarashi and Miyazaki, 2013). A number of studies have shown that advantages of Perilla are related to the metabolites contained therein (Ghimire *et al.*, 2017). To date, very limited information exists regarding the adaptability and management of chemical, organic and bio-fertilizer of Perilla in Iran.

Organic matter effects on physicochemical properties and health of soil. It also affects the efficiency of fertilizer application, pesticides and herbicides. One of the most effective organic fertilizers on the growth of

plants and improvement of soil status is humic acid. Humic acid can be obtained from any material such as organic matter, coal, or well-decomposed compost. This material plays an important role in increasing soil moisture, absorbing micronutrients and contributing to carbon sequestration (Spaccini *et al.*, 2002).

Another organic material that plays an important role in soil fertility is compost and it comes from plant and food residues. Some experimental researches have shown the important role of compost in crop production (Adugna, 2016). Compost retains moisture in the soil, slow release nutrients to crops and finally increase the crop yield. Application of compost obtained from plant remains leads to increased fertility, soil nutrients and increases water retention in the soil. Zemanek (2011) also confirmed that application of 50 t/ha and 100 t/ha compost has a positive effect on soil moisture retention.

Moreover, biologic fertilizers assists well in mineralization and channelization of nutrients leading to enhanced plant productivity (Fischer *et al.*, 2007; Ansari *et al*., 2017). Biologic fertilizers adopt various possible ways to accelerate the rate of crop production (Rizvi *et al*., 2015; Ansari *et al*., 2017). They increase and improve plant growth by increasing access to nutrients in the root rhizosphere. These fertilizers provide nutrients through biological processes such as biological nitrogen fixation, phosphorus solubilization, and plant growth stimulation. Also, they help to natural nutrient cycle and build soil organic matter (Kapoor *et al*., 2015). Use of biologic fertilizers ensures healthy plants growth, while enhancing the sustainability and the vigor of the soil. These biologic fertilizers play a special role in increasing plant nutrition and fertility of soils (Vessey, 2003).

A number of studies have shown that the chemical composition of secondary metabolites in the Perilla plant is influenced by various factors such as soil conditions, temperature, growth season (Kiazolu *et al*., 2016), geographic region (Ruberto *et al*., 2002), and phenological stages (Saeb and Gholamrezaee, 2012). Therefore, this study was carried out to evaluate the yield and physiological traits of Perilla cultivars under different fertilizer treatments in two regions. Based on the work, attempt was also made to provide amicable solutions to address the challenges of organic farming with the help of Perilla cultivation in two different locations in dry region of Iran.

2. Materials and Methods

The field experiments were conducted at two locations in Esfahan Province: 1-Mashhad Ardehal (latitude 34° North, longitude 51° East, Altitude 1800 m above mean sea level) 2- Sensen, Iran (latitude 33°, longitude 51°, Altitude 945 m above mean sea level) during 2015. Mashhad Ardehal is located in warm and dry condition and Sensen is located in the mountainous and dry region. The meteorological data recorded during the period of plant cultivation are given in Table 1.

In order to determine the physical and chemical characteristics of experimental fields, two weeks before planting, the soil samples were taken. Physicochemical properties of experimental field soil are presented in Table 2.

This experiment was conducted as split-plot factorial based on a randomized complete block design with three replications at two experimental sites in the year 2015. The main factor was three chemical fertilizer levels (50, 100, 200 kg/ha) plus control, and sub plots were different kinds of organic fertilizer (humic acid, and compost application), plus control; inoculation with *Piriformospora indica* (inoculation and without inoculation) was also evaluated.

Table 1 - Weather characteristics in Mashhad Ardehal and Sensen

Year	Temperature mean (°C)		Precipitation mean (mm)		Humidity mean (%)	
	Sensen	Mashhad Ardehal	Sensen	Mashhad Ardehal	Sensen	Mashhad Ardehal
2015	19.8	18.4	89.3	90.8	42	49
Average of 5 years	20.86	19.88	143.36	133.42	40.6	46.6

Table 2 - Soil analysis of the experimental site

Parameter	Location	
	Sensen	Mashhad Ardehal
Total Nitrogen (%)	0.09	0.28
Phosphorous availability (mg/kg)	15.56	12.18
Potassium availability (mg/kg)	245.6	209.7
pH	7.93	7.83
EC ($dS.m^{-1}$)	2.82	0.89
Organic carbon (%)	0.53	1.63
Clay (%)	14.3	10.3
Silt (%)	33.3	43.4
Sandy (%)	52.4	46.3

Foliar-applied humic acid (95% purity) was obtained from Humic Strong company (70% w/w, pH 5.17, EC: 4.80 mS/cm) and added to the plots at 4 stages of the plant growth. Compost fertilizer was prepared from Barij Essence Company (including 1.5% nitrogen, 1.1% phosphorous, 0.9% potassium, 50% organic matter). Compost fertilizer was used as a strip one week after plantation.

Perilla seeds were purchased from Barij essence Company, Isfahan, Iran. First of all, the seeds were germinated in the laboratory and then transplanted to the pots with 10 cm diameter and 15 cm height at the 4-leaf stage. The mycorrhizal-like fungus *Piriformospora indica* was prepared in mycology laboratory of Sari University, Iran and then prepared biologic fertilizer was placed two weeks in incubator with 20-25˚C and 50 rpm under dark condition. Before planting of Perilla plants in the field, half of the plants in pots were inoculated with biologic fertilizers and then translocated to the field. Each plot was 2×2 meter and had 4 furrows with 25 plant per plot.

The recommended dose of chemical fertilizer (0-50-100 and 200 kg/ha) in the form of urea, triple super phosphate and sulfate potassium was applied to grow the crop. Nitrogen was applied in three splits, the first along with phosphorus and potassium fertilizer at the time of soil preparation while the second part at the time of transplanting of plants and the third part at the flowering of crops. Date of transplanting of both experimental sites was 20 of March.

After plant growth, different traits were studied at the suitable stage. A furrow irrigation system was applied for both experimental sites. Hand weeding of the experimental area was performed as required. In both experimental sites, after flowering five plants from each plot were harvested from two central rows and then plant height, fresh weight and dry weight of plants were measured. Samples dried in an oven at 80°C for 24 hours and the mean of dry weight for each treatment at each replicate was determined.

Rosmarinic acid determination by HPLC

The dried seeds of Perilla were pulverized (60 mesh) for 3 min using an HR 2860 coffee grinder (Philips, Drachten, Netherlands), and each sample (1.0 g) extracted in 30 ml of 80% methanol for 6 h at room temperature in a shaking incubator. The supernatant was centrifuged at 3000g for 3 min and then filtered through a 0.45 lm syringe filter (Whatman Inc., Maidstone, UK) prior to HPLC analysis. For quantification, the peak areas of the isolated compounds were integrated from the HPLC chromatogram at 330 nm using the Dionex software. The stock solutions were prepared by dissolving in methanol to obtain a 1 mg/ml concentration. Calibration curves were obtained with methanol at eight different concentrations (0.5, 1, 2, 5, 10, 25, 50, and 100 g/ml). All calibration curves had coefficients of linear correlation r^2>0.998.

Stomatal conductance

Twenty days after plantation stomatal conductance was measured in the shade-enclosure with saturated light using Promoter (Model KR1301, KOREA TECH) Stomatal conductance (mmol H_2O m^{-2} s^{-1}) was measured at 12:00-14:00 hours on a clear, cloud-less day in fully expanded, healthy, turgid, three flat and uniform in color and size leaves of each samples (Barbieri *et al.*, 2012).

DPPH radical-scavenging activity

This test was used for the determination of the free radical-scavenging activity of the extracts (Ebrahimzadeh *et al*., 2008). DPPH test was performed following the method proposed by Ebrahimzadeh *et al*. (2008). Three young fully developed leaves were selected from each replication.

Polyphenoloxidase (PPO) Activity

Crude extract was prepared by homogenization of frozen plant sample in buffer medium. Leaves of Perilla plant which were stored at -20°C was used for the enzyme extraction. 10 g of the sample were cut quickly into thin slices and homogenized in 50 mL of 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM ascorbic acid and 0.5% (w/v) polyvinylpyrrolidone for 5 min at 4°C. The homogenate was filtered through three layers of cheesecloth and then the filtrate was centrifuged at 5,000 x g for 15 min, and the supernatant was collected.

Enzymatic activity was assayed by determining the rate of increase in absorbance at 420 nm and 25˚C in a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer (Shimadzu Corp., Tokyo, Japan). The reaction mixture contained 3.0 mL of catechol substrate, the solution freshly prepared in 0.05 M sodium phosphate buffer at pH 6.5 and a fixed quantity of PPO. The reference cuvette contained only the catechol substrate solution. The reaction was conducted at 25°C. The PPO activity was defined as a change of 0.001 in absorbance at the conditions of the assay (Pizzocaro *et al*., 1993).

Catalase activity assay

CAT activity was measured by monitoring the H_2O_2 decomposition at 240 nm in 3 mL of reaction

mixture containing 50 mmol/l phosphate buffer (pH 7.0), 15 mmol/L H_2O_2, 100 mL enzyme extract and 0.1% (v/v) Triton X-100 (Aebi, 1984). The activity was expressed in terms of mmol H_2O_2 reduced min/mg/protein.

Statistical analysis

The data were tested for homogeneity and normality of residuals using the Bartlett tests and Kolmogorov-Smirnov, respectively. A combined ANOVA was used to compare treatments for 2 location using PROC GLM of SAS 9.1 software. Means were separated by application of LSD test when the *F* test proved significant at $P \leq 0.05$ and 0.01.

3. Results and Discussion

Combined statistical analysis of two studied location is presented in Table 3. Also, the analysis of variance of Mashhad Ardehal and Sensen locations are shown separately in Table 4 and 5. In Mashhad Ardehal location, the maximum plant yield (157.6 g/m^2) was achieved in 100 kg/ha chemical fertilizer plus humic acid treatment and the minimum amount (90.68 g/m^2) was produced in zero levels of chemical fertilizer and without application of organic treatment (Fig. 1, Table 3). While in Sensen location, the maximum plant yield (126.9 g/m^2) was achieved with the application of 50 kg/ha chemical fertilizer plus humic acid (Fig. 1).

Between treatments of chemical × organic × biologic fertilizer interaction, the maximum plant yield (147.2 g/m^2) was produced in 100 kg/ha along with humic acid and application of biologic fertilizer (Table 6). Differences between plant yield (147.2 g/m^2 and 89.86 g/m^2) showed the importance of fertilizer application in improving yield in this plant. In all of the chemical fertilizer treatments, the plant yield was increased with application of organic and biologic fertilizer, especially with organic fertilizer. So, the integrated fertilizer application was very positive. In the present investigation, application of organic fertilizer especially humic acid significantly increased plant

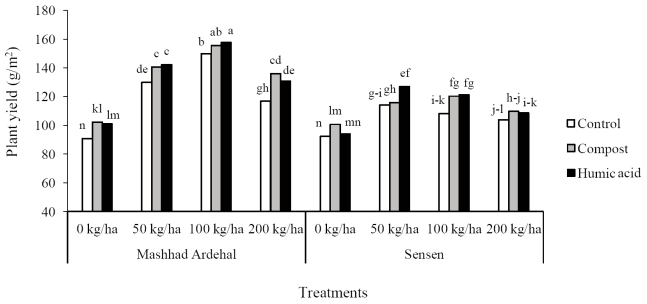

Fig. 1 - Effect of chemical and organic fertilizers on plant yield of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

Table 3 - ANOVA of some morphologic, yield and essence traits of Perilla under different fertilizer systems in Sensen and Mashhad Ardehal in Kashan regions

S.O.V.	d.f.	Means squares						
		Plant yield	Rosmarinic acid content	Rosmarinic acid yield	Stomatal conductance	DPPH	Catalase activity	Polyphenol oxidase
Location (L)	1	14012.64 **	13.80 **	8.87 **	248.11 **	224.60 **	0.00357 *	0.000935 **
Replication (Location)	4	56.75	0.70	0.01	4.02	18.56	0.00047	0.000021
Chemical fertilizer (C)	3	10188.25 **	244.44 **	13.92**	1504.81 **	99.75 **	0.03396 **	0.010834 **
L×C	3	1899.70 **	27.75 **	2.53**	184.57 **	19.74	0.01396 **	0.000478 **
Ea	12	28.48	1.18	0.04	5.74	44.51	0.00063	0.000072
Organic fertilizer (O)	2	1435.58 **	27.93 **	1.69**	76.66 **	75.18 *	0.00178	0.000454**
Biologic fertilizer (B)	1	308.17 **	8.41 **	0.53**	213.84**	0.24	0.00003	0.000146 *
C × O	6	73.04	4.75 **	0.06*	8.46	47.80 *	0.00170 *	0.000217 **
C × B	3	130.19 *	1.05	0.06	2.01	2.82	0.00257 **	0.000022
O × B	2	236.97 **	1.31	0.09*	2.47	19.55	0.00014	0.000008
L × O	2	71.26	4.79 **	0.17**	13.49	2.83	0.00253 *	0.000275 **
L × B	1	88.64	4.42 *	0.22**	1.92	22.75	0.00026	0.000003
L × C × O	6	83.62 *	2.89 **	0.03	10.55	5.98	0.00244 **	0.000180 **
L × C × B	3	18.09	0.16	0.001	3.44	0.20	0.00178 *	0.000009
L × O × B	2	95.96	0.77	0.11*	9.61	0.13	0.00075	0.000010
C × O × B	6	191.38 **	2.38 *	0.11 **	2.76	18.59	0.00068	0.000004
L × C × O × B	6	66.33	1.42	0.04	3.05	6.50	0.00009	0.000006
Eb	80	37.44	0.79	0.02	5.84	19.15	0.00059	0.0000224
CV (%)	-	5.11	4.69	6.66	3.64	2.42	5.48	3.06

*, ** significant at 5% and 1% probability levels, respectively.

yield in all levels of chemical fertilizer. However, there was not clear trend about application of biologic fertilizer. Previously, researchers (Ciarkowska *et al.*, 2017) had highlighted the important role of humic acid, especially in root formation.

Rosmarinic acid concentrations in Perilla were significantly affected by location, chemical and organic fertilization (Table 3) and, at both locations and all chemical fertilization levels, the humic acid application increased rosmarinic acid content in Perilla. This fact exhibited the importance of humic acid on rosmarinic acid content in Perilla. The maximum rosmarinic acid (25.01 mg.g DM) was achieved in Mashhad Ardehal along with using 100 kg/ha chemical fertilizer and application of humic acid (Fig. 2).

Application of organic fertilizer especially humic acid with/without biologic fertilizer in all chemical fertilizer levels increased rosmarinic acid concentration in Perilla. The highest rosmarinic acid (23.48 mg.g DM) was acheved in 100 kg/ha chemical fertilizer plus humic acid and without application of biologic fertilizer (Table 6). Similarly, with plant yield, rosmarinic acid concentration was affected by fertilizer application.

These results are in agreement with Hendawy *et al.* (2015) on *Mentha piperita*. They reported foliar application of humic acid increased growth characteristics and finally possessed the best oil percentage and yield in mint plant. Zaghloul *et al.* (2009) reported also the application of humic acid increased oil content of *Thuja orientalis.*

At both of studied location and with/without application of biologic fertilizer, the maximum rosmarinic acid yield was achieved by using humic acid. The maximum rosmarinic acid yield (2.967 g/m^2) was observed in Mashhad Ardehal and application of

Table 4 - ANOVA of some morphologic, yield and essence traits of Perilla under different fertilizer systems in Mashhad Ardehal in Kashan regions

S.O.V.	d.f.	Means squares						
		Plant yield	Rosmarinic acid content	Rosmarinic acid yield	Stomatal Conductance	DPPH	Catalase activity	Polyphenol oxidase
Replication	2	61.95	0.46	0.01	5.24	24.06	0.0006	0.000040
Chemical fertilizer (C)	3	10082.10 **	195.60 **	13.42**	1256.35 **	540.52 **	0.0411 **	0.00749 **
Ea	6	10.21	1.61	0.04	5.48	58.46 **	0.0003	0.00013 **
Organic fertilizer(O)	2	1049.35 **	27.60 **	1.46**	42.86 **	581.28 **	0.0012	0.00071**
Biologic fertilizer (B)	1	363.69 **	12.52 **	0.71**	87.60 **	13.86	0.0002	0.00005
C × O	6	50.03 *	3.84 **	0.07**	13.03 **	11.17	0.0006	0.00037 **
C × B	3	42.09	0.63	0.03	2.73	1.39	0.0027 **	0.00001
O × B	2	240.40 **	1.60	0.18**	1.28	8.78	0.0006	0.00001
C × O × B	6	89.83 **	1.67	0.08 **	5.60	5.38	0.0009 *	0.000005
Eb	40	21.24	0.86	0.02	3.16	14.71	0.0004	0.00003
CV (%)	-	3.56	4.79	5.65	2.63	2.32	4.48	3.64

*, ** significant at 5% and 1% probability levels, respectively.

Table 5 - ANOVA of some morphologic, yield and essence traits of Perilla under different fertilizer systems in Sensen in Kashan regions

S.O.V.	d.f.	Means squares						
		Plant yield	Rosmarinic acid content	Rosmarinic acid yield	Stomatal Conductance	DPPH	Catalase activity	Polyphenol oxidase
Replication	2	51.56	0.96	0.01	2.80	13.07	0.00030	0.000003
Chemical fertilizer (C)	3	2005.86 **	76.59 **	3.03**	433.02 **	326.18 **	0.00677 **	0.003819 **
Ea	6	46.75	0.76	0.04	5.99	30.56	0.00090	0.000009
Organic fertilizer(O)	2	457.49 **	5.13 **	0.39**	47.28 **	521.70 **	0.00307 *	0.000019
Biologic fertilizer (B)	1	33.13	0.31	0.03	128.16**	9.14	0.00005	0.000095 **
C × O	6	106.63	3.80 **	0.01	5.98	36.55	0.00351 **	0.000025
C × B	3	106.20	0.58	0.02	2.72	1.62	0.00156	0.000019
O × B	2	92.53	0.48	0.02	10.79	10.91	0.00030	0.000006
C × O × B	6	167.87 *	2.15 *	0.06 **	0.21	19.72	0.00065	0.000006
Eb	40	53.64	0.74	0.02	8.51	23.59	0.00077	0.000012
CV (%)	-	6.67	4.59	7.88	4.48	2.98	6.37	2.28

*, ** significant at 5% and 1% probability levels, respectively.

Table 6 - Means comparison of some morphologic, yield and essence traits of Perilla under different fertilizer systems in Sensen and Mashhad Ardehal in Kashan regions

Chemical fertilizer	Organic fertilizer	Biologic fertilizer (Inoculation)	Plant yield (g/m^2)	Rosmarinic acid content (mg/g D.M.)	Rosmarinic acid yield (g/m^2)
0 kg/ha	Control	(-)	93.12 lm	14.40 lm	1.34 l
		(+)	89.86 m	13.94 m	1.25 l
	Compost	(-)	99.40 kl	15.31 l	1.52 k
		(+)	103.40 jk	17.29 jk	1.67 ij
	Humic acid	(-)	99.91 kl	16.44 k	1.64 jk
		(+)	95.17 lm	17.31 jk	1.65 jk
50 kg/ha	Control	(-)	115.70 gh	19.52 def	2.25 gh
		(+)	128.40 de	19.38 defg	2.54 ef
	Compost	(-)	129.50 de	19.56 def	2.52 ef
		(+)	126.90 de	20.11 ced	2.54 e
	Humic acid	(-)	127.90 de	18.92 fgh	2.42 efg
		(+)	141.20 ab	20.37 cd	2.89 bc
100 kg/ha	Control	(-)	131.40 cd	21.05 bc	2.79 d
		(+)	126.70 de	21.58 b	2.75 d
	Compost	(-)	138.00 bc	21.7 b	3.02 bc
		(+)	137.70 bc	21.63 b	3.00 bc
	Humic acid	(-)	131.70 cd	23.48 a	3.11 b
		(+)	147.20 a	23.02 a	3.43 a
200 kg/ha	Control	(-)	107.20 ij	17.52 ij	1.88 i
		(+)	113.50 hi	18.4 ghi	2.09 h
	Compost	(-)	126.20 def	18.2 hij	2.30 g
		(+)	119.40 fgh	19.13 egh	2.29 g
	Humic acid	(-)	116.90 gh	19.33 efg	2.27 gh
		(+)	122.70 efg	19.07 fgh	2.35 fg

Different letters in each column denote a significant differences at p≤0.05 (LSD test).

humic acid beyond biologic fertilizer (Fig. 3). Also, between chemical × organic × biologic fertilizer treatments, the maximum rosmarinic acid yield (3.432 g/m^2) was observed in 100 kg/ha along with humic acid and biologic fertilizer application (Table 6). Integrated application of chemical, organic and biological fertilizers improved soil physical and chemical properties and resulted in the increase in availability of nutrients and ultimately the yield and quality of plants. Darzi *et al.* (2007) reported the application of mycorrhiza and vermicompost and phosphate solubilizing biologic fertilizers determined increased essential oil yield in fennel.

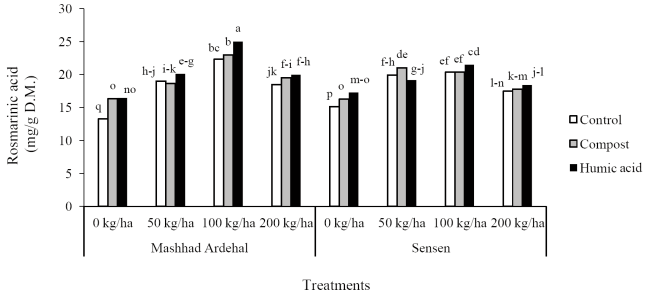

Fig. 2 - Effect of chemical and organic fertilizer on rosmarinic acid content of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

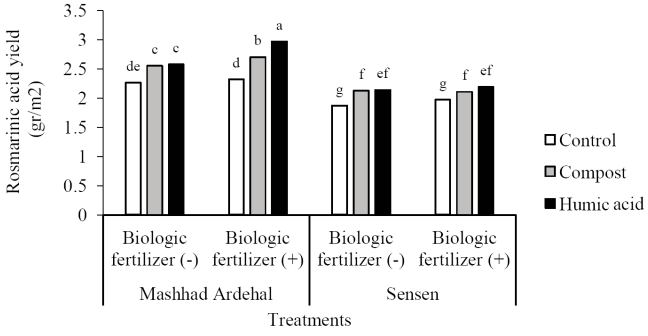

Fig. 3 - Effect of organic and biologic fertilizer on rosmarinic acid yield of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

Stomatal conductance

Stomatal conductance is one of the most important factor in determining the photosynthesis amount. The study of stomatal conductance between location and chemical fertilizer treatments showed the highest stomatal conductance (75.88 mmol H_2O m^{-2} s^{-1}) in Mashhad Ardehal with application of 100 kg/ha chemical fertilizer (Fig. 4). At both of studied location especially in Mashhad Ardehal, application of chemical fertilizer increased the stomatal conduc-

tance of Perilla leaves. Increasing of stomatal conductance increases the photosynthesis activities and finally improved the plant yield (Atteya, 2003). The results of stomatal conductance are agreement with result of plant yield. This result showed the increasing of stomatal conductance can be useful to increasing of plant yield especially with 100 kg/ha application of chemical fertilizer.

The highest stomatal conductance was obtained

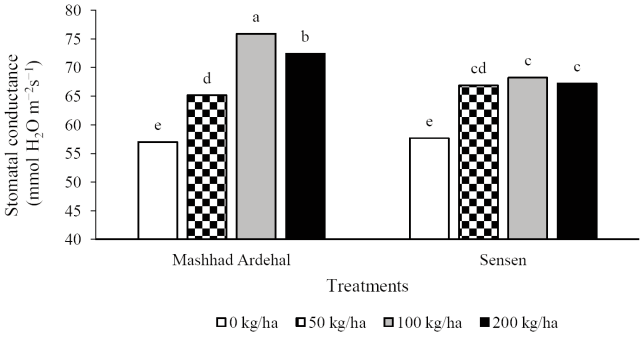

Fig. 4 - Effect of chemical fertilizer on stomatal conductance of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

with application of compost fertilizer (67.67 mmol H_2O m^{-2} s^{-1}) (Table 7). Organic fertilizers can provide better moisture conditions by increasing the absorption and preservation of water and the availability of water and food, and this can contribute to the favorable conditions for photosynthesis in the plant. If there is enough stomatal conductance, CO_2 gas will enter the stomata more easily and sufficient photosynthesis will be performed. Also means comparison showed inoculation with mycorrhiza improved stomatal conductance as compared to without inoculation (Table 7).

The physiological effects of mycorrhizal fungi symbiosis include aboveground modifications of water relations and physiological status in terms of leaf water potential, relative water content, stomatal conductance, CO_2 assimilation, and efficiency of photosystem II as compared to non-mycorrhizal plants (Barzana *et al.*, 2012). Mycorrhizal fungi increase the contact surface with soil and moisture around the plant roots by 10 to 1,000 times, thus increasing the plant's ability to use the resources in its surroundings (Sharma, 2002).

DPPH test

Many researchers have used DPPH test to express the antioxidant status of plants (Dasgupta and De, 2007; Sahu *et al*., 2013). The results showed the antioxidant activity of Perilla plant in Mashhad Ardehal (165.29 ug/ml) was higher than Sensen (162.79 ug/ml) location (Table 7). Perilla is very sensitive to free radicals productions. In a study in Korea on different species of Perilla, different species for DPPH test showed a significant difference (Choi *et al*., 2002).

Increasing levels of chemical fertilizer reduced the plant's efficiency in inhibiting free radicals. The highest amount of DPPH test was obtained (169.47 µg/ml) under control condition and the lowest (55.85 µg/ml) was obtained from the highest level of fertilizer application (200 kg/ha) (Table 7). Omar *et al*., (2012) reported the antioxidant activity determined using the DPPH was high with the application of organic fertilizer compared to chemical fertilizer in cassava tubers.

Organic fertilizers, especially compost (277.17 µg/ml), increased the antioxidant activity and inhibited free radicals (Table 7). Uthairatanakij *et al.* (2017) reported an increase in antioxidant activity and inhibition of free radicals due to the application of organic fertilizers. Organic fertilizers with plant sources activated defense mechanisms against pests, diseases and other tensions (Brandt and Molgaard, 2001).

Table 1 - Weather characteristics in Mashhad Ardehal and Sensen

Treatments	Stomatal Conductance (mmol H_2O $m^{-2}s^{-1}$)	DPPH (ug/ml)	Polyphenol oxidase (µmol/min)
Location			
Mashhad Ardehal	67.61 a	165.29 a	0.156 a
Sensen	64.98 b	162.79 b	0.152 b
Chemical fertilizer			
0 kg/ha	57. 36 d	169.48 a	0.130 c
50 kg/ha	65.99 c	161.73 c	0.152 b
100 kg/ha	72.05 a	166.41 b	0.167 a
200 kg/ha	69.80 b	158.55 d	0.168 a
Organic fertilizer			
Control	66.03 b	158.54 b	0.152 b
Compost	67.68 a	167.27 a	0.158 a
Humic acid	65.19 b	166.31 a	0.153 b
Biologic fertilizer (Inoculation)			
(-)	65.08 a	164.08 a	0.153 b
(+)	67.52 b	164.00 a	0.155 a

Different letters in each column denote a significant differences at p≤0.05 (LSD test).

Catalase activity

The highest catalase activity (0.5332 nmol H_2O_2/min) was observed in Mashhad Ardehal region with the application of 100 kg/ha fertilizer and with-

out application of biologic fertilizer. The lowest amount was obtained from Mashhad Ardeal area without using of chemical and biologic fertilizer. Also, in the Sansen location, the highest catalase activity was obtained from 200 kg/ha chemical fertilizer and biologic fertilizer application (Fig. 5).

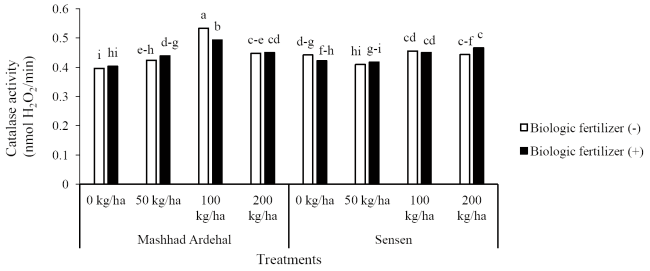

Fig. 5 - Effect of chemical and biologic fertilizer on catalase activity of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

Among the chemical and organic fertilizer treatments in two studied locations, the highest catalase activity was obtained from 100 kg/ha of chemical fertilizer with compost application in Mashhad Ardehal region (0.5182 nmol H_2O_2/ min) and the lowest amount (0.338 nmol H_2O_2/ min) was obtained without using of chemical and organic fertilizer control in Mashhad Ardehal location (Fig. 6). Logan *et al.* (1999) stated that nitrogen had a significant effect on the activity of enzymes involved in photosynthesis, such as Ribulose-1,5-bisphosphate. In another study the researchers reported increased nitrogen levels caused to production of antioxidant enzymes like APX, SOD, CAT, and POD in the *Populus yunnanensis* plants (Lin *et al.*, 2012). Our result indicated that sources of fertilizer had a significant influence on the level of catalase activity in field grown Perilla.

Polyphenol oxidase activity

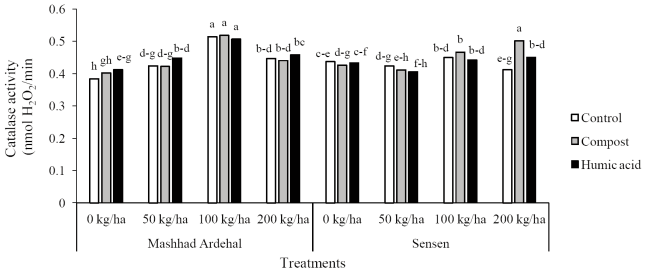

Fig. 6 - Effect of chemical and organic fertilizer on catalase activity of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

Among the treatments of chemical and organic fertilizer in different locations, similar to catalase activity, the highest polyphenol oxidase activity was obtained from 100 kg/ha with compost application in Mashhad Ardeal location (0.193 µmol/min) and the lowest amount (0.129 µmol/min) was obtained from without application of chemical and organic fertilizer in Mashhad Ardehal area (Fig. 7). At all levels of chemical fertilizer in both locations, application of organic fertilizers increased the activity of polyphenol oxidase activity, and in most cases, organic matter compost was superior to humic acid. Also, the application of biologic fertilizer led to an increase in polyphenol oxidase activity (Table 7). Similar to our results, the application of biologic fertilizer increased the amount of polyphenol oxidase activity in triticale (Kheirizadeh-Arough *et al*., 2016).

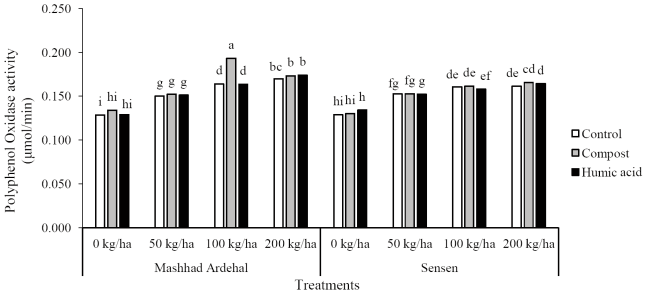

Fig. 7 - Effect of chemical and organic fertilizer on polyphenol oxidase activity of Perilla in Mashhad Ardehal and Sensen regions. Different letters in each column denote a significant difference at p≤0.05 (LSD test).

4. Conclusions

In the present experiment, the results showed that proper nutritional management of Perilla medicinal plant has a special role in improving quantitative and qualitative traits. Among individual fertilizer treatments, 50 and 100 kg/ha of fertilizer and among organic fertilizers, humic acid played a more effective role in improving the studied indices, but the application of biologic fertilizer separately did not have a very significant effect on this plant. Integration with other fertilizers has been shown to be more effective. Mashhad Ardehal area has better conditions for cultivating Perilla due to its soil characteristics and climatic characteristics. Also, the combined application of different fertilizer sources in comparison with the single application of each of them in both studied regions significantly improved the growth characteristics, as well as essential oil yield and biochemical

indices of Perilla. Furthermore, organic fertilizers like compost and humic acid discharge nutrients very slowly to the plants. Hence, an integrated approach, combining application of compost with an application of chemical fertilizer is a good strategy for increasing crop productivity. This will reduce the cost of chemical fertilizer and improve soil fertility.

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AHS
Advances in Horticultural Science

The use of organic nano-supplements of fertilizer for lily forcing period

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Key words: amylases, chitosan, forcing, flowering, magnetite, magnetic supplement of fertilizer, sugars.

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

Competing Interests:
The authors declare no competing interests.

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Abstract: *Lilium* is one of the most important ornamental plants after roses, carnations and chrysanthemum in the world that is requested as potted of cut flowers. It is so important to consider its quality along with its production rate in terms of yield (quantity). However, it always needs to intend the product costs aside from quantity and quality. Fertilizer utilization is so important and this may be improved by compounds that promote it and also have synergetic effects themselves. We examined both carboxymethylated chitosan (CMC) and magnetic nano-carbocymethylated chitosan (MNCC) to produce *Lilium* bulb and advised them especially magnetic chitosan. In this study, these compounds (magnetic and non-magnetic chitosan) at concentrations of zero, 2.5, 5, 10 and 15 mg/l were examined during lily forcing in three cultivars, including Cherbourg, Navona, Brunello, which are from Asiatic and Oriental lilies. The results showed that highest concentrations (10 and 15 mg/l) among between examined concentrations regardless of compounds types and cultivars did not make toxicity and had significant effects on plants biology and physiology [contents of carbohydrates and enzymes affecting these carbohydrates (amylases)]. However, for observing morphological changes may be need to use higher concentrations of these compounds. Note that this needs to examine the non-toxicity of higher concentrations in future studies.

1. Introduction

Flowers have always had a valuable place among different classes of society and various ceremonies and rituals. *Lilium* is one of the most important ornamental plants after roses, carnations and chrysanthemum in the world that is mostly used as cut flowers throughout the year. Furthermore, it is used as potted plants in gardens and green landscapes (Shafiee-Masouleh *et al.*, 2014). It is necessary to consider the nutrition of lilies like other plants in the greenhouse production. And also, optimization and improvement of photosynthetic efficiency of plants can be effective in increasing photosynthetic storages and thus increasing its quanti-

tative and qualitative yields in terms of flower number, plant size and vase life. Different groups of lilies (Asiatic, Oriental and *L. longiflorum*) have different nutritional requirements. For example, Asiatic lilies has been reported that have the best growth when are fertilized by lower levels during forcing (Treder, 2003).

Chitosan particles in nano-scale can be important in the delivery of drugs in medicine, because these compounds promote the absorption of active molecules or compounds through the cell membrane and allow organs to have bioavailability to molecules. Chitosans can be used as encapsulated nanoparticles or used directly and therefore, the surface of these particles with chelating structure of the modified chitosan can play an important role in sustainable agriculture (Kashyap *et al.*, 2015). Magnetic chitosan nanoparticles with the effect of chelating elements in the experiment conducted by Shafiee-Masouleh *et al.* (2014) increased the biomass of *Lilium* and the storage organs (bulbs) during bulb production. The effect of magnetic fluids on living systems has been studied both in medicine and in the world of plants, and it has been shown that magnetic fluids can be effective in the production of calluses and metabolic activities. The effects of magnetism on plants by Earth's magnetic field, pulsed and inductive constant magnetic fields, an electromagnetic field, and effects of magnetic nanoparticles have been studied by researchers and theirs roles have been confirmed to enhance photosynthesis and growth and development of plants (Pavel *et al.*, 1999; Răcuciu and Creangă, 2007 b; Răcuciu *et al.*, 2009; Shafiee-Masouleh *et al.*, 2014). It has also been shown that superparamagnetic nanoparticles, which are as permanent magnets effected by external magnetic fields or ambient temperature of their environment, affect membrane systems and membrane ion exchange (Pavel and Creangă, 2005; Faeghi and Seyedpour, 2013). Many researchers investigated the effects of chitin and the chitosan polymers and oligomers as spraying, soil application or seed treatment and fertilizer treatments, they reported that photosynthesis and plant growth were enhanced (El-Tantawy, 2009; Dzung *et al.*, 2011; Farouk and Amany, 2012).

The allocation of carbon in plants is important that is affected by various factors such as the level of photosynthetic compounds, the number and size of competing sink (flowers, seeds, fruits, bulbs and tubers, etc.) and their location in plants and the potential for initial storage in the leaves and re-translocation in plants. Understanding the components that affect the initiation and development of organs and the balance between source and sink organs are essential to regulate the allocation of assimilates (Du Toit, 2001). The *chl* content of the leaf affects photosynthesis, and *Chl* a/b is the best index to understand the photosynthetic capacity and direct information on the activity of the enzymes involved in the photosystem II in the chloroplast membrane (Răcuciu and Creangă, 2007 b; Răcuciu *et al.*, 2009). Iron deficiency reduces the amount of photosynthetic pigments. In addition, the electron transfer in the photosystems I and II undergoes a change. Also, the activity of 1, 5-D-phosphate-carboxylase and the photosynthetic function of plant decrease. Therefore, it is necessary to provide iron for the plant, because the biochemical properties of iron and its effect on the metabolic pathway of the plant are important. Uptaking iron in alkaline soils is difficult; this is due to the formation of ferric hydroxide in the presence of oxygen, because plant cannot uptake it (Thoiron and Briat, 1999). The main component in reducing NO_3^- absorption is iron deficiency. Ferrous enzymes (nitrite and nitrate reductases) affect the NO_3^- absorption. In addition, growth of plants decreases due to decreased NO_3^- absorption and the synthesis of metabolites (proteins, nucleic acids, chlorophylls, etc.) (Borlotti *et al.*, 2012). The use of chelating agents is useful to remove iron uptake problems by plants (Abadía *et al.*, 2011). Magnetic fluids at suitable concentrations have a positive effect on the photosynthetic capacity of plant. Iron ions in the structure of magnetic fluids can be an important source of iron for the development of plants (Răcuciu and Creangă, 2007 b). Iron is not in the *chl* structure, but it is one of the essential elements to synthesize *chl* (Răcuciu *et al.*, 2009). In general, magnetic fluids are nanoparticles dispersed in water or in a hydrocarbon fluid such as citrate. Biocompatibility of magnetite (Fe_3O_4) has been confirmed (Răcuciu *et al.*, 2009). However, treating plants with electromagnetism or any other magnetic field with these particles is destructive and changes the genotype and phenotype of plants and causes chromosomal deviations (Pavel *et al.*, 1999; Pavel and Creangă, 2005; Răcuciu and Creangă, 2007 a, b; Răcuciu *et al.*, 2009).

Chitosan is one of deacetylated derivatives of chitin. As a natural polymer is abundant and can be degraded by biological agents and it can be used in agriculture. This molecule is environmentally friendly and non-toxic that is used in the formulation of slow release fertilizers (Wu and Liu, 2008). In vitro use of chitosan in a suitable concentration in *Vitis vinifera* L.

stimulated the photosynthesis and increased plant growth with increasing root and shoot biomasses. It also protected the plant against *Botrytis cinererea* fungus and cytological changes (Barka *et al.*, 2004). We utilized the effects of chitosan, as nanoparticle with magnetic properties on plant growth and development in lily bulb production (Shafiee-Masouleh *et al.*, 2014) and cucumber (the data were not published yet).

Modern agriculture should look for factors that while having positive effects as well as its use to be easy, affordable and reasonable. It is not possible to use a large magnetic field in farms. Also, use of macromolecule of chitosan as foliar application will cause stomata obstruction and reduce gas exchange and photosynthesis. Therefore, the achievement of a soluble compound or nano-structured composite with the synergic effects of two compounds (magnetite and chitosan) will be very valuable at the same time. To achieve these purposes, this research studies the following: i) Increasing yield and post-harvest quality of cut lily by increasing the efficiency of nutrient uptake and photosynthetic performance of the plant; ii) Institutionalization of chitosan use at the nanoparticle scale during forcing of lily flowers; iii) Investigating the effect of magnetic nanoparticles on yield and quality of lily flowers; and iv) Physiological and morphological understanding of the effect of chitosan nanoparticles on the yield and quality cut flowers of lily.

2. Materials and Methods

Plant material and cultivation conditions

In this study, bulbs of three cultivars of *Lilium,* including Brunello (Asiatic, 16-18 cm in circumferences), Navona (Asiatic, 16-18 cm in circumferences) and Cherbourg (Oriental, 18-20 cm in circumferences) were used to study effects of two organic supplements of fertilizer (OSFs).

After melting of vernalizing substrate of bulbs, the bulbs were disinfected with 0.1% benomyl fungicide for 15 min and dried in the air for one day. Thirty uniform bulbs in size were selected per cultivars to force. The culture substrate contained cocopeat and perlite (1:1, v/ v) that was completely homogeneous. The bulbs were planted in one-kilogram plastic bags with some pores into 10 cm depth and irrigated with tap water until emergence. After emergence every 2-3 days depending on the weather and substrate conditions were fertigated.

Greenhouse experiments in the research greenhouse of the Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran in latitude of 37° 12' 3.94' N and longitude of 49 38' 55.78' E were performed.

Preparation of fertilizer supplements

Carboxymethylation of chitosan. The carboxymethylated chitosan (CMC) was prepared from chitosan [poly (d-glucosamine), $(C_6H_{11}NO_4)_n$, deacetylated chitin] with low molecular weight 100,000-300,000 (Acros, Acros Organics, Geel, Belgium) to increase solubility of chitosan by carboxymethylation. The procedure of preparing of CMC was presented by Shafiee-Masouleh *et al.* (2014). An IR (IR-470, Shimadzu, Kyoto, Japan) (Fig. 1) was used to analyze the produced H-form CMC (not salt containing Na ion).

Preparation of nano-particles magnetite

According to Shafiee-Masouleh *et al.* (2014), nano-particles of Fe_3O_4 were synthesized. $FeCl_3$ (Merck, Germany) and $FeCl_2.4H_2O$ (Merck, Germany) were used to prepare Fe_3O_4. The whole procedure of preparing Fe_3O_4 can be observed at the mentioned paper.

Preparation of magnetic nano-carboxymethylated chitosan

The magnetic nano-carboxymethylated chitosan (MNCC) was prepared with encapsulation of Fe_3O_4 by carboxymethylated chitosan (CMC) according to

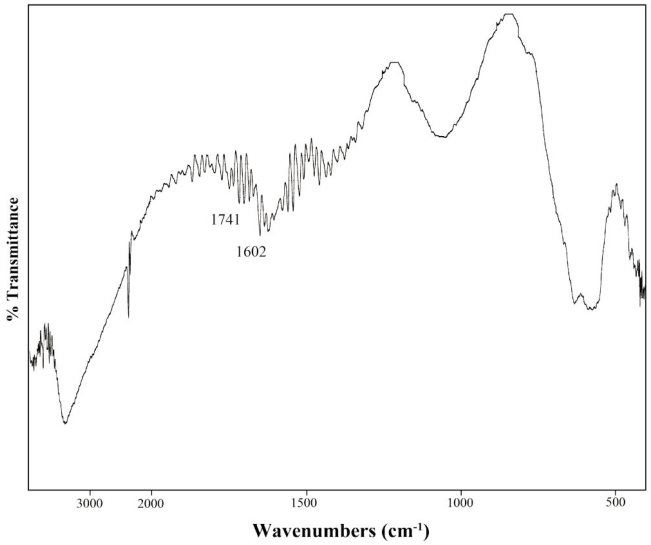

Fig. 1 - IR spectrum of carboxymethylated chitosan. This spectrum shows that the carboxylmethyl groups (-CH3COOH; 1741 cm-1; stretching vibration feature of C=O groups) resulted from monochlroacetic acid salt are linked to O atom in -OH group, and -NH_2 exists on carboxymethylated chitosan.

Shafiee-Masouleh *et al.* (2014). This was carried out by carbodimiide (Merck, Germany) and saline phosphate buffer in sonication conditions. The details of laboratory procedures can be studied in the mentioned paper. Analyses of the size and morphology of nano-particles were performed with SEM (Fig. 2), and IR and XRD were used to identify the coating and structure of MNCC (Figs. 3 and 4).

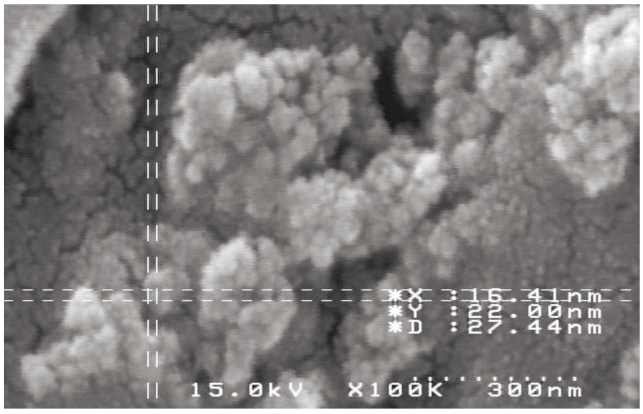

Fig. 2 - The image of magnetic nano-carboxymethylated chitosan particles by SEM microscopy. Size and morphology of Fe_3O_4 coated by carboxmethylated chitosan.

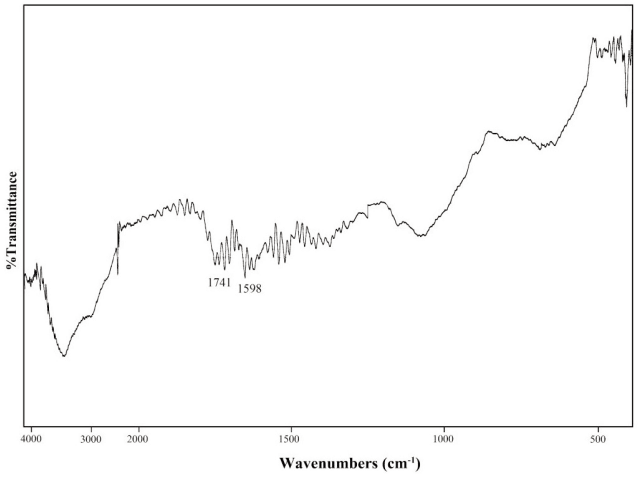

Fig. 3 - IR spectrum of magnetic nano-carboxymethylated chitosan. Transferring the absorbance band of -NH2 group from 1589 cm^{-1} to 1602 cm^{-1} and increasing absorbance intensity in wavelength number 1741 cm^{-1} show ester bond into some parts of carboxy groups in carboxymethyl chitosan on the surface of Fe_3O_4 nanoparticles.

Statistical design

A split plot factorial experiment was conducted in a completely randomized design with 15 main plots and 9 (1 + 8) sub plots. The main factors included three *Lilium* cultivars and sub factors were two types of compounds as organic supplements of fertilizer (OSFs); including carboxymethylated chitosan (CMC)

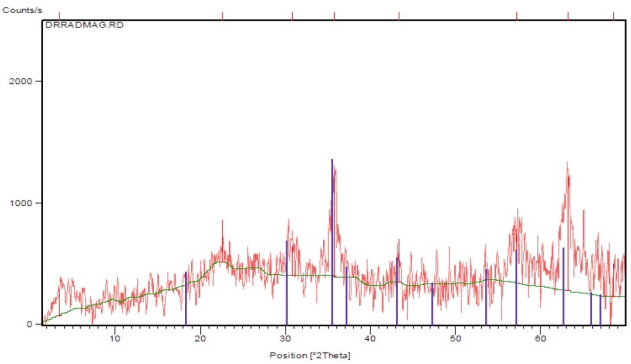

Fig. 4 - XRD pattern of magnetic nano-carboxymethylated chitosan. The widths of large peaks report crystal properties of magnetic nanoparticles. The average of particles sizes based on Debye Scherrer equation was calculated 10 nm.

and magnetic nano-carboxymethylated chitosan (MNCC) both of them at concentrations of 0, 2.5, 5, 10 and 15 mg/l. One group of zero concentration was considered as control treatment for both of compounds. The experiments were carried out by 5 replications and with 135 pots in the research greenhouse of the Faculty of Agricultural Sciences, University of Guilan, Iran. At the end of forcing period, the growth and morphological characteristics were measured for each plant (in puffy bud stage: when first flower bud showed the color and little opening in top of bud) and were harvested at a height of 45 cm from the final flower bud and transferred to the laboratory. After the stems were cut diagonally under water, they were placed into a 1000 ml bottles containing 500 ml distilled water and transferred to the growth chamber to study the post-harvest life and biochemical properties.

Variance analysis of data was measured using SAS software (version 9.1, 2003). Mean comparisons were performed with Tukey's test at probability levels of 5 or 1% according to variance analysis.

3. Results and Discussion

Magnetic nanoparticles (Pavel *et al.*, 1999; Pavel and Creangă, 2005; Răcuciu and Creangă, 2007 a, b; Răcuciu *et al.*, 2009) in maize and barley, constant magnetic field (Dhawi *et al.*, 2009) in a palm tree, pulsed magnetic field (Radhakrishnan and Kumari, 2012) in soybeen, chitosan (Górnik *et al.*, 2008) in grape and chitosan oligosaccharides (Dzung *et al.*, 2011) in coffee seedlings have been investigated. All of researchers reported an increase in mineral uptak-

ing by the effects of magnetism or chitosan, separately.

Based on the results of 3-way ANOVA test (Table 1), effects of OSFs on vegetative development of three lily cultivars due to the uniform variances of data around the mean and Type I Error did not show a significant differences for 3-way interactions between cultivars, compounds and their concentrations. Only a significant 2-way interaction (between cultivar and concentrations of OSFs) was observed on the *chl* intensity by ANOVA test. And also, it was observed significant differences between cultivars (Table 1).

Unlike variance analysis, significant differences between three factors were observed on the vegetative length, total length and *chl* intensity. Differences between treatments on vegetative development showed that morphological characteristics are less affected by the experimented OSFs and their concentrations or these compounds may have decreasing effects on these characteristics. In Cherbourg cultivar, the highest length of vegetative stem was observed in the absence of OSFs or at low concentration (5 mg/l) of CMC, which showed a significant difference with application of 2.5 mg/l CMC in Navona. Of course, these differences were not significant com-

Table 1 - Effects of OSFs on vegetative development of three lily cultivars

Cultivars (A)	Treatments		Vegetative length (cm)	Total length (cm) [w]	Chl intensity (SPAD index)
	OSFs (B)	Cons of OSFs (mg/l) (C)			
Cherbourg	CMC	0	56.08±2.39 a	84.16±2.51 a	46.62±1.76 efg
		2.5	54.48±5.14 ab	81.36±5.40 ab	48.52±2.43 defg
		5	57.70±2.14 a	84.60±3.52 a	42.46±2.74 ef
		10	52.30±3.31 ab	79.80±3.98 ab	42.16±2.22 ef
		15	54.10±1.49 ab	81.40±3.34 ab	46.70±2.35 efg
	MNCC	0	56.08±2.39 a	84.16±2.51 a	46.62±1.76 efg
		2.5	55.30±2.91 ab	82.00±3.29 ab	46.50±3.21 efg
		5	53.00±3.41 ab	81.40±4.41 ab	46.32±1.99 efg
		10	53.12±1.36 ab	80.60±1.93 ab	39.98±2.83 g
		15	50.84±2.73 ab	82.20±2.65 ab	46.02±1.99 efg
Navona	CMC	0	48.30±2.23 ab	81.20±3.01 ab	59.52±0.31 abcd
		2.5	47.60±2.01 b	78.00±1.92 ab	60.16±1.54 abcd
		5	50.80±2.00 ab	81.80±1.57 ab	61.60±0.58 abc
		10	50.30±1.51 ab	81.90±2.65 ab	61.98±1.15 ab
		15	46.20±1.83 ab	76.00±2.07 ab	61.40±1.57 abc
	MNCC	0	48.30±2.23 ab	81.20±3.02 ab	59.52±0.31 abcd
		2.5	39.60±0.80 ab	69.90±0.48 ab	57.10±3.82 abcde
		5	49.30±2.22 ab	80.00±1.25 ab	60.16±1.13 abcd
		10	45.60±1.83 ab	75.30±2.33 ab	62.72±1.83 a
		15	50.10±1.86 ab	80.00±3.58 ab	57.86±0.97 abcde
Brunello	CMC	0	50.90±2.24 ab	70.70±2.29 ab	53.38±3.54 abcdefg
		2.5	49.10±4.14 ab	68.50±5.34 ab	51.10±2.14 abcdefg
		5	50.90±3.12 ab	69.20±4.53 ab	50.12±1.74 abcdefg
		10	52.60±4.01 ab	72.30±5.27 ab	53.76±1.81 abcdef
		15	49.50±1.90 ab	66.80±2.81 b	49.34±0.50 bcdefg
	MNCC	0	50.90±2.24 ab	70.70±2.89 ab	53.38±3.54 abcdef
		2.5	54.50±2.23 ab	77.30±1.72 ab	49.70±0.61 bcdefg
		5	49.90±2.14 ab	69.80±2.18 ab	51.84±1.78 abcdefg
		10	50.90±3.60 ab	69.60±3.14 ab	51.60±1.68 abcdefg
		15	52.80±3.18 ab	71.80±3.99 ab	49.22±2.08 cdefg
ANOVA (3-way)					
A			**	**	**
B			NS	NS	NS
C			NS	NS	NS
A×B			NS	NS	NS
A×C			NS	NS	*
B×C			NS	NS	NS
A×B×C			NS [y]	NS [y]	NS [y]
CV (%)			10.46	8.68	8.58

N=5. The means with similar letters have not any significant differences at $HSD_{0.01}$.

[w] $HSD_{0.05}$

[y] Type I Error

**, *, NS = Significance at p≤0.01 and p≤0.05 and non-significance, respectively.

pared to other treatments. Total length showed similar reaction with vegetative length to treatments but the least value was observed in Brunello treated by 15 mg/l CMC. However, *chl* intensity under effects of treatments showed the most value in Navona treated by 10 mg/l MNCC and the least were observed in Cherbourg treated by 10 mg/l MNCC (Table 1). It seems that morphological characteristics of cultivars treated by two types of OSFs in different concentrations are under dominant effect of cultivar; on the other hand, morphological characteristics of different lily cultivars show the various reactions to OSFs and their concentrations. Generally, from the above observations on vegetative development, it can be interpreted that firstly, size of the plant is affected by genotype, and secondly, magnetism may increase the synthesis of *chl* and *chl* density, but it does not affect the size of the shoots and this needs to be investigated more with higher concentrations.

Nguyen Van *et al.* (2013) stated that chitosan nanoparticles can easily penetrate into plant cells and increase the biological activity of cells. They pointed to an important effect of chitosan nanoparticles on the biophysical properties of the coffee plant such as increasing *chl* content and plant growth and development. Răcuciu and Creangă (2007 b) used low-density (less than 100 µl/l) ferrofluids without electromagnetic treatment or any other magnetic field for corn seedlings. They observed that due to their super-paramagnetic effects (a special effect of magnetic nanoparticles) on the structure of photosynthetic enzymes, growth was enhanced. Răcuciu and Creangă (2007 a, b) and Răcuciu *et al.* (2009) used two fluids of magnetic nanoparticles of Fe_3O_4 coated with or without tetraethylammonium hydroxide in Petri dishes for germination of corn seeds without the use of a magnetic field (50 µl per liter). They observed that plant height increased, but when the concentration increased, toxicity was reported. They referred that magnetic iron is a source for plant growth. In addition, magnetism has a good effect on photosynthesis. El-Sayed (2014) reported the increasing effect of magnetic water treatment on plant growth of *Vicia faba*, *chl a* and *b*, and carotenoids, and contents of gibberellic acid and kinetin It is known that kinetin (cytokinins) is effective in preservation and prevention of the chlorophylls destruction (Taiz and Zeiger, 2002).

Farouk and Amany (2012) reported an increase in *chl a* and total *chl* by spraying the chitosan (250 mg/l) on chickpea compared to low concentrations (50 and 125 mg/l), and their interpretation about increasing the content of photosynthetic pigments was the increase of cytokinin level and stimulation of the synthesis of chlorophyll, and they interpreted this as the role of amino groups in chitosan on the mentioned processes. Chlorophyll density below 40 (Spad index) indicates a disruption to photosynthetic process (Netto *et al.*, 2005). However, health of the photosynthetic apparatus in all treatments, especially high concentrations of two OSFs (magnetic and non-magnetic) can be seen. Răcuciu *et al.* (2009) reported that when concentration of Fe_3O_4 magnetic nanofluid was increased up to 300 µl/l, content of *chl a* decreased by about 20 mg/l of fresh weight. In addition, Răcuciu and Creangă (2007 b) stated that the magnetic nanofluid of Fe_3O_4 coated with tetramethylammonium hydroxide (50 µl per liter) produced more pigments (*chl a* and *b* and carotenoid), and higher concentrations of fluids destroyed the photosynthetic process. Limpanavech *et al.* (2008) reported that chitosan affected the expression of chloroplast genes in *Dendrobium* orchid and altered the chloroplast size. Dzung *et al.* (2011) sprayed chitosan at appropriate concentrations on coffee seedlings and reported an increase in content of chlorophyll and also in uptake of mineral elements (nitrogen, phosphorus, potassium, calcium and magnesium), and enhancement of photosynthesis as well as the synthesis of chlorophyll of leaves.

As can be seen in Table 2, 3-way interactions between cultivars, OSFs and their concentrations in ANOVA test due to Type 1 Error could not affect reproductive development of lily plants. However, mean comparisons showed significant effects at $HSD_{0.01}$ on these characteristics. In Cherbourg, the most inflorescence lengths were observed in the plants treated with 15 mg/l MNCC, but in Navona without significant different with Cherbourg, the most lengths of inflorescence was observed in control and 10 mg/l CMC. The shortest length of inflorescence was observed in Brunello. However, this cultivar with the least concentration of magnetic OSF had the most length and generally, it seems that on this characteristic, the effect of genotype was dominant toward OSFs and their concentrations. How to make a reaction of a plant as flower bud number affected by 3-way interactions of factors shows dominant effects of genotype toward OSFs and their concentrations. We can statistically analyze each cultivar separately to observe 2-way interactions and/or single effects of OSFs on the responses of lily plants. Cherbourg and Navona could not show response to OSFs and their concentrations for bud number.

However, Brunello reaction to OSFs and their concentrations was obvious. It can be said that this cultivar could respond to the OSFs compared with two other cultivars, because produced the most bud number with 2.5 mg/l of magnetic compound. In the case of bud length, although 3-way interaction showed significance, it can be seen for this morphological characteristic that the effect of genotype was dominant again, and Cherbourg produced larger buds compared with two others. Furthermore, this response was observed about flowering date, and Cherbourg flowered later (Table 2). From the observations of the effects of three factors, including cultivar, OSFs and concentrations of them, it can be interpreted that this range of the tested concentrations could not affect vegetative and reproductive morphology, and it seems that more concentrations of OSFs must be investigated in future research.

It is known that one of the factors that contribute to growth of the reproductive part (flower bud length) is potassium. Amino groups in the carboxymethylated chitosan and its derivatives can

Table 2 - Effects of OSFs on reproductive development of three lily cultivars

Cultivars (A)	Treatments		Inflorescence length (cm)	Bud No.	Bud length (mm)	Flowering date (days)
	OSFs (B)	Concentration of OSFs (mg/l) (C)				
Cherbourg	CMC	0	28.08±0.56 abc	6.80±0.58 abc	116.63±0.24 a	94.60±0.24 a
		2.5	26.88±0.84 abcdef	6.80±0.37 abc	116.31±0.77 a	93.00±0.77 a
		5	26.90±1.57 abcdef	6.20±0.20 b	123.27±0.49 a	93.20±0.49 a
		10	27.50±1.47 abcd	7.00±0.32 abc	116.14±0.55 a	92.5±0.55 a
		15	27.30±1.87 abcde	6.40±0.40 abc	118.64±0.37 a	93.80±0.37 a
	MNCC	0	28.08±0.56 abc	6.80±0.58 abc	116.64±0.24 a	94.60±0.24 a
		2.5	26.70±1.09 abcdef	6.40±0.51 abc	122.06±0.51 a	93.60±0.51 a
		5	28.40±1.41 ab	6.80±0.37 abc	120.39±0.58 a	93.80±0.58 a
		10	27.48±0.88 abcd	6.80±0.37 abc	110.43±0.68 a	93.40±0.68 a
		15	31.36±2.06 a	7.00±0.58 abc	119.86±0.81 a	94.40±0.81 a
Navona	CMC	0	32.90±1.61 a	9.00±0.32 a	82.87±0.68 b	59.40±0.68 b
		2.5	30.40±0.53 ab	9.50±0.29 ab	80.02±0.73 b	60.20±0.73 b
		5	31.00±2.26 ab	9.20±0.49 a	79.80±0.49 b	59.80±0.49 b
		10	31.60±1.35 a	9.00±0.63 a	81.16±0.63 b	59.00±0.63 b
		15	29.80±1.11 ab	9.40±0.24 a	78.72±0.49 b	58.80±0.49 b
	MNCC	0	32.90±1.61 a	9.00±0.32 a	82.87±0.68 b	59.40±0.68 b
		2.5	30.30±0.89 ab	9.20±0.97 a	76.04±0.58 b	59.20±0.58 b
		5	30.70±1.07 ab	9.00±0.32 a	77.69±0.60 b	59.60±0.60 b
		10	29.70±1.07 ab	10.00±0.71 a	78.95±1.02 b	59.20±1.02 b
		15	29.90±2.34 ab	8.40±0.68 a	80.34±0.60 b	59.60±0.60 b
Brunello	CMC	0	19.80±1.41 cdefg	5.40±0.60 c	78.91±0.97 b	60.20±0.97 b
		2.5	19.40±1.37 defg	5.80±0.97 b	86.39±1.47 b	61.60±1.47 b
		5	18.30±1.52 g	5.80±0.66 b	79.46±1.71 b	62.20±1.71 b
		10	19.70±1.38 defg	5.80±0.97 b	80.04±1.56 b	62.80±1.56 b
		15	17.30±1.28 g	5.60±1.20 c	84.75±1.38 b	61.00±1.38 b
	MNCC	0	19.80±1.41 cdefg	5.40±0.60 c	78.91±0.97 b	60.20±0.97 b
		2.5	22.80±1.23 bcdefg	6.40±0.75 abc	84.40±1.59 b	62.20±1.59 b
		5	19.90±0.89 cdef	5.40±0.75 c	78.47±1.17 b	60.60±1.17 b
		10	18.70±0.85 fg	4.60±0.24 c	76.75±0.20 b	61.20±0.20 b
		15	19.00±0.96 g	5.50±0.96 b	80.59±1.72 b	61.60±1.72 b
ANOVA (3-way)						
A				**	**	**
B				NS	NS	NS
C				NS	NS	NS
A×B				NS	NS	NS
A×C				NS	*	**
B×C				NS	NS	NS
A×B×C				NS [Y]	NS [Y]	NS [Y]
CV (%)				17.61	6.69	1.63

N=5. The means with similar letters have not any significant differences at $HSD_{0.01}$.

[W] $HSD_{0.05}$

[Y] Type I Error

**, *, NS = Significance at p≤0.01 and p≤0.05 and non-significance, respectively.

serve as a place for the absorption of some metal cations. This compound is used to remove heavy metals and water purification and this shows its chelating role (Chang and Chen, 2005; Chang *et al.*, 2006). Chelating sites of chitosan may be effective in absorbing essential metals ions such as manganese, iron (Fe^{+2} and Fe^{+3}), potassium, magnesium, etc. for plants. The abilities of polymer or oligomer chitosan to stimulate plant growth under in vitro conditions; *Vitis vinifera* L. (Barka *et al.*, 2004); orchid *Dendrobium phalaenopsis* (Nge *et al.*, 2006); and the growth of protocorm like bodies in the *Dendrobium* orchid (Pornpienpakdee *et al.*, 2010) have already been reported. Pornpienpakdee *et al.* (2010) used chitosan macromolecules, which had been deacetylated up to 70%, at a concentration of 10 mg/l under *in vitro* culture conditions for *Dendrobium* orchid and produced plants with more length. In addition, Limpanavech *et al.* (2008) when used the polymer or oligomer chitosan (with deacetylated degrees of 70, 80 or 90%) at concentrations of 1-100 mg/l, they observed that polymers at concentrations of 1-10 mg/l and oligomers at concentrations of 50-100 mg/l resulted in more inflorescences production. Gornik *et al.* (2008) applied a type of commercial compound of chitosan, Biochikol 02.PC containing 2% chitosan, at concentration of 0.5% on grape cuttings and reported that root system expanded and it increased the number of new branches and their lengths. Radhakrishnan and Kumari (2012) reported an increase in leaf number and growth of soybean, and an increase in pod length and grain weight under the influence of pulsed magnetic field. El Sayed (2014) reported that magnetic water treatment increased the sink yield in bean (seed and number of seeds per plant). He suggested this effect may be due to increased photosynthetic function of the plant under influence of magnetic treatment. Ohta *et al.* (1999) reported an increase in the photosynthetic reservoir (flower number) in lisianthus with plant seed treatment by chitosan acid solution. Limpanavech *et al.* (2008) also demonstrated that mechanism of chitosan effect on increasing the number of plant photosynthesis reservoirs (number of flowers) in the *Dendrobium* orchid may be caused by effect on the development of photosynthetic apparatus and increasing the size of chloroplasts after chitosan spraying. Kananont *et al.* (2010) reported that different types of chitosan as poly- and oligosaccharides with varying degrees of deacetylation at a concentration of 10 mg/l are effective for *in vitro* culture medium of seed germination of *Dendrobium* orchids and growth of the protocorm like bodies. They stated that chitosan amino groups may be effective factor on the growth of the protocorm like bodies. Pornpinpakdee *et al.* (2010) described role of concentration of different types of chitosan on growth rate of orchid *Dendrobium*.

The results of 3-way ANOVA (Table 3) show significant differences in content of soluble carbohydrates of terminal bud of inflorescence in three cultivars of lily treated with OSFs and their concentrations. The highest content of glucose was significantly observed in Brunello treated with 15 mg/l OSFs regardless of their magnetic or non-magnetic properties. Navona was in second rank in response to treatments compared to Brunello. However, Cherbourg under effects of OSFs and their levels showed the least glucose content regardless of being magnetism or not compared with two other cultivars. Fructose synthesis in the terminal bud had different reaction to the type of OSFs based on genotypes. Generally, the most concentrations of OSFs regardless of their structures had the most effect on fructose content (Table 3). Sucrose approximately had similar response to glucose toward treatments, but about this carbohydrate after 'Brunello', 'Cherbourg' had second score for the most content not 'Navona'.

Starch and other sugars and proteins (enzymes) are photosynthetic products that are transported to storage organs (flowers, seeds or bulbs). Iron acts as a cofactor for many enzymes, forming part of cytochromes and involves in biochemical reactions, including respiration, photosynthetic material transfer, nitrate synthesis, nitrogen fixation, and DNA synthesis (He *et al.*, 2011). El Sayed (2014) reported that photosynthetic function of bean plant increases with magnetic water treatment. He reported that contents of glucose and sucrose as well as polysaccharides in the leaves, stems and the whole plant of bean were higher toward irrigation without magnetic field. In addition, positive effect of chitosan on plant growth may be due to its effect on increasing the phosphorus content. Phosphorus is an essential element in the biosynthesis and carbohydrate transfer for cell division and the formation of DNA and RNA (Farouk and Amany, 2012).

According to Table 4, variance analysis of OSFs effects (type and concentration) on the α-amylases activities of three cultivars of lily showed the significance of 3-way interactions. OSFs effects on α-amylase in 'Navona' compared with two other cultivars showed the highest activity of this enzyme in the highest tested concentration of non-magnetic OSF.

Table 3 - Effects of OSFs on soluble carbohydrates into the terminal bud tissue of inflorescence in three lily cultivars

Cultivar (A)	Treatments		Glucose	Fructose	Sucrose
	OSFs (B)	Concentration of OSFs (mg/l) (C)			
Cherbourg	CMC	0	0.80±0.11 g	2.20±0.13 ghij	3.21±0.19 ghi
		2.5	1.95±0.24 fg	2.21±0.18 ghij	5.70±0.28 def
		5	3.54±0.18 de	3.34±0.17 efg	8.21±0.35 ab
		10	4.50±0.23 bcd	3.33±0.20 efgh	8.19±0.28 abc
		15	3.50±0.24 de	4.61±0.21 bcd	5.70±0.23 def
	MNCC	0	0.80±0.11 g	2.20±0.13 ghij	3.21±0.19 gh
		2.5	0.89±0.13 g	3.33±0.16 efghi	5.71±0.24 def
		5	1.98±0.23 fg	3.27±0.18 efghi	5.67±0.15 def
		10	3.50±0.11 de	4.60±0.28 bcd	8.23±0.28 ab
		15	2.01±0.29 g	5.51±0.23 ab	8.22±0.44 ab
Navona	CMC	0	2.64±0.22 ef	2.81±0.18 fghi	1.53±0.26 i
		2.5	2.66±0.24 ef	4.03±0.24 cde	3.03±0.20 hi
		5	3.49±0.22 de	4.03±0.20 cde	3.07±0.19 hi
		10	5.03±0.31 abc	4.83±0.26 bc	4.53±0.25 fgh
		15	5.02±0.28 abc	6.05±0.31 a	5.81±0.17 de
	MNCC	0	2.64±0.22 ef	2.81±0.18 fghi	1.53±0.26 i
		2.5	3.51±0.23 de	1.54±0.13 jk	1.59±0.15 i
		5	5.02±0.32 abc	2.60±0.11 fghij	3.10±0.16 ghi
		10	5.84±0.23 ab	2.75±0.19 fghi	3.06±0.19 hi
		15	5.88±0.23 ab	4.02±0.17 cde	4.49±0.23 fgh
Brunello	CMC	0	1.54±0.17 fg	0.82±0.12 k	4.73±0.23 fgh
		2.5	2.72±0.18 ef	0.83±0.14 k	6.80±0.34 bcd
		5	4.03±0.25 cde	2.12±0.14 ij	06.80±0.31 bcd
		10	4.81±0.23 abcd	3.65±0.19 cdef	9.81±0.41 a
		15	6.03±0.30 a	3.57±0.19 def	9.84±0.46 a
	MNCC	0	1.54±0.17 fg	0.82±0.12 k	4.73±0.23 efg
		2.5	4.02±0.23 cde	0.88±0.18 k	4.81±0.24 efg
		5	4.81±0.25 abcd	2.13±0.18 hij	6.46±0.19 cde
		10	4.84±0.21 abcd	3.58±0.20 def	9.82±0.40 a
		15	6.02±0.26 a	4.70±0.25 bcd	9.79±0.36 a
ANOVA (3-way)					
A			**	**	**
B			NS	**	**
C			**	**	**
A×B			**	**	**
A×C			**	**	**
B×C			NS	NS	**
A×B×C			**	**	**
CV (%)			10.88	10.28	7.96

N=3. The means with similar letters have not any significant differences at $HSD_{0.01}$.

[W] $HSD_{0.05}$

[y] Type I Error

**, *, NS = Significance at p≤0.01 and p≤0.05 and non-significance, respectively.

While, the highest activity recorded for this enzyme in 'Brunello' was observed in magnetic OSF (MNCC) unlike 'Navona'. Cultivar Cherbourg unlike two other cultivars showed the highest activity of α-amylase regardless of being magnetic of OSF with the high concentrations of two OSFs, in tissue of the last flower bud of inflorescence. Therefore, responses types of cultivars toward being magnetic or not of OSFs were different. On the other hand, the amount of activity of this enzyme in 'Navona' was higher regardless of the type of OSF and their concentrations.

According to 3-way ANOVA, β-amylase activity in tissue of the terminal flower bud of inflorescence was significantly affected by three factors (Table 4). The activity of this enzyme unlike α-amylase was more in 'Brunello', and like α-amylase, the activity of this enzyme was more with the highest concentration of magnetic OSF. The β-amylase activity in 'Cherbourg' was like α-amylase in the same cultivar. However, in 'Navona', this enzyme showed the highest activity affected by the both of magnetic and

non-magnetic OSFs with different manner toward α-amylase in the same cultivar.

The Earth's magnetic field affects orientation of the ferromagnetic particles and modulations of reactions. It has been reported that the magnetic field has an effect on the biochemical processes and stimulation of the activity of proteins and enzymes on increase of the seed vigor (Dhawi *et al.*, 2009). Tham *et al.* (2001) reported an increase in shoot growth of rice seedling in the hydroponic medium using acid solution of chitosan. Magnetic fields have been reported to increase sugar content in sugar beet roots (*Beta vulgaris*) and the content of gluten in wheat (*Triticum aestivum*) (Dhawi *et al.*, 2009). Radhakrishnan and Kumari (2012) in their experiment on soybeans indicated a positive effect of a pulsed magnetic field on the increased activity of the α- and β-amylases. In our experiment, MNCC with its synergetic effects of magnetism, chitosan, and iron, was remarkably increased the photosynthetic structures,

Table 4 - Effects of OSFs on amylases (N=3) into the terminal bud tissue of inflorescence and vase life (N=5) of three lily cultivars

Cultivars (A)	Treatments		α-amylase	β-amylase	Vase life (days)[wz]
	OSFs (B)	Concentration of OSFs (mg/l) (C)			
Cherbourg	CMC	0	4.48±0.21 gh	5.98±0.49 g	7.00±0.00 ab
		2.5	4.42±0.21 gh	7.97±0.30 g	8.20±0.97 ab
		5	6.61±0.33 ef	12.31±0.90 de	7.00±0.71 ab
		10	6.61±0.20 ef	13.49±0.68 d	7.20±0.37 ab
		15	9.00±0.32 cd	12.33±0.99 de	6.00±0.63 ab
	MNCC	0	4.48±0.21 gh	5.98±0.49 g	7.00±0.00 ab
		2.5	6.56±0.21 ef	6.01±0.43 g	6.60±0.51 ab
		5	7.14±0.17 def	8.04±0.30 g	6.67±0.67 ab
		10	8.99±0.49 cd	12.04±0.95 def	8.00±0.00 ab
		15	11.07±0.44 ab	8.07±0.33 g	6.40±0.51 ab
Navona	CMC	0	5.60±0.27 fg	9.06±0.36 efg	7.80±0.37 ab
		2.5	8.09±0.40 cde	9.11±0.45 ef	7.20±0.49 ab
		5	8.02±0.41 cde	12.15±0.63 de	7.80±0.20 ab
		10	9.63±0.34 bc	15.21±0.65 cd	8.60±0.51 a
		15	12.04±0.53 a	15.11±0.73 cd	8.20±0.49 ab
	MNCC	0	5.60±0.27 fg	9.06±0.36 efg	7.80±0.37 ab
		2.5	3.07±0.20 hi	12.10±0.50 def	6.80±0.37 ab
		5	5.57±0.22 fg	14.92±0.68 cd	8.80±0.58 a
		10	5.59±0.27 fg	18.09±0.79 abc	7.80±0.37 ab
		15	8.03±0.34 cde	18.13±0.74 abc	9.25±0.48 a
Brunello	CMC	0	2.04±0.14 i	6.52±0.37 g	5.80±0.58 ab
		2.5	2.18±0.12 i	8.16±0.44 fg	4.80±0.73 b
		5	4.27±0.24 gh	13.38±0.53 d	6.20±0.37 ab
		10	6.82±0.32 ef	15.27±0.75 cd	7.00±0.77 ab
		15	6.81±0.35 ef	19.30±0.69 ab	7.67±0.67 ab
	MNCC	0	2.04±0.14 i	6.52±0.37 g	5.80±2.58 ab
		2.5	2.09±0.07 i	12.99±0.61 de	6.20±0.20 ab
		5	4.18±0.22 gh	15.43±0.81 bcd	7.60±0.51 ab
		10	6.80±0.24 ef	15.11±0.70 cd	5.80±1.02 ab
		15	9.29±0.50 bc	19.41±0.79 a	5.80±1.02 ab
ANOVA (3-way)					
A			**	**	*
B			**	*	NS
C			**	**	NS
A×B			**	**	NS
A×C			**	**	NS
B×C			**	**	NS
A×B×C			**	**	NS[y]
CV (%)			7.84	7.99	19.18

[z] The means with similar letters have not any significant differences at $HSD_{0.01}$.

[W] $HSD_{0.05}$ and N=5

[y] Type I Error

**, *, NS = Significance at p≤0.01 and p≤0.05 and non-significance, respectively.

the transfer of photosynthetic products and the storage of carbohydrates; so that Takeda *et al.* (1983) stated that the increase of substrate promotes the activity of amylase enzymes, especially α-amylase.

According to Table 4, the longer vase life was observed in 'Navona' in the high concentrations of OSFs (magnetic and non-magnetic) based on 3-way interactions compared with two other cultivars. Hajnorouzi *et al.* (2011) reported that using a combination of Earth's magnetic field and weak pulsed electromagnetic field on corn seedlings can increase the growth rate and decrease the iron content of the plant and maintain the membrane's health and decrease oxidative burst.

Since the magnetic field is the natural property of the earth, plants and other living creatures are permanently responding to the magnetic field during their lives. Earth acts as a magnet with its northern and southern poles, and natural effects of the magnetic field can change the growth and yield of plants on the ground. In particular, the electromagnetic spectrum of solar radiation stimulates plant growth through the process of photosynthesis. The possible mechanism is the changing of the electrostatic balance of the plant system at the membrane surface of the cell, which is the primary site for any plant growth restriction or promotion (Radhakrishnan and Kumari, 2012). Therefore, the role of superparamagnetic nanoparticles can be interpreted in our experiment based on the influence of the Earth's magnetic field.

It can be stated, instead of the iron salt (which is often difficult to uptake by plants), a chelating agent in the nutrition solution can be used both as the iron source for the plant and also is suitable for the absorption of other elements. Between two tested compounds, MNCC is a more suitable compound than CMC because it has a positive synergic effect in addition to providing iron in the plant, which is effect of both chitosan and magnetism. In addition, the characteristics of particle size in this type of supplement, i.e. nano- size, affect the biophysical characteristics and biological activities of the plant.

4. Conclusions

Our experiment showed that utilizing the magnetic composite of chitosan (MNCC) or modified and chelating macromolecule of chitosan (CMC) in horticulture can significantly affect the growth and development physiology of plants. We introduced this compound, especially magnetic compound, as fertilizer supplement in production period of lily bulb (Shafiee-Masouleh *et al.*, 2014); now in this experiment for three cultivars forcing period of lily shows that high concentration of both OSFs can be used as a supplement in nutrition solution. The highest concentrations (10 and 15 mg/ l) regardless of OSF types and the cultivars response caused significant physiological effects on the content of carbohydrates and also the enzymes that are influence on carbohydrates (amylases). It seems that observing the remarkable morphological changes needs to use the higher concentrations of both OSFs without toxic effects. However, this subject (the use of higher concentrations) must be examined in horticultural plants. The examined cultivars (Cherbourg, Navona and Brunello) showed almost different responses to OSFs, but generally magnetic OSF can be advised and for security utilization needs to be examined with higher concentrations at future research.

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Foliar application of asparagine and casein on biochemical and morphological attributes of garden cress (*Lepidium sativum* L.) under greenhouse conditions

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Key words: Foliar spray, nitrogen content, phosphorus content, vegetative stage.

Abstract: In this study, the effect of foliar application of Asparagine (ASN) and Casein (CSN) during vegetative stage at four rates (0, 50, 100 and 150 mg l^{-1}) was investigated on garden cress (*Lepidium sativum* L.). The results showed that asparagine application, especially at a high level, could significantly increase the morpho-physiological traits such as plant height, leaf and stem fresh weights and leaf and stem dry weights, leaf pigments (chlorophyll a and chlorophyll b) and leaf nutrients content (Nitrogen and Phosphorus). Also, the results indicated that casein application at 50 mg l^{-1} rate had the best performance through in stem and root fresh weights, stem dry weight and diameter of main stem traits. Casein application at rate 100 mg l^{-1} had the highest leaf nitrogen and phosphorus content. Generally, our findings suggest that the use of asparagine and casein can be considered as an appropriate growth regulator in garden cress cultivation.

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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

Garden cress (*Lepidium sativum* L.) is an edible herb and a member of the Cruciferae (Brassicaceae) family. It is commonly cultivated throughout the temperate regions of India and Pakistan (Nadkarni, 1954). The plant is cultivated as culinary vegetable all over Asia (Nadkarni, 1976). Garden cress is an annual standing plant, growing up to 30 cm. It is a well known cookable herb and the leaves are widely used as a garnish in salads. In addition to its leaves that have medicinal properties, the seeds are aperients, diuretic, tonic, demulcent, aphrodisiac, carminative (Chopra *et al*., 1986). Moreover, the seeds, which are used in folk therapies, have many activities like thermogenic, depurative, rubefacient, tonic, aphrodisiac, abortive, ophthalmic, diuretic (Gokavi *et al*., 2004; Dugasani *et al*., 2009).

Intensive farming practices, which produce high yields and quality, require the extensive use of chemical fertilizers that are both costly and

create environmental problems. Therefore, there has been a recent resurgence of interest in environmentally friendly, sustainable and organic agricultural practice (Orhan *et al*., 2006). Thus, it is necessary to supply the plant requirement to nutrient through proper procedure. There are different ways to supply plant nutrient's requirement such as soil feeding and foliar application. Of these, foliar feeding is an effective method for improving soil deficiencies and overcoming the soils inability to transfer nutrients to the plant. It has reported the foliar feeding can be 8 to 10 times more effective than soil feeding and up to 90% of a foliar fed nutrient solution can be found in the smallest root of a plant within 60 minutes of application (Garcia and Hanway, 1976).

Amino acids are the major building element for proteins (Andrews, 1986). Amino acids is a well-known biostimulant which has positive influence on plant growth, yield and significantly decrease the damages caused by abiotic stresses (Kowalczyk and Zielony, 2008). ASN is widely used as a source of organic nitrogen in the media upon which certain bacteria are grown (Long and Seibert, 1926). ASN is thought to play a clearly important role in the transportation and storage of nitrogen (Lehmann and Ratajczak, 2008), because of their relatively stable nature and high N⁄C ratio (Ireland and Lea, 1999; Masclaux-Daubresse *et al*., 2006). In plants, ASN, one of the most prevalent amides, has been reported to be the primal source of Nitrogen for protein synthesis, particularly in actively growing tissues (Brouquisse *et al*., 1992). ASN aggregation in plants in response to environmental stress could be an ammonium detoxification mechanism and a means to stock up Nitrogen when protein synthesis is impaired in plants due to stressful environments (Herrera-Rodríguez *et al*., 2007).

CSN is a very rich source of essential amino acids (Sarode *et al*., 2016). There are four different CSN proteins (α_{s1}, α_{s2}, β and K), which are different in their amino acid composition (Dalgleish, 1989). Among proteins, CSN has been vastly used in artificial rearing diets because it contains all the essential amino acids, is soluble in water, and does not coagulate or precipitate after heating (Parra, 1979). CSN also contains important substances such as fatty acids, cholesterol, sugars, vitamins, and minerals (Vanderzant, 1974). CSN contains 0.7-0.9% phosphorus. So, CSN is known as a phosphoprotein (Sarode *et al*., 2016). Several pre-harvest factors like climatic conditions and available nutrients can influence on yield and quality of vegetables (Lee and Kader, 2000).

In spite of wide rang of properties, there are a few study about the effect of amino acids on medicinal plants. So this study was conducted to evaluate the effects of ASN and CSN foliar application on vegetative and reproductive growth, physiological and biochemical traits of garden cress.

2. Materials and Methods

In order to investigate the response of *Lepidium sativum* to foliar application of asparagine (ASN) and casein (CSN) two separate pot experiments were conducted at research greenhouse of University of Guilan, Iran. The experiments were carried out based on two randomized complete design with three replications. The treatments in this study were different levels of ASN and CSN (0, 50, 100 and 150 mg l^{-1}). Foliar application of experimental solutions was started from the four-leaf stage and done once every two weeks in the morning, up to the early May. In each spraying date, 20-25 ml of corresponding solution was applied per pot. Garden cress seeds which were prepared from Isfahan-PakanBazr Company were planted in 6th January. The sowing was done in pots with the 33 cm diameter and 22 cm height. The seed bed was a mixture of soil, cow manure and sand with the ratio of 2:1:1. The main properties of soil used in the pots are shown in Table 1. Except for cow manure, no fertilizer was used during the plants growth cycle. Plants were irrigated every 10 days. Plant thinning was performed 15 days after emergence, so that, 6 plants were kept in each pot. During plant growth the greenhouse temperature was 25˚C on the day and 15°C during the nights. In addition, CO_2 concentration was 350 ppm, relative humidity was 40% and photoperiod adjusted as 16 hours' light and 8 hours of darkness.

Table 1 - Some chemical indices of soil used in pots for garden cress cultivation

Organic carbon (%)	Nitrogen (%)	Calcium (%)	Phosphorous (%)	Potassium (%)	pH	Texture
1.2	1	0.48	0.19	0.55	7.1	Loam sandy

In 4th May, four plants were lifted form the soil in each pot for determination of some above- and below-ground vegetative indices. The measured indices were plant height, leaf weight, root weight, stem weight and main stem diameter (by a ruler). The remained two plants were used to measure some reproductive traits including number of florets. The pigments content (carotenoid, chlorophyll a and chlorophylls b) was measured by the method has been explained by Minguez-Mosquera and Prez-Galvez (1998). To determine the leaf nitrogen, phosphorous and calcium percentages, the method of Jones (2001) was used.

The method of Bakhshi and Arakava (2006) also was used for the extraction of leaf extract to determine phenolic compounds and antioxidant capacity. Then, phenols were analyzed by using the method of Folin-Ciocalteau reported by Tavarini *et al*. (2008). For this purpose, the absorbance at 760 nm was measured using a spectrophotometer (T80+PG Instrument UV/Vis Spectrometer) and the values were expressed as mg gallic acid/100 g fresh weight. Antioxidant capacity was determined using DPPH free radical scavenging method which has been described by Sanchez-Moreno *et al*. (1999). Finally, data analysis was done using SAS 9.2 software and means were separated via Tukey's (honest significant difference) test at the 5% level of probability.

3. Results and Discussion

Vegetative and reproductive growth

The analysis of variance of the investigated morphological traits was presented in Table 2. The results revealed that both ASN and CSN significantly affected the plant length, leaf and stem fresh weight, leaf and stem dry weight. So that, the greatest plant height was observed at a rate of 150 mg l^{-1} of ASN. Moreover, applying ASN at rate of 100 mg l^{-1} had the highest leaf and stem fresh weights and leaf and stem dry weights.

Casein application significantly affected the stem and root fresh weights, stem dry weight and diameter of main stem (Table 2). So that, the plants grown under CSN spray at rate of 50 mg l^{-1} had the best performance due to stem dry and fresh weight, root fresh weight and main stem's diameter.

Asparagine and CSN foliar spraying could improve the morphological characteristics in garden cress. These results are in agreement with the previously studies by Kaya *et al.* (2013) on maize, Rasmia *et al.* (2014) on palm, Saeed *et al.* (2005) on soybean, Akladious and Abbas (2013) and El-Desouky *et al*. (2011) on tomato, Shafeek and Helmy (2012) on onion. Also, El-Zohiri and Asfour (2009) on potato found that spraying of amino acids at 0.25 ml/L significantly increased vegetative growth expressed as plant height and dry weight of plant. Their finding indicated that amino acids could improve the vegetative and reproductive traits in plants. These ability my be due to their important role in plant metabolism and protein assimilation which is necessary for cell formation and consequently increase the fresh and dry matter (Fawzy *et al*., 2012).

Amino acids contribute to the synthesis of growth hormones; therefore, it can be concluded that an increase in cell division and cell enlargement is the reason behind enhanced growth parameters (Shafeek and Helmy, 2012). The positive effect of amino acids on growth was stated by Goss (1973) who indicated that amino acids can serve as a source

Table 2 - Mean squares for the effect of different levels of Aspargine (ASN) and Casein (CSN) on vegetative and reproductive indices of garden cress

Traits	Source of Variation					
	Treatment		Error		CV	
	ASN	CSN	ASN	CSN	ASN	CSN
Plant height	24.22 *	5.86 NS	3.75	10.41	5.75	10.38
Leaf fresh weight	0.66 *	1.008 NS	0.09	0.25	5.09	7.97
Leaf dry weight	0.27 **	0.04 NS	0.03	0.02	11.69	12.28
Root fresh weight	0.17 NS	0.2 **	0.06	2	8.52	5.22
Root dry weight	0.002 NS	0.004 NS	0.01	0.005	8.54	5.94
Stem fresh weight	2.65 **	2.001 **	0.04	0.16	3.62	6.83
Stem dry weight	0.5 **	0.22 *	0.02	0.04	9.66	14.13
Diameter of main stem	0.04 NS	0.11 *	0.05	0.01	10.98	5.87
Number of florets	0.22 NS	0.75 NS	0.66	0.5	11.66	10.47

NS, *, ** Non-significant, significant at 5%, and 1% probability level, respectively.

of carbon and energy when carbohydrates become deficient in the plant's releasing the ammonia and organic acid form which the amino acid was originally formed (Table 3). The organic acids then enter Kerb's cycle, to be broken down to release energy through respiration (Goss, 1973). Serna *et al*. (2012) found that the spray of pepper plants with a mixture of amino acids led to raise the efficiency of photosynthesis, and thus, give the best vegetative growth.

Biochemical traits and nutrients content

The mean squares of physiological and mineral content are presented in Table 4. Total phenolic compounds and antioxidants activity were not affected by foliar spraying of ASN and CSN (Table 5). Chlorophyll a and b content significantly increased in response to foliar ASN treatments (Table 4). The highest contents of chlorophyll a and b observed in plants grown under spraying of ASN at rate of 100

Table 3 - Means comparison for the effect of different levels of Asparagine and Casein on some vegetative and reproductive growth parameters of garden cress

Treatments (mg l^{-1})	Plant height (cm)	Leaf fresh weight (g $plant^{-1}$)	Leaf dry weight (g $plant^{-1}$)	Root fresh weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Stem fresh weight (g $plant^{-1}$)	Stem dry weight (g $plant^{-1}$)	Diameter of main stem (mm)	Number of florets
Aspargine									
0	29.66 b	5.47 b	1.18 b	2.87 a	1.20 a	4.77 c	1.15 c	1.99 a	7.00 a
50	34.66 ab	6.05 ab	1.81 a	2.97 a	1.25 a	5.73 b	1.44 bc	2.08 a	6.66 a
100	34.00 ab	6.57 a	1.83 a	3.42 a	1.26 a	6.96 a	2.06 a	2.25 a	7.00 a
150	36.33 a	6.31 a	1.62 ab	3.12 a	1.26 a	6.38 ab	1.86 ab	2.02 a	7.33 a
Casein									
0	29.66 a	5.47 a	1.18 a	2.87 b	1.2 a	4.77 b	1.15 b	1.99 b	7.00 a
50	31.00 a	6.75 a	1.45 a	3.39 a	1.22 a	6.64 a	1.80 a	2.37 a	6.33 a
100	30.66 a	6.23 a	1.38 a	2.82 b	1.15 a	6.22 ab	1.58 ab	2.01 b	6.33 a
150	33.00 a	6.65 a	1.43 a	3.14 ab	1.25 a	6.16 ab	1.55 ab	2.3 ab	7.33 a

Means with the same letter(s) within a column are not significantly different (P≤0.05) based on Tukey's test.

Table 4 - Mean squares for the effect of different levels of Aspargine (ASN) and Casein (CSN) on leaf nutrient and pigments content and some biochemical parameters in garden cress

Traits	Source of variation					
	Treatment		Error		CV	
	ASN	CSN	ASN	CSN	ASN	CSN
Chlorophyll a	0.13 **	31.66 NS	0.006	0.008	4.9	6.75
Chlorophyll b	0.05 **	0.01 *	0.001	0.002	5.83	7.89
Carotenoid Content	0.0004 NS	0.003 NS	0.001	0.002	8.32	12.96
Leaf Nitrogen Content	0.09 **	0.18 **	0.006	0.01	4.79	6.72
Leaf Phosphorous Content	0.01 *	0.03 **	0.003	0.002	8.86	6.66
Leaf Calcium Content	0.007 NS	0.0007 NS	0.007	0.003	9.45	6.95
Total Antioxidants	21.41 NS	6.97 NS	12.75	6.16	6.64	4.7
Total Phenol	31.66 NS	6.97 NS	7.33	17.33	4.99	7.81

NS, *, ** Non-significant, significant at 5%, and 1% probability level, respectively.

Table 5 - Means comparison for the effect of different levels of Asparagine and Casein on biochemical parameters of garden cress

Treatments (mg l^{-1})	Chlorophyll a	Chlorophyll b	Carotenoid content	Leaf nitrogen content	Leaf phosphorous content	Leaf calcium content	Total antioxidants	Total phenol
Aspargine								
0	1.38 b	0.56 c	0.39 a	1.45 b	0.57 b	0.89 a	51.6 a	50.3 a
50	1.53 b	0.73 b	0.4 a	1.6 ab	0.66 ab	0.83 a	53 a	52.6 a
100	1.87 a	0.89 a	0.37 a	1.81 a	0.71 ab	0.95 a	57.6 a	57.3 a
150	1.64 ab	0.71 b	0.35 a	1.81 a	0.73 a	0.87 a	52.6 a	56.3 a
Casein								
0	1.36 a	0.56 b	0.39 a	1.45 b	0.57 c	0.89 a	51.66 a	50.3 a
50	1.41 a	0.73 a	0.37 a	1.62 ab	0.60 bc	0.86 a	52 a	54.3 a
100	1.45 a	0.70 a	0.44 a	2.01 a	0.78 a	0.82 a	55 a	54.6 a
150	1.38	0.65 ab	0.43 a	1.88 a	0.74 ab	0.87 a	52.3 a	53.6 a

Means with the same letter(s) within a column are not significantly different (P≤0.05) based on Tukey's test.

mg l^{-1} (Table 5). Generally, application of ASN had a significant effect on N and P% content, However, there was no significant difference in leafs calcium content (Table 4). Meanwhile, the highest amount of N% was belonging to application of ASN at rates of 100 and 150 mg l^{-1}. Moreover, the highest leaf content of P% was observed at rates of 150 mg l^{-1} ASN (Table 5).

Casein application could influence significantly on chlorophyll b content and leaf's Nitrogen and phosphorous content (Table 4). Lowest chlorophyll b content were observed in plants that treated with distilled water, while the highest chlorophyll b content were in plants that treated with 50 and 100 mg l^{-1} of CSN (Table 5). The plant treated with 100 and 150 mg l^{-1} CSN had the highest amount of nitrogen, and the highest Phosphorous content were in plants treated with 100 mg l^{-1} of CSN (Table 5). These results are in Conformity with previous study in maize (Cazetta *et al*., 1999; Kaya *et al*., 2013), palm (Rasmia *et al*., 2014), almond (Youssefi *et al*., 2000), Datura (Hussein *et al*., 1992) and strawberry (Abo Sedera *et al*., 2010).

Youssefi *et al*. (2000) reported that leaf-nitrogen concentrations were related positively to concentrations of applied amino acids (especially asparagine and glutamine). On the other hand, increasing the nitrogen supply led to increase in the activity of certain enzymes, starch and the levels of nitrogen compounds (total nitrogen, soluble protein and free amino acids) and decreased the levels of carbon metabolites (sucrose and reducing sugars) in the tested plant (Cazetta *et al*., 1999). Exogenous application of ASN was improved the phosphorus content of maize (Kaya *et al*., 2013).

This increase in chlorophyll contents might be due to the availability of higher levels of amino acids in treated plants, because amino acids help to increase the chlorophyll content and this may lead to the increase in different growth criteria (Awad *et al*., 2007).

4. Conclusions

The major problem with the use of conventional chemical fertilizers is the way in which ions enter the plant cell. The ions dissipate calcium from the cell wall and damage it, while amino acid chelates enter the space in the cell with the least resistance and without damaging the cell membrane. At the last stage, the binding of the chelate and desired atom is broken down into the plant cell, and the nutritional needs of the cells are provided with the lowest losses. Results of the current study revealed that foliar application of ASN and CSN could increase the growth and yield of garden cress. It could be recommended that spraying garden cress plants by ASN (100 and 150 mg l^{-1}) increased the vegetative growth traits (plant height, leaf fresh weight, number of florets), nutrients uptake (leaf nitrogen and phosphorus content) and pigments concentration (chlorophyll a and chlorophyll a) and also application of CSN at 50 mg l^{-1} level could improved some morphological and biochemical traits of garden cress such as: root fresh weight, stem fresh weight, Chlorophyll b, Leaf nitrogen and phosphorus content in comparison control treatment. Therefore, it can be concluded that application of ASN and CSN is a good strategy in garden cress cultivation, but their effectiveness must be also evaluated under environmental stresses, in the future studies. Also spraying different nutrient solution in combination with each other may show interaction and contrary behavior in plants in comparison applying them solely, so further research with higher concentrations and also combined spraying of these amino acids, applying on different vegetables in greenhouse and farm conditions is suggested.

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Impact of light quality on the physiological characteristics of *Capsicum chinense* seeds

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Key words: germination, LEDs bulbs, pepper, vigor.

Abstract: The objective of this work was to evaluate the physiological quality of *Capsicum chinense* seeds submitted to different light spectral qualities. It was used a completely randomized design, in a 4x5 factorial scheme, with four pepper cultivars [BRS moema biquinho yellow (Biq. Yellow), Airetama biquinho red (Biq. Red), Boyra Habanero red (Boyra Hab. Red), BRS Seriema tupã bode red (Tupã Bode Red)] and five light spectral qualities, being blue LEDs (B-LEDs); red LEDs (R-LEDs); blue+red LEDs (BR-LEDs); white LEDs (W-LEDs) and fluorescent lamp (FL) carried out germination and vigor analysis, with four replicates of 50 seeds. For this, the seeds were conditioned inside gerbox® boxes and kept in a growth room. The Biq. Yellow and Boyra Hab. Red peppers showed the highest potential of germination and vigor, respectively, indicating high physiological quality. In general, the light spectral qualities provide differentiated responses in the initial development of the pepper cultivars, being the reduction of the percentage of dead seeds favored by the spectrum BR-LEDs and W-LEDs. The root fresh mass is increased by all lights, except R-LEDs. The fresh mass of the aerial part presents positive results in the FL lamps. Shoot length is favored by the R-LEDs.

1. Introduction

Capsicum peppers are closely related to the Brazilian richness culture and are a valuable part of the biodiversity heritage, being cultivated an immense variety, sizes, colors, flavors and pungences (Neitzke *et al.,* 2008). The Brazilian production is around 11,071 tons (Conab, 2015), being the state of São Paulo considered the largest producer.

Among the factors that regulate plant production, the light plays an important role because it is an important regulator of growth and development of the plant, as it regulates morphological characteristics and acts as an energy source in the primary metabolism and in the photosynthetic process (Simlat *et al.,* 2016). The qualitative or quantitative characteristics of growth and morphogenesis are influenced by the quality of the supplied light, affecting the plants development, mainly, by photomorphogenic alterations (Heo *et al.,* 2002; Rezende *et al.,* 2008).

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In recent years, light-emitting diodes (LED) have been widely used as alternative light source potential for plants (Simlat *et al.,* 2016). Among the advantages of LEDs systems are visible light emission and low heat production for long periods, with a specific wavelength, color and lighting flexibility, reduction of electrical consumption and toxic substances, as well as improved lifetime (Carvalho, 2007; Yeh and Chung, 2009). This technology becomes promising for the growth of plants in a controlled environment, such as in tissue culture and also in the supplementation of growth chambers and greenhouses (Yeh and Chung, 2009).

Different wavelengths of light can trigger a variety of responses in plants (Simlat *et al.,* 2016). For example, red, blue, green and white LEDs lights were tested in different species, mainly forest ones, demonstrating the promotion of seed germination and subsequent development (Gonçalves *et al.,* 2006; Victório and Lage, 2009). Red light may promote seed germination and root development (Daud *et al.,* 2013), shoot elongation (Kim *et al.,* 2004; Araújo *et al.,* 2009), fresh mass increment (Sorgato *et al*., 2016), and an increase in shoot length (Cybularz-Urban *et al*., 2007), among others. Combinations of blue and red LEDs may promote biomass increase (Gu *et al.,* 2012; Maluta *et al.,* 2013; Da Silva *et al.,* 2016) and increase of the root system (Gu *et al.,* 2012). The blue wavelengths tend to improve stomatal conductance (Hogewoning *et al.,* 2010), affect phototropism (Johkan *et al.,* 2010), and increase the rate of photosynthetic pigments production. However, plants exhibit a wide range of morphological and phytochemical plasticity in response to each type of wavelength of light (Macedo *et al.,* 2011).

Many studies report that LEDs can modify seed germination and plant growth and development (Gonçalves *et al.,* 2006; Victório and Lage, 2009; Daud *et al.,* 2013; Da Silva *et al.,* 2016). Although there are reports on the development of seedlings of the genus *Capsicum spp*. in light qualities (Da Silva *et al*., 2016), the effects of the light spectral qualities on the germination and vigor of *Capsicum chinense* have not yet been analyzed. Thus, the objective of this work was to verify the impact of the light spectral qualities on the physiological quality of *Capsicum chinense* seeds.

2. Materials and Methods

Plant material and conduction of the experiment

The work was conducted at the Plant Tissue Culture Laboratory of the Universidade Federal de Santa Maria, campus Frederico Westphalen - RS (Federal University of Santa Maria, campus Frederico Westphalen - RS), in november 2016.

The experiment was conducted in a completely randomized design, in a 4x5 factorial scheme, with four pepper cultivars (*Capsicum chinense*) and five light spectral qualities, totaling 20 treatments, with four replicates of 50 seeds each tratament, totalizing 4000 seeds tested. Four cultivars of pepper were used [BRS Moema biquinho yellow (Biq. Yellow), Airetama biquinho red (Biq. Red), Boyra Habanero red (Boyra Hab. Red) and BRS Seriema tupã bode red (Tupã Bode Red)], and five light spectral qualities [TEC-LAMP® blue LEDs - (450 nm) B-LEDs; red LEDs (660 nm) R-LEDs; blue (450 nm) + red (660 nm) BR-LEDs in the ratio of 40% and 60%, respectively; white LEDs W-LEDs; and special daylight type fluorescent FL (Osram®, Brazil)].

The seeds were placed inside gerbox® boxes with lids (11x11x3 cm) containing two sheets of Germitest® paper (in box dimensions), moistened with 0.2% KNO_3 solution (dissolved in distilled water), in proportion to 2.5 times the dry paper weight, as described in the Regras Analis de Sementes (Rules for Seed Analysis) (MAPA, 2009). The gerbox boxes were maintained in a growth room under temperature of 25±2°C and a luminous intensity of 36 µmol m^{-2} s^{-1} for 14 days.

Analyzed variables

For the germination test, counting was performed by seven and 14 days after the test installation. At the first count (FC), the normal seedlings were counted and the values expressed as a percentage (%), at 14 days the following variables were analyzed: percentage of germination (PG), percentage of abnormal seedlings (PAS), percentage of hard seed (PHS) and percentage of dead seeds (PDS) (MAPA, 2009). According to MAPA (2009), dead seeds are the seeds that do not germinate at the end of the test, are neither hard nor dormant, and are usually softened, attacked by microorganisms and show no signs of germination. Already, the hard seeds are those that remain without absorbing water for a longer period than normal and are therefore at the end of the test with the appearance of seeds newly placed on the substrate.

For the root length (RL) and shoot length (SL) variables, 10 seedlings of each replicate were measured for all light qualities, being measured with a digital caliper. For the fresh mass of the aerial part (FMAP) and root fresh mass (RFM), the same seedlings were

used for the SL and RL measurement, with the values referring to the 10 seedlings. Afterwards, the seedlings were conditioned in paper bags and kept in a forced air oven at 60°C, until constant weight was reached, to determine the dry mass of shoot (DMS) and dry mass of the root (DMR).

The germination speed index (GSI) was calculated by the sum of the number of germinated seeds per day, divided by the number of days between sowing and germination, following the Maguire's methodology (1962).

$$GSI = (G1 / N1) + (G2 / N2) + \cdots + (Gn / Nn) \quad (1)$$

Where GSI = G_1, G_2, ..., Gn = number of seedlings computed in the first, second, third and last count; N_1, N_2, ..., Nn = number of days of sowing to the first, second, third and last count.

The obtained data were submitted to analysis of variance, and the interaction between pepper cultivars and light spectral qualities was evaluated, and when they were significant, the averages were compared by the Tukey's test, at 5% of error probability, using the statistical program Assistat 7.7 beta.

3. Results

The analysis of variance showed significant interaction for the pepper cultivar factors x light spectral qualities only for the fresh mass of the aerial part (FMAP) and shoot length (SL) variables. The variables of the first count (FC), percentage of germination (PG), normal seeds (NS), abnormal seeds (AS), hard seeds (PHS), germination speed index (GSI), root fresh mass (RFM) and root length (RL) were significant only for the pepper cultivars factor. Root length (RL), root fresh mass and percentage of dead seeds (PDS) variables were significant for the light spectral qualities factor. On the other hand, the dry mass of the aerial part (DMAP) and dry mass of the root (DMR) variables were not significant (data not shown) by the F test, at 5% of error probability.

The pepper cultivars differed for the percentage of normal seeds (NS) and abnormal seeds (AS). The highest percentages of NS were observed for Biq. Yellow pepper with approximately 88%, being the same as Biq. Red pepper (85%), and differing from the others ($p<0.05$) (Fig. 1A). For the variable AS, the Tupã Bode Red cultivar presented the highest values, with 12.30% of abnormality, being higher than the others, and the lowest percentages were verified for Biq. Yellow pepper, with 4.40% (Fig. 1B).

Regarding the percentage of hard seeds, the Boyra Hab. Red cultivar had the highest values, with an average of 11.8%, differing significantly from the others (Fig. 1C). The Biq. Yellow pepper cultivar showed the highest percentages of germination in the evaluation of the first count (FC), with 94.8%, differing significantly from the Boyra Hab. Red cultivar (Fig. 1D).

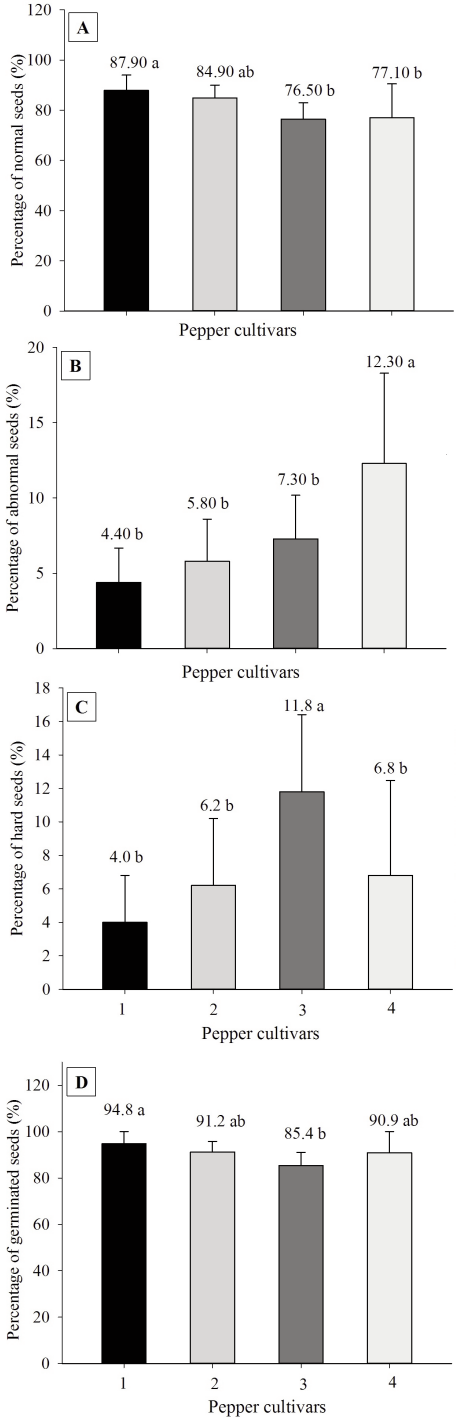

Fig. 1 - Percentage of normal seeds (NS-A), abnormal seeds (AS-B), hard seeds (HS-C) and germinated (GS-D) of four pepper cultivars, being Biq. Yellow, Biq. Red, Boyra Hab. Red and Tupã Bode Red, submitted to different light spectral qualities. *Different letters represent significant statistical difference (Tukey's Test $P<0.05$; Bars=SD).

For the root length variable, the Boyra Hab. pepper presented the highest values with approximately 51 mm in length, being statistically different from the other cultivars. The lowest averages were observed in Biq. Yellow and Biq. Red peppers with 36 and 35 mm, respectively (Fig. 2A). The Boyra Hab. Red pepper again stood out for the root fresh mass variable, with 0.072 gram for 10 seedling differing significantly from the other ones evaluated (Fig. 2B).

For the germination speed index (GSI), the Biq. Yellow pepper stood out, presenting 23.70, being significantly similar to the Biq. Red and Tupã Bode Red peppers, differing only from Boyra Hab. Red pepper (Fig. 2C). For the first counting of normal seeds the Biq. Yellow pepper showed the highest values with 84.1% of normal seedlings, differing from the other ones (Fig. 2D).

The light spectral qualities of BR-LEDs and W-LEDs provided higher root length (RL), with 43.37 and 42.97 mm respectively, differing statistically from the FL (Fig. 3A). For the root fresh mass variable, it was observed that the red spectrum promoted mass

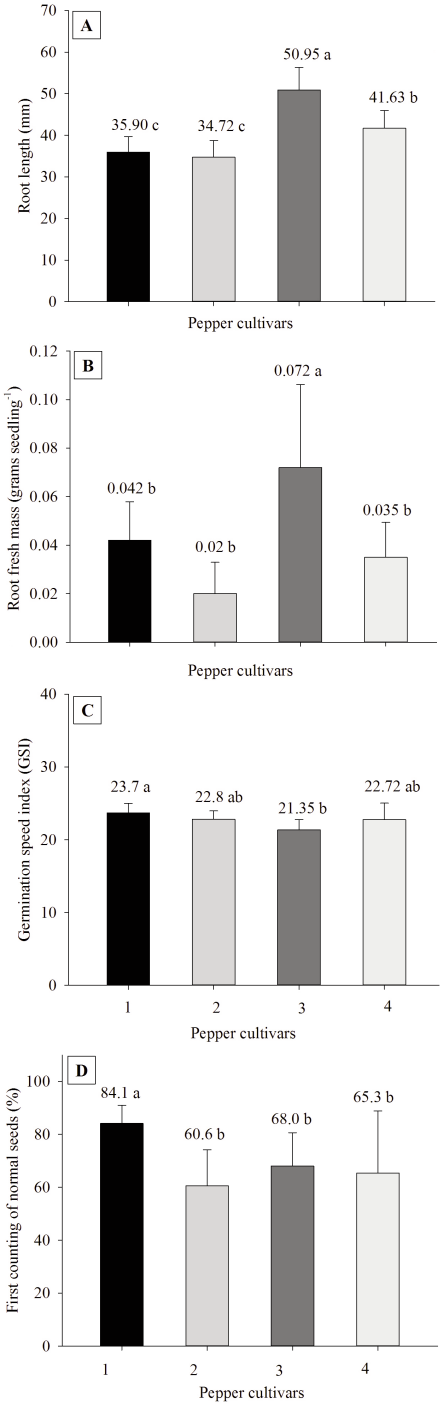

Fig. 2 - Root length (A), root fresh mass (g for 10 seedlings) (B), germination speed index (C) and first counting of normal seeds (D) of four pepper cultivars, being Biq. Yellow, Biq. Red, Boyra Hab. Red and Tupã Bode Red, submitted to different light spectral qualities. *Different letters represent significant statistical difference (Tukey's Test P<0.05; Bars=SD).

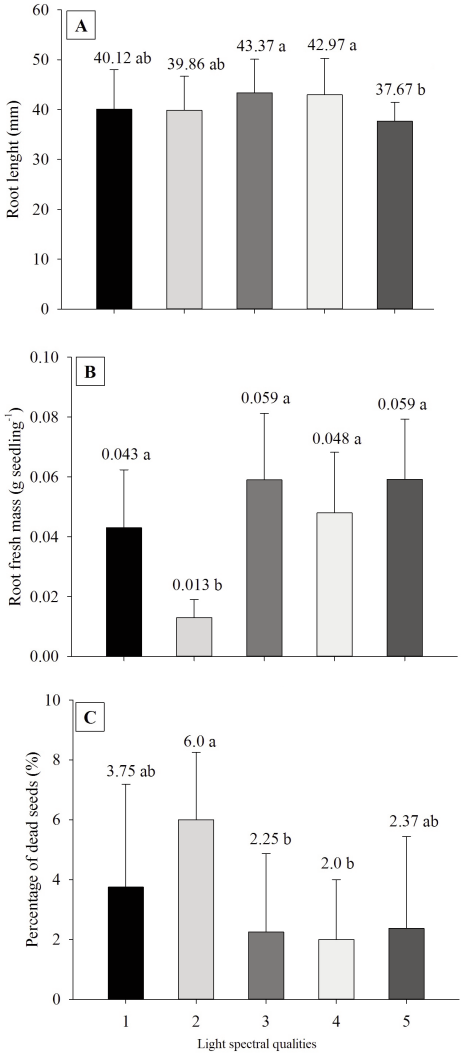

Fig. 3 - Root length (A), root fresh mass (g for 10 seedlings) (B) and percentage of dead seeds (C) of four pepper cultivars submitted to different light spectral qualities, being B-LEDs, R-LEDs, BR-LEDs, W-LEDs and FL lamps. *Different letters represent significant statistical difference (Tukey's Test P<0.05; Bars=SD).

reduction, with 0.013 gram for 10 seedling, differing statistically from the other light spectra, which presented higher mass (Fig. 3B). Correspondingly, it was observed that the red spectrum conditioned the highest percentage of dead seeds, with 6%, differing statistically from the BR-LEDs and W-LEDs. The BE-LEDs and W-LEDs spectra conditioned low percentage of seed mortality (Fig. 3C).

For the Biq. Yellow pepper cultivar the light spectral qualities of W-LEDs, FL and BR-LEDs showed the highest values of fresh mass of the aerial part (FMAP), differing significantly from R-LEDs (Table 1). For Biq. Red and Boyra Hab. Red peppers, the FL light provided greater accumulation of fresh mass, presenting 0.194 and 0.283 gram for 10 seedlings, respectively, being statistically higher to the others (Table 1). As for the Tupã Bode Red pepper, the W-LEDs light conditioned the largest fresh mass of the aerial part, differing significantly from the B-LEDs and R-LEDs (Table 1).

It was observed that the BR-LEDs and FL lights provided a greater increment of fresh mass for Boyra Hab. Red pepper, with 0.236 and 0.283 g for 10 seedling, respectively, differing significantly from the R-LEDs spectrum. The tested peppers presented similar responses in relation to the W-LEDs light spectrum, not statistically different from each other (Table 1).

R-LEDs spectral quality provided the highest averages for the shoot length variable, being higher to the other spectra. In this spectrum, the Boyra Hab. Red and Tupã Bode Red were superior to the other peppers. For Biq. Yellow pepper the R-LEDs, FL lamp and BR-LEDs spectra conditioned the larger shoot length, statistically differing from the B-LEDs and W-LEDs. For Biq. red pepper, the R-LEDs spectrum was statistically superior to the others (Table 2).

The Boyra Hab. Red pepper presented superior performance to the others, presenting the highest average in shoot lenght (SL), being favored by the LEDs spectra and in disadvantage by the FL, differing significantly. Shoot length of Tupã Bode Red cultivar was favored by the R-LEDs light spectra qualities, differing significantly from the other spectra (Table 2).

4. Discussion and Conclusions

The Biq. Yellow pepper cultivar was superior to GSI, FC, NS, PG, presenting the lowest percentage of abnormal (AS) and hard (HS) seeds (Fig. 1). However, the Boyra Hab. Red pepper cultivar has been highlighted for the RL (Figs. 2A, 3A), SL (Table 2), FMAP (Table 1), RFM (Figs 2B, 3B) variables. The results sug-

Table 1 - Fresh mass of the aerial part (g for 10 seedlings) of four pepper cultivars, being Biquinho Yellow, Biq. Red, Boyra Hab. Red and Tupã Bode Red, submitted to different light spectral qualities, B-LEDs, R-LEDs, BR-LEDs, W-LEDs and FL lamps

Cultivars	Fresh mass of the areal parts				
	B-LEDs	R-LEDs	BR-LEDs	W-LEDs	FL
Biq. Yellow	0.144 bAB	0.114 bB	0.182 bA	0.191 aA	0.192 bA
Biq. Red	0.1233 bC	0.139 abBC	0.179 bAB	0.169 aABC	0.194 bA
Boyra Hab. Red	0.196 aBC	0.176 aC	0.236 aAB	0.204 aBC	0.283 aA
Tupã Bode Red	0.099 bC	0.138 abBC	0.160 bAB	0.200 aA	0.183 bAB
CV			15%		

* Different lowercase letters in the column or uppercase letters in the row represent significant statistical difference (Tukey's Test P<0.05).

Table 2 - Shoot lenght of four pepper cultivars, being Biquinho Yellow, Biq. Red, Boyra Hab. Red and Tupã Bode Red, submitted to different light spectral qualities, being B-LEDs, R-LEDs, BR-LEDs, W-LEDs and FL lamps

Cultivars	Shoot lenght (mm)				
	B-LEDs	R-LEDs	BR-LEDs	W-LEDs	FL
Biq. Yellow	28.66 abB	34.28 bA	30.62 abAB	29.26 bcB	34.15 aA
Biq. Red	26.56 bC	37.65 bA	30.34 bBC	30.65 abBC	33.33 aB
Boyra Hab. Red	30.74 aB	43.14 aA	34.59 aB	33.92 aB	25.52 bC
Tupã Bode Red	29.075 abC	42.10 aA	33.89 abB	26.18 cC	26.53 bC
CV			6.71%		

* Different lowercase letters in the column or uppercase letters in the row represent significant statistical difference (Tukey's Test P<0.05).

gest that Biq. Yellow pepper has a higher germinative potential, while Boyra Hab. Red pepper has greater vigor. The characteristics of germination and vigor are individual for each cultivar and variables between them. For most of them, the speed, uniformity and germination rate depends on external and internal factors to the seed (Plue *et al.,* 2010; Demotes-Mainard *et al.,* 2016), such as temperature, humidity, light, viability of the embryo and genetic factors, characterizing in this way, the differences verified between the pepper cultivars. In general, the use of high vigor seeds results in a good performance of the crops in the field through better establishment of seedlings and survival of seedlings. In this way, germination and vigor tests are important in order to choose the best pepper to be used.

Specific light spectra can act positively stimulating the germination process in some species (Gonçalves *et al.,* 2006), or can be indifferent to others. In general, species whose seeds present sensitivity to the light quality, the positive photoblastics, are considered pioneers in nature, since they require light stimulus to initiate their germination process (Rebouças and Santos, 2008). For the *Capsicum chinense* pepper plant, as observed in this study, only the percentage of dead seeds was influenced by the luminous spectra tested where the R-LEDs spectrum caused a high number of dead seeds whilst the BR-LEDs and W-LEDs reduced the percentage of mortality. The other germination variables evaluated did not present responses to the spectra. Red light has been reported to stimulate seed germination and root development (Bewley and Black, 1994; Abdullateef and Osman, 2011; Daud *et al.,* 2013).

In addition to the quality, the luminous intensity in which the seeds and plants are submitted can also promote differentiated responses in the plant, as verified for *Capsicum chinense* Habanero, which presented increase in growth with the light intensity of 28 µmol m^{-2} s^{-1} (Barrales-López *et al.,* 2015). The luminous spectra used in this experiment were larger, with 36 µmol m^{-2} s^{-1}, which may have masked the plant response. It is known that light in excess can result in reduction of the net photosynthetic rate, causing oxidative damage to the foliar tissues; only under appropriate light plants can be fully self-regulated to obtain the best status for absorption and transformation of light energy (Yao *et al.,* 2017). It is known that excess light can also promote photovoltaic changes in plants, leading to the production of reactive oxygen species (ROS), which may have promoted mortality in seeds submitted to the red spectrum.

After germination, it was possible to observe changes in the morphological characteristics due to the different light spectral qualities in which they were submitted. Fluorescent light (FL), for example, provided a higher increase of FMAP in the evaluated peppers. This spectral quality is the most used for *in vitro* growth of plant species. Positive results in the increase of fresh mass of the aerial part were already found for *Curcuma longa* in fluorescent light, followed by red light (~ 625-440nm) and yellow light (~565-590nm) (De Souza Ferrari *et al.,* 2016). Naturally plants develop themselves under varied lights composed of a mixture of quality and quantity, which promotes the activation of several photoreceptors, among them phytochromes (Rockwell *et al.,* 2006).

The highest values of shoot length were verified in R-LEDs spectral quality for *Capsicum chinense* peppers, corroborating with other studies, which found an increase in root formation in cultures such as *Jatropha curcas* and *Protea cynaroides* (Daud *et al.,* 2013; Wu and Lin, 2013), and *Stevia rebaudiana* (Simlat *et al.,* 2016). Kim *et al*. (2004), observed stretching of the aerial part of chrysanthemums cultivated *in vitro*, under light in the red band. When the plants were submitted to the R-LEDs spectrum, some authors verified elongation in *Cattleya loddigesii* (Araújo *et al*., 2009), increase of fresh mass in *Dendrobium phalaenopsis* (Sorgato *et al.,* 2016) and increase of shoot length for *Cattleya* (Cybularz - Urban *et al.,* 2007), corroborating with the results found in this work.

Red light is effective for photosynthesis as the red emission spectrum fits perfectly with the photon energy required to reach the first excited status of *a* and *b* chlorophyll (Singh *et al.,* 2015). Lights in blue and red spectra too are strongly absorbed by phytochrome through specific photoreceptors (Mathews, 2010). These photoreceptors activate enzymes associated with the synthesis of auxins, growth hormone, and greater photosynthetic efficiency (Sun *et al.,* 1998), promoting an increase in growth, justifying the results found. In this way, plants that grow under these conditions have a good initial development, such as a well-formed root system, which allows for faster acclimation and better survival rates in the field (Chandra *et al.,* 2010; Gruszecki *et al.,* 2010).

Positive results for root fresh mass and root length were observed in BR-LEDs spectrum. Some studies indicate that combinations of LEDs with blue (30%) and red (70%) spectrum promoted an increase

in biomass of *Fragaria* x *ananassa* and *Saccharum officinarum* (Nhut *et al.,* 2003; Maluta *et al.,* 2013). Results for *Anthurium andraeanum* favored gains in fresh and dry matter in the combination of RB-LEDs, followed by white light (Gu *et al.,* 2012). When RB-LEDs have been used *in vitro* propagation, a large part of the works observed a mass gain in both the root and the aerial part (Gu *et al.,* 2012; Maluta *et al.,* 2013; Da Silva *et al.,* 2016).

For chili culture (*Capsicum annuum* L. cv. Rubi Gigante), shoot length and collar diameter were favored by treatment with BR-LEDs light compared to white fluorescent light (FL) (Da Silva *et al.,* 2016). For this species, the authors also point out that the different qualities of the light spectrum have little effect on the growth and development of seedlings.

The photosynthetic pigments absorb light in the red and blue range, the red quality increas the photosynthetic rate, while blue quality improves the chloroplast development, chlorophyll biosynthesis and stomatal opening, thus, increasing the content of photosynthetic pigments (Johkan *et al.*, 2010; Hogewoning *et al*., 2010; Daud *et al*., 2013). These luminous spectra influence the primary and secondary metabolism in plant development, however, plants exhibit a wide range of morphological plasticity, and phytochemical in response to a given type and wavelength of light (Macedo *et al*., 2011). In this way, some cultures are favored by the supply of these wavelengths, increasing their growth.

The tested peppers showed good results for the root length and root fresh mass variables when submitted to the W-LEDs spectrum. Positive results with the use of W-LEDs were obtained by Wilken *et al.* (2014), in which they found more vigorous growth of *Musa spp*. compared to the use of fluorescent lamps. For sugarcane plants, W-LEDs lamps promoted a higher number of shoots, in addition to a higher content of chlorophylls and carotenoids (Ferreira *et al*., 2017).

The emitted photons in the combination of BR-LEDs, suppose that more photoreceptors of the pepper seedlings received stimuli, which may have triggered some morphogenetic mechanism in more photoreceptor cells than when exposed to only one spectrum. Similar assumptions were made by Shimokawa *et al.* (2014) and Chen *et al.* (2017). In this way, the positive results found for RFM and RL and the low seed mortality (SM), in the BR-LEDs combination were explained.

Light is a signal that is received by photoreceptors, which regulate plant differentiation and growth (Li *et al.,* 2013). The quality of the emitted light by LEDs has promoted significant improvements in morphogenesis and differentiation in different species grown *in vitro* (Gupta and Jatothu, 2013). However, the effects and mechanisms associated with light quality may be peculiar to each species or cultivar (Li *et al.,* 2013; Da Silva *et al.,* 2016). Some explanations may be generalized, however, this specificity seems to be related to the different responses that were found for the vigor of the pepper cultivars.

The Biq. Yellow and Boyra Hab. Red present high potential of germination and vigor, respectively, indicating high physiological quality.

In general, the light spectral qualities provide differentiated responses in the initial development of the peppers, being the reduction of the percentage of dead seeds favored by the spectrum BR-LEDs and W-LEDs. The root fresh mass is increased by the all lights, except R-LEDs. The fresh mass of the aerial part presents positive results in the FL lamps. Shoot length is favored by the R-LEDs.

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Selection of open pollination progenies in some pear species in order to achieve dwarf and drought tolerant rootstocks

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Abstract: One of the important products in Iran and Isfahan province is the pear that its cultivation has been limited by drought stress and global warming in recent years. The use of drought-tolerant rootstocks is one of the available solutions for pear orchards in semi-arid regions. In addition, the lack of dwarf or semi-dwarf rootstocks, which are appropriate and compatible with Iran climatic conditions, limited high density pear orchards. In order to obtain drought tolerant rootstocks, in this research fruit of *Pyrus glabra*, *Pyrus syriaca,* and *Pyrus salicifolia*, along with *P. communis* cv. Spadona, Dargazi, as well as Khoj n. 1 and n. 2 species were collected from different regions of Iran in August and September of 2016. The seeds were separated from the flesh and dried at room temperature. The seeds were cultivated in uniform and light texture of soil in November in the field condition to break the seed dormancy. Seedlings were irrigated regularly for three months in order to establish in the soil; drought stress began in July. In order to apply drought stress, irrigation time was considered based on 80% of allowed water depletion. Morphological traits of seedlings were recorded before stress and at the end of the stress period (late September). The viability percentage of seedlings after drought stress was between 14.28% (*P. communis* cv. Dargazi progenies) to 82.55% for *P. salicifolia*. Comparison of the means and cluster analysis, among populations showed that the three populations of *P. salicifola*, *P. glabra* and *P. communis* cv. Khoj n. 2 had the lowest height and were placed in the same group. After studying single genotypes in these three populations, genotypes no. 31, 32, 41, 57 and 12 from *P. salicifolia*, genotypes 10, 11, 7, 3 and 9 of *P. glabra* and genotype 4 of *P. communis* cv. Khoj n. 2 populations were selected as drought tolerant and dwarf genotypes and were taken to the propagation phase for future evaluation.

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1. Introduction

Pear is from the *Rosaceae* family and the *Pyrus* genus. This species has been cultivated in Iran since ancient times, and Iran is one of the earliest

areas of pear distribution and variation in the world (Abdollahi, 2011). Drought stress is the most important environmental stress that occurs annually with extreme damage to crops, especially in arid and semi-arid regions (Xoconostle-Cazares *et al*., 2010). In dry and semi-arid regions of Iran, the amount of annual evaporation is higher than precipitation. In recent years, the cultivation of fruit trees, such as pears, has been limited due to climate change and reduce rainfall. Considering that the drought tolerance of the rootstocks is transmitted to the scion (Landsberg and Jones, 1981), the choice of drought tolerant rootstocks with a low water requirement is one of the solutions to drought problem of pear orchards in arid and semi-arid regions such as Iran (Cheruth *et al*., 2009).

Another problem in the cultivation of pears in Iran is the lack of suitable dwarf or semi-dwarf rootstocks that are compatible with cultivars and climatic conditions of the country for high density orchards. The pear and quince are used as rootstocks for pear trees. The quince rootstocks are very dwarf (EM-C) and semi-dwarf (BA29), but these rootstocks could not overcome the problem of graft incompatibility with some pear cultivars. These rootstocks are not also tolerant to winter frosts and chlorosis caused by iron deficiency in the calcareous soils (Harotko, 2007). In recent years, a number of clonal rootstocks have been introduced into Iran such as Pyrodwarf, Fox-11 and some of the American rootstocks of the Old home × Farmingdale series (Abdollahi, 2011). The use of native pear species as a seed or clonal rootstocks can be a solution for access to proper rootstocks with good adaptability to the climatic conditions of the country. Some of these species are dwarf and have a high tolerance to unfavorable environmental conditions that can be used to produce clonal rootstocks (Abdollahi *et al*., 2012).

In the pear genus, 22 species have been identified in the world that are mainly native to Europe, Africa and Asia. Of the 22 identified species, there are 12 species in Iran (Sabeti, 1995). In some parts of the world, these species are used as the rootstock for pear commercial cultivars. For example, a large number of pear cultivars in Turkey are grafted on *P. elaeagrifolia*, in Syria and Lebanon on *P. syriaca*, in ancient Yugoslavia, Turkey and Greece on *P. amygdaliformis*, in the south of Russia on *P. salicifolia* and in Algeria and Morocco are grafted on *P. longipes* (Henareh, 2015).

Wild germplasm has evolved in natural dry ecosystems for tolerance of stress conditions such as high temperatures, drought and salinity. Identification of wild pear germplasm is very important to use as a rootstock in semi-arid regions (Zarafshar *et al*., 2014). According to this, three wild pear genotypes from *P. syriaca* were exposed to four irrigation treatments. The results showed that the Coile wild genotype (*P. syriaca*) was more tolerant to drought conditions compared with other genotypes due to its high relative water content during drought stress and non-decreasing dry weight. Drought stress reduced leaf photosynthesis, stomatal conductance and transpiration in a number of pear species (Javadi and Bahramnejad, 2011).

In another study, drought tolerance was evaluated in three populations of wild pear germplasm (*P. boisseriana*) in greenhouse conditions. Among the three populations studied, the collected population from semi-arid regions showed higher drought tolerance than the other two populations that were collected from semi-humid areas, and recovered more rapidly after irrigation. These seedlings were introduced as promising sources for use as rootstock for commercial pear cultivars in drought conditions (Zarafshar *et al*., 2014). In a research carried out by Ghasemi *et al*. (2014), chlorophyll index was significantly different between studied pistachio rootstocks. In these rootstocks, chlorophyll index in stress conditions was lower than drought conditions. So, the reduction of chlorophyll content can be caused by chlorophyll degradation under drought stress conditions, which leads to reduced pure photosynthesis.

The rootstocks of European pear species are from very vigor to relatively dwarf. These rootstocks are compatible with all the pear tree cultivars and have considerable tolerance to the adverse conditions of soil and fire blight disease. Other species of *Pyrus* genus, such as *P. syriaca* Boiss is compatible with commercial pear cultivars and is currently used in some countries (Radnia, 1996).

Seed cultivation after cross or open pollination is one of the breeding methods that is frequently used in breeding programs. In this way, it is possible to achieve a wide variation for choice of new rootstocks and cultivars. For example, the Manon cultivar was obtained from open pollination of Beurre Bosc cultivar. The Pib-BU3 dwarf pear rootstock was obtained from open pollination of *P. longipes*, while Pi-BU4 and Pi-BU7 rootstocks were obtained from open pollination of the *P. pyrifolia* species (Mohan Jain and Priyadarshan, 2009).

Currently, most pear cultivars in Iran are grafted on different seedling rootstocks of the *P. communis*

species. Considering that wild pear genotypes grow on rocks and dry or low moisture soils, as well as some of them have a small growth in the form of shrubs, so some of them can be used as drought tolerant and dwarf rootstocks for commercial pear cultivars (Ashraf and Karimi, 1991; Henareh, 2015). The aim of this research was an evaluation of drought stress tolerance in some wild pear species, and then selection of the dwarf genotypes among the drought tolerant genotypes.

2. Materials and Methods

Plant materials

In this research, seeds from open pollination of *Pyrus syriaca* Boiss., *Pyrus salicifolia* Pall. and *Pyrus. glabra* Boiss. along with *P. communis* L. cv. Spadona and Dargazi as well Khoj including large fruit (Khoj no. 1) and small fruit (Khoj no. 2) were evaluated. *P. syriaca* Boiss. is distributed from west Azarbaijan to Fars in the Zagros Mountains and northwest of Iran (Abdollahi, 2011). This species has shown compatibility with Spadona and Kochia pear cultivars and have improved the growth of the scion in calcareous soils. It is currently used as a rootstock in some countries (Fallouh *et al*., 2008). *P. glabra* species is known as Anchuchek in Iran, and has spread mainly in the Zagros Mountains. The seeds of this species are large and are consumed as snacks in Fars province (Abdollahi, 2011). *P. communis* is more commonly known as Khoj and is scattered in the forests of the north, West Azarbaijan, Sardasht and Baneh in Kordestan province. The fruit of this species is very diverse. Two types of native Khoj including large fruit (Khoj n. 1) and small fruit (Khoj n. 2) were studied in this research (Moazedi *et al*., 2014). *P. communis* cv. Dargazi is native commercial cultivar of Iran that has been introduced as tolerant rootstock (Mansouryar *et al*., 2017). *P. communis* cv. Spadona is high yielding and has high resistance to chlorosis due to iron deficiency. It is also relatively tolerant to psylla and fire blight. *P. salicifolia* species spread in the northwest of Iran, including west and east Azerbaijan provinces (Abdollahi, 2011).

Cultivation of seeds

Fruits of studied species were collected in August and September of 2016 from different regions of West Azarbaijan and Isfahan provinces as well as northern regions of Iran and transferred to the laboratory. The seeds were separated from the flesh and kept in a cool and dry place in paper bags after washing and drying, In order to eliminate the chilling requirement, seeds were cultivated in separate rows in the nursery soil with a sandy loam texture at Isfahan Agricultural and Natural Resources Research Center, in December. From each of these species, 500 seeds were cultivated at intervals of 2-3 cm in the nursery and each produced seedlings were studied as a genotype. During chilling period, adequate moisture was provided and prevented from drying of the culture bed.

Irrigation of seedlings

After emergence of seeds, the seedlings were irrigated for three months in order to establish in the nursery soil. Determination of irrigation time based on allowed water depletion of pear trees is after a 50% decrease in humidity, but because of the seedling roots had not yet been sufficiently developed, and needed enough moisture for better growth, so allowed water depletion was considered 35%. In the irrigation intervals, soil moisture at various depths of the soil was measured up to a depth of 100 cm by the Time Domain Reflectometry device (TDR, Trase6050X1) (Doorenbos and Pruitt, 1977; Alizadeh, 2006).

To obtain the amount of irrigation (irrigation volume), first, net irrigation depth was calculated according to formula 1.

$$In = \sum [(\theta FCi - \theta BLi) \times Di] \quad (1)$$

In this formula, In is the net irrigation depth (mm), θFCi is the moisture content of the field capacity for each layer, θBLi is the soil moisture before irrigation for each layer, Di is the root development depth (mm), and i is the number of each soil layer. Then, according to formula 2, gross irrigation depth was calculated.

$$Ig = In/(1-Lr) \times Ei \quad (2)$$

In this formula, Ig is the gross irrigation depth (mm), In is the net irrigation depth (mm), (1-Lr) is the amount of leaching and Ei is the irrigation efficiency (usually 80-90% for drip irrigation).

Irrigation volume was obtained from the multiplication of gross irrigation depth in the irrigation plot area. This volume was controlled by the installed counter on the pipe before irrigation plot. For irrigation of seedlings leaked tubes with drippler at 10 cm intervals were used. The outlet flow of each drippler was measured in one liter per hour at an appropriate pressure. In this study, the efficiency of the drip system and the leaching requirement was considered 90% and 10% respectively.

Drought stress

Drought stress began in July, which coincided with the beginning of the stress period in Isfahan region. Irrigation frequency was changed in order to apply drought stress, and irrigation time was considered based on 80% of allowed water depletion. Table 1 shows the volume of pure and impure irrigation water and number of irrigation per month.

Evaluated traits

The morphological traits of seedlings were recorded separately for each genotype at the end of the stress period (late September). These traits included height and diameter of seedlings, number and length of internode, crown width, number of suckers and branches, chlorophyll index and leaf dimensions. Before applying stress, seedling height and diameter at 5 cm above the soil surface were recorded. The difference in both seedling height and diameter before and after stress were also calculated. Qualitative traits, including leaf chlorosis and trichome, as well as seedling growth vigor were recorded after stress using national guideline for distinctness, uniformity and stability in pear (DUS guileline) in pears. The abbreviation and measurement method of evaluated traits are given in Table 2.

Data analysis

Mean comparison was calculated with SAS software (version 9.1). Descriptive statistics including mean, minimum, maximum and coefficient of variation and also cluster analysis were performed by Ward method based on Squared Euclidean Distance with SPSS software (version 15).

3. Results

Viability percent

Viability percent of seedlings of the studied pear species after the stress was shown in Table 3. According to the results, *P. salicifolia* showed the highest survival in drought stress conditions. After that, *P. communis* cv. Khoj n. 1 and 2 were placed in the next rank. The lowest percentage of seedling via-

Table 3 - Seedlings viability percent of pear cultivars and species after drought stress

Species	Viability percent
Pyrus communis cv. Spadona	14.77
Pyrus communis cv. Dargazi	14.28
Pyrus communis cv. Khoj n. 1	55.17
Pyrus communis cv. Khoj n. 2	43.79
Pyrus syriaca	20.33
Pyrus salicifolia	82.55
Pyrus glabra	17.74

Table 1 - Number of irrigation, net and gross volume of irrigation water

Irrigation	February	March	April	May	June	July	August	September	October	November	Total
Number of irrigation	3	3	5	7	8	1	1	1	1	1	32
Net volume of irrigation water ($m^3.ha^{-1}$)	540	540	900	1260	1440	180	180	180	180	180	5580
Gross volume of irrigation water ($m^3.ha^{-1}$)	666.6	666.6	1111.1	1555.5	1777.7	222.2	222.2	222.2	222.2	222.2	6888.2

Table 2 - Symbol and measurement method for recorded traits (based on DUS guideline)

Characteristic	Symbol	Unit	Measurement method
Leaf chlorosis	LC	Code	No chlorosis (1), low chlorosis (3), medium chlorosis (5), high chlorosis (7), very high chlorosis (9)
Leaf trichome	LT	Code	No trichome (1), low trichome (3), medium trichome (5), high trichome (7), very high trichome (9)
Seedling growth vigor	GV	Code	Very low (1), low (3), medium (5), high (7), very high (9)
Seedling height	SE	cm	Ruler
Seedling diameter	SD	mm	Caliper
Height difference	HD	cm	Calculation
Diameter difference	DD	cm	Calculation
Internode number	IN	Number	Counting
Internode length	IL	cm	Ruler (average of internodes in branches)
Crown width	CW	cm	Meter
Number of secondary branches	NSB	Number	Counting
Number of suckers	NS	Number	Counting
Chlorophyll index	CI	-	Chlorophyll meter (Spad)
Leaf length	LL	cm	Ruler (average of 10 leaves)
Leaf width	LW	cm	Ruler (average of 10 leaves)

bility during drought stress belonged to *P. communis* cv. Spadona and Dargazi.

Traits before applying stress

The mean comparison of recorded traits among populations before applying drought stress is presented in Table 4. According to the results, *P. communis* cv. Spadona had the highest seedling height. The seedling height in the wild species of *P. glabra*, *P. salicifolia* and *P. communis* cv. Khoj n. 2 was the lowest. *P. communis* cv. Khoj n. 2 and *P. glabra* produced seedlings with the lowest diameter. The largest seedling diameter belonged to *P. communis* cv. Spadona and *P. syriaca* (Table 4). Generally, without drought stress conditions, growth of *P. glabra*, *P. salicifolia* and *P. communis* cv. Khoj n. 2 were lower than *P. communis* cv. Spadona, Khoj n. 1, Dargazi and *P. syriaca*.

Table 4 - The mean comparison of the seedling height and diameter among populations of pear species before applying stress

Species	Seedling height (cm)	Seedling diameter (mm)
Pyrus communis cv. Spadona	33.52 a	3.44 a
Pyrus communis cv. Khoj n. 1	23.38 b	3.15 ab
Pyrus communis cv. Dargazi	24.23 b	3.15 ab
Pyrus syriaca	20.92 b	3.31 a
Pyrus salicifolia	12.80 c	2.62 bc
Pyrus communis cv. Khoj n. 2	11.83 c	1.74 d
Pyrus glabra	7.54 c	2.25 cd

Similar letters in each column indicate no significant difference (LSD).

Traits after applying stress

The mean comparison of recorded traits among populations after stress is presented in Table 5. *P. communis* cv. Spadona had the highest seedling height (36.73 cm) after stress. The seedling height of *P. glabra*, *P. salicifolia* and *P. communis* cv. Khoj n. 2 were lower than other species. Similarly, *P. communis* cv. Spadona showed the highest height difference before and after applying stress.

The seedling diameter of *P. communis* cv. Spadona, Khoj n. 1, Dargazi and *P. syriaca* was more than the other seedling diameter, So that *P. glabra* and *P. communis* cv. Khoj n. 2 had the lowest seedling diameter. No significant difference was observed among the seedling diameter of the species before and after drought stress.

P. communis cv. Spadona had the highest number of internode after the end of drought stress. The lowest number of internode belonged to *P. glabra* and *P. communis* cv. Khoj n. 2. *P. salicifolia* produced the crown with the widest (24.71 cm) width, compared to other species. It should be noted that *P. communis* cv. Spadona, Khoj n. 1 and *P. syriaca* did not show any significant difference with *Pyrus salicifolia*. *P. communis* cv. Khoj n. 2 had the lowest crown width (4.75 cm).

P. salicifolia did not produce a secondary branch. There were no significant differences in the number of secondary branches among the other species. *P. salicifolia* showed the highest chlorophyll index, but did not show significant differences with *P. communis* cv. Spadona, Khoj n. 2, *P. syriaca* and *P. glabra*. The lowest chlorophyll index belonged to *P. communis* cv. Dargazi (Table 5).

P. communis cv. Spadona , with a length of 6.06 cm and a width of 2.7 cm had the highest leaf length and width compared with other species. After *P. communis* cv. Spadona, *P. communis* cv. Khoj n. 1 had the highest leaf length and width. The lowest leaf length was related to *P. communis* cv. Khoj n. 2 with an average of 2.01 cm. *P. syriaca* also produced the leaves with the lowest width (1.37 cm).

P. communis cv. Spadona and Dargazi had the longest internode, but did not show significant differences with *P. communis* cv. Khoj n. 1 and *P. syriaca*.

Table 5 - Mean comparison of some traits in the seedling populations of pear species after applying stress

Species	Seedling height (cm)	Height difference (cm)	Diameter (mm)	Diameter difference (mm)	Internode number	Crown width (cm)	Number of secondary branches	Chlorophyll index	Leaf length (cm)	Leaf width (cm)	Internode length (cm)
Pyrus communis cv. Spadona	36.73 a	3.20 a	4.34 a	0.89 a	20.23 a	10.03 ab	0.23 ab	44.99 ab	6.06 a	2.70 a	1.91 a
Pyrus communis cv. Khoj n. 1	26.65 b	2.80 ab	4.00 a	0.84 a	14.56 b	10.62 ab	0.68 a	42.48 b	5.45ab	2.23 b	1.21 ab
Pyrus communis cv. Dargazi	25.83 b	1.60 ab	3.81 a	0.66 a	14.66 b	7.83 b	0.66 a	42.02 b	4.73 bc	2.10 bc	1.93 a
Pyrus syriaca	21.60 b	1.41 ab	4.02 a	0.70 a	12.91 bc	9.16 ab	0.25 ab	49.77 ab	4.50 c	1.37 d	1.34 ab
Pyrus salicifolia	14.21 c	0.67 b	3.56 ab	0.94 a	10.05 cd	11.24 a	0.00 b	53.83 a	4.30 c	1.73 c	0.94 b
Pyrus communis cv. Khoj n. 2	13.67 c	0.84 b	2.63 c	0.88 a	8.31 de	4.75 c	0.21 ab	46.53 ab	2.01 d	1.97 bc	0.71 b
Pyrus glabra	10.22 c	0.68 b	2.92 bc	0.66 a	5.27 e	8.31 b	0.36 ab	48.85 ab	3.98 c	2.10 bc	0.73 b

Similar letters in each column indicate no significant difference (LSD).

The lowest internode length belonged to *P. salicifolia*, *P. glabra*, and *P. communis* cv. Khoj n. 2 (Table 5).

Cluster analysis of populations

According to the results of cluster analysis, species were classified into three groups at five squared Euclidean distance basis of the Ward method (Fig. 1). *P. communis* cv. Spadona and Dargazi were placed in the first group. *P. syriaca* and *P. communis* cv. Khoj n. 1 in the second group, while *P. communis* cv. Khoj n. 2, *P. salicifolia* and *P. glabra* species in the third group.

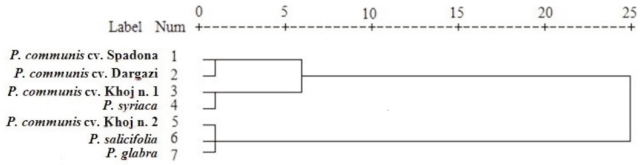

Fig. 1 - Grouping of pear species based on measured characteristics by Ward method.

Study of single genotypes

The mean and the range of traits for each of the examined genotypes, as well as the coefficient of variation in each trait are presented in Table 6. Each genotype was identified by a number and genotypes with minimum and maximum values were presented. Internode length (66.12%), seedling height before stress (60.45%) and seedling height after stress (56.85%) had the highest and chlorophyll index (14.34%) had the lowest coefficient of variation.

In order to achieve dwarfing genotypes, the trait of seedling height was considered. The three pear populations, including *P. salicifolia*, *P. glabra* and *P. communis* cv. Khoj n. 2 had a lower mean seedling height than other populations, so the height of single genotypes of them after stress was shown in figures 2, 3 and 4, respectively. In *P. salicifolia*, genotypes no. 31 (4.5 cm), 32 (5 cm), 41 (5.5 cm), 57 (6 cm) and 12 (6 cm) had the lowest seedling height. Genotypes

Table 6 - Mean, Range and coefficient of variation in studied traits

Characteristics	CV (%)	Standard deviation	Mean	Minimum		Maximum	
				Genotype (number of genotypes)	Rate	Genotype (number of genotypes)	Rate
Seedling height before stress	60.45	9.83	16.26	*P. glabra* (42)	1.4	*P. communis* cv. Spadona (23)	53.9
Seedling diameter before stress	28.35	0.76	2.68	*Py. communis* cv. Khoj n. 2 (33)	0.75	*P. communis* cv. Spadona (64)	6.3
Seedling height after stress	56.85	10.28	18.08	*P. glabra* (10 & 11)	3	*P. communis* cv. Spadona (2)	55
Seedling diameter after stress	26.47	0.94	3.55	*P. communis* cv. Khoj n. 2 (8)	1.6	*P. salicifolia* (8)	7.28
Height difference	55.32	2.19	1.82	*P. salicifolia* (24 & 28)	0	*P. communis* cv. Khoj n. 1 (9) & *P. syriaca* (3, 4,5,6 & 7)	19
Diameter difference	55.17	0.48	0.87	*P. salicifolia* (47)	0.04	*P. salicifolia* (66)	3.06
Internode number	44.65	5.00	11.20	*P. glabra* (7,10 & 11)	3	*P. communis* cv. Khoj n. 1 (14)	26
Crown width	30.67	3.11	10.14	*P. communis* cv. Khoj n. 1 (5) & *P. glabra* (10)	4	*P. communis* cv. Khoj n. 1 (8)	30
Number of secondary branches	37.22	0.67	0.18	Many genotypes	0	*P. communis* cv. Khoj n. 2 (4)	4
Chlorophyll index	14.34	7.81	54.46	*P. communis* cv. Khoj n. 1 (8)	23.2	*P. salicifolia* (36)	69.8
Leaf length	31.93	1.37	4.29	*P. communis* cv. Khoj n. 2 (15)	1	*P. communis* cv. Spadona (13)	7.2
Leaf width	26.70	0.51	1.91	*P. syriaca* (5)	1	*P. communis* cv. Spadona (2)	3.3
Internode length	66.12	0.82	1.24	*P.salicifolia* (30)	0.3	*P. communis* cv. Spadona (1)	10

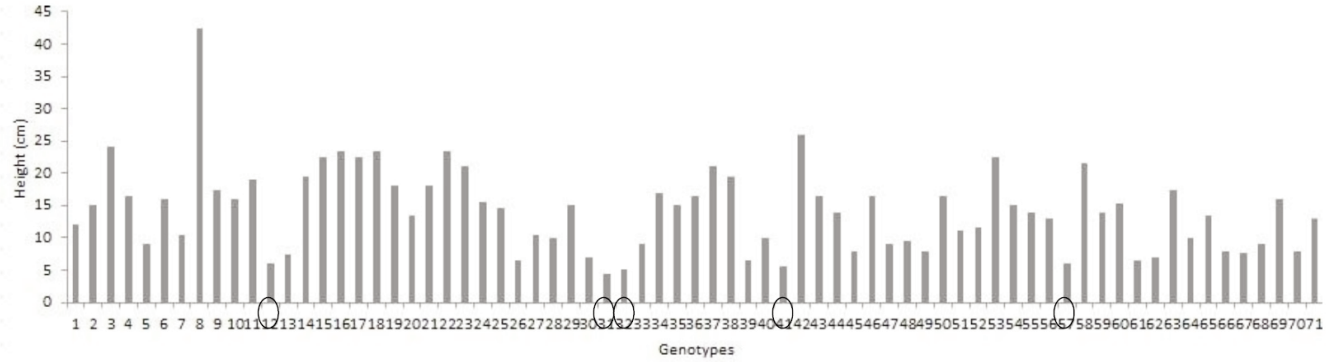

Fig. 2 - Height of single genotypes in *P. salicifolia* after stress.

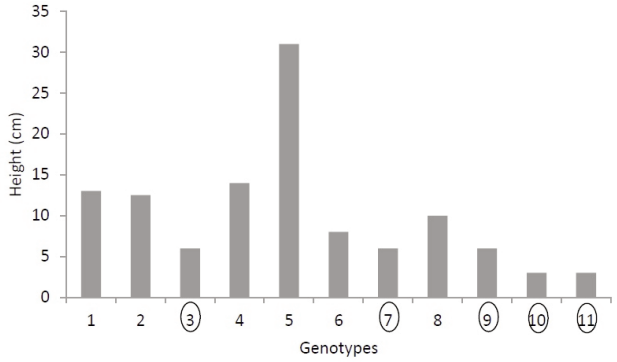

Fig. 3 - Height of single genotypes in *P. glabra* after stress.

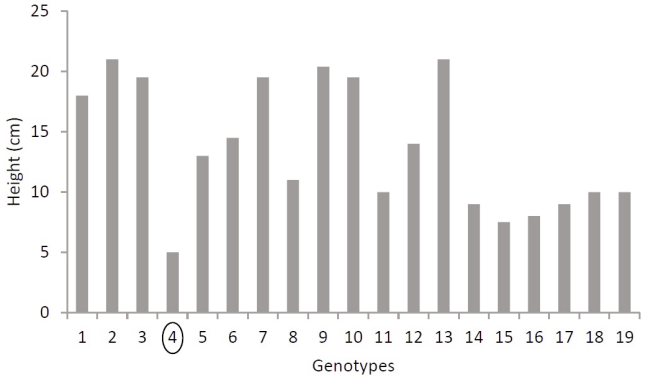

Fig. 4 - Height of single genotypes in *P. communis* cv. Khoj n. 2 after stress.

no. 10 (3 cm), 11 (3 cm), 3 (6 cm), 9 (6 cm) and 7 (6 cm) showed the lowest seedling height in the *P. glabra* species. Genotype no. 4 had the lowest height from *P. communis* cv. Khoj n. 2 population.

Cluster analysis of single genotypes based on all studied traits

Cluster analysis among the single genotypes of the *P. salicifolia*, *P. glabra* and *P. communis* cv. Khoj n. 2 populations was shown in figures 5, 6 and 7. Genotypes no. 12, 31, 32, 41 and 57 of *P. salicifolia* (Fig. 5) and genotypes no. 10, 11, 3, 9 and 7 of *P. glabra* (Fig. 6) were placed in the groups close to each other.

Qualitative traits for selected genotypes were presented in Table 7. All genotypes had a small trichome and had low or very low growth potentials. Leaf chlorosis was not observed in genotypes except for the genotype no. 31 from *P. salicifolia* and genotype no. 3 from *P. glabra* that had low chlorosis. Genotypes no. 12 and 32 of *P. salicifolia* were green and very green, respectively. Genotypes no. 9, 7, 10 and 11 of *P. glabra* and genotype no. 4 of the *P. communis* cv. Khoj n. 2 preserved their green color after applying drought stress.

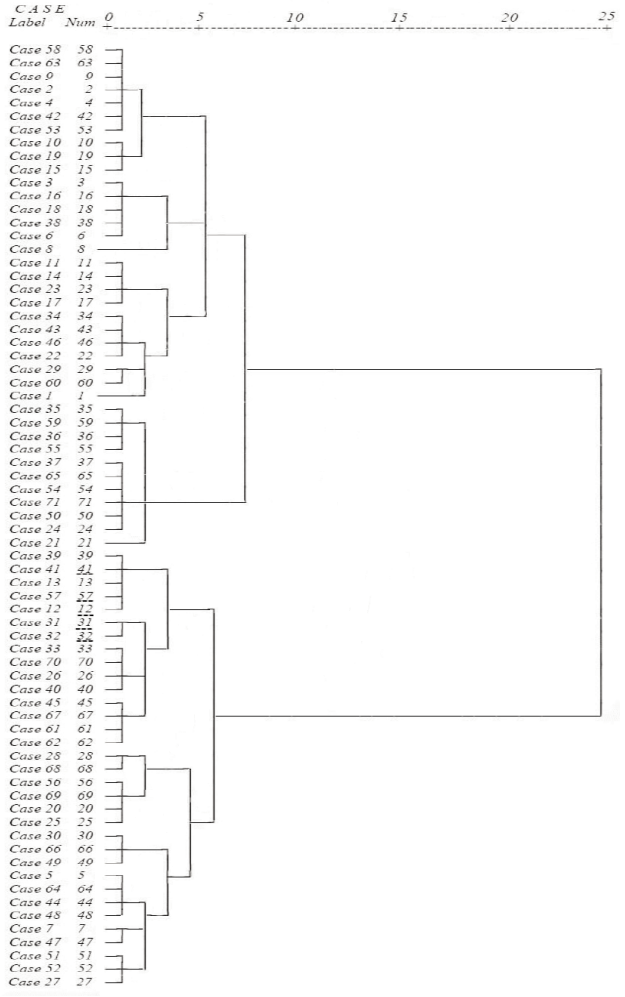

Fig. 5 - Grouping of drought tolerant genotypes in *P. salicifolia* species based on measured traits by Ward method.

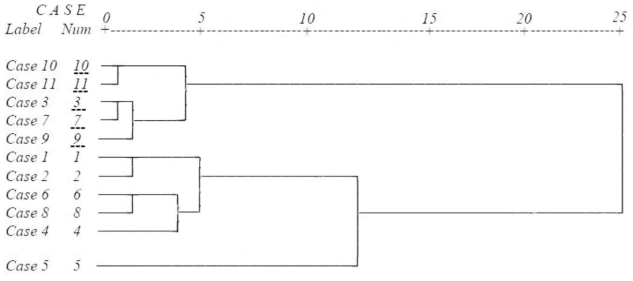

Fig. 6 - Grouping of drought tolerant genotypes in *P. glabra* species based on traits measured by Ward method.

4. Discussion and Conclusions

Viability percent

After the drought stress period, 35.51% of the genotypes survived, and the rest of them were dried. Most surviving genotypes belonged to *P. salicifolia* species. *P. communis* cv. Spadona and Dargazi

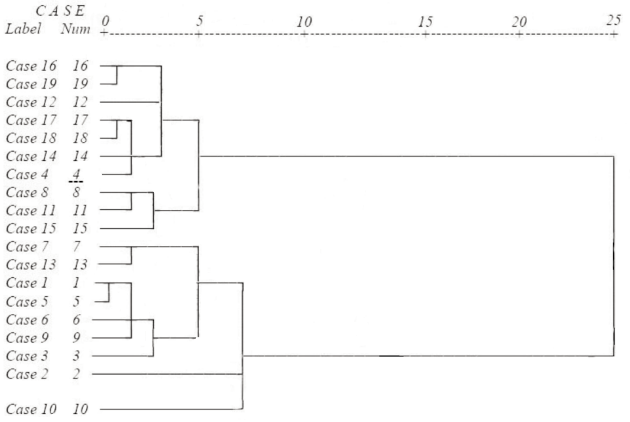

Fig. 7 - Grouping of drought tolerant genotypes in *P. communis* cv. Khoj n. 2 based on the measured traits by the Ward method.

showed the lowest percentage of survival (Table 3). For a long time, wild pear genotypes have been considered in Iran's plateau due to the tolerance to biotic and abiotic stresses (Javadi *et al*., 2005). The adaptation of wild pears with rocky areas and dry or low moisture soils can lead to more tolerance of them under drought stress conditions compared with domestic and commercial rootstocks (Henareh, 2015).

Traits before and after applying stress

The analysis of variance showed that the investigated species had a significant difference in most of the studied traits, which is due to the diversity among populations, so it is possible to select species for different values of a trait. According to table 4, in normal conditions, seedling height and diameter of *P. glabra*, *P. salicifolia* and *P. communis* cv. Khoj n. 2 were less than *P. communis* cv. Spadona, Khoj n. 1, Dargazi and *P. syriaca*. Similarly, after applying drought stress, the seedling height and diameter of *P. glabra*, *P. salicifolia* and *P. communis* cv. Khoj n. 2 species were lower than other populations (Table 5). Morphological adaptations in plants can be one of the adaptive mechanisms under drought stress (Pire *et al*., 2007). The first reaction of plants against drought stress is a reduction in their vegetative growth. Drought stress affects the vegetative characteristics of trees, including their height (Higgs and Jones, 1990). Due to height difference among populations before and after stress, it seems that the effect of drought stress on the seedling height trend of these populations *before and after applying the stress is almost* same.

Before drought stress, the seedling diameter of *P. communis* cv. Spadona, Khoj n. 1, Dargazi and *P. syriaca* was higher than the seedling diameter of *P. salicifolia*, *P. glabra* and *P. communis* cv. Khoj n. 2, while the diameter difference before and after drought stress among compared species did not show a significant difference, therefore, it can be concluded that the effect of drought on the seedling diameter among populations was not the same. Other results also showed that the negative effect of drought stress on seedling diameter was less than its effect on seedling height (Haghighatnia *et al*., 2013).

Growth of branch and internode length is an appropriate index for detecting the effect of drought stress on the plants, so that the occurrence of drought stress can be observed even before the change in the water potential of the leaves (Grimplet *et al*., 2007). Among the remaining genotypes after the stress, *P. communis* cv. Spadona had the highest and *P. glabra* and *P. communis* cv. Khoj n. 2 had the lowest number and length of internode.

The chlorophyll meter indicates the relative

Table 7 - Growth vigor, trichome and chlorosis of leaf in selected genotypes

Species	No. genotype	Leaf chlorosis	Leaf trichome	Seedling growth vigor
Pyrus salicifolia	12	No chlorosis	Low trichome	Low
	31	Low chlorosis	Low trichome	Low
	32	No chlorosis	Low trichome	Low
	41	No chlorosis	Low trichome	Low
	57	No chlorosis	Low trichome	Low
Pyrus glabra	3	Low chlorosis	Low trichome	Low
	9	No chlorosis	Low trichome	Low
	7	No chlorosis	Low trichome	Low
	10	No chlorosis	Low trichome	Very low
	11	No chlorosis	Low trichome	Very low
Pyrus communis cv. Khoj n. 2	4	No chlorosis	Low trichome	Low

chlorophyll concentration, based on the difference between the light transmittance in two red and infrared wavelengths, which correlates with the chlorophyll content of the leaves (Hoel and Solhaug, 1998). In the present study, *P. salicifolia* and *P. communis* cv. Dargazi showed the highest and the lowest chlorophyll index, respectively. Preservation and not decomposition of chlorophyll in *P. salicifolia* during drought stress indicates the tolerance of that species to this stress (Tarahomi *et al*., 2010).

Cluster analysis of populations

Drought tolerance in plants has a direct or indirect relationship with a complex of traits, therefore, all the traits should be considered for selection of tolerant plants. For this reason, in this research cluster analysis was used to facilitate the selection of drought tolerant species. Cluster analysis classified species into three groups at five squared Euclidean distance (Fig. 1). *P. communis* cv. Spadona and Dargazi were placed in the first group that had the lowest survival rate after drought stress. The highest seedling diameter belonged to the plants of this group. In the second group, *P. syriaca* and *P. communis* cv. Khoj n. 1 were placed, which showed low to moderate survival rate after drought stress. In total, the most crown width, primary and secondary seedling height and diameter, as well as the highest number of internode were observed in the species of the first and second groups. *P. communis* cv. Khoj n. 2, *P. salicifolia* and *P. glabra* species formed the third group. These populations had the lowest seedling diameter and height. The lowest seedling growth vigor was also found in these species; therefore, selection of dwarf and drought tolerant genotypes in this group was more possible than other groups. It seemed that traits such as seedling height and diameter were the most effective grouping traits. In the research carried out by Aran *et al*. (2012), seedling height was also one of the important traits in seedling grouping. Of course, different values of some traits were seen in this group. For example, the crown width and viability percentage were observed at low, medium and high levels in this group.

Study of single genotypes

The mean, the range of changes, and the coefficient of variation of each trait were shown in Table 6. Coefficient of variation shows the extent of variability in relation to the mean of the population. In the traits with a high coefficient of variation has provided a higher selection range. Genetic variation helps the plant to overcome environmental changes and also provides more chance for selection of new cultivars (Liu, 2006). In this research, high variation was observed for internode length and seedling height before and after stress. The variability of some traits in 15 cultivars of *Vitis vinifera* L. was previously investigated by Mousazadeh *et al*. (2014). They reported that leaf traits had the highest diversity among the studied traits. Doulati Baneh *et al*. (2013) and Tahzibihagh *et al*. (2012) also observed a high variation in the morphological characteristics of the leaves of grape and pear cultivars. In the current research, the coefficient of variation in leaf dimensions was not high compared to other traits, because of less scatter in relation to the mean of the population. The high diversity coefficient for internode length and seedling height before and after stress indicates the high range of variation in these traits among the studied seedlings, so it is possible to select genotypes based on these two traits.

Survived genotypes after drought stress were selected in order to dwarfing. For this purpose, seedling height of each genotype was investigated separately. Considering that the average height of three populations including *P. salicifolia*, *P. glabra* and *P. communis* cv. Khoj n. 2 were lower than other populations, therefore selection was carried out among genotypes of these populations. The highest number of genotypes was selected from *P. salicifolia*. The least seedlings height was belonged to *P. glabra*. Only a dwarf genotype was selected from the *P. communis* cv. Khoj n. 2 population (Figs. 2, 3 and 4). Finally, 11 dwarf genotypes were selected from these species.

Cluster analysis of single genotypes

Cluster analysis was performed for three populations with a lower seedling height. According to this, five selected genotypes of *P. salicifolia* (Fig. 5), five genotypes of *P. glabra* (Fig. 6) and a genotype of the *P. communis* cv. Khoj n. 2 population (Fig. 7) were classified in the same subgroups. These genotypes had lower seedling height and internode number. Leaf width was also lower in these genotypes.

Qualitative traits of 11 selected genotypes showed that all genotypes had low trichome. The survival and tolerance of these genotypes to drought stress was not along with the increase of leaf trichome. The growth vigor of genotypes was low, and two genotypes 10 and 11 from *P. glabra* had very low growth vigor. These genotypes also had the lowest seedling height. In fact, their growth vigor was correlated with the seedling height. This positive correlation was observed between seedling height and

growth vigor in bitter cherry seedlings (Mahlab) (Ganji-Moghadam and Khalighi, 2006). Under drought stress, studied genotypes did not show chlorosis, only genotypes 3 of *P. salicifolia* and 31 of *P. glabra* were greenish-yellow after drought stress. Selected genotypes were transferred to a propagation phase through cutting and layering.

After studying single genotypes in these three populations, genotypes no. 31, 32, 41, 57 and 12 from *P. salicifolia*, genotypes 10, 11, 7, 3 and 9 of *P. glabra* and genotype no. 4 of *P. communis* cv. Khoj n. 2 populations were selected as drought tolerant and dwarf genotypes and were taken to the propagation phase for future evaluation.

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AHS
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TuMV as an efficient transient vector for expressing heterologous proteins in *Nicotiana tabacum* and *N. benthamiana*

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Abstract: **Nowadays the production of recombinant proteins such as drugs and commercial protein compounds in plants is called molecular farming. It has some benefits such as fast and large quantity production of recombinant proteins with low cost. In this research, the green fluorescent protein (GFP) was transiently expressed in two tobacco species via turnip mosaic virus (*TuMV*) derived vector, a virus which can infect a wide range of plant species. Florescence microscopy results indicated that *TuMV* could infect tobacco plants and accumulate GFP protein in plant leaves. In addition, RT-PCR, Dot-Blot and ELISA assays demonstrated the recombinant gene transcription, translation and stability. This is the first report of using *TuMV*-based viral vectors for producing recombinant proteins in tobacco. Optimized *TuMV*-based viral vectors could be used for producing recombinant proteins in tobacco.**

1. Introduction

Various expression systems, such as bacteria, yeast, plants, insects and mammalian cell cultures can produce recombinant proteins. The benefits of expressing recombinant proteins in plants include economic, agricultural scale, safe and authentic production (Sijmons *et al.*, 1990; Ma *et al.*, 2003; Mardanova *et al.*, 2015;). Molecular farming (also known as molecular pharming or biopharming) uses genetically engineered plants for the production of biopharmaceutical products, vaccine subunits, industrial enzymes therapy peptides and other compounds of interest (Boothe *et al.*, 1997; Wang and Ma, 2011; Yarbakht *et al.*, 2015).

Recombinant proteins in plants may be gained by stable genetic transformation (nuclear or plastid) or through transient expression. Transient expression is usually used for fast and flexible expression of genes of

interest (GOI), evaluation of expression system performance and components such as promoter and enhancers (Chiera *et al.*, 2008). In plants, a number of virus-based vectors are utilized for the transient expression of foreign genes, such as tobacco yellow dwarf virus (*TYDV*) for transient expression of chalcone synthase in *Petunia hybrida* (Atkinson *et al.*, 1998), tobacco mosaic virus (*TMV*) for transient expression of *GFP* (jellyfish, *Aequorea victoria* green-fluorescent protein) in tobacco (Shivprasad *et al.*, 1999), bean pod mottle virus (*BPMV*) for expression the *GFP* in the soybean (Zhang *et al.*, 2010), wheat streak mosaic virus (*WSMV*) for expression the *GFP* in cereals (Tatineni *et al.*, 2011) etc.

Turnip mosaic virus (*TuMV*) belongs to *Potyviridae* family and infects a wide range of plant species especially cruciferous (*Brassicaceae* family). It is a positive-sense single stranded RNA virus with a linear and monopartite genome and average length of 720 nm (Brunt *et al.*, 1996). Previously Beauchemin *et al.* (2005) strongly expressed *GFP* and *GUS* (bacterial β-glucuronidase) reporters genes in *Brassica perviridis* plants via *TuMV* virus. Furthermore, Chen *et al.* (2007) introduced *GFP* in some *Brassica* hosts such as *B. campestris* and *B. juncea* and high levels of the recombinant protein expression were observed. Therefore, in this study, to investigate the performance of recombinant protein production, the *GFP* reporter gene was introduced into the tobacco (*Nicotiana tabacum* and *N. benthamiana*) plants by using *TuMV* vector.

2. Materials and Methods

Plant material and growth conditions

Nicotiana tabacum cv. Xanthi and cv. Samsun and *N. benthamiana* seeds were grown in pots containing autoclaved soil, including 40% farm soil, 30% peat moss and 30% perlite. They were kept at 25°C in a phytotron under a 16-hour photoperiod (16:8 h L: D).

Plasmid and viral constructs

The *TuMV-GFP* construct (Fig. 1) was kindly provided by Dr. Shyi-Dong Yeh, Plant Pathology Department, National Cheng Hsing University, Taichnug, Taiwan. The plasmid contains a cauliflower mosaic virus 35S promoter (CaMV 35S) and *GFP* coding sequence between the NIb (nuclear inclusion protein b) and CP (coat protein) positions. Recombinant viral construct, p*TuMV-GFP*, was transferred into bacterial (*Escherichia coli* DH5α) competent cells (Sambrook and Russell, 2001). Bacteria were grown in 200 ml Luria-Bertani medium and then, p*TuMV-GFP* was extracted (Engebrecht *et al.*, 1991).

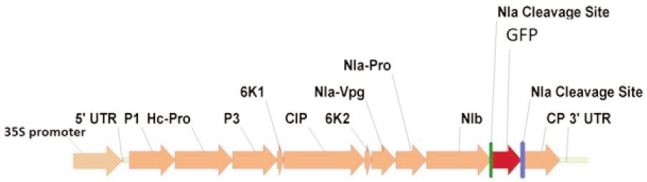

Fig. 1 - Schematic representation of the viral construct containing *GFP* under the *35S* promoter that was used in this expression analysis. The foreign gene insertion site is between NIb (nuclear inclusion protein b) and CP (the virus coat protein gene) provided by *NcoI* and *NheI* restriction endonuclease enzymes.

Plant Rub-inoculation with TuMV-derived vector

Wild-type TuMV (for control plants) and p*TuMV-GFP* was mechanically inoculated on upper surface of two top leaves (10 µg in 10 µl per leaf), using a cotton stick and carborandum powder according to Hosseini *et al.* (2013). Systemically infected (non-inoculated leaves) were used for further analysis.

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The presence of the *GFP* gene in inoculated leaves was determined by RT-PCR. Total RNA was extracted from inoculated and control plant leaves (five independent samples) by Qiagene kit (South Korea) twelve days after incubation according to the manufacturer's instruction. RNA was extracted from non-inoculated leaves for confirming replication and movement of the virus. After treating with DNase I (Thermo Fisher Scientific, USA), cDNA was synthesized using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific, USA) and *GFP* reverse primer (5' –TTG TAC TCC AGC TTG TGC CC-3') according to the producer's instructions. RT-PCR was conducted using the cDNA and the following forward and reverse primers under the following cycling conditions: forward (5'- ACG ACG GCA ACT ACA AGA CC -3') and reverse (5'- TTG TAC TCC AGC TTG TGC CC -3'). PCR cycling conditions were as follows: 94°C for 3 min for initial denaturation; 35 cycles of 94°C for 30 s, 51°C for 30s, and 72°C for 30 s; and 72°C for 10 min for a final extension. Then PCR products were analyzed by 1% TAE agarose gel.

Fluorescence microscopy

Leaves from TuMV-based vector inoculated and control plants were observed under an Olympus fluorescent microscope 6 (version IX71, Tokyo, Japan).

The fluorescence photographs were taken using a mounted high-resolutionm7 Olympus DP70 DP71 digital camera at 12 days post-inoculation (dpi).

Protein extraction and GFP analysis

Proteins were extracted from 0.5g tissues of control and inoculated tobacco leaves (five independent samples) with extraction buffer, including 0.2M Tris-HCl (pH 8.0), 5mM ethylenediaminetetraacetic acid (EDTA), 100 mM sucrose, and 0.1 mM 2-mercapthoethanol (Abdoli-Nasab *et al.*, 2013) and the concentration was assessed by Bradford's assays (Bradford, 1976). Dot-Blot (Stott, 1989) and indirect enzyme-linked immunosorbent assay (ELISA) (Wang and Gonsalves, 1990) were carried out to quantitative detection of the GFP protein in the inoculated tobacco plants.

Statistical analysis

All experiments were done according to a completely randomized design at five independent samples. Data analyses were performed using *Microsoft Excel* program software and *SPSS version 22*. When significant differences were found least significant difference (LSD) test at $P<0.05$ was applied to separate means.

3. Results and Discussion

The main benefits of the plant made proteins (PMPs) are lower costs and potential to produce a very large scale of recombinant proteins. Viral vectors have the ability to express transgenes in hosts and they are suitable and rapid platform for production high-level of recombinant proteins.

In this research, we utilized a *TuMV* viral vector (Fig. 1) under the control of the CaMV 35S promoter for transient expression of the *GFP* in tobacco plants. Although systemic symptoms of *TuMV* were not observed on infected plants, *GFP* was detected by the fluorescence microscopy (Fig. 2) twelve days post-inoculation.

This research has displayed for the first time that recombinant protein (GFP) can accumulate in tobacco plants via *TuMV* based viral vector with CaMV 35S promoter. *TuMV* can infect tens different plant species (Chen *et al.*, 2007) (to compare common viral vectors which can affected specific plant species), therefore, *TuMV* based viral vector can be economical.

In this study, two different tobacco species, *N. benthamiana* and *N. tabacum* (two different cultivars Xanthi and Samsun) were investigated. Fluorescence

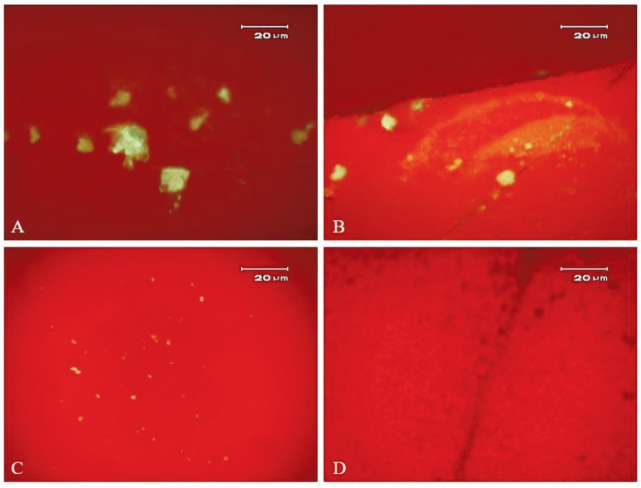

Fig. 2 - Fluorescence microscopy analysis of GFP expression in tobacco plant's leaves which infected by pTuMV-GFP. (A) *Nicotiana benthamiana*, (B) *N. tabacum* cv. Xanthi, (C) *N. tabacum* cv. Samsun (D) Negative control (tobacco plant infected with wild-type TuMV). Green color indicated GFP expression and the red indicated chlorophyll autofluorescence.

microscopy analysis of *GFP* (Fig. 2), RT-PCR (Fig. 3), Dot-Blot analyses (Fig. 4) and ELISA assay (Fig. 5) indicated that recombinant protein expression in tobacco plants leaves occrued. RT-PCR (Fig. 3) showed that, as expected, 160 bp bands were found in infected plants, while not observed in the negative control (wild type). It shows that the *TuMV* virus can infect the plant and replicate its genome. Viruses (like *TuMV* from Potyviridae family) have developed proteins such as Helper Component Proteinase (HCPro),

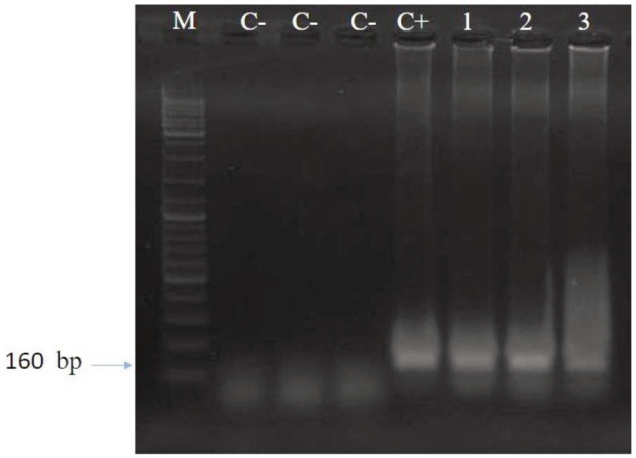

Fig. 3 - RT-PCR amplified a 160 bp fragment from the GFP with specific primers in 1% agarose gel. C- 1= negative control (water template), C- 2= negative control (RNA template), C- 3= negative control (Wild type (non-inoculated plant)), C+= positive control, Lane 1= *Nicotiana benthamiana* transformed plants, Lane 2= *N. tabacum* cv. Xanthi, Lane 3= *N. tabacum* cv. Samsun transformed plants, M= molecular weight marker (1 kb standard GeneRuler).

which suppress the plants silencing defense (Voinnet, 2001). Furthermore, HCPro has protease activity and it is necessary for virus genome replication and viral movement and transmission (Klein *et al.*, 1994; Chiera *et al.*, 2008).

Dot-Blot assay (Fig. 4) indicated that GFP protein was recognized by specific antibody and developed brown color in transformed plants and positive control. ELISA assay indicated that expression levels of GFP was estimated approximately ≤0.5% of total soluble protein (TSP) of fresh weight of tobacco leaves. These results show lower accumulation of recombinant proteins compared with a number of previous studies which expressed by viral vectors such as Artichoke mottled crinckle virus (Lombardi *et al.*, 2009), Beet curly top virus (Kim *et al.*, 2012) etc. Some strategies, such as codon-optimization (Love *et al.*, 2012) and the use of improved viral vector elements including strong viral promoters (Gleba *et al.*, 2007) can increase the expression of recombinant protein.

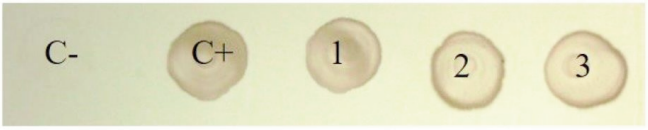

Fig. 4 - Dot-Blot analysis of GFP transient expression in *Nicotiana benthamiana* (spot 1), *N. tabacum* cv. Xanthi (spot 2) and *N. tabacum* cv. Samsun (spot 3), C-= negative control [Wild type (non-transformed plant)] and C+= Purified GFP protein.

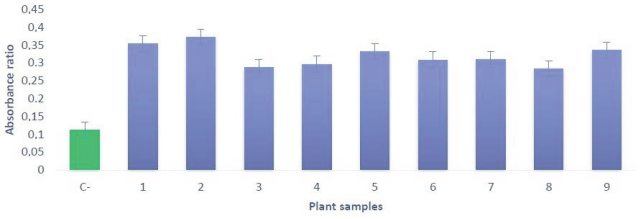

Fig. 5 - Analysis of GFP expression in tobacco plants by ELISA assay. C-= negative control (Wild type TuMV-infected plant), 1-3= *Nicotiana benthamiana* transformed plants, Lane 4-9= *N. tabacum* transformed plants. The data represent means ± SE from five independent infected samples.

It seems that, *N. benthamiana* lacks RNA-dependent RNA polymerases (RdRPs) which required for defense against viruses, therefore *N. benthamiana* infected plant displays more strong symptoms and its products more than do other tobacco species (Yang *et al.*, 2004).

In many previous studies (Kumar and Kirti, 2010; Sasaki *et al.*, 2015; Vojta *et al.*, 2015; Park *et al.*, 2016), *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) delivery systems has been used to express the transient expression of recombinant protein using a viral vector. However, in this study, a direct virus inoculation system via dusted with carborundum has been used. Our study indicated that this method is useful to accelerate the production of recombinant proteins in tobacco plants.

4. Conclusions

In conclusion, our results showed that *TuMV*, as a virus that could infect a wide range of plant species, could be used to produce recombinant proteins in tobacco. In this investigation, all inoculated plants expressed GFP protein. Results showed that incubated *N. benthamiana* has more accumulated recombinant protein compared to the two *N. tabacum* cultivars. Although the level of expression is low and should be optimized for future studies.

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Effects of crop system and genotype on yield, quality, antioxidants and chemical composition of organically grown leek

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

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The authors declare no competing interests.

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Abstract: The research was carried out in order to assess the effects of nine cultivars in factorial combination with open field or greenhouse growing on yield, quality indicators, antioxidants and elemental composition of leek in Moscow region. Greenhouse management resulted in higher yield compared to open field cultivation, due to higher mean pseudo-stem weight, and cultivar Giraffe gave the highest production. Pseudo-stem dry matter was better affected by greenhouse cultivation, whereas the content of monosaccharides, total sugars, nitrates, ascorbic acid and polyphenols was enhanced by open field growing. The cultivars Vesta and Summer breeze showed the highest dry matter and total sugar content, whereas Goliath had the highest antioxidant, selenium and potassium concentration. Among the mineral elements, K and Mg in pseudo-stems were better affected by greenhouse conditions, whereas Ca attained a higher concentration under open field growing. The antioxidant system of *Allium porrum* was characterized by significant positive correlations between Se, polyphenols, ascorbic acid and potassium.

1. Introduction

Leek (*Allium porrum* L.) is a major crop among *Allium* species and it is mainly grown in Indonesia, Turkey and, within Europe, in France and Belgium for producing edible pseudo-stems. The latter have high nutritional value, also due to the high content of potassium and iron (Koca and Tasci, 2016), and high biological activities connected with the remarkable concentration of antioxidants comparable with that of *Allium cepa*

(Sekara *et al.*, 2017), such as polyphenols (Ben Arfa *et al.*, 2015), glucosinolates, S-alkenyl-L-cysteine sulfoxides and pectic polysaccharides (Ozgur *et al.*, 2011). Accordingly, leek shows antimicrobial, cardio-protective, hypo-cholesteremic, hypoglycemic, anti-rheumatic, hypotensive, antianemia, and anticancer action, improves liver, gastro-intestinal and brain efficiency, decreases blood pressure, inhibits platelets aggregation and prevents neural tube defects as well as prostate diseases (Radovanović *et al.*, 2015).

Protected cultivation may be appropriate to organic horticulture which is more susceptible to the environmental unbalances due to the milder farming practices and is usually more profitable than the conventional management (Caruso *et al.*, 2012; Conti *et al.*, 2015). Within the crop system, cultivar assessment in terms of content of antioxidants as well as macro- and micro-elements in leek pseudo-stems raises the interest of establishing the relations between the mentioned substances and, accordingly, identifying the most interesting genotypes, also based on their yield. Due to the fragmented investigations relevant to varietal differences in biologically active compounds (Bernaert *et al.*, 2012) and elemental composition (Koca and Tasci, 2016), we carried out research aiming to evaluate the effect of both crop system and cultivar on yield, quality, antioxidant content and elemental composition of *A. porrum* grown either in greenhouse or in open field.

2. Materials and Methods

Plant material and growth conditions

Research was carried out on leek (*A. porrum* L.) grown in greenhouse at the experimental fields of Federal Scientific Center of Vegetable Production, in Odintsovo (Moscow, Russia, 55°40' N, 37°12' E) in 2015 and 2016 on a clay-loam soil, with pH 6.8, 2.1% organic matter, 108 mg kg^{-1} N, 450 mg kg^{-1} P_2O_5, 357 mg kg^{-1} K_2O, exchangeable bases sum as much as 95.2%. Mean temperature values from May to October were: 13.0, 16.1, 19.8, 18.6, 12.3, 6.4°C in open field; 20.4, 21.4, 23.7, 20.0, 14.5, 8.3 in greenhouse. The experimental protocol was based on the factorial combination between two crop systems (open field, greenhouse) and nine cultivars (Goliath, Summer breeze, Premier, Casimir, Kalambus, Camus, Vesta, Giraffe, Bandit), using a split-plot design with three replicates.

The sowing was performed on 5 December in 8 x 8 cm trays and the plantlets were transplanted in the field on 14 May, spaced 15 cm along the rows, the latter being 40 cm apart. Leek crops were preceded by organically grown vegetables in the previous four years, such as carrot, bean, rape and pea. Prior to planting, plough at 30 cm depth, hoeing at 15 cm and fertilization with 180 kg ha^{-1} N, 80 P_2O_5 and 120 K_2O were practiced; during the growing period, 40 kg ha^{-1} N were supplied in three times at two-week intervals, starting at bulbification stage, and just in the last N application 7 kg ha^{-1} of P_2O_5 and of K_2O were also provided. Drip irrigation was activated at 80% soil available water. The organic farming practice complied with EC Regulation 834/2007 and 889/2008. Plant protection was achieved by applying copper oxychloride against rust and azadirachtin against aphids.

Harvests of ripe plants were performed from 5 to 10 October in greenhouse and from 12 to 19 October in open field, when the pseudo-stems had reached their maximum growth, and the leaf blades were trimmed at 15 cm length for obtaining the marketable product. In each plot, determinations were made of the marketable product weight (pseudo-stems with 15 cm long leaf blades) and the mean pseudo-stem (with 15 cm long leaf blades) weight on twenty-plant samples. Further plant samples were collected, gently washed with water to remove surface contaminants and dried with filter paper. Pseudo-stems and leaves were separated, cut with plastic knife, dried to constant weight and homogenized; the resulting powders were subjected to laboratory analysis.

Dry matter

The dry matter content in leaves and pseudo-stems of *A. porrum* was assessed after dehydration of the fresh samples in an oven at 70°C, until they reached constant weight.

Sugars

Monosaccharides were determined using ferricyanide colorimetric method, based on the reaction of monosaccharides with potassium ferricyanide (Swamy, 2008). Total sugars were determined after acidic hydrolysis of water extracts with 20% hydrochloric acid (Swamy, 2008). Fructose was used as an external standard.

Polyphenols

The concentrations of the total polyphenols in each sample of leaves and pseudo-stems were determined in 70% ethanol extract (1 hour at 80°C) using the Folin-Ciocalteu colorimetric method, according to

Golubkina *et al.* (2018 b) by Unico 2804 UV (USA) spectrophotometer. The polyphenol content was expressed as milligrams of gallic acid equivalents per 100 grams of dry weight (mg GAE 100 g^{-1} d.w.).

Ascorbic acid

The ascorbic acid content in leek leaves and pseudo-stems was assessed by visual titration of fresh plant extracts in 6% trichloracetic acid with Tillmans reagent (Caruso *et al.*, 2009; AOAC, 2012).

Antioxidant activity

The antioxidant activity of leek leaves and pseudo-stems was assessed using redox titration method (Maximova *et al.*, 2001; Golubkina *et al.*, 2018 b), via titration of 0.01 N $KMnO_4$ solution with ethanolic extracts of leaves and pseudo-stems. The values were expressed in mg GAE 100 g^{-1} d.w.

Nitrates

The nitrate content was assessed in fresh pseudo-stems using ion selective electrode on ionomer Expert-001 (Econix, Russia).

Elemental composition

The content of Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, I, K, Li, Mg, Mn, Na, Ni, P, Pb, Se, Si, Sr, V and Zn in leek pseudo-stems was assessed using ICP-MS on quadruple mass-spectrometer Nexion 300D (Perkin Elmer Inc., Shelton, CT 06484, USA) in the Biotic Medicine Center in Moscow (Golubkina *et al.*, 2017).

Statistical analysis

Data were processed by analysis of variance and mean separations were performed through the Duncan multiple range test, with reference to 0.05 probability level, using SPSS software version 21. The data expressed as a percentage were subjected to angular transformation before processing.

As the year of research had no significant effect on the yield, quality, antioxidant and elemental composition variables examined, both as main factor or in interaction with the experimental factors "crop system" and "cultivar", the results are reported as average values of the two years of investigation.

3. Results and Discussion

Growth, yield and quality indicators of pseudo-stems

The crop system showed significant effects on leek plant biomass, pseudo-stem yield and mean weight, as these variables attained higher values in greenhouse compared to open field (Table 1); as reported in the previous section, the plant commercial ripeness was anticipated by 8 days on average in the protected environment. These trends are consistent with those recorded in previous research (Conti *et al.*, 2015). Differences between the varieties were recorded with regard to: biomass, which was highest in cultivar Summer breeze and lowest in Premier; mean pseudo-stem weight and, accordingly, yield which ranged from 22.0 to 36.1 Mg ha^{-1}, with the cultivar Giraffe showing the best performance, Premier and Kalambus the worst.

The greenhouse growing resulted in higher concentration of dry matter, ash and nitrates, but lower monosaccharides and total sugars in pseudo-stems, compared to those detected in open field (Table 1), similarly to previous reports (Conti *et al.*, 2015). Notably, the values of dry matter recorded in our

Table 1 - Growth and yield indicators, and content of dry matter, sugars and nitrates in *A. porrum* pseudo-stems

Treatment	Plant biomass (Kg m^{-2} d.w.)	Marketable pseudo-stems						
		Yield (Mg ha^{-1})	Mean weight (g)	Dry matter (%)	Monosaccharides (g 100 g^{-1} d.w.)	Total sugars (g 100 g^{-1} d.w.)	Ash (%)	Nitrates (mg kg^{-1} f.w.)
Crop system								
Open field	11.0	25.3	162.8	16.7	3.81	12.0	4.8	44.7
Greenhouse	15.8	30.8	185.7	19.7	3.41	10.7	5.2	66.3
	*	*	*	*	*	*	*	*
Cultivar								
Goliath	8.7 ef	27.6 de	177.0 de	12.1 e	5.11 a	7.5 e	8.5 b	48.0 d
Premier	8.3 f	22.0 g	132.6 g	14.6 d	4.62 ab	10.6 c	12.4 a	45.9 d
Bandit	12.0 d	31.0 bc	192.8 bc	14.9 d	3.73 c	8.9 d	4.7 c	65.3 a
Kalambus	9.9 e	22.3 g	134.6 g	17.1 c	4.13 bc	10.5 c	2.8 e	43.1 d
Cazimir	11.6 d	24.3 f	150.1 f	18.4 c	2.98 d	11.0 bc	4.1 cd	57.5 bc
Giraffe	18.6 b	36.1 a	225.4 a	19.8 b	3.58 c	11.2 bc	3.1 e	54.7 c
Camus	16.0 c	29.9 cd	188.0 cd	20.6 b	2.62 d	12.3 b	3.2 e	64.9 a
Vesta	15.4 c	26.3 ef	163.8 ef	22.6 a	2.74 d	14.6 a	2.9 e	60.1 ab
Summer breeze	20.3 a	33.1 b	204.5 b	23.7 a	2.97 d	15.5 a	3.4 de	60.5 ab

* significant at P≤0.05. Within each column, means followed by different letters are significantly different according to Duncan test at P≤0.05.

research fell within the 12.1 to 23.7 % range (Table 1) which is much wider than that relevant to *A. porrum* grown in Czech Republic (9-11%) (Lundegardh *et al.*, 2008). Moreover, the cultivars with high dry matter content (Summer breeze and Vesta) have a long shelf-life and are even suitable as dry spice source, whereas the varieties showing low dry matter (Goliath, Premier and Bandit) better fit the salad industry target.

Significant varietal differences in ash content were recorded (Table 2), with the ratio between leaf and pseudo-stem related to this variable decreasing as follows: Summer breeze > Cazimir > Vesta > Giraffe > Bandit > Kalambus > Camus > Premier > Goliath.

The higher nitrate accumulation in pseudo-stems grown in greenhouse is connected with the lower light intensity occurring in the protected environment compared to open field conditions, which limits the nitrate reductase activity; however, it was much lower (105 mg kg^{-1} f.w.) than that relevant to the top-accumulator species (Caruso *et al.*, 2011) and referred to previous reports (Santamaria, 2006).

Antioxidants

Ascorbic acid and polyphenols highly affect plant antioxidant activity (Proteggente *et al.*, 2002); in our research, the open field conditions resulted in higher content of both antioxidant compounds in leek pseudo-stems compared to greenhouse (Table 2). The crop system did not affect the selenium concentration either in pseudo-stems or leaves of *A. porrum*.

The high average content of ascorbic acid recorded in our research presumably makes the product safe and healthy, as ascorbic acid participates in producing essential nitrogen oxide for human organism, thus preventing nitrosamine formation from nitrate accumulating in plants (Santamaria, 2006). Moreover, wide varietal differences were found in ascorbic acid concentration, unlike the polyphenol content which was characterized by higher stability in pseudo-stems and even more in leaves (Table 2). Among the cultivars examined, Goliath showed the highest content of ascorbic acid, polyphenols and selenium in pseudo-stems (Table 2). Notably, these cultivars from domestic selection are characterized by lower levels of polyphenols compared to literature references, which may be connected with the different crop cycle and harvest time (Biesiada *et al.*, 2007). Bernaert *et al.* (2012) also reported a higher polyphenol concentration in pseudo-stems of thirty leek cultivars grown in Belgium (7.3 to 11.3 mg GA g^{-1} d.w.) compared to our values (3.3 to 6.3), but a lower content of ascorbic acid ranging between 90 to 350 mg 100 g^{-1} d.w.

In the latter research no correlation was recorded between ascorbic acid and polyphenol concentration in leek pseudo-stems, whereas a significant positive relationship has been found in our investigation ($r = 0.94$ at $P<0.01$). The lack of correlation relevant to the thirty leek cultivars grown in Belgium (Bernaert *et al.*, 2012) presumably depends on varietal hetero-

Table 2 - Concentrations of ascorbic acid, polyphenols and selenium in leek

Treatment	Ascorbic acid in pseudo-stems (mg 100 g^{-1} f.w.)	Polyphenols (mg GA 100 g^{-1} d.w.)		Selenium (µg kg^{-1} d.w.)	
		Pseudo-stems	Leaves	Pseudo-stems	Leaves
Crop system					
Open field	57.6	466.5	887.1	71.9	61.2
Greenhouse	47.2	376.8	699.5	76.2	64.4
	*	*	*	NS	NS
Cultivar					
Goliath	169.3 a	626.1 a	827.8 a	106.4a	14.1 e
Premier	72 b	497.6 b	751.8 b	79.1 b	64.8 c
Bandit	50.6 c	455.6 bc	739.8 b	74.1 bc	47.2 d
Kalambus	32.1 de	368.6 de	858.5 a	70.6 bc	74.8 b
Cazimir	37 d	334.1 e	843.1 a	59.7 e	76.9 ab
Giraffe	31.3 ef	386.1 d	760.8 ab	72.9 bc	48.4 d
Camus	27.1 fg	401.6 cd	786.3 ab	63.1 de	81.4 ab
Vesta	24.3 g	345.3 de	855.5 a	68.1 cd	85.4 a
Summer breeze	28.0 fg	379.7 de	716.3 b	72.1 bc	72.3 bc

NS not significant; * significant at $P\leq0.05$. Within each column, means followed by different letters are significantly different according to Duncan test at $P\leq0.05$.

geneity, consequent to genotype selection based on morphological types (light-green summer type, dark-green winter type and intermediate autumn type).

Interestingly, in our research the concentration of polyphenols in leek leaves has always been higher than the pseudo-stem one, raising the issue of possible crop waste valorization as a source of these antioxidants.

Among the components of plant antioxidant system, selenium also plays a significant role. Indeed, though it is not an essential element for plants, selenium is able to provide a powerful antioxidant defense to plants against drought, salinity, frost, flooding, UV light and herbivore (Malagoli *et al.*, 2015). Notably, *Allium* species belong to the secondary selenium accumulators, which show a remarkable tolerance to high concentration and consequent accumulation of this element due to Se ability to substitute sulfur in natural compounds, as previously reported in leek (Koca and Tasci, 2016).

In our research, *A. porrum* grown in Moscow region showed a Se accumulation range from 60 to 107 µg kg^{-1} d.w., which is much lower than the values recorded in Turkey (Koca and Tasci, 2016). This suggests the significant effect of selenium status in the environment on plant ability to concentrate this microelement. The adverse correlation between selenium content in leaves and pseudo-stems (r= - 0.95 at P≤0.01), similar to that recorded for polyphenols, entails a rather stable level of selenium accumulation in plant.

Reports relevant to selenium in plant secondary metabolites, as well as to polyphenols particularly in absence of selenium uptake are rather scarce and often controversial. In this respect, a positive correlation between selenium and polyphenol content was found in wheat (Lachman *et al.*, 2011) and a negative correlation between quercetin and selenium was recorded in *A. cepa* (Golubkina *et al.*, 2016). However, moderate doses of selenium are deemed to enhance the content of antioxidants such as polyphenols, flavonoids and carotenoids (Malagoli *et al.*, 2015).

In our research, the nine leek genotypes examined showed significant correlations between the components of the antioxidant system, i.e. selenium, ascorbic acid and polyphenols: Se and ascorbic acid (r= 0.93 at P≤0.01); Se and polyphenols (r= 0.92 at P≤0.01); ascorbic acid and polyphenols (r = 0.94 at P≤0.01). The latter correlations relevant to leek pseudo-stems may be significantly useful in leek selection based on high antioxidant content.

Elemental composition

The beneficial effect of many mineral elements to human health has arisen a remarkable interest to the chemical composition of vegetable crops, such as leek (Koca and Tasci, 2016). Investigations of element content in *A. porrum* plants have disclosed this species ability to accumulate high concentrations of minerals, but so far assessments of the leek whole profile relevant to mineral elements and to the varietal features connected to their accumulation have been lacking.

The analysis of twenty-five element content in leek pseudo-stems (Tables 3-5) has allowed to assess the varietal differences in elemental profile. The concentration of calcium was higher in pseudo-stems grown in greenhouse, whereas potassium and magnesium attained higher levels in open field. Sodium,

Table 3 - Macroelement concentration in *A. porrum* pseudo-stems (g kg^{-1} d.w.)

Treatment	Calcium	Potassium	Magnesium	Sodium	Phosphorus
Crop system					
Open field	4.2	22.5	1.0	0.27	2.83
Greenhouse	4.8	18.9	0.8	0.28	2.73
	*	*	*	NS	NS
Cultivar					
Goliath	3.7 bc	58.7 a	0.8 c	0.32 bc	3.12 b
Premier	3.1 cd	5.4 e	0.8 c	0.36 b	2.74 bc
Bandit	10.4 a	26.3 b	2.1 a	0.75 a	2.53 cd
Kalambus	4.0 b	15.2 c	0.6 d	0.16 e	2.43 cd
Cazimir	4.3 b	9.0 d	0.6 d	0.15 e	2.08 d
Giraffe	2.6 d	17.9 c	0.7 cd	0.12 e	2.49 cd
Camus	4.4 b	19.6 c	1.1 b	0.16 e	4.07 a
Vesta	3.9 bc	16.9 c	0.7 cd	0.18 de	2.77 bc
Summer breeze	4.4 b	17.7 c	0.8 c	0.26 cd	2.80 bc

NS not significant; * significant at P≤0.05. Within each column, means followed by different letters are significantly different according to Duncan test at P≤0.05.

phosphorus and all the microelements and heavy metals analyzed were not significantly affected by the crop system (Tables 3-5). Moreover, the potassium concentration was positively correlated with the ash content (r= 0.78 at P≤0.01).

From the comparison between the nine leek cultivars it arose that the three cultivars Premier, Goliath and Cazimir had contrasting features from each other. Indeed, Goliath was characterized by the highest content of K, Fe, B, Zn and Se, and the lowest of Cd. Premier accumulated preferably Co, I, Al, As, Cd, Ni, Pb and Sr, but poorly Cu and Zn. Cazimir showed the highest concentration of Na and Zn, but the lowest of K, I, Se, Cr and Ni.

Among the minerals examined, the highest correlation coefficients were recorded between Al and As, Pb, V, Co and Li. Indeed, the physiological role of Al in plants has not been completely understood so far, though this element is supposed to both activate at low doses some enzymes and control membrane permeability (Ahn and Matsumoto, 2006).

Lithium also showed wide varietal differences, consistently with previous reports (Kabata-Pendias and Pendias, 2010). The correlations recorded in our research are in agreement with those found in five species grown both in ecological unpolluted and in oil-polluted areas of Nigeria (Essiett *et al.*, 2010). As for selenium, though the leek varietal differences are rather low compared to other elements, the significant correlation recorded between Se and K is a remarkable characteristic of this *Allium* species and it has been very scarcely investigated so far.

In spinach, the fertilization with sodium selenate increased the potassium content in the female plants but not in the male ones (Golubkina *et al.*, 2017), whereas in other research (Põldma *et al.*, 2011) garlic biofortification led to selenium antagonistic activity towards K. Taking into account that potassium participates in plant protection against all forms of biotic and abiotic stress along with selenium and other antioxidant compounds (Wang *et al.*, 2013), the close relationship between the two minerals in leek suggests intensive interactions between all components of the defense system. Indeed, potassium was predominant in leek elemental composition, showing significant correlations with both ash (r= 0.78 at P≤0.01) and polyphenols (r= 0.96 at P≤0.01). The known ability of potassium to decrease the activity of polyphenol oxidase in plants and enhance polyphenol accumulation (Mudau *et al.*, 2007) may be a good explanation of the positive correlation between polyphenols and potassium in leek plants. The active

Table 4 - Microelements concentration in *A. porrum* pseudo-stems (mg kg^{-1} d.w.)

Element	Goliath	Cazimir	Premier	Vesta	Kalambus	Summer breeze	Bandit	Giraffe	Camus
B	20.5 a	14.8 bc	16.3 b	9.5 de	8.5 e	9.4 de	9.4 de	12.3 cd	10.7 d
Co	0.08 b	0.05 d	0.10 b	0.03 d	0.29 a	0.04 d	0.04 d	0.04 d	0.10 b
Cu	4.7 df	4.4 ef	3.4 g	5.7 bc	6.4 ab	5.0 ce	6.8 a	4.0 fg	5.3 cd
Fe	206 a	110 c	168 b	97 bd	74 e	80 de	93 ce	100 cd	215 a
I	0.06 bc	0.04 c	0.31 a	0.04 c	0.06 bc	0.06 bc	0.04 c	0.08 b	0.08 b
Li	0.11 b	0.04 c	0.15 a	0.03 c	0.01 c	0.03 c	0.02 c	0.03 c	0.11 b
Mn	12.2 c	12.0 c	22.6 a	9.8 c	6.3 d	9.5 c	10.6 c	19.2 b	21.7 ab
Si	14.1c	10.5 d	27.3 a	9.2 e	12.8 cd	13.1 cd	11.0 de	18.8 b	15.4 c
Sn	0.15 c	0.22 b	0.02 d	0.16 c	0.48 a	0.18 bc	0.51 a	0.22 b	0.23 b
Zn	23.5 ab	26.7 a	11.8 f	18.1 de	16.0 e	19.2 ce	21.5 bc	22.2 bc	21.2 bd

Within each row, means followed by different letters are significantly different according to Duncan test at P≤0.05.

Table 5 - Heavy metal concentration in *A. porrum* pseudo-stems (mg kg^{-1} d.w.)

Element	Goliath	Cazimir	Premier	Vesta	Kalambus	Summer breeze	Bandit	Giraffe	Camus
Al	78.0 c	29.5 d	126.0 a	21.0 df	7.7 g	24.1 de	12.2 fg	19.3 ef	88.4 b
As	0.03 b	0.02 bc	0.05 a	0.02 bc	0.01 c	0.02 bc	0.01 c	0.02 bc	0.06 a
Cd	0.08 d	0.10 bc	0.18 a	0.11 b	0.08 d	0.06 d	0.11 b	0.17 a	0.11 b
Cr	0.13 c	0.08 g	0.48 a	0.10 df	0.10 eg	0.12 cd	0.09 fg	0.15 b	0.11 ce
Ni	1.05 a	0.46 c	0.96 ab	0.98 ab	0.56 c	0.57 c	0.83 b	0.60 c	1.08 a
Pb	0.34 b	0.28 bc	0.83 a	0.10 e	0.10 e	0.13 de	0.20 cd	0.12 e	0.83 a
Sr	28.3 ab	25.3 c	30.4 a	24.6 c	28.2 ab	17.3 d	28.8 ab	26.4 bc	28.5 ab
V	0.21 b	0.07 cd	0.28 a	0.07 cd	0.04 d	0.09 c	0.07 cd	0.07 cd	0.30 a

Within each row, means followed by different letters are significantly different according to Duncan test at P≤0.05.

participation of potassium in the antioxidant defense system of this *Allium* species is also characterized by positive correlation of the element with the ascorbic acid content (r= 0.95 at P≤0.01). In this respect, the results of the present work reveal the close relationship between the main components of the leek antioxidant system, including polyphenols, ascorbic acid, selenium and potassium.

In our research, the lowest negative correlation coefficients were recorded between selenium, chromium and iodine (Table 6). Se is known as an antagonist of Cr and its protective role towards Cr has been previously reported (Qing *et al.*, 2015). The interaction between Se and I is more complex; both the elements are not essential for plants, but at low concentrations they may improve plant growth, development and protection from biotic and abiotic stresses (Pilon-Smits, 2015). Separate plant fortification with Se and I showed the possibility of mutual stimulation by the two elements (Golubkina *et al.*, 2018 a). The selective accumulation of selenium in the spinach male plants and of iodine in the female ones suggests the participation of phytohormones in the interactions between selenium and iodine (Golubkina *et al.*, 2017).

With regard to heavy metals, highly significant correlations were found between V and Al, As, Co, Pb and Fe (Table 6).

4. Conclusions

From research carried out in Moscow region with the aim to assess the effects of open field or greenhouse conditions on yield and quality performances of nine leek (*A. porrum*) cultivars under organic farming, useful remarks have been drawn. The genotypes examined had a uniform behavior with both the crop systems, showing higher yield and dry matter when grown in the protected environment, but better quality and antioxidant performances in the open field conditions. Taking into account that Giraffe was the highest-yielding cultivar, whereas Goliath displayed the best overall quality and antioxidant features, the identification of best-performing genotypes within a crop system is target dependent.

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Table 6 - Correlation coefficients between mineral elements in *A. porrum* pseudo-stems

	As	Ca	Co	Cr	Fe	I	K	Li	Mg	Mn	Pb	V
Al	0.93 ***	0.71 *	0.95 ***	0.74 *	0.85 **	0.77 **	0.50	0.99 ***	0.75 *	0.74 *	0.92 ***	0.98 ***
K	0.23	0.13	0.39	0.21	0.63	0.17	1.00					
Li	0.90 ***	0.70 *	0.93 ***	0.73 *	0.86 **	0.76 *	0.56	1.00				
Se	0.05	0.14	0.19	-0.72 *	0.42	-0.71 *	0.95 ***	0.42	0.18	-0.03	0.04	
V	0.94 ***	0.60	0.98 ***	0.61	0.91 ***	0.65	0.51	0.97 ***	0.64	0.75 *	0.94 ***	

*** P≤0.001; ** P≤0.01; * P≤0.05.

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Nanosilver, salicylic acid and essential oils effects on water relations of gerbera 'Rosalin' cut flowers

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Key words: clove, hydraulic conductivity, peppermint, thyme, vase life.

Abstract: **The effects of pulse and permanent treatments were studied on vase life, water content and hydraulic conductivity of gerbera cut flower cv. Rosalin. This study was conducted as a factorial experiment based on completely randomized design with three replications. The first factor was pulse treatments, using nanosilver (NS) 5 and 10 mg/L, salicylic acid (SA) 50 and 100 mg/L and distilled water as control, and the second factor was permanent treatments applying distilled water, sucrose, peppermint, thyme and clove essential oils (EO). The results showed that NS 10 mg/L + peppermint EO 100 mg/L and NS 10 mg/L + thyme EO 100 mg/L treatments had the best effect on longevity and maintaining the water content and hydraulic conductance of Rosalin cut flower, compare to other treatments. These solutions enhanced life of gerbera cut flowers to about 14 days. Flower water content was high (about 90%) except in 4% sucrose permanent treatment flowers which decreased more rapidly during the vase life. The effective hydraulic conductivity was observed in NS 10 mg/L + peppermint EO 100 mg/L (0.16 cm/min) and NS 10 mg/L + thyme EO 100 mg/L (0.21 cm/min) solutions and had nearly stable trend even in day 8 after pulsing.**

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1. Introduction

Gerbera (*Gerbera jamesonii* Bolus, Asteraceae) commonly known as Transvaal Daisy, Barberton Daisy or African Daisy, is one of the ten most popular and important commercial cut flowers grown in a wide range of climatic conditions. Gerbera is a perennial, tropical, herbaceous plant with colorful and attractive flowers that are widely used as a decorative garden plant or cut flowers. Cut gerbera flowers consist of a terminal composite floral head (inflorescence), called the capitulum, and a stem, which called scape and has no leaves (Dole and Wilkins, 2006). Gerbera has the fourth place in the international cut flower market. The flowers are hardy and resist against transport conditions. However, the most important problem of the gerbera cut flowers is short vase life. The end of vase life of cut gerbera flowers is often due to bending of the scape, which precedes wilting of the ray florets (Nair *et al*., 2003; Van Son, 2007; Ansari *et al*., 2011; Perik *et al*., 2012; Kilic and Cetin, 2014; Aghajani and Jafarpour, 2016). However, postharvest life of cut flowers could be affect-

ed by the application of various chemicals as preservatives (Nair *et al*., 2003; Prashanth *et al*., 2010).

Insufficient water uptake is one of the main reasons for water deficit and wilting during the vase life (Knee, 2000; Van Ieperen *et al*., 2002). Stem end blockage is a main factor in the imbalance between water uptake and water loss from cut flowers (He *et al*., 2009). Researches showed that bacteria (microbes) (Van Meeteren, 1978; He *et al.*, 2006) or bacteria and decay products (Liu *et al.*, 2009) cause the blockage of cut gerbera flower. Bacteria in vase water can block the vessels in the surface of cut stems (Ferrante *et al.*, 2007). Bacterial inhibitors such as silver nanoparticles or salicylic acid could extend vase life of cut flowers (Loubaud and Van Doorn, 2004; Solgi *et al*., 2009; Vahdati Mashhadian *et al*., 2012). Physiological substances such as lignin, mucilage or gum (Van Doorn and Cruz, 2000; Loubaud and Van Doorn, 2004, Wang *et al*., 2014) and cavitation (Van Meeteren *et al*., 2006) decrease the vase life of some cut flowers. The air emboli or cavitation would be reduced if cut flowers were put into water after cutting immediately (Van Ieperen *et al*., 2002). Cut flowers are sensitive to microbial contamination at the stem end and this determines a reduction of their vase life (Van Meeteren, 1978, Van Doorn and De Witte 1994, Balestra *et al*., 2005).

Nano technology is based on engineered particle of 1-100 nm (diameter). Nanosilver (NS) is included in this technology and can have more chemical and biological activities in order to reduce size. In recent years, NS is being used as a new antiseptic for many industrial processes like medical industry, water purification and vegetable disinfection (Rai *et al*., 2009). In addition, NS treatment has been proposed for improving the postharvest life of cut flowers (Liu *et al*., 2009; Solgi *et al*., 2009; Ansari *et al*., 2011; Danaee *et al*., 2013).

The role of salicylic acid (SA), as an internal growth regulator and a natural phenolic compound, has been completely proved in multiple physiological processes like ethylene biosynthesis, stomatal conductance, respiration, senescence and the activation of defense systems against different pathogens. By activating antioxidant enzymes, SA delays the process of senescence in flowers. In addition, SA inhibits ethylene synthesis and action (Raskin, 1992; Hayat *et al*., 2010; An and Mou, 2011; Jamshidi *et al*., 2012).

Exogenous supply of carbohydrate can play an important role in lengthening the vase life and postharvest conditions of cut flowers. The gerbera cut flowers have short postharvest life. Sucrose effect on enhancing the vase life of cut flowers is associated with water balance. The application of sucrose treatment and sugars accumulated in the flowers increase the sugar and osmotic concentration, improve water absorption and flower turgidity (Reddy and Singh, 1996; Prashant *et al*., 2010; Bhanusree *et al*., 2015). Researches also showed that the combined use of NS 5 mg/L with 4% sucrose and 2.5 mg/L gibberellic acid increase postharvest life of gerbera (Ansari *et al.*, 2011).

Many chemicals have been used in cut flowers vase solutions for inhibiting microorganisms' growth and extending the vase life by improving water uptake. These chemicals include silver nitrate, 8-hydroxyquinoline sulfate and 8-hydroxyquinoline citrate, which are expensive and harmful for the environment and human health (Nowak *et al*., 1990; Ichimura *et al*., 1999; Nair *et al*., 2003; Motaghayer and Esna-Ashari, 2009; Solgi *et al*., 2009; Ansari *et al*., 2011). It is crucial to use natural, safe and inexpensive compounds for the large-scale application of preservatives improving cut flower vase life (Kilic and Cetin, 2014). Essential oils (EO) are organic, natural, safe and eco-friendly substances that have strong anti-inflammatory, antibacterial, antifungal, antioxidant and anticarcinogenic effects. These properties are attributed to the high levels of phenolic compounds (Solgi *et al*., 2009; Bayat *et al*., 2011; Raut and Karuppayil, 2014).

The application of different medicinal plants EOs on increasing the vase life of cut flowers have been studied by many researches. The effect of peppermint (*Mentha pipperita* L.) EO has increased freshness and quality of flower color and prevented the discoloration in alstroemeria (Babarabie *et al*., 2016), flower's quality and delay of leaf and flower senescence of tuberose cv. Pearl (Hoseini and Korehpaz, 2015) and vase life of 'Utopia' rose cut flowers (Saghazadeh *et al*., 2014). Thyme (*Thymus vulgaris* L.) EO (Solgi *et al*., 2009) and water extract of thyme (Amini *et al*., 2014) was added to the preserving solution for extending the vase life of gerbera 'Dune' cut flower and essence containing *Thymus vulgaris* and *Cuminum cyminum* increased solution uptake and quality of gerbera 'Sorbet' cut flowers (Dareini *et al*., 2014). Clove (*Eugenia caryophyllata* Thunb.) EO increased lisianthus cut flower vase life (Kazemi *et al*., 2014) and clove EO and water extract increased gerbera 'Ecco' vase life (Ziyaei Movahed *et al*., 2010).

There are two different ways for treating cut flowers; pulse and permanent treatment. Pulsing is a

short-term treatment that can be done by producers and it helps postharvest vase life and flowering after storage period. Permanent treatment mostly is a long-term treatment, which can be done by consumers for enhancing cut flower vase life (Abdel-Kader and Rogers 1986; Nowak *et al.*, 1990; Arora and Singh, 2002).

Sucrose can maintain the cell's turgor pressure and provide energy for cellular respiration, also is an important nutrient for microorganisms. Therefore, it should not be used without anti-microbial agents in preservatives (Nowak *et al.*, 1990). The effect of NS (Liu *et al.*, 2009) and SA (Jamshidi *et al*., 2012) treatments alone on extending cut flowers vase life was assessed in different researches. Since the effects of different concentrations of various preservative solutions on the postharvest life of cut flowers are altering depend on plant species, the applied chemicals and interaction of their compounds in vase solution and the method of treatment, the determination of the effective preservatives as well as the method of application is very important. Therefore the aim of this study was to screen the effects of NS and SA as pulse treatment and sucrose and thyme, clove and peppermint EOs as permanent treatment on vase life and hydraulic conductivity of gerbera 'Rosalin' cut flowers.

2. Materials and Methods

Plant growth conditions and treatments

Gerbera (*G. jamesonii* cv. 'Rosalin') flowers were grown in standard hydroponic greenhouse conditions in Ferdowsi University of Mashhad, Iran. The flowers were harvested during morning by pulling out the stems from the plants when 2-3 rows of stamens of the bisexual disc florets were mature. Stems were pulled, not cut and the base of stem was removed before hydration (Dole and Wilkins, 2006). The stems were taken immediately to the laboratory and recut under water to 35 cm length. The cut flowers were immediately immersed individually into 500 ml vase solutions. In order to simulate the domestic use, the vase solutions were not changed and the stems were not recut during the experiment. The end of gerbera cut flower vase life was considered as the time in which more than one third of the outer petals of inflorescence start to be brown or wilted or curled or stem bending (≥90°) or breaking was occurred (Dole and Wilkins, 2006). This study was conducted as a factorial experiment based on completely randomized design with three replications and four stems in each replicate. The first factor was pulse treatments: distilled water (D), salicylic acid (SA) 50 and 100 mg/L (Merck Company), Nanosilver (NS) 5 and 10 mg/L (Nanocid Company, Iran). The second factor was permanent treatments: distilled water, sucrose 4% (Merck Company), peppermint EO 100 mg/L, thyme EO 100 mg/L, clove EO 300 mg/L (Zardband Company, Iran) (Table 1). Pulse treatments were applied for 24 h. Treated stems were then stood into vases containing permanent treatments. Vase solutions were freshly prepared at the beginning of the experiment and not renewed during of the study.

The EOs constituents were determined by Zardband Company (Iran) using GC-MS analysis

Table 1 - Pulse and permanent treatments used in the experiment

Pulse treatment/Permanent treatment
Distilled water/Distilled water
Distilled water/Sucrose
Distilled water/Peppermint EO
Distilled water/Thyme EO
Distilled water/Clove EO
SA 50 mg.L^{-1}/Distilled water
SA 50 mg.L^{-1}/Sucrose
SA 50 mg.L^{-1}/Peppermint EO
SA 50 mg.L^{-1}/Thyme EO
SA 50 mg.L^{-1}/ Clove EO
SA 100 mg.L^{-1}/Distilled water
SA 100 mg.L^{-1}/Sucrose
SA 100 mg.L^{-1}/Peppermint EO
SA 100 mg.L^{-1}/Thyme EO
SA 100 mg.L^{-1}/ Clove EO
NS 5 mg.L^{-1}/Distilled water
NS 5 mg.L^{-1}/Sucrose
NS 5 mg.L^{-1}/Peppermint EO
NS 5 mg.L^{-1}/Thyme EO
NS 5 mg.L^{-1}/ Clove EO
NS 10 mg.L^{-1}/Distilled water
NS 10 mg.L^{-1}/Sucrose
NS 10 mg.L^{-1}/Peppermint EO
NS 10 mg.L^{-1}/Thyme EO
NS 10 mg.L^{-1}/ Clove EO

(Table 2). GC-MS analysis revealed that the major constituents of the EOs were: thymol (53.5%) in thyme EO; 1-menthol (41.22%) and menthone (24.01%) in peppermint EO and eugenol (62.4%) in clove EO.

Table 2 - Major chemical constituents of the EOs

Peppermint EO		Thyme EO		Clove EO	
GCMS Analysis (%)		GCMS Analysis (%)		GCMS Analysis (%)	
Limonene	2.25	Terpinene gamma	7.20	Alpha Copaene	0.04
Cineole	4.59	Para-Cymene	27.4	Beta Caryophyllene	3.79
Menthone	24.01	Thymol	53.50	Alpha Humulene	0.45
Isomenthone	3.83			Oxyde De Caryophyllene	0.29
1-Methyl acetate	4.38			Eugenol	81.83
Neomenthol	2.84			Isoeugenol	0.13
1-Menthol	41.22			Acetate De Eugenyle	12.50
Pulegone	1.56			Methyl Eugenol	0.01
Menthofuran	2.98				
Density (20°C)	0.9036	0.923		10.636	
Refractive Index(20°C)	14.605	1.502		15.335	
Optical Rotation (°)	-23.68	-1.0		-0.35	
Batch	13/47/23	45109		68382	

Measuring hydraulic conductivity

Hydraulic conductivity was measured by a slight modification in the method of Melcher *et al*. (2012) (Fig. 1). A piece of 15 cm of flower stem end was cut with a sharp blade under distilled water. The upper part of the stem (part 1 in Fig. 1) was inserted into a silicon tube (part 2 in Fig. 1) (internal diameter 4 mm) filled with degas distilled water and the basal part of the stem (part 3 in Fig. 1) was kept in the degas distilled water. Using a three-way glass valve (part 4 in Fig. 1), the silicon tube was connected from one side to the degassed distilled water tank (part 5 in Fig. 1) and from the other side was attached into a U-shaped pipe (part 6 in Fig. 1) below the stems end. The whole set (stem, three-way glass valve, degas distilled water tank and U-shaped pipe) was fixed (Van Ieperen *et al*., 2002). The stem vase was placed on a digital scale (part 7 in Fig. 1) connected to the computer and the stem and degas distilled water weight changes were recorded at time regular intervals (30 minutes). Fifty cm head pressure of water (*h*; which made 5 kPa pressure) was applied, so that water had passed through the segments. The flow rate was then determined by measuring the volume of the passed water. Three stem segments were used for each treatment (Ichimura *et al*., 2005).

Measurements of stem hydraulic conductivity involves measuring the flux for a given driving force ($Q/\Delta P$; where ΔP is the pressure drop across the segment), normalized by the length of the stem segment and referenced either to the cross-sectional area of the stem (Melcher *et al*., 2012). In equation 1, K (cm/min) is hydraulic conductivity, Q (cm^3/min) is the recorded flux (gravimetric or volumetric flow rate), L (cm) is the length of the measured segment, A (cm^2) is the cross-sectional area of the stem segment and h (cm) is head pressure of water height. The data were collected at days 2, 4, 6 and 8 after pulse treatment.

$$K = Q_L/Ah) \quad (1)$$

To observe the microscopic effects of chemicals and EOs on stem closure and hydraulic conductivity during the vase life, 2 cm of treated stem was used for histological study. The cut stem segments (3-5 cm in length) were stored in a solution of FAA (formalin (40%): glacial acetic acid (50%): ethyl alcohol (70%):

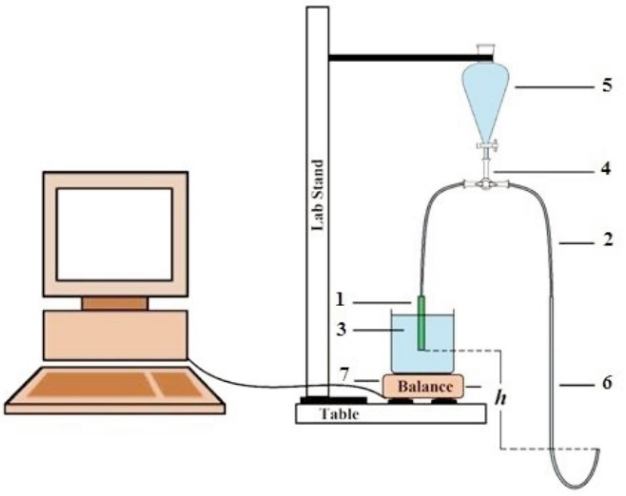

Fig. 1 - Hydraulic conductivity system scheme; the upper part of the stem (1); silicon tube (2); the basal part of the stem (3); the three-way glass valve (4); the degas distilled water tank (5); the U-shaped pipe (6); the digital scale (7); h: water height.

[13:5:200]) to preserve the tissue before sectioning. Stem transverse sections, at 16-µm thickness, were made using a manual rotary microtome (Leitz 1512, Germany) after fixing in FAA and permanent mounts were prepared in paraffin wax. Cross sections were stained with Safranin O/Fast Green Stain method and embedded on microscope slides. Digital images were made at 10X magnification with a digital camera (Olympus DP71, Japan) attached to a light microscope (Olympus BH2, Japan) and computer.

Flower water content

Flower water content (*WC*) was measured as mentioned in equation 2. Flower fresh weights (*FW*) were assessed at the beginning of the experiment and flower dry weights (*DW*) were recorded after drying to constant weight in an oven for at least 48 h at 85°C. Water content was calculated for three replicates (He *et al*., 2006; Lu *et al*., 2012).

$$WC = (FW-DW)/DW * 100 \quad (2)$$

The experiment was conducted in the laboratory at 20-22°C, 40-50% RH, and 15 µmol/m^{2}s light intensity (cool white florescent tubes) under a daily light period of 12 hours. The obtained data were analyzed using MSTAT-C program and mean comparison was done using LSD range test.

3. Results

Flower vase life

Results of this study showed that in single effect of applied treatments, all NS pulse treatments markedly (P<0.01) extended vase life of gerbera 'Rosalin' cut flowers. The 10 mg/L NS pulse treatment gave the longest vase life (12.20 days) as compared to the other treatments (Table 3). SA 100 mg/L (9.08 days) pulse treatment significantly increased flower vase life compared to the control (8.13 days). However, there was no significant difference between NS 5 mg/L and SA 100 mg/L.

The single effect of applied treatments indicated that peppermint and thyme EOs (100 mg/L) application in preservative solutions as permanent treatment (Fig. 2) could extend the vase life of gerbera cut flowers to 9.98 and 10.35 days respectively (Table 3). However, there was no significant difference among these two treatments and control in extending vase life. Flowers placed in sucrose 4% and clove EO 300 mg/L had the least (8.83 days) vase life (Table 3).

The results showed that the interaction of pulse and permanent solutions had remarkable effect on flower longevity. The best treatments were NS 10 mg/L + thyme EO 100 mg/L (14.25 days) and NS 10 mg/L + peppermint EO 100 mg/L (14 days) which had significant difference with other treatments (Table 4). It has been observed that SA 50 mg/L + 4% sucrose was less effective than control on flower postharvest life and sucrose had negative effect on gerbera flowers life (Table 4).

Flower water content

Flower water contents for the pulse treatments with distilled water and 50 mg/L SA were a little higher than other treatments (Table 5). In 4% sucrose per-

Table 3 - The simple effect of pulse and permanent treatments on 'Rosalin' gerbera cut flower vase life (day)

Treatments	Vase life (Day)
Pulse treatment	
Distilled water	8.13 d
SA 50 mg.L^{-1}	8.30 cd
SA 100 mg.L^{-1}	9.08 bc
NS 5 mg.L^{-1}	10.02 b
NS 10 mg.L^{-1}	12.20 a
Permanent treatment	
Distilled water	10.02 a
Sucrose	8.55 b
Peppermint EO	9.98 a
Thyme EO	10.35 a
Clove EO	8.83 b

The means showing similar letters in each column have no significant difference according to the LSD range test (P<0.01).

Fig. 2 - Gerbera 'Rosalin' cut flowers treatment by NS (10 mg/L) and peppermint EO (100 mg/L). During pulse treatment, vase solution was covered by dark plastic coverage to prevent undesirable light reaction in NS.

manent treatment flowers, water content declined more rapidly during the vase life period and was significantly different from the others. Generally, the interaction of pulse and permanent treatment showed that 4% sucrose had negative effect especially after 10 mg/L NS application as pulse treatment (Table 4).

Hydraulic conductivity

The hydraulic conductance of the stem end segments did not change over the first 2 days after pulsing and had very low rates. Thereafter it changed over time and increased slightly on day 4. The rate of stem flower hydraulic conductivity sharply increased at day 6 and 8 after pulse treatment application. Hydraulic conductivity of stems treated with 5 and 10 mg/L NS pulse markedly showed lower rate during the experiment compared to other treatments (Table 6).

In addition, permanent treatments had significant effect on hydraulic conductance throughout assessment. Result showed that peppermint EO 100 mg/L had lower look rate in hydraulic conductance than other solutions (Table 7).

However, hydraulic conductance of the stem segment was nearly the same at the initial day of the experiment. The interaction of pulse and permanent treatment determined that NS 10 mg/L + peppermint EO 100 mg/L and NS 10 mg/L + thyme EO 100 mg/L had the lowest rate even in day 8 after pulsing (Table 8). In the NS 10 mg/L + peppermint EO 100 mg/L flowers, the hydraulic conductance of the stem segments slightly increased thereafter. Hydraulic con-

Table 4 - The effect of different treatments' interactions on 'Rosalin' gerbera cut flower vase life (day) and water content (%)

Treatment	Vase life (Day)	Water content (%)
Distilled water/Distilled water	9.58 efg	90.95 ab
Distilled water/Sucrose	8.08 i	86.15 c
Distilled water/Peppermint EO	7.83 ij	91.29 a
Distilled water/Thyme EO	8 i	91.39 a
Distilled water/Clove EO	7.17 jk	91.21 a
SA 50 mg.L^{-1}/Distilled water	9.08 fgh	91.31 a
SA 50 mg.L^{-1}/Sucrose	7 k	85.02 c
SA 50 mg.L^{-1}/Peppermint EO	8.08 i	91.09 a
SA 50 mg.L^{-1}/Thyme EO	8.92 gh	90.97 a
SA 50 mg.L^{-1}/ Clove EO	8.42 hi	91.15 a
SA 100 mg.L^{-1}/Distilled water	9.83 def	90.54 ab
SA 100 mg.L^{-1}/Sucrose	8.58 hi	83.44 d
SA 100 mg.L^{-1}/Peppermint EO	9.17 fgh	90.45 ab
SA 100 mg.L^{-1}/Thyme EO	9.83 def	90.73 ab
SA 100 mg.L^{-1}/ Clove EO	8 i	89.46 b
NS 5 mg.L^{-1}/Distilled water	9.67 efg	90.55 ab
NS 5 mg.L^{-1}/Sucrose	8.58 hi	82.68 d
NS 5 mg.L^{-1}/Peppermint EO	10.83 c	90.81 ab
NS 5 mg.L^{-1}/Thyme EO	10.75 c	90.56 ab
NS 5 mg.L^{-1}/ Clove EO	10.25 cde	90.42 ab
NS 10 mg.L^{-1}/Distilled water	11.92 b	90.52 ab
NS 10 mg.L^{-1}/Sucrose	10.50 cd	81.1 e
NS 10 mg.L^{-1}/Peppermint EO	14 a	91.36 a
NS 10 mg.L^{-1}/Thyme EO	14.25 a	91.22 a
NS 10 mg.L^{-1}/ Clove EO	10.33 cde	90.99 a

The means showing similar letters in each column have no significant difference according to the LSD range test (P<0.01).

Table 5 - The effect of pulse and permanent treatments on 'Rosalin' gerbera cut flower water content (%)

Treatment	Water content (%)
Pulse Treatment	
Distilled water	90.20 a
SA 50 mg.L^{-1}	89.91 a
SA 100 mg.L^{-1}	88.93 b
NS 5 mg.L^{-1}	89.00 b
NS 10 mg.L^{-1}	89.04 b
Permanent treatment	
Distilled water	90.77 a
Sucrose	83.68 b
Peppermint EO	91.00 a
Thyme EO	90.97 a
Clove EO	90.65 a

The means showing similar letters have no significant difference according to the LSD range test (P<0.01).

Table 6 - The effect of pulse treatments on trends of hydraulic conductivity of gerbera cut flower stem on day 2, 4, 6 and 8 of the experiment

Pulse treatment	K (cm/min)			
	Day 2	Day 4	Day 6	Day 8
Distilled water	0.57 d	5.42 a	7.58 b	8.95 c
SA 50 mg.L^{-1}	1.04 b	5.35 a	12.96 a	9.17 c
SA 100 mg.L^{-1}	1.72 a	6.44 a	10.42 a	15.47 a
NS 5 mg.L^{-1}	0.84 c	2.50 b	4.45 c	13.08 b
NS 10 mg.L^{-1}	0.57 d	0.91 c	2.05 d	4.97 d

The means showing similar letters in each column have no significant difference according to the LSD range test (P<0.01).

ductivity of other treated flowers increased sharply after day 6 during the rest of the vase life (Table 8).

The survey of slope trend of each treatment during the experiment's period, showed that the lowest slope was observed in NS 10 mg/L + peppermint EO 100 mg/L and NS 10 mg/L + thyme EO 100 mg/L respectively. While in other treatments, slope trend was enhanced so that the most slope trend was considered in 50 mg/L SA + 4% sucrose.

In addition, histological study showed that in the NS 10 mg/L + peppermint and thyme 100 mg/L EOs treated flowers stem remained healthy for longer period while the other stems became hollow after a few days (Fig. 3 and 4).

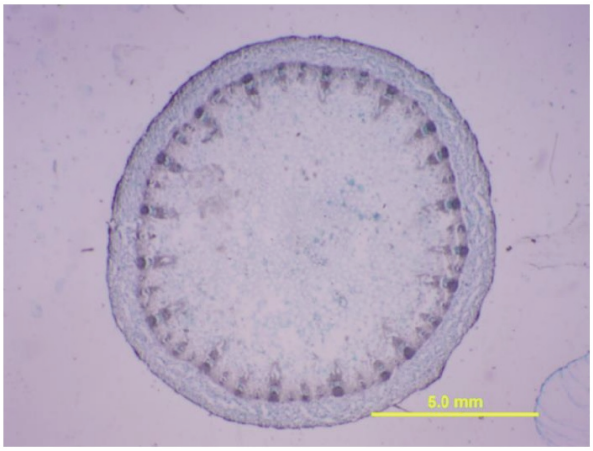

Fig. 3 - The gerbera cut flower healthy stem.

Table 7 - The effect of permanent treatments on trends of hydraulic conductivity of gerbera cut flower stem on day 2, 4, 6 and 8 of the experiment

Permanent treatment	K (cm/min)			
	Day 2	Day 4	Day 6	Day 8
Distilled water	0.81 c	5.14 a	8.66 b	14.62 a
Sucrose	1.46 a	4.93 ab	13.50 a	12.76 a
Peppermint EO	0.84 c	2.74 c	3.61 d	6.25 c
Thyme EO	0.69 d	3.65 bc	6.00 c	8.77 b
Clove EO	0.94 b	3.52 bc	5.07 cd	9.25 b

The means showing similar letters in each column have no significant difference according to the LSD range test (P<0.01).

Table 8 - The trends of hydraulic conductivity of gerbera cut flower stem in all treatment on day 2, 4, 6 and 8 of the experiment

Treatment	K (cm/min)				Slope trend
	Day 2	Day 4	Day 6	Day 8	
Distilled water/Distilled water	0.42 ghi	5.60 bcd	9.41 cde	17.04 cd	53.664
Distilled water/Sucrose	0.64 fg	5.96 abcd	8.88 def	14.04 ef	43.117
Distilled water/Peppermint EO	0.21 hi	4.89 cde	4.62 ghi	0.00 j	5.16
Distilled water/Thyme EO	0.62 fg	4.52 de	6.41 fg	0.00 j	52.384
Distilled water/Clove EO	0.96 de	6.14 abcd	9.23 cde	13.69 f	41.263
SA 50 mg.L^{-1}/Distilled water	0.76 ef	7.91 a	11.28 cd	14.95 def	45.931
SA 50 mg.L^{-1}/Sucrose	1.14 d	7.58 ab	43.58 a	0.00 j	16.337
SA 50 mg.L^{-1}/Peppermint EO	1.51 bc	1.94 fgh	2.74 ij	0.00 j	12.728
SA 50 mg.L^{-1}/Thyme EO	0.57 fg	4.39 de	9.54 cd	14.21 ef	46.081
SA 50 mg.L^{-1}/ Clove EO	1.19 cd	5.67 bcd	12.34 c	16.71 cde	53.215
SA 100 mg.L^{-1}/Distilled water	1.53 b	7.30 ab	11.33 cd	14.69 def	43.499
SA 100 mg.L^{-1}/Sucrose	1.67 ab	6.87 abc	21.41 b	21.51 a	74.065
SA 100 mg.L^{-1}/Peppermint EO	1.83 ab	5.17 cd	6.60 efg	20.43 ab	57.224
SA 100 mg.L^{-1}/Thyme EO	1.67 ab	7.95 a	10.99 cd	20.72 ab	60.198
SA 100 mg.L^{-1}/ Clove EO	1.94 a	5.10 cd	5.31 gh	0.00 j	5.769
NS 5 mg.L^{-1}/Distilled water	1.04 de	4.22 de	8.54 def	18.74 bc	5.741
NS 5 mg.L^{-1}/Sucrose	1.91 a	3.18 ef	5.28 gh	22.59 a	64.126
NS 5 mg.L^{-1}/Peppermint EO	0.52 fgh	2.27 fg	4.69 ghi	10.40 g	32.056
NS 5 mg.L^{-1}/Thyme EO	0.45 ghi	1.97 fgh	4.26 ghi	7.48 hi	23.375
NS 5 mg.L^{-1}/ Clove EO	0.40 ghi	1.07 ghi	0.67 k	6.19 i	16.963
NS 10 mg.L^{-1}/Distilled water	0.40 ghi	1.51 ghi	3.93 hi	7.68 ghi	24.278
NS 10 mg.L^{-1}/Sucrose	1.91 a	1.86 fgh	4.79 ghi	5.65 i	14.158
NS 10 mg.L^{-1}/Peppermint EO	0.16 i	0.12 i	0.28 k	0.41 j	0.0904
NS 10 mg.L^{-1}/Thyme EO	0.21 i	0.57 hi	0.94 jk	1.42 j	0.4012
NS 10 mg.L^{-1}/ Clove EO	0.23 hi	0.67 hi	1.02 jk	9.65 gh	28.601

The means showing similar letters in each column (Day) have no significant difference according to the LSD range test (P < 0.01).

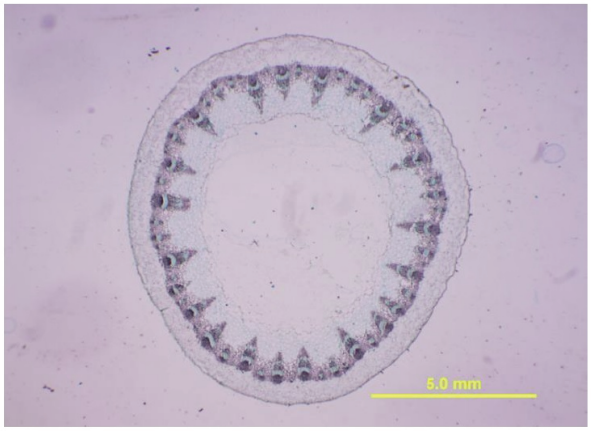

Fig. 4 - The gerbera cut flower hollow stem.

3. Discussion and Conclusions

Flower vase life

NS particles enter into cell, tissue and organs, so they can replace with silver salts (such as silver nitrate or silver thiosulfate) in preservative solutions. NS inhibits the respiration and electron transfer system and material transfer in microbial cell membrane (Paull and Lyons, 2008). Various researches indicated that flowers treated with NS solution, had more vase life. Silver ions, because of small size, have more contact with outer space and influence more on their environment. NS, in comparison with silver ions, showed antimicrobial property at inferior concentration (Solgi *et al*., 2009; Ansari *et al*., 2011)

Different studies determined antimicrobial effects of main components of thyme (Nikolić *et al.*, 2014 a), peppermint (Kazem Alvandi *et al.,* 2011; Nikolić *et al.*, 2014 b) and clove EO (Boukaew *et al.,* 2017). In addition, Amini *et al*. (2014) reported that thyme EO in pulsing with distilled water treatment showed the best results for extending cut gerbera flower vase life and preventing more weight loss. Hydrophobicity is an important characteristic of thyme and peppermint EOs. This enables them to separate the lipid components of the bacterial cell membrane and mitochondria, binding to membrane proteins and releasing lipopolysaccharides, which results in disturbing cell wall structures (Solgi *et al*., 2009).

Researches indicated also that SA pulse treatment, followed by NS as permanent solution (Danaee *et al*., 2013), SA utilization as permanent treatment (Jamshidi *et al*., 2012) significantly promoted the vase life of gerbera cut flowers. However, in this study, SA had no special effect on flower longevity. Although Ziyaei Movahed *et al*. (2010) reported that clove EO increased gerbera vase life. In this study, it had the least effect on postharvest life of gerbera cut flowers. Despite of sucrose important role in extending the vase life of cut flowers, Ansari *et al.* (2011) reported a negative effect on 'Rosalin' gerbera cut flowers. The main reason could be severe bacterial growth in vase solution.

Flower water content

Cut flowers and foliage can have limited commercial value because they dehydrate during vase life because of water uptake decrease. Water deficit could develop even when cut flowers are placed in water (Nazari Deljou, *et al*., 2012). Gerbera cut flowers stem break of is mainly caused by water shortage in the flowers due to the increased difficulty of water flow from the water source to petals. It is also supposed to be a competition for available water between flower heads and stems. The increase in flow resistance leads to stem break as a result of microbial activity in the vase water (Balestra *et al*., 2005). It could be concluded from the results of this study that in all treatments with antimicrobial agents such as NS, SA and thyme, peppermint and clove EOs flower water contents were high and had no harmful decrease.

Hydraulic conductivity

Many experiments were performed to find out the cause of stem bend in gerbera cultivars. Research showed that removal of the floral head prevented stem bending, indicating that bending is physically due to the gravitational pull on the floral head. Stem bending in cut gerbera can be due to lack of mechanical support. Bending might relate to lack of wall thickening, particularly in the xylem. At least two other factors might contribute to mechanical stem strength. The first factor is gerbera stems elongation during vase life. Elongation zones usually have weakly developed xylem and sclerenchyma. Since the stems are usually placed in water under an angle, stem elongation will increase the gravitational pull of the floral head result in earlier stem bending. The second factor is the presence of a cavity in the center of the gerbera stem. Observations showed the cavity at the time of harvest, in several cultivars (Perik *et al*., 2012). Other factors affecting stem bending could be adverse water relations such as lack of turgor. After a few days of vase life, there are many bacteria in the vase solution. Stem bending can be due to xylem blockage by bacteria, which results in low water uptake. As transpiration is not inhibited as

much as water uptake, net water loss occurs followed by the loss of turgor and stem bending (Van Meeteren, 1978).

Generally, in cut flowers fresh weight decreased before stem bent occurred, and this is accompanied by a decline in absorption of water by the flowers. Stem break could be prevented by pretreatment of the stems with NS by adding to the vase water. Van Meeteren (1978) suggested that there are two different pathways for water uptake: a direct one through the xylem vessels at the cut surface and an indirect one through the cavity in the stem. Only the direct water uptake is strongly inhibited by growth of bacteria in the vase water. Stem bend occurs when the direct water uptake is inhibited by bacterial activity. Van Meeteren (1978) suggested that the minimum concentration of silver nitrate could avoid stem bending and inhibit bacterial growth in the water. There is an association between a high population density of bacteria in the water and scape bending (Van Doorn and De Witte, 1994). The results of this experiment also showed that bacteria would block the main water pathway (xylem vessels) over the time and the stem would become hollow (Fig. 3 and 4). So that active water uptake is effectively prevented. Bacterial activity could be significantly inhibited by adding NS (as pulse treatment), peppermint (1-menthol and menthone) and thyme (thymol) EOs (as permanent treatment) to vase solution.

Based on the results of this study, new antimicrobial agents such as NS, thyme and peppermint EOs had a positive effect on flower vase life and water content. It might be due to this fact that these are very effective antimicrobial agents, which inhibited the microbial growth and prevented bacterial plugging in conducting tissues. However, exogenous supply of sugars can increase water balance and osmotic concentration and plays an important role in lengthening the vase life of cut flowers, but in this research, sucrose had negative effect on flower water contents and gerbera 'Rosalin' vase life period. This issue can be related to the negative effect of sucrose on microbial growth. Although, there was no available data about the effect of EOs on stem hydraulic conductivity changes in cut flowers, this research showed that EOs could improve hydraulic conductance and keep it in a normal and stable condition for longer period.

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First insight into *Araucaria araucana* (Molina) K. Koch under its southernmost European growing condition: a proposed descriptor list for morphological characterization

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Key words: germplasm, monkey puzzle, phenology, phenotyping, climate, Pistoia's nurseries.

Abstract: *Araucaria araucana* is a south American endemic Conifer of conservation concern. After its introduction to Europe, this species has been often planted for ornamental purposes in parks and gardens, where its unusual appearance was admired. After the mid-1970s this tree received increasing attention from the nursery growers of Pistoia (Tuscany) and became of considerable economic importance. However, being one of the iconic threatened trees listed in CITES Appendix I, the international trade of these species is rigorously regulated. This study was aimed at developing a first morphological descriptor list for further phenotyping of *in loco* produced plant material. A first step of the research focused on the description and a better understanding of *A. araucana* phenological phases inferred from Mediterranean climate conditions. The second phase regarded the analysis of observed or measured morphological characteristics of the tree, branches, scales, inflorescences, fruits and seeds observed on a subset of 4 selected putative populations over a 3-year period of vegetative growth. The results allowed to select 39 most discriminant descriptors, which are presented together with their range of variability and classes. The achieved descriptor list represents a suitable tool for the selection of genotypes and for the breeding of *A. araucana*.

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1. Introduction

Araucaria araucana (Molina) K. Koch, commonly known as 'monkey puzzle tree' or 'pehuèn', is an endangered conifer species native to south-central Chile and south-western Argentina, where it has a relatively limited distribution, split between the main area spanning both sides of the Andes and two other disjunct small subpopulations in the Coastal Cordillera of Chile (Donoso, 1993, 2006; Donoso *et al.,* 2008; Drake *et al.,* 2009). The present distribution is a remnant of a more extensive former distribution, which has been severely diminished by logging, human-set

fires and land clearance since European colonization in the mid-19th century (Veblen, 1982; Burns, 1993; Rechene, 2000). In particular, the intense human seed collecting and animal grazing have led to a lack of natural reproduction by seed, and when any regeneration occurs it is principally asexual with tree sprouting from roots (Schilling and Donoso, 1976; Gallo *et al.,* 2004). Since 1976, this species has been protected in Chile under the status of a Chilean National Monument and, since 1997, it is also protected internationally under the Convention on International Trade in Endangered Species of Wild Fauna and Flores (CITES) (Farjon and Page, 1999; Herrmann, 2006).

Araucaria araucana was first introduced in England by the Scottish naturalist Archibald Menzies in 1795. Its unusually straight cylindrical bole and whorled branches as well as its 10-15 cm thick tortoise-shell-like bark made it internationally popular as an ornamental plant. The following decades saw a rapid spread of this impressively large and long-lived conifer throughout all the European continent. It was introduced in Italy from Paris in 1822 and the first *A. araucana* tree was planted in the garden of the Marquis Pucci's favourite property in Florence, Tuscany. This Italian region represents the southernmost limit of its distribution area. Thanks to its perfect adaptability to the Tuscan soil and climate conditions, this majestic slow-growing tree was included in the important ornamental horticulture district of Pistoia (NW from Florence) starting from the II post-world war period. Thereafter, there was a notable and rapid increase of *A. araucaria* commercial propagation in the Pistoia district in line with the steadily-growing demand. This tree became of considerable economic importance and the period between the 1970s till the early 2003 saw the maximum expansion of commercialization rate and tree planting in private properties and public gardens. Due to its constantly declining distribution, together with its slow growth and its limited dispersal ability, in 2003, according to the Regulation (CE) n. 1497, the listing of this species was transferred from Appendix II to Appendix I of CITES (http://www.cites.org/eng/app/appendices.html; valid from October 4th, 2017), which strictly regulates the trade in its timber and seeds, and listed in the 2008 IUCN Red List of Threatened Species (http://www.iucnredlist.org; March 2017) as an endangered species currently on risk of extinction. As a consequence, plant nurseries had to adopt a mandatory stock register of alive and dead specimen, where both entries and exits (including origin, quantity, causes of death, etc.) had to be specified. A progressive and ongoing reduction of monkey puzzle tree propagation in the Pistoia district has resulted from increased regulation, complex management, high risk of penalties, increased costs for staff training, and risks from plant diseases associated with climate change. Ultimately, the number of nurseries holding and propagating *Araucaria* plants conspicuously declined during the last fifteen years.

The identification of a new Tuscan variety could offer the opportunity to disengage from the procedural constraints imposed by the CITES Convention. Hence, the production of a descriptor list for the characterization of Tuscan selected germplasm is a necessary first step towards the definition of genetic diversity based on morphological variation and for varietal identification in *Araucaria*. The descriptor list might represent the first attempt at achieving an unified documentation system thereby enabling through a standardized format an easier exchange of information between researchers and collection curators. Although there is a high demand for new descriptor lists to be developed for many forest conifer species, up to our knowledge there is only an UPOV descriptor list available for *Picea abies* L. (https://www.upov.int/test_guidelines/en/fulltext_tgdocs.jsp?q=Picea; copyright © 2011, UPOV).

Despite the conservation interest in this species, little is known of its phenotypic and genetic variation. The genetic diversity of monkey puzzle between Andean and coastal Chilean populations has been investigated in previous studies by Delmastro and Donoso (1980) and Rafii and Dodd (1998). More recently, advanced biotechnologies, such as RAPDs, Isozymes, microsatellite and RFLP analysis, were used to characterize genetic heterogeneity within and among some South American populations (Bekessy *et al.,* 2002; Ruiz *et al.,* 2007; Marchelli *et al.,* 2010; Martín *et al.*, 2012). However, no reports were found in the world literature on morphological traits.

This study was part of the CARAVIV project 'Characterization of *Araucaria araucana* germplasm selected by the nursery industries of the Pistoia's district for commercial development', supported by the Ministero delle Politiche Agricole Alimentari e Forestali (MiPAAF-OIGA), aimed at contributing to a better understanding of *Araucaria* growing and to enhance the commercial exploitation of local genetic resources. This paper provides a brief account of *Araucaria araucana* phenological phases under Italian climatic conditions and is focused on the development of a first descriptor lists in order to

characterize *in loco* produced plant material and make information available to other growers in a systematic and unambiguous form.

2. Materials and Methods

Plant material

The plant material used in this study was the *A. araucana* germplasm available in the Pistoia' nursery district, covering an area of approx. 965 sq km, ranging from 50 m to 550 m above sea level, located in northern Tuscany.

Phenology

Throughout three-year growing cycles (2015-2017), the phenological phases (onset of flowering, full bloom, fertilization, fruit ripening and seed production) of *A. araucana* trees belonging to 8 putative populations were observed every two weeks from March to June (during the flower maturation), and monthly in the rest of the year. Taking into account the peculiar structure of the reproductive buds of this species, we decided to consider as onset of flowering the inflorescence appearance and as full bloom the inflorescence maturity, i.e. the pollination phase. Four out of the selected populations belong to Pistoia's hinterland in a plain area (43°53' N; 10°55' E; 60 m a.s.l.), while the remaining four are located in high hills (44°0' N; 10°52' E; 550 m a.s.l.). The number of plants for each population ranged from 10 to more than 200, varying in age, gender and sexual maturity.

Morphological descriptor list

Twenty/twenty-five-year-old specimens belonging to a subset of 4 putative populations were randomly defined in order to perform a morphological description. The considered populations were derived from seeds of different origin (unknown, Dutch fair, Spanish fair, local selected progeny) and grown in three private nurseries, located very close to each other in a plain area under the same organic regime. Local selected progeny refers to seeds collected from a couple of old trees located in Villa Lodolo (S. Marcello Pistoiese, 44°03' N; 10°47' E; 623 m a.s.l.). These trees were introduced from Argentina in 1920 and represent the main genetic source of *A. araucana* local germplasm. Climatic data, i.e. atmospheric pressure (AP), medium (AVG T), maximum (MAX T) and minimum (MIN T) air temperatures, relative air humidity (RH), wind run (WR), global horizontal irradiation (GHI), rainfall (RAIN), evaporation (EV), for the same area were collected monthly from January 2015 to December 2017 (Table 1).

Minimum, maximum, average, standard deviation and coefficient of variability (%) values of each morphometric character were calculated for the whole set of plants by using one-way analysis of variance (ANOVA). This in turn led to the definition of classes for all the measured traits by subdividing the total range into intervals 2 times the standard deviation, i.e. whether above or below the average value of each parameter as described in Bassi (2003). Statistical analysis was performed using SPSS 20 software (Chicago, IL, USA).

Table 1 - Seasonal averages (2015-2017) of main meteorological data collected in Pistoia's nursery area[a]

Year	Season	AP (mbar)	AVG T (°C)	MAX T (°C)	MIN T (°C)	RH (%)	WR (km)	TSI (kWh/m²)	RAIN (mm)	EV (mm)
2015	Winter	1009.33	8.33	14.73	3	68.33	86.7	1.53	2.7	1.6
	Spring	1011	18.7	26.67	11.03	62.33	73.37	3.93	1.73	4.9
	Summer	1009	24.77	33.13	16.87	57	69.6	4.17	1.23	6.5
	Fall	1016.33	11.5	18.07	6.97	78.33	33.73	1.17	3.67	1.07
2016	Winter	1007.33	9.1	14.9	4.47	74	77.6	1.37	5.9	1.4
	Spring	1007	16.83	25.2	9.83	65	77.9	3.7	2	5
	Summer	1010.67	24.2	33.03	16.37	57.67	66.9	4.2	1.57	6.5
	Fall	1014.67	10.43	18.3	5.1	78	24	1.3	3.57	1.03
2017	Winter	1012.33	8.47	15.77	2.43	67.33	66.4	1.67	3.53	1.77
	Spring	1009.67	19.5	27.7	11.53	60	76.03	4.07	1.4	6.3
	Summer	1009.33	23.67	32.47	15.43	57	65.6	4.03	1.7	6.43
	Fall	1011.33	9.93	18	4.3	76	28.3	1.27	5.13	1.1

AP= atmospheric pressure; AVG T= medium air temperature; MAX T= maximum air temperature; MIN T= minimum air temperature; RH= relative air humidity; WR= wind run; GHI= global horizontal irradiation; RAIN= rainfall; EV= evaporation.

[a] Processed from Ce.Spe.Vi database, Pistoia (http://www.cespevi.it/meteo.htm; Paolo Marzialetti © 1996/2018 Ce.Spe.Vi. - Pistoia).

3. Results

Phenology

The results of our observations throughout the growing cycles of male and female *A. araucana* trees in the Pistoia district are shown in figure 1. The main collected phenological data (flowering onset, flower maturation, fertilization, fruit ripening and seed production) were compared with those found in previous published surveys on native Andean populations (Table 2).

Morphological descriptor list

Starting from field observations over a three-year growing cycle, a total number of 39 descriptors were developed for further germplasm phenotyping, divided into 6 sections: 1) tree, 2) branches, 3) leaves, 4) male inflorescences, 5) female strobiles, 6) seeds and productivity. These descriptors apply to twenty/twenty-five-year-old trees (i.e. approx. the age of first fruit bearing) grown in nurseries as ornamental plants. The descriptor list has been enriched by images and drawings for a better understanding

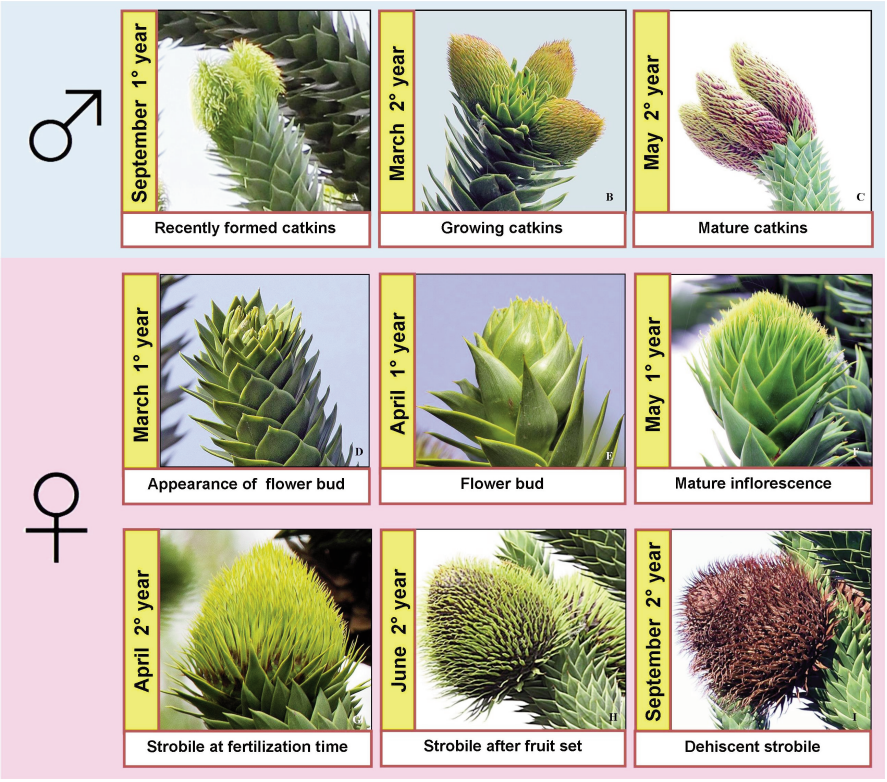

Fig. 1 - *Araucaria araucana* male (♂) and female (♀) phenological stages under Tuscan climate conditions.

Table 2 - Differences between the maturation stages of female and male inflorescences and seed production in *Araucaria araucana* populations in their origin area and in Italy

Phenological phases	Chile/Argentine[a]		Italy	
	♀	♂	♀	♂
Flowering onset	Nov. I	Aug. I / Sep. I	Mar. I	Jul. /Aug. I
Maturation	Dec. I	Dec. I	May I	May II
Fertilization	Jan. II	-	Apr. II	-
Seed production	Dec. II / Feb. III	-	Sep. / Oct. II	-

I = first year; II second year; III = third year.

[a] Tortorelli, 1956; Montaldo, 1974; Donoso and Cabello, 1978; Rodriguez *et al.*, 1983; Marticorena, 1995.

of the less familiar and discernible traits.

Descriptor list EXAMPLES [a]

TREE

1. Size (height) (m)
□ Low <3
□ Medium 3-8
□ High >8

2. Trunk diameter, 1m from collar (mm)
□ Small <70
□ Medium 70-110
□ Large >110

3. Shape of canopy
□ Globose (3a)
□ Elliptic (3b)
□ Pyramidal (3c)
□ Columnar (3d)

3a 3b 3c 3d

4. Density of canopy
□ Sparse (4a)
□ Medium (4b)
□ Dense (4c)

4a 4b 4c

5. Distance among scaffold branches (mm)
□ Low <250
□ Medium 250-400
□ High >400

6. Colour of bark
□ Light grey (6a)
□ Greyish-green (6b)
□ Greyish-brown (6c)

6a 6b 6c

7. Density of scales on the trunk
□ Sparse
□ Medium
□ Dense

8. Insertion angle of the scales on the trunk
□ Angle ≤ 30°(8a)
□ Angle >30° (8b)
□ Angle variable (8c)

8a 8b 8c

9. Homogeneity of the trunk scales
□ Homogeneous shape (9a)
□ Inhomogeneous shape (9b)

9a 9b

10. Lenght of the trunk scales (mm)
□ Short <35
□ Medium 35-50
□ Long >50

11. Widht of the trunk scales (mm)
□ Narrow <15
□ Medium 15-20
□ Large >20

BRANCHES

12. Length of the first internode on the primary branch (mm)
□ Short <25
□ Medium 25-50
□ Long >50

13. Length of the median internode on the primary branch (mm)
□ Short <15
□ Medium 15-25
□ Long >25

14. Density of branching: number of secondary branches on primary branch
□ Sparse <8
□ Medium 8-14
□ Dense >14

15. Density of scales on the secondary branch
□ Sparse (15a)
□ Medium (15b)
□ Dense (15c)

15a 15b 15c

16. Average number of branches per scaffold

17. Apparent diameter of the primary branch including scales (mm)

Wide section area	Narrow section area
□ <8	□ <6
□ 8-12	□ 6-9
□ >12	□ >9

18. Apparent diameter of the secondary branch including scales (mm)

Wide section area	Narrow section area
□ <6	□ <4
□ 6-8	□ 4-6
□ >8	□ >6

19. Uniformity of the apparent diameter on the primary branch
□ Uniform
□ Difform
□ Very difform

20. Uniformity of the apparent diameter on the secondary branch
□ Uniform (20a)
□ Difform (20b)
□ Very difform (20c)

20a 20b 20c

21. Insertion angle of the scales on the primary branch
□ Angle ≤ 45° (21a)
□ Angle >45° (21b)


21a


21b

LEAVES (scales)

22. Maximum length including mucron (mm)
□ Short <28
□ Medium 28-40
□ Long >40

23. Maximum width (mm)
□ Narrow <10
□ Medium 10-16
□ Wide >16

MALE INFLORESCENCES (catkins at the mature stage)

24. Catkin lenght (mm)
□ Short <81
□ Medium 81-123
□ Long >123

25. Catkin thickness (mm)
□ Thin <40
□ Medium 40-54
□ Thick >54

26. Catkin bending
□ Attenuated (26a)
□ Medium (26b)
□ Marked (26c)


26a


26b


26c

FEMALE STROBILES (pine-cones at harvest)

28. Pine-cone max. diameter (mm)
□ Small <170
□ Medium 170-200
□ Large >200

29. Maximum length of the scale including appendix (mm)
□ Short < 36
□ Medium 36-44
□ Long >44

30. Maximum width of the scale (mm)
□ Narrow <7
□ Medium 7-9
□ Wide >9

SEEDS and PRODUCTIVITY

31. Number of catkins

32. Number of female cones

33. Total number of seeds

34. Number of fertile seeds

35. Color of fertile seeds [b]
□ Reddish brown
□ Greenish brown
□ Dark brown

36. Length of the seed appendix excluded (mm) [b]
□ Short <37.8
□ Medium 37.8-45.6
□ Long >45.6

37. Width of the seed (mm) [b]
□ Narrow <12.3
□ Medium 12.3-16.5
□ Wide >16.5

38. Length of the appendix [b]
□ Short <33.3
□ Medium 33.3-48.3
□ Long >48.3

39. Prevailing shape of the seed
□ Elongated (39a)
□ Oblong (39b)
□ Obconical (39c)


39a


39b


39c

[a] Araucaria draws have been taken and modified from the website http://www.eryprihananto.com (© Ery Prihananto, Indonesia).
[b] Mean of 25 seeds.

4. Discussion and Conclusions

Very few studies have examined botanical aspects in *A. araucaria* following individual plants over their lifetimes, and all are rather dated and referred to plants grown under South America climatic conditions (Montaldo, 1974; Donoso and Cabello, 1978; Hoffman, 1982; Rodríguez *et al.,* 1983). No data concerning the phenology and growing of this species under both European and Italian environmental conditions have been reported elsewhere, except for a very old contribution to the understanding of cytology and sexual reproduction in *A. araucana* plants grown in Northern France (Favre-Duchartre, 1960). Søndergaard (2003) reported about new introductions of monkey puzzle to Scandinavia and the West coast of Norway, while Kubus *et al.* (2014) evaluated hardiness of *A. araucana* trees grown in open ground in Poland. However only data on annual shoot growth, tree height and degree of frost damages were given. On the other hand, phenological observations are some of the most sensitive data in identifying how plant species respond to regional climate conditions and to climatic changes.

Like all Gymnosperm, monkey puzzle has extremely simple flowers without any ornamental value. The male and female reproductive structures are carried by 'cones' and are as a rule separated, in

fact monkey puzzle is usually dioecious (Martinez, 1957; Bekessey *et al.,* 2002). Nevertheless, although being basically female, 4 out of 15 flowering trees were found to produce both male and female cones during recording in southwestern of Norway; moreover, on completely isolated male and female trees, a couple of cones of the other sex were observed in some years in the northernmost area (Søndergaard, 2003). The Author suggested a possible correlation between stress (by isolation and climatically exposed situations) and monoecious behavior in *A. araucana*. In our census, only one monoecious tree was found among all considered specimen (approx. 700 plants) of the Pistoia province. The ratio of females to males in the examined populations grown in plain area was 1:4 (on a total of approx. 25% differentiated trees) being biased towards the male sex, while a rather balanced sex ratio was observed in older (approx. forty-five-year-old) totally differentiated trees grown in high hills (Fig. 2). This finding is almost in accordance with sex occurrence reported by Søndergaard (1975), who determined half female and half male trees in a population with 76% flowering trees in the West coast of Norway. No other comparison with literature was possible for sex ratio, which has never been reported in elsewhere published data. Since the relationship between tree growth and climate appeared to be sex-dependant, in that male trees were more sensitive to land precipitation and female trees appeared more sensitive to air surface temperature during the prior period of growth (Hadad and Roig Juñent, 2016), it is probably realistic to assume that gender imbalance in favor of male or female at the beginning of sexual differentiation might vary according to different climatic environments, such as those found in Norway and Italy. On the contrary, day length did not seem to have a strong influence on the growth and development of the monkey puzzle (Søndergaard, 2003); as a matter of fact, day length dependant trees would never survive Scandinavian latitudes, since growth would begin too early and cease too late causing extensive frost damage and eventually killing the plant.

Fig. 2 - Totally differentiated *Araucaria araucana* female (A) and male (B) trees grown in Pistoia's high hills, showing strobiles and catkins formed in consecutive years.

Under the Tuscan environmental conditions, male catkins start out erect in July-August, stop growing during the winter season and then become elongated in shape, pendant and reddish-brown at maturity in May of the following year. Formed by many small scale-shaped leaflets, called microsporophyllus, they gave rise to a large quantity of pollen. In barely adult trees male caktins are found mostly in groups of 1-3 cones (Fig. 3), while older adult trees have groups of 2 up to 7-8 cones.

Fig. 3 - *Araucaria araucana* mature catkins during pollination.

First differences in the apex of potentially vegetative or female flower buds become visible at the end of March (Fig. 1D). In flower buds the apex develops into a round dome of 3-4 cm, with more elongated and less tight scales provided with long yellow-orange appendixes. Female inflorescences, having fertile scales which contain the ovules, called macrosporophylls, are distinguishable in April (Fig. 1E), grouped in light green strobiles at the extremity of the new sprouts. Greatest frequency of full bloom

and pollination was observed in May (Fig. 1C and 1F). Some differences were found in flower appearance among growing areas. Generally, flower buds developed two up to three weeks later when trees were grown at higher altitudes and experienced rigid winters. After fruit set, that occurs in April of year II (Fig. 1G), the sessile and generally solitary and immature female strobiles are erect, globular, with a symmetrical shape, and green colored. They take usually about 4-5 months to develop into ripe globular dark brown mature cones (Fig. 1I) and remain closed until the complete maturation of the seeds. Scales usually fall off at maturity in August-September of the same year, although an ongoing trend towards the postponement of fruit drop towards November-late December was observed as a consequence of the gradual rise in mean temperatures (Fig. 4). As already assessed in previous studies (Søndergaard, 2003; Sanguinetti, 2014), cones occurrence was found to vary in relation with sun exposure; more in details,

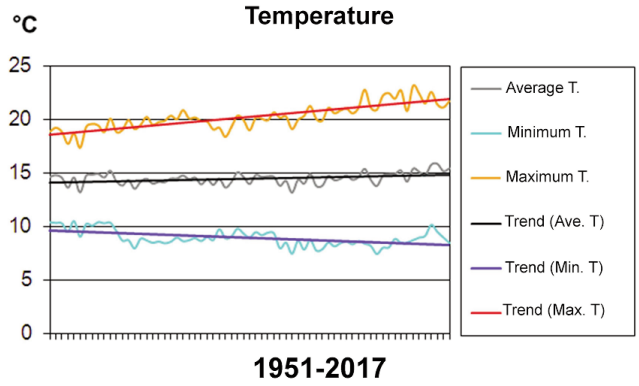

Fig. 4 - Minimum, maximum and average temperature values and trends in Pistoia (years 1951-2017) (http://www.cespevi.it/meteo.htm; Paolo Marzialetti © 1996/2018).

cones were most abundant in the South part of the crown. Almost all trees showed a marked alternate bearing; especially in males, yearly fluctuation in cone number seemed too large to be imputable only to climatic variations among years, particularly evident for rainfall (Fig. 5). Alternate bearing was found to be much more pronounced in barely sexually differentiated trees compared to plants older than fifty years, suggesting that in young plants alternate bearing represents a strategic mechanism to save nutrient reserves for significant vegetative growth.

Approx. 200-250 seeds, reddish to brown and oblong to obconical in shape, were released from each strobile; these range of seeds is fully included in those found out from the literature (150-300) (Montaldo, 1974; Donoso and Cabello, 1978; Salazar *et al.,* 2000). Negative effects of heavy rainfall on pollination and seed production have been reported (Sanguinetti *et al*., 2002).

Fructification began when plants were twenty-twenty-five-year-old, partially in agreement with reproductive organs appearance reported by Salazar *et al.* (2000) in Chilean *A. araucana* plants, while trees in Neuquén-Argentina have been reported to become sexually mature after thirty years of age, once the trunk has reached a diameter larger than 20 cm (Muñoz Ibáñez, 1984). Conversely, according to Søndergaard (2003), flowering was not initiated before the trees were forty/fifty-year-old in northern Europe and this discrepancy might be related to the altitude and latitude difference.

The ontogenetic stages presented here (Fig. 6) were in accordance with the *A. araucaria* biological

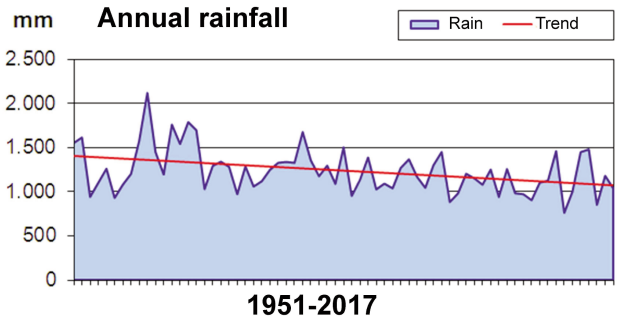

Fig. 5 - Annual rainfall values and trends in Pistoia (years 1951-2017) (http://www.cespevi.it/meteo.htm; Paolo Marzialetti © 1996/2018)

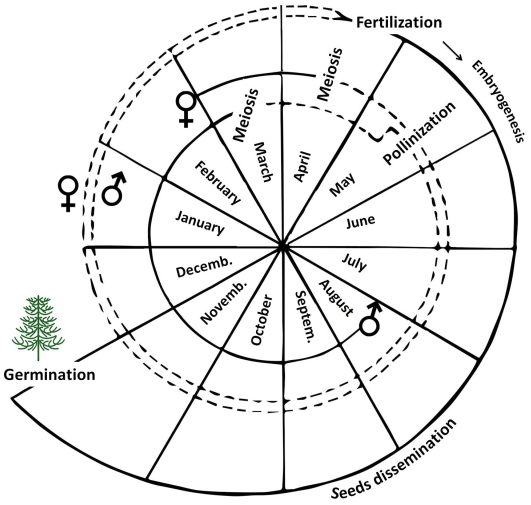

Fig. 6 - Schematic representation of the ontogenetic cycle of *Araucaria araucana*. Solid lines represent diploid phases (female and male gametogenesis) and embryogenesis after fertilization, while dashed lines represent haploid phases (ovules and pollen). (From Favre-Duchartre, 1960, modified).

cycle highlighted by Favre-Duchartre (1960) in northern France; moreover, differences in springtime temperatures between France and Norway corresponded fairly well to the differences in time (about one month) for release of pollen and seedfall at the two sites, as reported by Søndergaard (2003). On the contrary, as shown in Table 2, *A. araucana* phenology was found to differ strongly from that observed in its natural distribution in the Andes Mountains. Obviously, differences between months were principally due to the reversal of the hemispheres and consequentially of the seasons: when it is spring in Europe, it is autumn in the Andes and vice versa. However, changes seemed probably to be associated also to other factors, such as altitude, climate changes, growing conditions, etc. The effect of climate on the phenology, and in particular on the timing of reproduction, is well known for plants and extensively documented (Beebee, 1995; Stenseth and Mysterud, 2002). These studies have demonstrated that onset of reproduction in spring may have advanced by a week or two due to recent changes in climate over much of Europe, but to a much lesser extend in South America (Walther *et al.,* 2002; Stenseth *et al.,* 2002). Contrary to expectations, it was noticed that in males the appearance of the catkins takes place approximately in the same months (August-September in Chile and July-August in Italy), which, however, correspond to the end of summer in Italy and the end of winter in Chile. Moreover, in Chile the male catkins reach full maturity within 3-4 months, whereas in our environmental conditions full bloom occurred within 9-10 months. This could be explained by the fact that in Italy the further development of male inflorescence is stopped during the winter, while in Chile, where the season turns towards spring, caktins keep growing without interruption. On the other hand, maturation seems to be related to the photoperiod coinciding in both hemispheres with long days in late spring (beginning of December in Chile and end of May in Italy).

Similar considerations can be drawn for females as well. The time between female flower appearance (March in Italy and November in Chile) and maturation (end of May in Italy and beginning of December in Chile, as for males) is about 3 months in our country and 1 month in Chile. In both cases, the entry of the pollen tube into the ovary takes place within a month from the pollination. On the other hand, in our experimental conditions the true fertilization has been reported to occur after approx. 11 months (April of year II) from pollination (Favre-Duchartre, 1960), whereas timing of fecundation has never been mentioned in the literature under Andean environments. There are contradictory records about the time of seed maturation in Chile, as it is unclear if fruits release the seeds after 11-13 months (Donoso and Cabello, 1978) or 16-18 months (Tortorelli, 1956; Montaldo, 1974) from pollination. Data herein obtained showed that gravity seed dispersal, which usually takes place at the end of the Summer (approx. 12-13 months after pollination), moved towards Autumn (14-15 months after pollination) in 2016, and then towards early Winter (16-18 months after pollination) in 2017.

Descriptor lists include the basic description of the traits, and the different classes of their expression (characterization) or how to measure the range of their variation (evaluation). Most of the descriptors for characterization and evaluation are species-specific, but should be preferentially evaluated under homogeneous growing conditions in order to obtain comparable data and to avoid potential environmental influence on the phenotype. In our study, in order to find the most appropriate descriptors able for distinguishing effectively between individual phenotypes without loss of discriminating power, repeated observations were made on all plant material available in the Pistoia's nursery district. It immediately became clear that there were no evident differences among the expression of growing characters of young potted seedlings (Fig. 7), despite the very high number of individuals within the various collections, whereas a high degree of between- and within-population variability was noted among older trees (Fig. 8). A first analysis of the available specimen clearly evidenced that plant habitus as well as various other vegetative traits consistently varied with plant age,

Fig. 7 - *Araucaria araucana* seedlings in Pistoia's nurseries (A: two-year-old seedlings; B: six-year-old seedlings).

being this species a long-lived and massive tree up to 50 m tall and 2 m in diameter and attaining maximum ages of at least 1300 years (Montaldo, 1974). Moreover, different climatic patterns and growing conditions, such as plant density and weed control, might have influenced plant growth and habitus as well.

The variability observed here is consistent with the outbreeding dioecious reproductive habit of this species and suggests that these populations should continue to be viable and able to respond to moderate levels of environmental change (Hadad and Roig Juñent, 2016). However, recent phytopathological analysis in Tuscany have revealed an increase in mortality, especially of female individuals, without a specific pathogen responsible (Rizzo, 2017, personal communication). The most widely accepted theory is that weaker plants, due to climate-related stress, are more vulnerable to damage caused by aspecific pathogens.

Fig. 8 - Habitus variability in *Araucaria araucana* trees grown in Pistoia's hinterland in a plain area.

A correct germplasm characterization should consider adult plants, being necessary the presence of flowers and fruits. However, as the aim of the present study is the phenotyping of young cultivated plants for sale, only trees sufficiently young for trade but at the beginning of sexual differentiation were suitable for characterization. This is why only twenty/twenty-five-year-old specimen belonging to a subset of 4 putative populations grown under similar environmental and agronomic conditions were considered. Plants over 25 year-old were discarded as well as plants grown in nearby locations.

With respect to the 39 descriptors detailed in the descriptor list, some very peculiar characters, i.e. insertion angle of the scales on the trunk (N. 8), uniformity of the apparent diameter on the primary and secondary branch (N. 19 and 20), catkin bending (N. 26), were individuated beside some of the most common traits, such as shape and density of canopy (N. 3 and 4), maximum length and width of the leaves (N. 22 and 23). Undoubtedly, one of the tree's most distinguishing feature is its scales, which are stiff, dark green and glossy with a spiny tip and completely cover each branch, closely overlapping each other. But we found surprisingly interesting the size together with the insertion angle of the scales on primary and secondary branches, resulting in evident dissimilar apparent diameters of branches. On the contrary, female inflorescence didn't show any distinctive feature among populations, therefore it was not taken into account as a descriptor. In fact, while the true flower represents the main distinctive character in Magnoliophyta, the Pinophyta flowers, that are extremely simple, are not particularly relevant for the description of the species.

In our study, morphological traits of *A. araucana* were observed, measured and documented for the first time under Tuscan growing condition. In particular, the research was developed using trees from putative populations growing in the Pistoia's nursery district. The comparison of the observed phenotypic characteristics showed a wide range of variability among and within the considered populations. The resulting data allowed to classify accessions, and to build a catalogue of specific descriptors with embedded biological information that is an essential step towards germplasm phenotyping (in particular new variety description), management or for direct use in agriculture. Limitations linked to the potential environmental influence on the phenotype are presented as well. Apart from this preliminary study, nothing is known of the patterns of morphological variation within this species. The development of this descriptor list will assist in the systematic and objective recording and exchange of information, which in turn will increase utilization of genetic resources along with a better screening and use of *A. araucana* biodiversity for breeding programs. In order to analyze relationships between individuals or groups of specimens within locally grown populations, the morphometric characterization of *A. araucana* trees is in progress based on the defined descriptor list and suitable statistical multivariate approaches. Genetic

analysis of the same populations are being performed and will be processed in order to validate the discriminating efficiency of the presented descriptor list.

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Short note

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Tobacco dust waste as an alternative medium to grow geranium (*Pelargonium x hortorum*) plants

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Key words: agricultural waste, EC, ornamentals, peat, pH.

Abstract: Tobacco dust (TD) waste is the typical lignocellosic agricultural residue of cigarette processing. In the region of Peloponnese, cigarette production is carried out by the leading company of Karelias S.A. The production of TD waste of approx. 2-3 tons/month is a major problem for the company. Plant growth media containing peat (P) + 0, 5, 10, 25 or 50% TD were prepared and tested on geranium plant growth and development. The use of TD increased EC and pH of the final medium. Plants of the cvs "ML Diego" and "ML Sailing '12" grown in P+5% or in P+10% TD had similar height, number of leaves, number of flowers, photosynthetic activity and transpiration rates to the P alone (control) indicating that solid agro-industrial waste of tobacco could be used to partially substitute peat in growing medium for floricultural crop production.

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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

Pelargonium x hortorum or ''zonal geranium'' is an ornamental species that originate from South Africa, perfectly adapted to the Mediterranean region. It is propagated by cuttings and it is a hybrid between *P. inquisaus* (L.) L'Herit and *P. zonale* (L.) L'Herit (Dole and Wilkins, 2005). Zonal geraniums attract an increased commercial interest as they are extensively used in landscape designs and terrace gardens (Berninger, 1993). Flowering is strongly dependent on growth stage (i.e. juvenility), temperature (i.e. cold requirement) and sunlight (i.e. intensity and duration), but zonal geraniums are not described as long- or short-day plants (Fonteno, 1992; Dole and Wilkins, 2005).

Agricultural waste such as cotton gin trash, olive mill and green waste have been used in combination with peat for cultivation of ornamental plants (Papafotiou *et al.*, 2004; 2005; Grigatti *et al.,* 2007). The residue from tobacco processing (i.e. the tobacco dust; TD) is buried to landfields, but in high w/w concentrations can be toxic due to its high tannin and alkaloid content (Briski *et al.,* 2003). Compared to other waste material, TD contains higher N and K and has pH values ranging between 5.0 and 6.0 (Aderidan *et al.,* 2003). The application of TD waste in soil cultivated with lettuce increased yield compared to control plants (Okur *et al.,*

2008). It was suggested that incorporation of TD waste as an alternative organic amendment might improve soil chemical and biological parameters, as well as crop yield in soils containing low organic matter content.

Apparently, no previous research has been conducted on utilization of tobacco byproducts as alternatives to grow ornamental plants. In the present study, we tested growing mediums of peat + increasing concentrations of TD on growth and flowering response of zonal geraniums.

2. Materials and Methods

Plant material, media preparation and experimental lay-outs

Zonal geranium (*Pelargonium x hortorum*) rooted cuttings (3-5 leaves; up to 12 cm height) of cvs 'ML Diego' (red inflorescences) and 'ML Sailing'12' (white inflorescences) were provided by Selecta-one ltd (Kavala, Greece). Single rooted cuttings were transplanted in plastic 2.5 L pots filled with growing mediums of peat + TD. TD was provided by Karelias S.A. (Kalamata, Greece) and samples were analyzed in a continuous flow analyzer (CFA AA3; Seal-Analytical Ltd., Germany) (Table 1). Peat (Hawita, Germany) was mixed with 0, 5, 10, 25 and 50% (w/w) TD (Table 2). Six-replicate pots per treatment with geranium plants were placed on the ground of a non-heated greenhouse at the premises of the University of Peloponnese (lat. 37° 2' 20'' N, long. 22° 6' 51'' E) in a completely randomized design. Two individual experiments were carried out (one for each cultivar) from November 2017 to February 2018.

Medium properties, plant assessments and statistical analysis

Medium pH and EC (mS/cm) were measured using a pH/mV Meter (Delta OHM HD 2105.2, Padova, Italy) and a Conductivity Handheld Meter (Eutech Instruments, EcoScan CON 5, Singapore), respectively. Plant height (cm), number of leaves and number of inflorescences were recorded weekly over the entire cultivation period of nine weeks. Chlorophyll fluorescence, net CO_2 assimilation (A_s; μmol m^{-2} sec) and transpiration (E; mmol m^{-2} sec) were recorded using a handheld fluorimeter (OS-30p, Opti-Sciences, Inc. U.S.A.) and a LCpro+ portable photosynthesis system (ADC Bioscientific ltd. Great Amwell, Herts, UK), respectively, on the 5th, 6th, 7th and the 8th week from transplanting (i.e. week-1). Data means were separated using Duncan's multiple range test at P = 0.05. Statistical analysis was performed in SPSS v. 21.

3. Results

Tobacco dust contained nicotine, TSS and small amounts of nitrates and ammonia (Table 1). EC and pH values ranged between 8.92 and 9.46, and between 5.3 and 5.6, respectively. After mixing peat with 0 - 50% TD, pH and EC of the final growing medium increased linearly to reach the values of 5.42 and 6.18 mS/cm, respectively (Table 2).

Table 1 - Means and range values of tobacco dust content

Tobacco dust content	Means	Range
Nicotine (mg/L)	158.18	157.84 -160.14
TSS (mg/L)	583.12	581.36 -584.04
Nitrates (mg/L)	32.06	30.15-33.02
Ammonia (mg/L)	6.38	6.25 - 6.52
EC (mS/cm)	9.17	8.92-9.46
pH	5.5	5.3-5.6

Tobacco dust samples were analyzed in a continuous flow analyzer

Table 2 - pH and EC (mS/cm) values of growing media after mixing peat with tobacco dust at 0 (control), 5, 10, 25 and 50% (w/w)

Growing media	pH	EC (mS/cm)
Mixing peat	4.00	0.06
Mixing peat + 5% Tobacco dust	4.51	1.30
Mixing peat + 10% Tobacco dust	4.85	3.22
Mixing peat + 25% Tobacco dust	5.09	4.84
Mixing peat + 50% Tobacco dust	5.42	6.18

Peat replacement with 5 or 10% TD in the growing medium positively affected growth and flowering of cvs. "ML Diego" and "ML Sailing'12" geraniums (Table 3). Plants of cv. "ML Diego" grown in P+5% or P+10% TD maintained similar or higher height, number of leaves, number of inflorescences, net CO_2 assimilation and transpiration rates to the control plants (i.e. P+0% TD) (Table 3). However, plants grown in P+25 or P+50% TD suffered reductions in growth and flowering compared to the controls. Plants grown in P+25 or P+50% TD showed reduced number of inflorescences and transpiration rates, compared to the plants grown in P+5 or P+10% TD (Table 3). Stunting growth and reduced flowering of plants cultivated in P+25 or P+50% TD were justified

by decreases in chlorophyll fluorescence (F_v/F_m) ratios (Table 3). The F_v/F_m ratios ranged between 0.571 and 0.893 for plants grown in P+25% TD and between 0.538 and 0.835 for plants grown in P+50% TD indicating damage in plants' photosystem (PS II) that induced stress responses.

Plants of cv. "ML Sailing'12" grown in P+5, P+10 or P+25% TD, showed similar or higher number of leaves, number of inflorescences, net CO_2 assimilation and transpiration rates to the control plants (Table 3). However, plants grown in P+50% TD showed reduced height, number of leaves, number of inflorescences, indicating damage in plants' photosystem (PS II) recorded as low F_v/F_m ratio ranging between 0.538 and 0.773 (Table 3). Plants in P+50% TD failed to reach minimum growth requirements and standard commercial size.

4. Discussion and Conclusions

TD successfully replaced part of peat in growth medium for zonal geranium plant production. Plants of cvs. "ML Diego" and "ML Sailing 12" responded well when grown in P+5, P+10 and in few cases in P+25% TD and, therefore, could potentially replace part of peat in the growth medium. The concept of peat replacement with agricultural waste material for the cultivation of ornamental plants has been examined the past 20 years. Papafotiou *et al.* (2004) showed that olive-mill waste composts (OWC) could partially replace peat for the production of *Euphorbia pulcherrima* (poinsettia), although, at concentration of >12.5% delayed growth compared to plants cultivated in peat/perlite medium. Tropical potted plants such as *Syngonium podophyllum*, *Ficus benjamina* and *Codiaeum variegatum* could be successfully grown in 75% OWC without showing symptoms of toxicity or other negative effects on growth and development (Papafotiou *et al.,* 2005). Cotton gin trash compost (CGTC) and rice hulls (RH) were tested as peat replacements for the production of *Nerium oleander*, *Pelargonium zonale*, *Dedranthema grandiflora* and *Lantana camara* (Papafotiou *et al.,* 2001). Replacing peat with 60% of GCTC resulted in plant height decrease of all species, except those of *P. zonale* and increase in number of flowers to all species, except those of *D. grandiflora*. The use of green waste and sewage sludge compost (WSSC) at 80% - 20% (v:v) as a 25%-replacement of white peat, had positive effects on growth and flowering of *Begonia semperflorens, Mimulus hybridus, Tagetes patula x erecta* and *Salvia splendens* (Grigatti *et al.,* 2007). All species grown in 25% WSSC showed greater height, number of flowers and plant dry weight compared to plants grown in 100% white peat. TD is a potent agricultural byproduct that could be used in concentrations of <25% without affecting growth and quality of ornamentals. Replacing peat with byproducts of the agricultural sector, merits an eco-biological prospect of environmental-friendly ornamental production. Further research is needed to test TD as peat replacement for cultivation of other ornamental species.

Table 3 - Effect of growing medium of peat amended with 0, 5, 10, 25 and 50% TD on number of leaves, plant height, number of inflorescences, chlorophyll fluorescence, net CO_2 assimilation and transpiration of *P. x hortorum* plants of cvs "ML Diego" and "ML Sailing'12"

Treatments	Plant height (cm)	Range (cm)	Number of leaves	Range	Number of inflorescences	Range	Net CO_2 assimilation (μmol m^{-2}.sec)	Range (μmol m^{-2}.sec)	Transpiration (mmol m^{-2}.sec)	Range (mmol m^{-2}.sec)	Chlorophyll fluorescence (F_v/F_m)	Range (F_v/F_m)
ML Diego'												
0	8.83±0.25 ab	4-16	11.82±0.41 c	4-24	0.85±0.07 a	0-3	2.18±0.26 a	0.34-6.11	1.00±0.087 a	0.61-1.92	0.805±0.001 a	0.778-0.834
5	8.89±0.27 ab	2-18	13.50±0.66 b	4-33	0.86±0.09 a	0-6	3.01±0.34 a	0.24-7.14	1.36±0.170 a	0.71-2.57	0.801±0.002 a	0.685-0.828
10	9.12±0.24 a	5-17	15.12±0.68 a	2-36	0.93±0.10 a	0-5	2.40±0.29 a	0.22-5.32	1.28±0.184 a	0.42-2.34	0.802±0.002 a	0.753-0.878
25	8.39±0.23 b	4-15	12.11±0.55 bc	4-28	0.60±0.08 b	0-3	2.08±0.20 a	0.28-4.00	0.65±0.060 b	0.33-1.06	0.786±0.004 b	0.571-0.893
50	7.01±0.19 c	4-18	5.79±0.32 d	2-20	0.31±0.05 c	0-2	2.23±0.68 a	0.14-4.90	0.37±0.082 c	0.13-0.61	0.731±0.006 c	0.538-0.835
ML Sailing'12'												
0	9.70±0.33 a	5-16	14.11±0.62 bc	5-24	0.83±0.10 ab	0-3	2.07±0.32 b	0.34-4.07	0.89±0.096 a	0.29-1.40	0.801±0.001 a	0.778-0.819
5	9.53±0.38 a	2-18	15.79±1.00 ab	4-33	0.76±0.11 ab	0-3	3.40±0.52 a	0.24-7.14	1.04±0.161 a	0.30-2.72	0.793±0.003 a	0.685-0.818
10	9.07±0.34 a	5-17	16.68±1.01 a	4-36	0.98±0.16 a	0-5	2.40±0.62 ab	0.22-5.32	1.17±0.232 a	0.32-2.27	0.799±0.001 a	0.777-0.824
25	7.64±0.30 b	4-14	12.94±0.83 c	4-28	0.53±0.11 b	0-3	1.77±0.40 b	0.28-3.69	0.48±0.057 b	0.26-0.71	0.779±0.005 a	0.668-0.893
50	6.25±0.14 c	4-9	5.01±0.16 d	3-7	0.20±0.06 c	0-1	-	-	-	-	0.678±0.008 b	0.538-0.773

Data are means ± SE of 9-week recordings and letters indicate the statistical differences according to Duncan's multiple range test at P = 0.05.

- not measured. Plants failed to reach minimum growth requirements.

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