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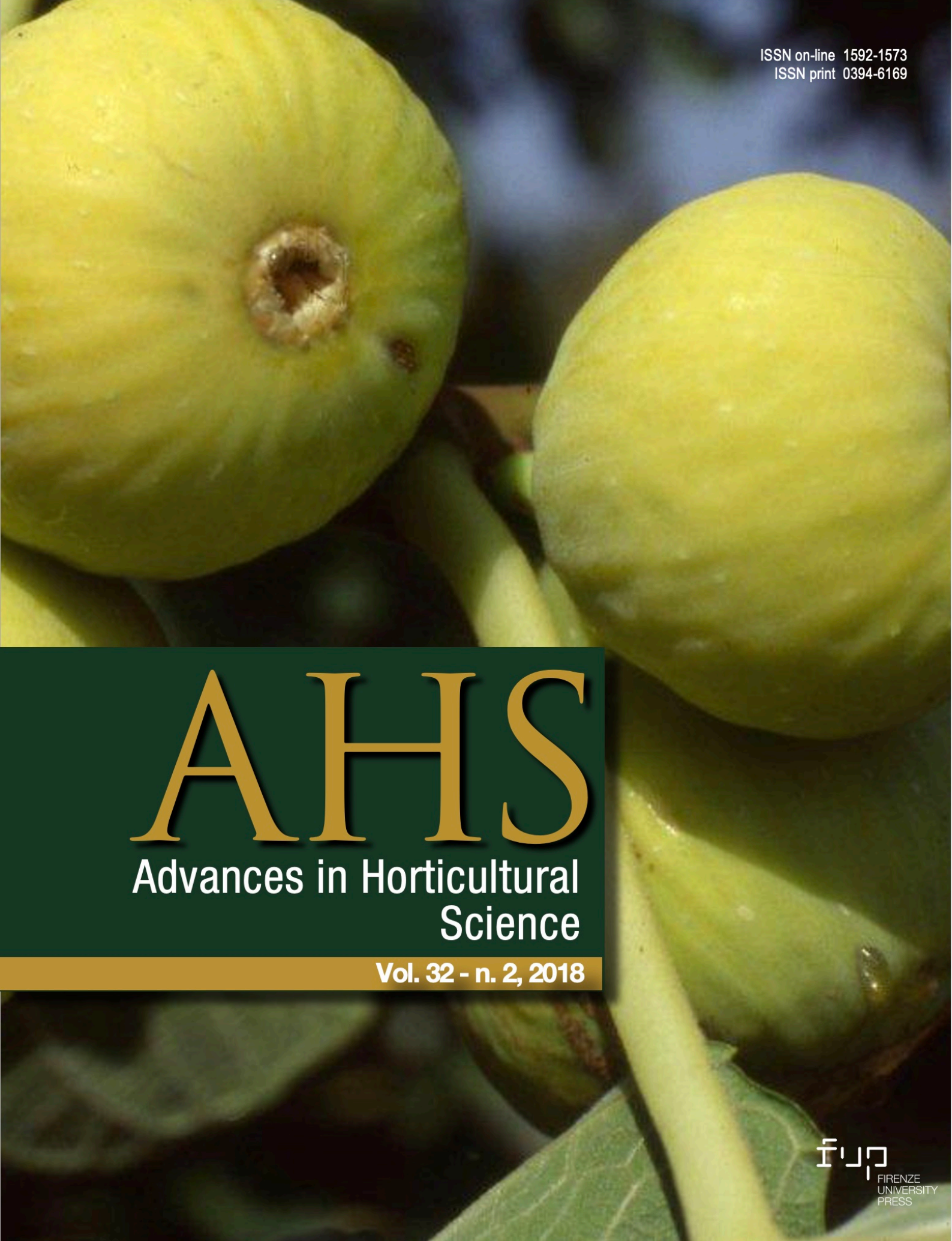

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Biochemical and physiological evaluations of common bermudagrass [*Cynodon dactylon* (L.) Pers.] Iranian accessions under cold stress

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Key words: cold tolerance, *Cynodon*, Iranian accessions, physiological characters.

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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: In this study, one foreign cultivar and forty-nine common bermudagrass accessions were collected from 18 provinces of Iran. Turfgrasses were grown at four temperature regimes (24/17, 7.5/0, -7.5/-12 and -15/-15°C day/night cycles) in a factorial experiment based on the completely randomized design with three replications. Physiological traits were evaluated to categorize all accessions as either cold sensitive or tolerant using Hierarchical Clustering with Ward's method in SPSS software. Our results revealed that cold-tolerant common bermudagrass accessions showed higher proline, protein, antioxidant enzymes, color, visual quality and chlorophyll content and cold-sensitive accessions showed more severe cell membrane damage (EL) under cold stress conditions. Fall in temperature from 24°C severely decreased chlorophyll content, visual quality and color in all accessions. The highest antioxidant enzymes activity, chlorophyll content, color and visual quality at -7.5°C were observed in Taft, foreign cultivar, Naein, Malayear, Aligoudarz, Safashahr and Gorgan accessions. The increase in POD, SOD, CAT and APX activity observed in this study led to protection against oxidative damage caused due to high ROS levels. The most cold-tolerant accessions at -15°C were Taft, Naein and Malayear. Great variations in freezing tolerance were observed between Iranian accessions of common bermudagrass. Further molecular studies are needed to clarify better these findings.

1. Introduction

Cold stress is the main serious problem that limits plant growth, agricultural productivity, survival, as well as geography of plant distribution. Common bermudagrass (*Cynodon dactylon* [L.] Pers.), from the grass (Poaceae) family, is a typical creeping grass grows in warmer parts of all continents between about 45 degrees north and 45 degrees south latitude (Harlan and de Wet, 1969; Anderson *et al*., 2003). This perennial, herbaceous, warm season, C4 grass is commonly known as 'Chaiar' or

'Margh' in Iran. Common bermudagrass's high density, recuperative ability, high tolerance to drought, heat, salinity, wear, flood and most of soils cause the species to be extensively used in tropical and subtropical regions of Iran. This species is a major turfgrass for livestock herbage, golf courses, sport fields, public parks and soil conservation. Despite its good characters, *C. dactylon* has a considerable tendency to be damaged or killed by frost, especially in transition zone (Munshaw *et al*., 2006). An important process in the winter perpetuity of common bermudagrass is acclimatization, which is an adaptation process to overcome the environmental stresses (Levitt, 1980). The most favorable temperature for root and shoot growth of cool-season turfgrass species varied from 10 to 18°C and 18 to 24°C, respectively. Warm season turfgrasses have C4 photosynthetic pathway and are best adapted to warm climatic region of the world and grows well at temperatures between 24 to 29°C and 27 to 35°C for root and shoot growth, respectively (Beard, 1973). During cold stress, plants exhibit different mechanisms to develop their cold hardiness and increase their freezing tolerance (Zhu *et al*., 2004; Knight and Knight, 2012). Some of these processes includes changes in the concentration of amino acids, sugars, proteins, compatible solutes, certain hormones, and changes in the degree of fatty acid saturation level and antioxidant capacity that affect the freezing tolerance (Karpinski *et al*., 2002; Munshaw *et al*., 2006; Zhang and Ervin, 2008).

Genetic resources and wild plant species that genetically related to cultivated variety have gross value in plant breeding programs (Hajjar and Hodgkin, 2007). Today, many investigations have focused on the naturally occurring genetic differences in stress tolerance of many plants such as *Lolium perenne* L., *Brachypodium distachyon* L. and *Festuca arundinacea* Schreb. (Luo *et al*., 2011; Hu *et al*., 2012; Salehi *et al*., 2013). For many years, improvement of warm season turfgrass quality and cold tolerance are the main goals in breeding programs. Natural populations of bermudagrass should have considerable genetic variation for tolerance to environmental stresses. Since bermudagrass is cosmopolite plant, its considerable genetic variation is predictable. There is great diversity among wild populations and cultivars of common bermudagrass for tolerance to freezing (Anderson *et al*., 2003) and other environmental stresses. In spite of that, there is little data about the cold tolerance of common bermudagrass, and many researches are being conducted to improve cold tolerance of this species (Zhang *et al*., 2011; Shi *et al*., 2015). The aims of the present research were to evaluate Iranian common bermudagrass accessions to find accessions with good freezing tolerance and examine physiological changes during cold stress.

2. Materials and Methods

Forty-nine accessions of natural common bermudagrass were collected from 18 provinces of Iran with different climatic conditions ranging from Shiraz city with subtropical condition and Tabriz city with temperate climatic condition (Fig. 1), and one foreign cultivar 'Blackjack' was used as control. All accessions were collected originally from grasslands, roadside, seaside, and around orchards and agriculture fields, and transferred to the School of Agriculture, Shiraz University, under natural greenhouse condition (52°32' E and 29°36' N, elevation

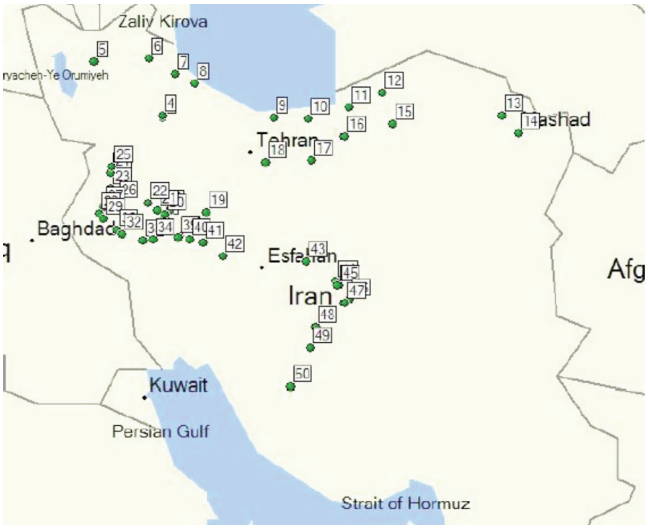

Fig. 1 - Map showing the sampling locations of common bermudagrass accessions from different regions of Iran. 1: Boroujerd, 2: Malayear, 3: Ghidar, 4: Zanjan, 5: Tabriz, 6: Sarein, 7: Talesh, 8: Anzali, 9: Nour, 10: Sari, 11: Gorgan, 12: Minoudasht, 13: Chenaran, 14: Mashhad, 15: Maiami, 16: Damghan, 17: Semnan, 18: Tehran, 19: Arak, 20: Malayear intersection, 21: Nahavand, 22: Firouzan, 23: Kamiaran, 24: Dehgolan, 25: Sanandaj Abidar, 26: Kermanshah Taghbostan, 27: Mahidasht, 28: Islamabad gharb, 29: Homail, 30: Ilam Saymareh bridge, 31: Holailan, 32: Poldokhtar, 33: Mamoulan, 34: Khoram abad, 35: Foreign cultivar, 36: Doroud Nahalestan, 37: Doroud Daneshjo park, 38: Doroud Siahvel, 39: Doroud Babahour, 40: Azna, 41: Aligoudarz, 42: Daran, 43: Naein, 44: Ardakan 1, 45: Ardakan 2, 46: Yazd, 47: Taft, 48: Abarkouh, 49: Safashahr, 50: Shiraz.

1810 m a.s.l.). Each accession was transplanted into 14 cm diameter pots filled with uniform mixture of 1:1:1 (v:v:v) of sand, loamy soil and decomposed manure. Turfgrasses were kept in natural greenhouse condition and were clipped to a height of 5 cm every 2 weeks. Low and freezing temperature treatments were conducted at 24/17, 7.5/0, -7.5/-12 and -15/-15°C day/night cycles and a 10 h light (300 µmol m^{-2} s^{-1}) for 7 days, using a controlled-environment chamber. After each temperature regime, physiological traits including: superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971), catalase (CAT) (Dhindsa *et al*., 1981), ascorbate peroxidase (APX) and peroxidase (POD) (Chance and Maehly, 1995) activities, proline (Bates *et al*., 1973), protein (Bradford, 1976), electrolyte leakage (Saadalla *et al*., 1990) and chlorophyll content (Saini *et al*., 2001) were measured. Turfgrass color and visual quality were rated visually after each treatment (Beard, 1973).

To extract antioxidant enzymes, fresh leaf or stolon samples (0.5 g) were collected and ground to a fine powder in a mortar by adding liquid nitrogen and then homogenized with an ice cold enzyme extraction buffer containing 0.5% polyvinylpyrrolidone (PVP), 3 mM EDTA, and 0.1 M potassium phosphate buffer (pH=7.5). The extracted samples were centrifuged for 10 min at 13500 rpm and 2-4°C and stored on ice until used. The resulting supernatant was used for enzyme analysis. SOD activity was determined according to the procedure used by Beauchamp and Fridovich (1971), CAT activity was determined as described by Dhindsa *et al*. (1981), ascorbate peroxidase and peroxidase activities were determined according to the method described by Chance and Maehly (1995). Proline was determined according to the method described by Bates *et al*. (1973). Using spectrophotometer (UV-120-20, Japan) at 520 nm wavelength, appropriate proline standards were included in calculation of its content in samples. The protein content was quantified using Bradford method with bovine serum albumin (BSA) as standard. Electrical leakage measured, as described by Saadalla *et al*. (1990), using an electrical conductivity meter (Metrohm 644, Swiss) and calculated with the following formula:

$$\text{Electrolyte leakage} = EC1/EC2 \times 100$$

Chlorophyll content was measured according to the method of Saini *et al*. (2001) using the following formula:

$$\text{mg Chl/g f.w.} = \frac{[(20.2(OD\ 645\ nm) + (8.02(OD\ 663\ nm)] \times V}{f.w. \times 1000}$$

where: OD is optical density, V is the final solution volume in ml, and f.w. is tissue fresh weight in mg.

Turfgrass color and visual quality were measured after each treatment on a 1 to 9 scale where 1 was very poor quality turf, 6 was minimally acceptable turf, and 9 was exceptional turf quality (Beard, 1973). This study was conducted in a factorial experiment based on completely randomize design (CRD) with three replications. Factors were fifty accessions and four different concentrations of low and freezing temperatures (24/17, 7.5/0, and -7.5/-12 day/night cycles). In the case of treatment with -15/-15°C day/night cycles, only the seven most cold tolerant accessions were evaluated. Mean comparisons were performed using the least significant difference (LSD) at P= 0.05 probability level. Physiological traits were evaluated for accession clustering to determine cold sensitive or cold tolerant using Ward's method of Hierarchical cluster analysis in SPSS software.

3. Results

The Ward cluster analysis based on physiological traits before low temperature treatments (at 24/17°C day/night cycles) grouped the 49 accessions and the foreign cultivar into two major groups (Fig. 2). The first group contained 18 accessions with low antioxidant enzymes activity, proline, protein and chlorophyll content including: Abidar Sanandaj, Boroujerd, Holailan, Malayear, Ghidar, Nour, Saymareh bridge, Anzali, Islamabad gharb, Tehran, Tagh bostan, Kermanshah, Homail, Maiami, Minodasht, Mashhad, Poldokhtar, Safashahr and Shiraz. The second group contained other accessions with more antioxidant enzymes activity, proline, protein and chlorophyll content. Two major groups were formed based on physiological characters after cold stress (at 7.5/0°C day/night cycles) (Fig. 3). The first group contained 36 accessions with low proline, protein, chlorophyll content and low antioxidant enzymes activity. Other accessions were in second group and had more proline, protein, chlorophyll content and more antioxidant enzymes activity included fourteen accessions: Arak, Doroud daneshjo park, Azna, Taft, Safashahr, Ardakan 2, Mahidasht, Mamoulan, Naein, Yazd, Chenaran, Semnan, Homail and Daran. The dendrogram from physiological characters after cold stress (at -7.5/-12°C day/night cycles) grouped the 50 accessions in two main clusters (Fig. 4). The dendrogram from physiological characters at -15/-15°C day/night cycles grouped the 7 most cold-tolerant genotypes into two main groups (Fig. 5). The first group contained 3 accessions with lower proline, pro-

tein, chlorophyll content and antioxidant enzymes activity than other elite accessions were: Aligoudarz, Gorgan and Safashahr. Other accessions were in second group and had more antioxidant enzymes activity, proline, protein, chlorophyll, color and visual quality, and the least EL included: foreign cultivar, Malayear, Naein and Taft.

POD, CAT, SOD, APX, proline, protein, EL, chlorophyll, color and visual quality were influenced by cold and freeze temperatures. With drop in temperature from 24 to 7.5°C, POD, CAT, SOD, and APX activities, and proline and protein content increased in all accessions; and from 7.5°C to -7.5°C significantly decreased the same parameters. The highest antioxidant enzymes activity, chlorophyll content, color and visual quality at -7.5°C were observed in Taft, foreign

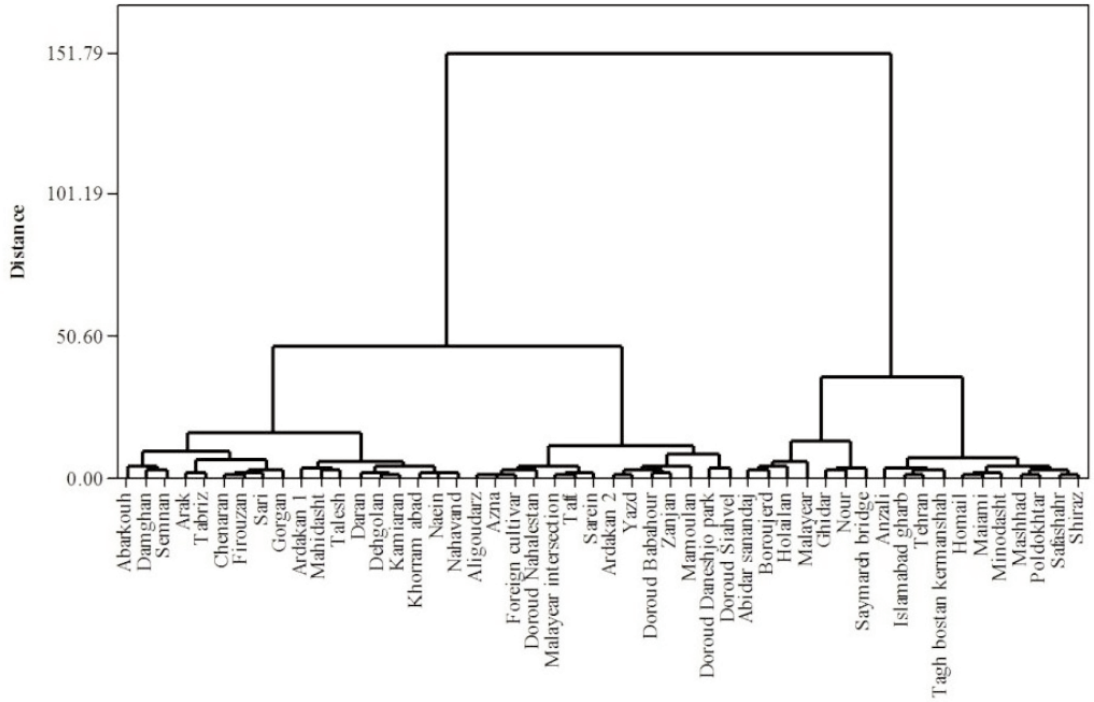

Fig. 2 - Dendrogram of the physiological relationships between 50 accessions of common bermudagrass and control cultivar before cold stress (at 24/17°C day/night cycles).

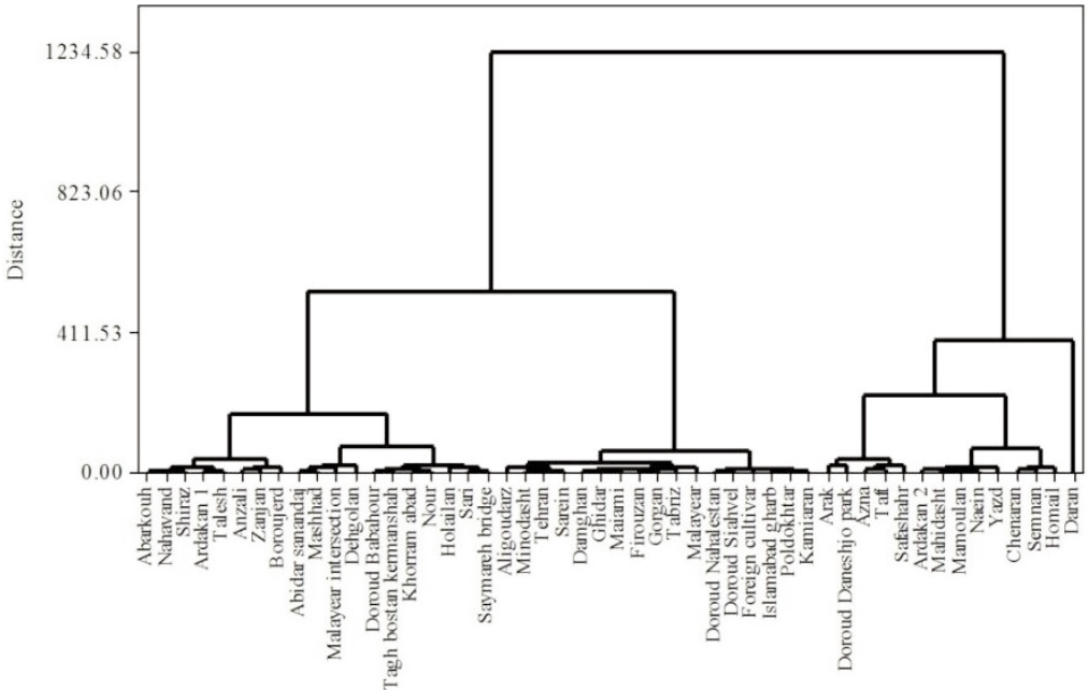

Fig. 3 - Dendrogram of the physiological relationships between 50 accessions of common bermudagrass and control cultivar aftrer cold stress at 7.5/0°C day/night cycles.

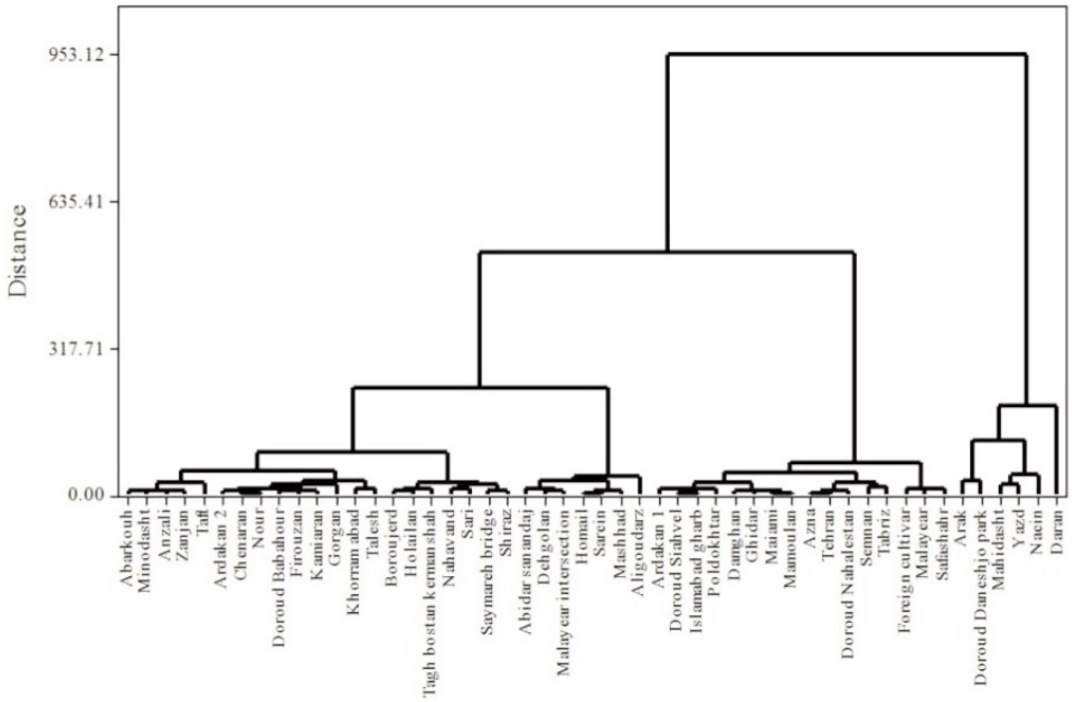

Fig. 4 - Dendrogram of the physiological relationships between 50 accessions of common bermudagrass and control cultivar after cold stress at -7.5/-12°C day/night cycles.

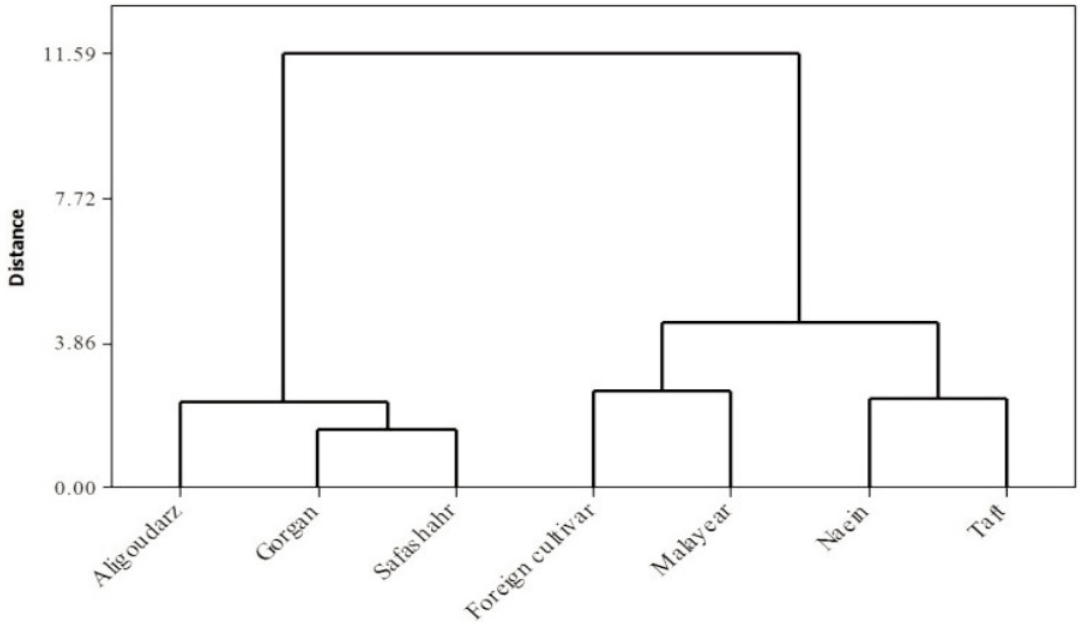

Fig. 5 - Dendrogram of the physiological relationships between 7 cold-tolerant accessions of common bermudagrass after cold stress at -15/-15 °C day/night cycles.

cultivar, Naein, Malayear, Aligoudarz, Safashahr and Gorgan accessions.

Peroxidase

The results of peroxidase assay at 24°C showed that its maximum and minimum activity were belonged to Gorgan and Arak accessions, respectively (Table 1). The highest peroxidase activity at 7.5°C and -7.5°C was observed in Malayear accession. The maximum peroxidase activity at -15°C was observed in Naein accession and the second rank was belonged to common bermudagrass accession collected from Taft.

Catalase

The results of catalase assay at 24°C showed that

its maximum activity was belonged to Ghidar and Nour accessions, and its minimum activity was belonged to Abarkouh accession (Table 1). The highest catalase activity at 7.5°C and -7.5°C was observed in Doroud daneshjo park and Taft accessions, respectively (Tables 2 and 3). The maximum catalase activity at -15°C was observed in Taft accession and the second rank was belonged to common bermudagrass accession collected from Malayear (Table 4).

Superoxide dismutase

The results of SOD assay showed that there was difference between SOD activities of different accessions in all temperature regimes. The maximum and minimum superoxide dismutase activity at 24°C was belonged to Doroud nahalestan and Mamoulan

Table 1 - Amount of POD, CAT, SOD, APX, CHL, Proline, EL, protein, color and visual quality of different Iranian accessions and foreign common bermudagrass before cold stress (at 24/17°C day/night cycles)

Location of accession	POD (U mg^{-1} f.w.)	CAT (U mg^{-1} f.w.)	SOD (U mg^{-1} f.w.)	APX U mg^{-1} f.w.)	CHL	Proline (µmol g^{-1} f.w.)	EL (%)	Protein (mg g^{-1} f.w.)	Color	VQ
Doroud Nahalestan	13.26	36.11	120.50	81.25	4.06	13.20	11.90	15.20	7.2	7.1
Abarkouh	13.17	34.12	119.10	79.12	3.68	13.10	13.40	14.30	6.3	6.5
Abidar sanandaj	12.45	35.62	118.60	59.85	3.95	13.20	11.80	14.60	7.1	7.2
Aligoudarz	13.54	36.11	119.50	79.56	4.02	13.40	11.30	14.80	7.0	7.1
Anzali	13.36	34.25	116.40	69.55	3.78	12.80	12.60	14.90	6.2	7.3
Arak	12.11	35.25	119.70	75.25	3.92	13.10	12.70	14.90	7.0	7.1
Ardakan 1	12.26	36.11	116.80	72.12	3.97	12.70	12.30	14.30	7.1	7.2
Ardakan 2	13.21	35.17	117.50	81.21	4.02	13.10	12.50	14.80	7.0	7.0
Azna	13.12	36.24	118.60	80.12	3.96	13.50	11.30	14.90	7.6	8.1
Boroujerd	12.25	35.24	118.50	59.21	4.36	12.10	10.50	14.30	8.5	9.0
Chenaran	12.18	35.14	118.90	78.56	4.35	13.40	11.10	14.30	8.4	8.6
Damghan	12.17	36.24	117.50	77.56	3.81	12.50	13.70	13.80	6.0	6.2
Daran	13.18	35.59	117.30	74.21	4.09	12.90	11.80	13.50	7.0	7.2
Dehgolan	13.11	34.35	117.90	74.32	3.89	13.10	11.60	14.50	7.6	7.2
Doroud Babahour	13.14	36.12	116.50	79.65	3.94	13.40	11.20	15.20	7.8	8.0
Doroud Daneshjo park	13.21	35.59	117.40	84.21	4.41	13.50	10.80	15.30	8.2	8.5
Doroud Siahvel	13.54	36.15	114.50	85.17	4.51	13.30	10.40	15.10	8.5	9.0
Firouzan	12.45	35.14	118.50	77.89	3.89	13.20	11.30	14.30	7.0	7.0
Foreign cultivar	13.55	36.25	119.50	79.81	4.47	13.10	11.20	15.10	8.7	8.5
Ghidar	13.31	36.28	115.80	67.18	3.85	12.70	12.30	13.80	7.7	7.5
Gorgan	13.65	35.17	116.50	77.21	4.38	13.10	10.60	13.90	8.2	8.3
Holailan	12.58	35.18	115.50	58.52	4.12	12.40	12.50	14.50	7.2	7.4
Homail	13.26	35.26	117.80	68.74	3.96	12.90	12.40	14.70	7.6	7.4
Islamabad gharb	13.14	34.16	118.60	69.52	3.76	13.10	13.40	15.30	6.1	6.2
Kamiaran	13.89	34.23	117.60	75.12	4.02	13.40	11.50	14.50	7.2	7.3
Khorram abad	13.23	35.35	115.40	74.25	4.39	12.50	11.90	14.70	8.1	8.4
Mahidasht	13.45	36.21	118.30	72.32	4.32	13.50	10.60	15.10	8.6	9.0
Maiami	12.54	35.25	118.20	68.25	3.98	12.90	11.60	13.90	7.4	7.5
Malayear	13.12	36.12	119.40	63.14	4.18	13.20	11.10	15.20	7.3	8.0
Malayear intersection	13.24	36.25	118.60	81.32	4.32	13.40	11.20	14.50	8.0	8.2
Mamoulan	13.14	35.21	113.70	81.23	3.92	12.30	12.40	14.60	7.4	7.2
Mashhad	13.11	36.15	119.20	69.65	4.32	13.10	10.80	14.40	8.1	8.2
Minodasht	13.14	36.15	117.70	69.54	4.06	13.20	10.90	13.80	7.2	8.4
Naein	13.85	35.16	115.30	75.16	4.06	13.20	11.20	14.20	7.3	7.1
Nahavand	13.55	36.12	116.50	75.14	4.07	13.10	11.70	15.10	7.6	7.4
Nour	12.51	36.28	115.30	65.23	4.41	11.90	13.10	14.80	8.0	7.6
Poldokhtar	12.57	35.18	119.40	69.85	4.37	12.10	11.70	14.80	8.0	8.2
Safashahr	13.35	35.12	118.70	71.21	4.05	13.10	12.20	15.10	7.4	7.5
Sarein	13.15	35.15	119.20	81.25	4.81	13.20	10.90	15.30	9.3	8.4
Sari	13.12	35.21	117.50	78.54	3.96	12.50	11.20	14.60	7.8	8.2
Saymareh bridge	13.21	35.62	117.50	64.21	4.09	12.60	13.10	14.60	7.0	6.7
Semnan	13.32	35.27	116.50	76.5	3.35	12.40	14.70	13.80	5.3	4.2
Shiraz	12.56	35.52	119.10	70.25	3.89	12.80	12.50	14.80	7.1	7.2
Tabriz	12.74	34.18	120.10	75.65	4.38	12.90	11.10	14.60	8.2	7.6
Taft	13.54	36.18	118.60	82.35	4.49	13.20	11.20	14.90	8.3	8.1
Tagh bostan kermanshah	12.46	35.23	117.60	68.65	3.81	12.80	13.10	14.10	6.2	5.7
Talesh	12.18	36.14	117.50	74.32	4.85	13.50	10.80	14.70	9.0	8.1
Tehran	13.21	34.56	118.80	69.95	3.89	12.50	13.80	14.10	6.5	6.2
Yazd	12.96	35.11	116.40	81.24	3.95	12.80	12.60	14.50	7.2	7.5
Zanjan	12.25	35.62	117.20	81.25	3.96	13.40	11.40	15.10	7.5	8.0
LSD (5%)	0.458	0.264	1.860	0.650	0.508	0.320	1.520	0.458	1.452	1.286

Turfgrass visual quality and color based on a scale of 1-9, 1= brown/dead turf, 6= minimal acceptable turf, 9= ideal green, healthy turf.

accessions, respectively. The highest superoxide dismutase activity at 7.5°C and -7.5°C was observed in Doroud daneshjo park accession (Tables 2 and 3). The maximum superoxide dismutase activity at -15°C was observed in Taft accession and the second rank was belonged to common bermudagrass accession collected from Naein (Table 4).

Ascorbate peroxidase

The results of ascorbate peroxidase assay showed that there was difference between ascorbate peroxidase activity of different accessions in all temperature regimes. The maximum and minimum ascorbate peroxidase activity at 24°C was belonged to Doroud siahvel and Holailan accessions, respectively. The

Table 2 - Amount of POD, CAT, SOD, APX, CHL, Proline, EL, protein, color and visual quality of different Iranian accessions and foreign common bermudagrass after cold stress (at 7.5/0°C day/night cycles)

Location of accession	POD (U mg^{-1} f.w.)	CAT (U mg^{-1} f.w.)	SOD (U mg^{-1} f.w.)	APX (U mg^{-1} f.w.)	CHL (mg g^{-1} f.w.)	Proline (µmol g^{-1} f.w.)	EL (%)	Protein (mg g^{-1} f.w.)	Color	VQ
Doroud Nahalestan	20.90	40.86	143.0	125.00	2.12	19.6	59.6	19.1	4.8	4.9
Abarkouh	21.73	42.29	136.0	108.57	2.28	21.9	57.8	21.8	5.7	5.6
Abidar sanandaj	25.64	40.86	132.5	59.29	2.17	20.8	59.3	19.0	5.0	5.1
Aligoudarz	22.18	42.75	138.5	148.57	3.11	26.4	52.5	21.2	7.0	7.1
Anzali	26.39	41.02	147.5	91.43	2.28	21.6	58.2	18.7	5.5	5.6
Arak	23.38	41.17	128.5	249.29	2.31	22.2	56.4	18.4	5.7	5.9
Ardakan 1	22.86	41.12	132.5	105.15	2.27	19.8	57.5	18.5	5.1	5.2
Ardakan 2	22.78	40.96	136.0	185.71	2.31	21.7	56.8	19.2	5.3	5.3
Azna	23.38	42.50	144.0	224.29	2.41	25.3	56.2	20.3	6.0	6.2
Boroujerd	15.19	41.48	140.0	91.43	2.24	21.3	57.4	19.8	5.2	5.3
Chenaran	24.36	43.16	136.0	156.43	2.31	21.9	57.5	18.9	5.8	5.9
Damghan	18.20	41.48	135.5	137.14	2.21	22.3	58.1	19.2	5.1	5.2
Daran	22.78	43.46	139.7	427.14	2.41	27.3	55.1	18.6	6.0	6.2
Dehgolan	23.53	44.69	137.0	42.14	2.34	21.9	58.2	18.6	5.7	5.9
Doroud Babahour	15.94	40.71	132.0	81.43	2.29	21.9	58.2	19.7	5.7	5.6
Doroud Daneshjo park	17.22	44.94	151.0	245.00	2.41	21.6	54.9	20.2	5.9	6.1
Doroud Siahvel	17.82	41.12	137.8	127.86	2.31	21.5	57.5	19.8	5.7	5.8
Firouzan	24.36	43.52	132.5	135.69	2.36	22.4	56.8	19.1	5.7	5.8
Foreign cultivar	22.11	42.24	137.5	122.86	3.28	27.9	51.1	21.5	7.5	7.5
Ghidar	20.08	42.44	138.5	132.71	2.26	21.5	58.5	18.7	5.4	5.5
Gorgan	24.14	41.99	129.5	135.00	2.35	27.3	56.5	18.8	6.4	6.5
Holailan	26.92	42.35	145.5	75.24	2.31	21.9	56.4	18.7	5.8	5.9
Homail	19.40	42.95	142.0	170.00	2.19	19.7	57.2	18.5	5.0	5.2
Islamabad gharb	21.82	41.85	141.4	120.24	2.15	19.5	57.5	18.8	5.0	5.1
Kamiaran	26.62	43.21	139.0	120.00	2.31	21.7	57.4	19.4	5.6	5.5
Khorram abad	24.59	42.55	130.0	85.71	2.35	21.8	57.5	18.7	5.7	5.8
Mahidasht	23.31	44.08	135.5	189.29	2.37	24.9	54.5	18.9	6.1	6.2
Maiami	22.41	41.37	136.0	132.72	2.35	23.2	55.5	18.5	6.0	6.1
Malayear	34.14	41.78	138.0	134.29	3.21	27.2	51.5	21.5	7.4	7.4
Malayear intersection	19.55	41.83	146.0	57.14	2.33	22.4	56.5	19.3	5.8	5.9
Mamoulan	22.78	43.98	139.5	191.43	2.39	24.3	55.5	19.1	6.0	6.1
Mashhad	22.56	42.95	136.0	62.86	2.28	21.5	57.2	19.1	5.7	5.8
Minodasht	20.38	42.09	147.0	140.00	2.25	21.5	57.9	19.1	5.2	5.4
Naein	23.08	42.29	139.0	191.43	3.26	27.5	51.2	18.7	7.5	7.6
Nahavand	24.74	41.22	135.5	110.71	2.26	21.8	57.2	20.8	5.5	5.4
Nour	24.66	42.90	133.5	89.14	2.21	20.7	57.1	18.6	5.0	5.3
Poldokhtar	22.33	40.81	139.2	121.43	2.37	22.5	54.5	18.6	6.0	6.2
Safashahr	21.95	41.53	132.5	212.86	2.38	27.1	53.5	21.3	6.4	6.3
Sarein	23.91	43.31	144.0	140.71	2.31	22.6	56.1	20.5	5.5	5.8
Sari	21.73	43.06	129.5	71.43	2.32	21.8	57.2	18.5	5.8	5.7
Saymareh bridge	25.86	40.96	129.5	75.00	2.34	19.8	57.8	19.4	5.7	5.1
Semnan	23.98	42.18	128.6	161.43	1.86	17.8	59.5	19.5	4.1	4.2
Shiraz	23.08	41.42	137.3	115.29	2.15	19.8	57.8	20.1	5.0	5.2
Tabriz	25.41	41.22	136.5	142.14	2.15	19.8	59.4	18.4	4.9	4.9
Taft	25.26	42.80	142.4	232.14	3.36	27.2	50.5	22.1	7.7	7.7
Tagh bostan kermanshah	21.23	41.46	134.5	81.74	2.14	26.4	59.8	17.1	5.0	4.9
Talesh	21.34	41.36	135.5	102.20	1.15	18.9	62.4	14.9	3.3	3.4
Tehran	19.85	41.27	143.5	140.71	2.24	21.4	57.3	18.7	5.2	5.2
Yazd	25.94	42.65	126.0	184.29	2.11	21.7	58.5	17.4	4.9	4.8
Zanjan	23.76	42.80	145.5	101.43	2.34	26.1	56.1	19.1	6.0	5.9
LSD (5%)	1.014	1.382	7.674	0.458	0.512	0.7279	4.086	1.825	0.988	1.027

Turfgrass visual quality and color based on a scale of 1-9, 1= brown/dead turf, 6= minimal acceptable turf, 9= ideal green, healthy turf.

highest ascorbate peroxidase activity at 7.5°C and -7.5°C was observed in Daran accession (Tables 2 and 3). The maximum ascorbate peroxidase at -15°C was observed in Taft accession and the second rank was belonged to common bermudagrass accession collected from Naein (Table 4).

Total protein

The results presented in Tables 2, 3 and 4 revealed that there was difference between total protein of different accessions in all temperature regimes. The maximum total protein at 24°C was belonged to Doroud daneshjo park, Sarein and

Table 3 - Amount of POD, CAT, SOD, APX, CHL, Proline, EL, protein, color and visual quality of different Iranian accessions and foreign common bermudagrass after cold stress (at -7.5/-12°C day/night cycles)

Location of accession	POD (U mg^{-1} f.w.)	CAT (U mg^{-1} f.w.)	SOD (U mg^{-1} f.w.)	APX (U mg^{-1} f.w.)	CHL (mg g^{-1} f.w.)	Proline (µmol g^{-1} f.w.)	EL (%)	Protein (mg g^{-1} f.w.)	Color	VQ
Doroud Nahalestan	20.90	40.86	143.0	125.00	2.12	19.6	59.6	19.1	4.8	4.9
Abarkouh	21.73	42.29	136.0	108.57	2.28	21.9	57.8	21.8	5.7	5.6
Abidar sanandaj	25.64	40.86	132.5	59.29	2.17	20.8	59.3	19.0	5.0	5.1
Aligoudarz	22.18	42.75	138.5	148.57	3.11	26.4	52.5	21.2	7.0	7.1
Anzali	26.39	41.02	147.5	91.43	2.28	21.6	58.2	18.7	5.5	5.6
Arak	23.38	41.17	128.5	249.29	2.31	22.2	56.4	18.4	5.7	5.9
Ardakan 1	22.86	41.12	132.5	105.15	2.27	19.8	57.5	18.5	5.1	5.2
Ardakan 2	22.78	40.96	136.0	185.71	2.31	21.7	56.8	19.2	5.3	5.3
Azna	23.38	42.50	144.0	224.29	2.41	25.3	56.2	20.3	6.0	6.2
Boroujerd	15.19	41.48	140.0	91.43	2.24	21.3	57.4	19.8	5.2	5.3
Chenaran	24.36	43.16	136.0	156.43	2.31	21.9	57.5	18.9	5.8	5.9
Damghan	18.20	41.48	135.5	137.14	2.21	22.3	58.1	19.2	5.1	5.2
Daran	22.78	43.46	139.7	427.14	2.41	27.3	55.1	18.6	6.0	6.2
Dehgolan	23.53	44.69	137.0	42.14	2.34	21.9	58.2	18.6	5.7	5.9
Doroud Babahour	15.94	40.71	132.0	81.43	2.29	21.9	58.2	19.7	5.7	5.6
Doroud Daneshjo park	17.22	44.94	151.0	245.00	2.41	21.6	54.9	20.2	5.9	6.1
Doroud Siahvel	17.82	41.12	137.8	127.86	2.31	21.5	57.5	19.8	5.7	5.8
Firouzan	24.36	43.52	132.5	135.69	2.36	22.4	56.8	19.1	5.7	5.8
Foreign cultivar	22.11	42.24	137.5	122.86	3.28	27.9	51.1	21.5	7.5	7.5
Ghidar	20.08	42.44	138.5	132.71	2.26	21.5	58.5	18.7	5.4	5.5
Gorgan	24.14	41.99	129.5	135.00	2.35	27.3	56.5	18.8	6.4	6.5
Holailan	26.92	42.35	145.5	75.24	2.31	21.9	56.4	18.7	5.8	5.9
Homail	19.40	42.95	142.0	170.00	2.19	19.7	57.2	18.5	5.0	5.2
Islamabad gharb	21.82	41.85	141.4	120.24	2.15	19.5	57.5	18.8	5.0	5.1
Kamiaran	26.62	43.21	139.0	120.00	2.31	21.7	57.4	19.4	5.6	5.5
Khorram abad	24.59	42.55	130.0	85.71	2.35	21.8	57.5	18.7	5.7	5.8
Mahidasht	23.31	44.08	135.5	189.29	2.37	24.9	54.5	18.9	6.1	6.2
Maiami	22.41	41.37	136.0	132.72	2.35	23.2	55.5	18.5	6.0	6.1
Malayear	34.14	41.78	138.0	134.29	3.21	27.2	51.5	21.5	7.4	7.4
Malayear intersection	19.55	41.83	146.0	57.14	2.33	22.4	56.5	19.3	5.8	5.9
Mamoulan	22.78	43.98	139.5	191.43	2.39	24.3	55.5	19.1	6.0	6.1
Mashhad	22.56	42.95	136.0	62.86	2.28	21.5	57.2	19.1	5.7	5.8
Minodasht	20.38	42.09	147.0	140.00	2.25	21.5	57.9	19.1	5.2	5.4
Naein	23.08	42.29	139.0	191.43	3.26	27.5	51.2	18.7	7.5	7.6
Nahavand	24.74	41.22	135.5	110.71	2.26	21.8	57.2	20.8	5.5	5.4
Nour	24.66	42.90	133.5	89.14	2.21	20.7	57.1	18.6	5.0	5.3
Poldokhtar	22.33	40.81	139.2	121.43	2.37	22.5	54.5	18.6	6.0	6.2
Safashahr	21.95	41.53	132.5	212.86	2.38	27.1	53.5	21.3	6.4	6.3
Sarein	23.91	43.31	144.0	140.71	2.31	22.6	56.1	20.5	5.5	5.8
Sari	21.73	43.06	129.5	71.43	2.32	21.8	57.2	18.5	5.8	5.7
Saymareh bridge	25.86	40.96	129.5	75.00	2.34	19.8	57.8	19.4	5.7	5.1
Semnan	23.98	42.18	128.6	161.43	1.86	17.8	59.5	19.5	4.1	4.2
Shiraz	23.08	41.42	137.3	115.29	2.15	19.8	57.8	20.1	5.0	5.2
Tabriz	25.41	41.22	136.5	142.14	2.15	19.8	59.4	18.4	4.9	4.9
Taft	25.26	42.80	142.4	232.14	3.36	27.2	50.5	22.1	7.7	7.7
Tagh bostan kermanshah	21.23	41.46	134.5	81.74	2.14	26.4	59.8	17.1	5.0	4.9
Talesh	21.34	41.36	135.5	102.20	1.15	18.9	62.4	14.9	3.3	3.4
Tehran	19.85	41.27	143.5	140.71	2.24	21.4	57.3	18.7	5.2	5.2
Yazd	25.94	42.65	126.0	184.29	2.11	21.7	58.5	17.4	4.9	4.8
Zanjan	23.76	42.80	145.5	101.43	2.34	26.1	56.1	19.1	6.0	5.9
LSD (5%)	1.014	1.382	7.674	0.458	0.512	0.7279	4.086	1.825	0.988	1.027

Turfgrass visual quality and color based on a scale of 1-9, 1= brown/dead turf, 6= minimal acceptable turf, 9= ideal green, healthy turf.

Islamabad gharb accessions, and minimum total protein was belonged to Daran accession. The highest total protein at 7.5°C and -7.5°C was observed in Taft accession (Tables 2 and 3). The maximum total protein at -15°C was also observed in Taft accession and the second rank was belonged to common bermudagrass accession collected from Malayear (Table 4).

Proline

Our results revealed that there was difference between proline content of different accessions in all temperature regimes. The maximum proline content at 24°C was belonged to Doroud daneshjo park, Azna, Mahidasht and Talesh accessions, and the minimum proline content was belonged to Nour accession. The highest proline content at 7.5°C and -7.5°C was observed in foreign cultivar and Taft accession, respectively (Tables 2 and 3). The maximum proline content at -15°C was observed in Naein accession and the second rank belonged to common bermudagrass accession collected from Taft (Table 4).

Electrolyte leakage

As shown in Tables 1, 2, 3 and 4, drop in temperature severely increased EL in all accessions. The highest EL at 7.5°C and -7.5°C was observed in Talesh and Abidar accessions, respectively, and the least was seen in Taft accession (Table 2 and 3). The minimum EL at -15°C was observed in Taft accession and the second rank was belonged to common bermudagrass accession collected from Naein (Table 4).

Chlorophyll content, color and visual quality

The highest chlorophyll content, color and visual quality were observed at 24°C and fall in temperature under 24°C severely decreased these characters in all accessions (Tables 1, 2, 3 and 4). The highest chlorophyll content, color and visual quality at -7.5°C were observed in Taft, foreign cultivar, Naein, Malayear, Aligoudarz, Safashahr and Gorgan (Table 3). The maximum chlorophyll content, color and visual quality at -15°C were observed in Taft, Naein and Malayear accessions.

4. Discussion and Conclusions

The 50 *C. dactylon* accessions were clustered into two major groups by Ward's method on the basis of physiological characters at all temperature regimes. Accessions with high POD, CAT, SOD, APX, proline, protein, chlorophyll, color and visual quality, fall in same group. No complete relationships were found between the clustering of the common bermudagrass accessions in dendrograms based on physiological characters and their geographical affiliations. These patterns of physiological variations within common bermudagrass accessions might be due to different genetic background because of various ploidy levels, cross pollination, genetic overlap, germplasm exchange and gene flow. Seven accessions collected from Taft, Naein, Malayear, foreign cultivar, Aligoudarz, Safashahr and Gorgan were the most cold-tolerant genotypes. Because, the highest antioxidant enzymes activity, chlorophyll content, color and visual quality at -7.5°C were observed in these accessions. Iran has a variable climate and we collected these species from different climatic regions of the country that shows its adaptation to wide ranges of climates. The results obtained from our physiological analysis demonstrated that the level of variation was great among Iranian *C. dactylon* accessions. The best color and visual quality in all common bermudagrass accessions were observed before cold stress. Reducing the temperature below 24°C severely decreased chlorophyll content, visual quality and color in all accessions. The results report-

Table 4 - Amount of POD, CAT, SOD,APX, CHL, Proline, EL, protein, color and visual quality of the most cold-tolerant accessions of bermudagrass from Iran after cold stress (at -15/-15°C day/night cycles)

Location of accession	POD (U mg^{-1} f.w.)	CAT (U mg^{-1} f.w.)	SOD (U mg^{-1} f.w.)	APX (U mg^{-1} f.w.)	CHL (mg g^{-1} f.w.)	Proline (µmol g^{-1} f.w.)	EL (%)	Protein (mg g^{-1} f.w.)	Color	VQ
Taft	10.12 a	31.35 a	96.00 a	71.45 c	1.85 a	16.350 a	78.1 d	14.20 a	6.20 a	6.30 a
Naein	10.81 a	26.25 bc	93.86 a	85.39 a	1.74 a	16.41 a	82.3 c	13.70 a	6.10 a	6.00 ab
Malayear	9.55 a	27.28 b	87.08 ab	74.65 c	1.36 e	14.21 ab	87.5 ab	13.90 a	5.36 b	5.30 ab
Foreign cultivar	11.16 a	24.24 cd	76.18 bc	81.26 b	1.24 bc	13.82 ab	85.6 bc	12.80 a	5.10 b	5.10 bc
Aligoudarz	10.13 a	23.36 d	71.22 c	67.25 d	0.94 cd	11.87 b	89.4 ab	11.85 a	4.30 c	4.30 c
Gorgan	8.21 a	23.57 d	74.39 c	52.39 f	0.89 cd	11.68 b	91.2 a	12.10 a	4.20 c	4.50 cd
Safashahr	9.17 a	22.11 d	69.00 c	57.48 e	0.75 d	11.89 b	90.8 a	12.80 a	4.10 c	4.10 d

In each column, means with the same letter are not significantly different according to Least Significant Difference (LSD) test at P= 0.05. Turfgrass visual quality and color based on a scale of 1-9, 1= brown/dead turf, 6= minimal acceptable turf, 9= ideal green, healthy turf.

ed by Esmaili and Salehi (2012) correspond to the results we obtained. The best temperature for growth and development of tropical grasses is ranging from 27 to 35°C (Beard, 1973). In warm-season turfgrasses, if temperature drops to 10-12.8°C their growth decrease considerably and enters dormancy at close to 0°C (Christians, 2004). McCarty (2001) suggested that a sudden air temperature decline to -5°C or a less rapid fall to below -12°C can cause damage to tropical turfgrasses. Photosynthetic apparatus directly affected by cold stress. In our study, the highest POD, CAT, SOD and APX activity were observed at 7.5°C. As temperature diminished from 7.5 to -15°C, antioxidant enzymes activity decreased. This is in agreement with Manuchehri *et al*. (2014) for *Cynodon dactylon* (California origin). The study made by Zhang *et al*. (2006) on common bermudagrass 'Riviera' and 'Princess-77' showed an increase in the SOD activity during the first seven days of cold acclimation and then after a decline was observed. Cold-acclimatized plants can tolerate freezing stress better than non-acclimated ones due to rapid development of metabolic defenses against freezing stress (Zhang *et al*., 2006). Overall, cold stress produces large amounts of ROS which causes oxidative injury to plants through vast destruction of proteins, carbohydrates, lipids, cellular membranes, DNA and major decline of ATP reserve, and finally cell death (Dionne *et al*., 2001; Gill and Tuteja, 2010). Plants protect their cells from ROS damage by raising the activity of antioxidant enzymes like APX, SOD, CAT and POD (Apel and Hirt, 2004). Results of many studies indicate a positive correlation between freezing tolerance in bermudagrasses and antioxidant enzymes activity (Zhang *et al*., 2006, Manuchehri *et al*., 2014). Over-production of ROS during chilling periods increase oxidative stress and can enhance the activity of antioxidant enzymes and stimulate synthesis of antioxidant metabolites (Karpinski *et al*., 2002). Our results regarding an increase in POD, SOD, CAT and APX activity found in this study is assumed to defense mechanism against oxidative damage caused by cold stress. The central role in the antioxidant defense system is perform by SOD via catalyzing $O_2^{\cdot-}$ into H_2O_2 and O_2, while other antioxidant enzymes are also essential for breakdown of H_2O_2 through various pathways (Mittler, 2002; Apel and Hirt, 2004). CAT can produce O_2 from H_2O_2. APX play a key role in ROS detoxification by conversion of H_2O_2 in to H_2O. The balance between ROS production and elimination is vital mean for the protection of plant cells, and APX plays a major role in maintaining this balance (Asada, 1992; Lin *et al*., 2004). Karpinski *et al*. (2002) showed that tolerant plants have higher antioxidant enzyme activity that has dilatory effect on photooxidative injury during cold stress periods. It is postulated that under cold stress condition, cold tolerant plants increase the activity of their antioxidant enzymes that support active photosynthesis and development of carbohydrate reservation and other compounds such as protein and proline with protective functions. Our findings revealed that higher antioxidant enzymes activity can be attributed to better cold tolerance in common bermudagrass. Decreasing the temperature increased EL, proline and protein content. This negative correlation between temperature and EL can be due to cell membrane damage caused by cold temperatures. The EL method is commonly used to quantify the degree of cell membrane damage induced by cold temperatures and to assay the cold stress tolerance of turfgrasses (Shashikumar and Nus, 1993; Anderson and Taliaferro, 2002). Our results indicated that leaf EL increased during cold acclimatization. Cold acclimatization may induce ROSs production and oxidative stress, and may cause slight damage to cell membrane and later will cause an increase in EL. The production of free radicals under cold stress conditions may initiate the signaling pathways of plant metabolic defense responses, which may reduce cell membrane disruption and EL (Zhang and Ervin, 2008). One of the most important differences between cold tolerant and cold sensitive accessions may be greater development of defense responses to scavenging ROSs and lowering the EL in cold tolerant accessions. Cold acclimation increase proline content in all accessions, but we observed much higher proline content in cold tolerant accessions. This results for proline content in our research, is in accordance with the results presented by Munshaw *et al*. (2004), where they reported a significant increase of stolon proline concentration of the common bermudagrass 'Princess-77' during cold acclimation period. Other supporting results include those of Munshaw *et al*. (2006) and Zhang *et al*. (2006), where they found that higher proline content in bermudagrass cultivars during the winter can be related to greater freezing tolerance. Many studies have pointed to the cryoprotectiveness of proline and their function as osmolytes or compatible solutes (Koster and Leopold, 1988; Santarius, 1992; Karpinski *et al*., 2002). Proline is a cryoprotectant for chloroplast membranes of spinach (*Spinacia oleracea* L.) and plays a crucial role in plant protection against freezing stress (Santarius, 1992). Proline contributes

to the acclimatization of plants to cold stress by increasing osmotic potential (reducing the water potential) and decreasing the freezing point of cells. Protein content of all accessions was high during cold acclimation. Higher protein content was observed in cold-tolerant accessions compared to cold-sensitive ones. One of the studies that confirm our results also, is a study by Zhang *et al*. (2006), with their study on *C. dactylon* 'Riviera' as a cold tolerant cultivar with higher protein content, and 'Princess-77' as a cold sensitive cultivar with lower protein content following acclimatization. During cold acclimation plants increase their capacity for synthesis of novel proteins (Cloutier, 1983). This increase in protein content can be attributed to their determinant role in freezing tolerance of common bermudagrass.

Our results showed rapid physiological alterations in common bermudagrass accessions in response to cold stress. Our results also revealed that cold-tolerant common bermudagrass accessions showed higher proline, protein, antioxidant enzymes, color, visual quality and chlorophyll content, and cold-sensitive accessions showed more severe cell membrane damage (EL) under cold stress conditions. We identified drastic natural variations in tolerance between common bermudagrass accessions of Iran in response to freezing stress. Comparative study between bermudagrass accessions based on morphophysiological traits is one of the best method for its cold tolerance improvement. According to our physiological investigations, accessions collected from Taft, Naein and Malayear were the most cold-tolerant genotypes compared to other accessions. Further molecular studies are in progress to clarify better these findings. This is the first report based on physiological characters of Iranian common bermudagrass accessions differences in cold tolerance and provides useful information for breeding programs.

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Application of calcium to decrease yellow sap contamination at different positions of *Garcinia mangostana* L.

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Abstract: The present research aimed at studying the effects of Ca application, through soil fertilization, on yellow sap contamination based on the position of the fruits on the canopy of the tree. The tree was divided into 6 sectors based on the differences in light exposure i.e. sector 1, 2, and 3 for shaded fruit positions and sector 4, 5, and 6 for well-exposed (to light) fruit positions. The present study used a Randomized Complete Block design (RCBD), consisting of 2 treatments i.e. 0 kg Ca/tree and 4.8 kg Ca/tree. The results revealed that Ca treatment lead to an increase in Ca-pectate content in pericarp. In addition, the exposed fruit position allegedly increase the absorption of Ca-pectate to the fruit. Thus, it is important to both apply Ca on the soil and ensure that the fruit, in the canopy, gets enough light to decrease the occurrence of yellow sap contamination. The well-exposed position of the fruit, in the 4.8 kg Ca/tree treatment during anthesis, had increased the Ca-pectate content of the pericarp which, in turn, resulted in a decrease in yellow sap contamination in segment, aryl, and rind of the mangosteen fruit.

1. Introduction

Yellow sap is a sap which is naturally produced in each organ of the mangosteen, excepts the root. Indeed, it constitutes the main constraint in the Indonesian mangosteen agribusiness industry due to the fact that it causes the fruit flesh (aryl) to have a bitter taste and a less attractive look (Osman and Milan, 2006). Statistical data (2015) revealed that only 14.8% of the total Indonesian mangosteen production is exported as a consequence of high percentage of yellow sap contamination.

Yellow sap is found in the yellow sap duct which is surrounded by typical epithelium cells (Dorly *et al*., 2008). It will contaminate the surface of the fruit (or aryl) if the epithelial cells of the secretory duct break as a result of Cadeficiency. The break of the epithelial cells is connected with the extreme changes in groundwater during the developmental process of the fruit (Pechkeo *et al.,* 2007), and the differences in growth rate

between the seed and the aryl with the fruit pericarp during the growing phase of the fruit (Poerwanto *et al*., 2010).

According to previous research, the break of yellow sap duct is related to the concentration of Ca. Indeed, Ca content of the pericarp of mangosteen contaminated fruit (by yellow sap) is lower compared to that of a normal fruit (Poovaradom, 2009; Kurniadinata *et al*., 2016). According to Marshner (2012), Ca structurally functions to strengthen the cell wall, plant tissues, and the stability of the membrane. Mortazavi *et al.* (2016) reported that Ca can minimize cell membrane injury. The increased effect of Ca application can be explained by its role in cell membrane structure. Meanwhile, Seligmann *et al.* (2009) stated that Ca in plant plays an important role regarding the strength of the mechanical tissue and the determination of the fruit quality. However, Ca is an immobile nutrient that could not be translocated from plant tissues. Thus, developing leaves and fruits fully depend on the transmission of Ca in the transpiration stream of the xylem. According to Qiang and Ling (2005), transpiration is the main factor that promotes Ca movements and a low transpiration rate will result in a low addition of Ca^{2+} to aerial organs.

Mangosteen is a small or medium height tree with a straight, symmetrically branched to form a conical and very tight canopy which leads to both a low light intensity and temperature in the shaded internal part compared to the well light-exposed parts (sector). The described architecture is believed to influence the microclimate of various parts of the plant canopy, causing differences in transpiration rate, which in turn, is believed to cause differences in Ca absorption rate to the fruit. Crisosto *et al*. (1995) report the greater the light interception by an individual fruit and its surrounding leaves the better its quality. Fruit that developed in the more shaded inner canopy positions have a greater incidence of internal breakdown than fruit from the high light, outer canopy positions. Erez and Flore (1986) reported that fruit exposed to light experienced a quality improvement by pigmentation of peach that is also assumed to relate to the sink strength of the fruit.

Research, on the relationship between both Ca and the fruit position in the canopy and the occurrence of yellow sap, become capital in determining the sector with a high contamination potential. The present research was aimed to determinate the effects of Ca on yellow sap contamination based on the position of the fruit in the tree canopy

2. Materials and Methods

Time and place

The present research was conducted in Tandolala village, Poso District, central Sulawesi, from October 2015 to April 2016. Tandolala village is situated at an altitude of 508 mdpl with a pH (soil) of 4.8, and a rainfall of 192.8 mm/month. The observations on yellow sap contamination were carried out in the laboratory of Natural science at the University of Sintuwu Maroso Poso. Analyses of total Ca and pericarp Ca-pectate contents were performed in the laboratory of soil chemistry and fertility, Bogor Agricultural University.

Materials

Ten productive plants, at an average 40 years old (with a planting distance ranging from of 4x4 m to 6x6 m), an average height of 16 meters and a canopy diameter of 4 m, were used in the present study. Dolomite [$CaMg(CO_3)_2$], containing 30% Ca, was used as Ca source.

The canopy of the tree was divided into 6 sectors as follows: sector 1 - inner bottom, sector 2 - outer bottom, sector 3 - inner middle, sector 4 - outer middle, sector 5 - inner top, and sector 6 - outer top. The tree was divided into sectors based on the modifications of Setiawan *et al*. (2012) techniques (Fig. 1).

Parameters, such as light intensity, temperature, and humidity, were measured on weeks 4, 8, and 12, on each sector by placing the measuring tool (thermohydrometer) in each sector. Afterwards, the light intensity was recorded as displayed on the measuring

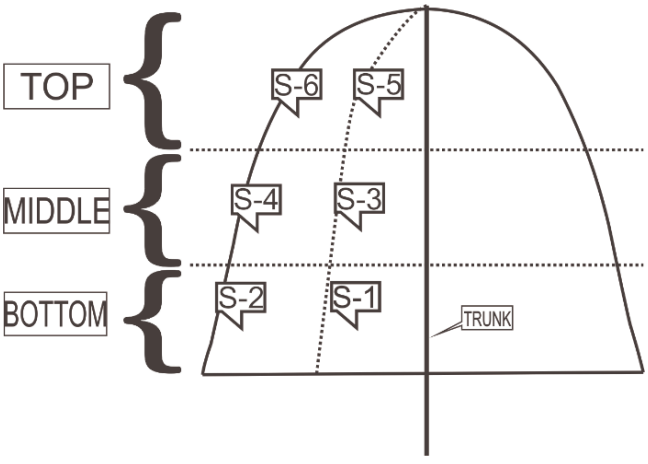

Fig. 1 - Fruit positions in the canopy of the tree. Adapted from Setiawan *et al*., 2012.
S= Sector.

tool. Light intensity, temperature and humidity averages for each sector are presented in Table 1.

Table 1 - Light intensity, temperature, humidity, and transpiration rate at different fruit positions (exposed and shaded)

Fruit positions	Light intensity (lux)	Temperature (°C)	Humidity (%)	Transpiration rate (Hpa/s)	
Sector 1	414.44	26.54	70.98	shaded	0.03
Sector 2	536.00	26.80	70.46		
Sector 3	492.76	26.08	71.54		
Sector 4	1581.51	27.04	69.77	well-exposed	0.06
Sector 5	944.60	26.74	72.04		
Sector 6	1163.11	27.26	70.97		

Experimental design

A Randomized Complete Block design (RCBD*),* consisting of 2 treatments i.e. 0 kg Ca/tree and 4.8 kg Ca/tree (equivalent to 16 kg dolomite/tree), was used as experimental design in the present study. Fertilization with Ca was carried out during anthesis by sowing in the path that was already made around the mangosteen tree (below the crown of the plant) and recovering it with soil.

Based on the light intensity measurement results in Table 1, the observations on yellow sap contamination were carried out on both shaded and well-exposed positions. A fruit was considered shaded if the perceived light intensity is low. Meanwhile, it was categorized as well-exposed if the average received light intensity is high. Shaded fruit positions were identified in sectors 1, 2 and 3, with an average light intensity of 481 lux, while well exposed fruit positions were identified in sectors 4, 5 and 6 with an average light intensity of 1229 lux. The total sample from each sector was 12 fruits (72 fruits per tree). Thus, a total of 720 mangosteen fruits were used as samples.

The transpiration rates, of the leaves, were measured at both shaded and well-exposed positions. The results of the above measurements will be used to estimate the transpiration rates of the fruits in various positions. The transpiration rate was measured by means of a lux meter.

Measurements

Harvesting was carried out 16 weeks post-anthesis (WPA). The observations on the percentage of yellow sap (PYS) were carried out in order to determine the percentage of contaminated fruit in the group of fruits that was observed. A fruit was considered contaminated although the yellow sap, that pollutes both the aryl and the rind just one spot (small patch). The percentages of contaminated aryl (PCA), contaminated rind (PCR), and contaminated segment (PCS) was calculated by means of the following equations:

PCA = (total yellow sap contaminated aryl /total fruit sample) x 100
PCR = (total yellow sap contaminated rind/total fruit sample) x 100
PCS = (total yellow sap contaminated segment/total fruit segment sample) x 100

Yellow sap contamination score shows the severity level of a fruit contaminated by yellow sap and ranges from 1 (very well) to 5 (very bad). Observations on aryl fruit and yellow sap contaminated rind scores referred to Kurniadinata *et al*. (2016) method as presented in Table 2 and 3.

Ca pectate analyses referred to an analysis method developed by Setyaningrum *et al*. (2011) which uses dry fruit sample. The samples were ground into parti-

Table 2 - Yellow sap contamination score on aryl

Score	Description
1	Very good, clean white aryl, no yellow sap between aryl and rind, and fruit vessels as well
2	Good, 1-2 yellow sap stains (small patch) on one end of the aryl, but does not make the fruit bitter
3	Good enough, the presence of some yellow sap stains (patch) on one end of the aryl or between the segments and the littering aryl
4	Bad, presence of yellow sap stains/blobs at the end of the segments, between the segments or the fruit vessels, making the fruit bitter
5	Very bad, the presence of large yellow sap stains/blobs at the end of the segments, between the segments or at the fruit vessels, making the fruit bitter with a clear colored aryl

Score 1= Very good/without contamination, up to score 5= very bad /high contamination score.

Table 3 - Yellow sap contamination score on rind

Score	Description
1	Very good, flawless rinds with no visible yellow sap.
2	Good, flawless rinds with 1-5 yellow sap stains (small patch) which dry without affecting the color of the fruit
3	Good enough, flawless rinds with 6-10 yellow sap drops which dry and do not affect the color of the fruit
4	Bad, flawed rinds due to medium/large yellow sap clumps, there are 1-2 yellowing streams
5	Very bad, flawed rinds with more than one large yellow sap clumps with lots of yellowing streams on the rind of the fruit and a dull fruit color

Score 1= Very good/without contamination, up to score 5= very bad /high contamination score.

cles, added with ion free water and shacked for 2 hours. Afterward, the solution was centrifuged for 15 minutes at a speed of 3000 rpm. The supernatant was then filtered to collect the pellet to which a 1 mol L^{-1} of NaCl was added, shacked for 2 hours and centrifuged for 15 minutes. The extraction result was analyzed Atomic Absorption Spectrophotometer (AAS) in order to obtain data on Ca pectate.

Statistical analysis

PCA, PCR, PCS, total Ca contents, and Ca-pectate were analyzed using SAS 9.1.3 program, which was followed by Duncan's post-hoc comparison (at a significance level of 5%) test. Meanwhile, data on fruit score, yellow sap contaminated aryl were analyzed by mean of a Kruskal Wallis test, which was followed by Dunn test.

3. Results

The application of Ca had an effect on the decrease in yellow sap contamination in mangosteen (Table 4). The lowest percentage of yellow sap contaminated segment was observed in the 4.8 kg Ca/tree (exposed fruit position), which significantly differed to those of other treatments. The percentage of yellow sap contaminated segment showed significant decline of 81% with application of 4.8 Ca/tree on exposed position compared to treatment with 0 kg Ca/tree on shaded position, 71% compared to treatment with 0 kg Ca/tree on exposed position and 73% compared to 4.8 kg Ca/tree on shaded position.

The percentage of yellow sap contaminated aryl, in the 4.8 kg Ca/tree treatment (exposed fruit position), showed an average percentage of 14.2%, which was not different to that of the 0 kg Ca/tree treatment (23.9%). No differences were observed among treatments application of 4.8 kg Ca/tree in terms of yellow sap contaminated rind percentage. The percentage of yellow sap contaminated rind was considerably high with treatment of 0 kg Ca/tree, which was 79.9% on shaded position and 73.9% on exposed position. While the application of 4.8 kg Ca/tree showed that yellow sap contamination was still considerably high at 61.3% on shaded position and 53.5% on shaded position. Although the numbers were still high, there was a decline of 33% with application of 4.8 Ca/tree compared to without Ca application on shaded position. According to Martias *et al.* (2012), percentage of yellow sap contaminated that was higher than 50% was considered very high.

Table 5 presents the yellow sap contamination scores of both aryl and rind. The best yellow sap contaminated aryl score was observed at the well-exposed fruit position with the application of 4.8 kg Ca/tree, and significantly differed to other treatments. Meanwhile, the yellow sap contaminated rind score in the 4.8 kg Ca/tree treatment revealed similar results for both shaded and well-exposed fruit positions (Table 5).

Accumulation of Ca in fruit pericarp was a representation of adsorbed soil Ca by plant. Total Ca content of the fruit pericarp did not significantly differ among treatments, but was significantly different to that of the Ca-pectate contents. The Ca-pectate con-

Table 5 - Score of yellow sap contamination in aryl and on rind (16 WPA)

Treatments	Yellow sap contaminated aryl and rind score in 1-5	
	Aryl	Rind
0 kg Ca/tree		
Shaded	1.99 a	2.38 a
Exposed	1.67 c	2.17 b
4.8 kg Ca/tree		
Shaded	1.77 b	1.90 c
Exposed	1.23 d	1.74 c
Dunn-test	*	*

Data were analyzed based on Kruskal Wallis test. Numbers followed by different letters within the same column showed significant differences based on Dunn test (1%). Score 1: Very good; score 2= Good; score 3= Good enough; score 4= Bad; score 5= Very bad.

Table 4 - Percentage yellow sap contamination in fruit segment, aryl and on rind (16 WPA)

Treatments	Fruits contaminated by yellow sap (%)			Pericarp Ca content (ppm)	
	Segment	Aryl	Rind	Total	Pectate
0 kg Ca/tree					
Shaded	21.3 a	45.7 a	79.9 a	1088.9	552.02 b
Exposed	13.6 a	23.9 b	73.9 ab	2100.0	528.87 b
4.8 kg Ca/tree					
Shaded	15.0 a	34.9 a	61.3 bc	1500.0	576.40 b
Exposed	4.0 b	14.2 b	53.5 c	1566.7	754.60 a
F-test	*		*	NS	*

Numbers followed by different letters within the same column showed significant differences in DMRT test (α= 5%). Shaded (sector 1, 2 and 3) with a light intensity of 481 lux, Exposed (sector 4, 5 and 6) with a light intensity of 1229 lux.

tent was higher in the Ca treated fruit (well-exposed position) (Table 6). The percentage of yellow sap contaminated rind and aryl was reduced because of increased Ca-pectate in fruit pericarp, proving that Ca-pectate had a role in strengthening cell wall epithelium which compile the yellow sap duct making the cell stronger and kept yellow sap from leaking and contaminate the aryl and rind.

Leaves transpiration rate measurement, on week 4, 8, and 12, resulted in average transpiration rates of 0.03 Hpa/s (shaded position) and 0.06 Hpa/s (well exposed position) (Table 1). This data supported the measurement result of light and temperature on Table 1, that the higher the temperature and light intensity, the higher the transpiration rate.

Table 6 - Calcium total and Ca-pectate content in pericarp and percentage ratio of Ca-pectate/total Ca at 16 WPA

Treatment	Ca content in pericarp (%)		Percentage pectate/total
	Total	Pectate	
0 kg Ca/tree			
Shaded	0.11	0.06 b	50.5
Exposed	0.21	0.05 b	23.8
4.8 kg Ca/tree			
Shaded	0.15	0.06 b	40.0
Exposed	0.17	0.08 a	47.1
F-test	NS	*	NS

Numbers followed by different letters within the same column showed significant differences in DMRT test (α= 5%). Shaded (sector 1, 2 and 3) with a light intensity of 481 lux, Exposed (sector 4, 5 and 6) with a light intensity of 1229 lux.

4. Discussion and Conclusions

Ca is an immobile nutrient, for its absorption rate follows the transpiration pathway in the xylem. Thus, a deficiency in Ca often occurs in fruits that do not transpire as much as the leaves. In the present study, the transpiration rate of the fruits was estimated by observing parameters such as light intensity, temperature and humidity in each sector, and also the transpiration rates of leaves at both the exposed and shaded positions.

Data shown in Table 4 showed that the percentage of yellow sap contamination on the rind was higher than aryl and segment. Yellow sap contamination was caused by the same factor which was the lack of Ca on epithelium cell of yellow duct sap, but triggered by different things. According to Dorly *et al*. (2008), yellow sap contamination on the aryl was caused by turgor and mechanical pressure, that was the pressure of aryl and seed growth outward during fruit enlargement. While contamination on the rind was caused by turgor pressure of pericarp cell, or by insect, fungal, or bacterial attack.

Fruits that were exposed to a high light intensity (Table 1) and treated with 4.8 kg Ca/tree had highest Ca-pectate content (Table 6) which was a consequence of the high light intensity received. Marschner (2012) stated that part of the pectin, in the leaves, is in the form of Ca-pectate in a high light intensity condition. Ca strengthens the main plant cell wall in a crosslinking with the pectin. Formation of calcium pectate due to the binding of calcium with pectins has been found beneficial to increase the strength of cell wall and middle lamella (Carpita and McCann, 2000). The high Ca-pectate content is believed to be a result of light intensity not only on the leaves but also on the fruit that possibly increases photosynthesis rate and transpiration rate, as demonstrated by the results of the present research.

Such fruits (well-exposed, treated with Ca, and showing highest Ca-pectate), showed also lowest yellow sap contamination levels. A high ratio of Ca-pectate over total Ca content (48% of the total Ca) (Table 6) could decrease yellow sap contamination through the strengthening of the epithelial cell walls in the yellow sap duct. A research, conducted by Setyaningrum *et al.* (2011), revealed that the Ca- pectate content represented 20% of the total Ca, which tend to reduce yellow sap contamination in mangosteen.

The improvement of fruits quality (low yellow sap contamination) is related to high light intensity of fruits exposition. Physiologically, light has a direct influence by photosynthesis and indirect influence throught plant's growth and development as the results of direct metabolic responses (Fitter and Hay, 1991). The improvement of fruits quality is caused by the distribution of photosynthate to the fruit exposed to light.

The high temperature and light intensity in the exposed tree canopy resulted in higher transpiration rate at exposed positions compared to the shaded ones (Table 1). Similar results were observed by Caleb *et al.* (2013) who demonstrated that temperature and humidity have significant effects on the transpiration rate. The high leaves transpiration rate at the exposed position is believed to be a consequence of higher Ca translocation from the root to parts of the leaf (including part of the exposed fruit), for the Ca is translocated along with water during the transpiration process. The high transpiration rate led to a high translocation of Ca from the root to the fruit, since Ca is translocated along with water during

the transpiration process. Hansen (1980) stated that Ca is transported in the fruit by means of water distribution through the xylem. Gilliham *et al.* (2011) demonstrated that the mechanism of Ca uptake is through the apoplast of the root along with the mass flow which follows the apoplast or the symplast pathway to the xylem. According to Qiang and Ling (2005) and White and Broadley (2003), the transport through the apoplastic pathway mainly depends on the transpiration, while the symplast pathway is selective in controlling Ca^{2+} to the xylem which depends on Ca^{2+} requirement at the canopy.

If there is a deficiency in Ca supply of the plant, the plant may experience damages at the cell level. Thus, the supplementation of Ca is required on soil with low Ca content. According to Martias *et al*. (2012), yellow sap contamination could be directly prevented by the Ca availability in the soil. Amor and Leo (2006) stated that the Ca concentration of the plant significantly decrease due to a low supply. In addition, Hocking *et al.* (2016) demonstrated that low supply and transport of Ca would result in a Ca deficiency, leading to damages of both membrane and cell walls of the fruit.

The results revealed that Ca treatment lead to an increase in Ca-pectate content in pericarp. In addition, the exposed fruit position allegedly increase the absorption of Ca-pectate to the fruit. Thus, it is important to both apply Ca on the soil and ensure that the fruit, in the canopy, gets enough light to decrease the occurrence of yellow sap contamination. The well-exposed position of the fruit, in the 4.8 kg Ca/tree treatment during anthesis, had increased the Ca-pectate content of the pericarp which, in turn, resulted in a decrease in yellow sap contamination in segment, aryl, and rind of the mangosteen fruit.

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Poultry manure application time on pistachio (*Pistacia vera* L.) trees

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Abstract: The effectiveness of poultry manure application time was studied on pistachios (*Pistacia vera* L.) trees. The experiment consisted of seven different poultry manure application time, including poultry manure application as one time in 1) last week of October 2) last week of December 3) last week of January 4) mid-March and dividing into two parts and use in fall or in winter, dividing into four parts and use in dormant seasons (fall and winter). Based on the results, there were significant differences among treatments. The highest number of fruit per cluster (27.4) was found in poultry manure applied in last week of October. The highest nut splitting percent (84.3%) and the lowest nut blanking percent (8.6%) were obtained in poultry manure applied by dividing into four parts. Half kernel nuts followed a similar trend with blanking percent. Weight of 1000 nuts increased and responded positively and number of pistachios nut per ounce decreased by manure application when divided into two parts and used in the winter. Application of poultry manure in the mid-March enhanced the nut protein (19.63%).

1. Introduction

Production technologies of horticultural crops including pistachio tree have undergone vast changes recently, and led to the extension of innovative technologies about nutrient management. The critical factor of nutrient management of nut trees like pistachio is to elevate the net yield and improve the quality of nut fruits. Pistachio blanking and flower bud abscission can be directly related to nutrition management (Mahmoudi Meimand and Ghanbari Odivi, 2013). Nowadays, fertilizers play a key role in nutrition of fruit trees. Because of harmful side effects of chemical fertilizers, (Ljoyah and Sophie, 2009) the use of bio fertilizers are increasing recently. Organic fertilizers, such as animal manure, have a long history of use by men (BayBordi and Malakuoti, 2003). Different animal manures such as sheep, cow and poultry manures have been used as natural crop

fertilizers for centuries. Poultry manure, because of its high level of nitrogen which is absorbable for pistachio trees, has been recognized as one of the main favorable manures. It has been documented that poultry manures also supply other essential pistachio nutrients and act as soil amendant by raising organic matter content, which helps improve the moisture level of soil and nutrient maintenance. According to Alimoradi (2011) nearly 5.3 million tons of poultry manure is consumed in Rafsanjan and Kerman pistachio orchards every year. These bio fertilizers are known as main source of manure for Iranian pistachio growers. Nutrients provided by poultry manures have been indicated to establish effects on different crops, including fruit crops (Mitchell *et al.,* 1993; Miller, 1996). It has been reported that poultry manure is composed of essential elements for fruit crops containing about 3% of nitrogen NH_4^+, 63.2% phosphorus P_2O_5 and 4.1% potassium K_2O (Reddy and Reddy, 1995). The main form of nitrogen in poultry manures is NH_4^+, which elevate the availability of nitrate to the plants for a longer period (Burmester, 1993; Crawford and Chalk, 1993; Touchton and Bosewell, 1980). On the other hand, organic manures application to decrease the use of chemical fertilizers in pistachio orchards and other fruit trees is an important goal in fruit production (Reganold *et al.,* 2001; Forge *et al.,* 2002). Organic manures increase the fertility of soil and the crop yield. Therefore, they can be helpful to achieve sustainable agriculture. It has been demonstrated earlier that poultry manure improved growth parameters, yield and quality in different crops (Ram and Rajput, 2002; Ingle *et al.,* 2003; Arancon *et al.,* 2003). Pimpini *et al.* (1992) revealed higher rate of ex-tractable sucrose by using 4 t $ha^{-1}$of poultry manure in crops. Increased content of total carbohydrate, protein and ascorbic acid were reported by Abusaleha and Dutta (1988) when poultry manure was used. Improving photosynthesis, plant biomass and glycosides content of *Stevia* were also demonstrated before (Xiangyang *et al*., 2010). Enhanced starch content, crude fiber, ash, crude protein, phosphorus, calcium and magnesium of *D. bulbifera* were reported by Ezeocha *et al.* (2014). Adekiya and Agbede (2017) showed that poultry application increased soil organic matter, leaf N, P, K, Ca, Mg contents, growth and yield in tomato. They also showed that the application of poultry manure at 3 weeks before transplanting had highest effects on leaf nutrient concentrations, growth and yield in tomato (Adekiya and Agbede, 2017). Now, pistachio growers are frequently using poultry manure as a source of plant nutrient. To our knowledge, there are no reports available to recommend favorable time for poultry manure application in orchard of pistachio trees. The aim of this investigation is to study the effect of poultry manure application time on nut yield and quality in pistachios trees.

2. Materials and Methods

The experimental orchard selected for this study is located at Khatam, Yazd province, Iran. It is located at 39.33°N latitude and 54.40°E longitudes, at an elevation of 1605 m above sea level. The average temperature of the zone is 18.5°C, the annual total chilling hours (≤7.2°C) is about 950; the average annual rainfall is 300 mm. The climate of this area is typically subtropical. Just before poultry manures application, soil sample of the experimental orchard were collected and chemically analyzed (Table 1). This research was conducted on 12 years old pistachio cv. Akbari grafted on 'Badamii Zarand' rootstock. Management factors such as irrigation regime, pruning practices, and weed control were followed according to local standards. Trees were trained with a modified central leader system and distance of trees was 2x6 m. To achieve better results, uniform trees were selected with uniform vigor and age and three uniform shoots were selected from different sides of the tree for data collection. Harvest index was considered when several nuts in the cluster were light colored and the hull was easily separated from shell. Characteristics of yield, percentage of splitting, weight of 1000 nuts, blanking, pistachio weight in

Table 1 - Soil fertility analysis report of the experimental orchard

Soil characteristics	Value
pH	7.6
EC ds m^{-1}	4
P ppm	28.1
K ppm	180
O.M. %	1.3
T.N.V. %	22.1
Sand %	52
Silt %	34
Clay %	14
Texture	Loam
ESP	17.9

ounce, dry weight and some other characteristics related to kernel and leaf were recorded. The experiment consisted of seven different poultry manure application time, with three replications based on the Randomized Complete Block Design (RCBD). The treatments were poultry manure application as one time in 1) last week of October 2) last December 3) last week of January 4) mid-March and dividing into two parts and use in fall or in winter, dividing into four parts and use in dormant seasons (fall and winter). These treatments were conducted beneath the tree canopy and mixed well with surface soil, 10 kg per tree. Data collecting was performed in next growth season. Statistical analysis was conducted using the SAS software (9.2) and means were compared by Duncan's Multiple Range test (P≤ 0.05).

3. Results and Discussion

Effect of poultry manure application time on yield parameters

Number of fruits per cluster. The study indicated that poultry manure application time had significant effects on number of fruits per cluster (P≤ 0.05) (Table 2). The highest number of fruit per cluster was found by poultry manure application in last week of October (27.4) followed by the last week of December (26.9) (Table 2). The minimum value (19.2) was observed in control treatment. The higher fruit per cluster with poultry manure application in last week of October (concurrently with tree deciduous) might be related with the positive role of nitrogen and other critical elements on cluster final development. Burmester (1993) Crawford and Chalk (1993) Touchton and Bosewell (1980) demonstrated the positive role and proportion ratio of elements on reproductive growth parameters.

Nut splitting percent. Also, our results demonstrated that poultry manure application time had significant effects on nut splitting percent (Table 2). Highest nut splitting percent (84.3%) was obtained in treatment of poultry manures divided into four parts and used in last weeks of October, December, January and mid-March followed by divided into two parts and used in fall (83.5%) and in winter (83.1%) (Table 2). The minimum value for nut splitting percent was reported in control treatment. Based on our results, the poultry manure application in four different times, thus involving a longer period of the year improved nut splitting percent. It has been demonstrated before that poultry manure was improved yield quality in different crops (Arancon *et al.,* 2003; Ingle *et al.,* 2003).

Number of pistachio nuts per ounce. Table (2) indicates that the use of poultry manure increased the pistachio ounce index or decreased number of inshell pistachio nuts per ounce significantly (P≤ 0.01). The results showed also that when a portion of poultry manure is allocated at the end of dormant season, increase fruit size is achieved. Previous findings showed that poultry manure improved yield component in different crops (Ram and Rajput, 2002; Arancon *et al.,* 2003).

Blanking percent. Based on the results, the lowest value (8.6%) for nut blanking was observed when treatment consisted in dividing poultry manures into four parts and using in last weeks of October, December, January and mid-March; this was followed by treatment divided into two parts and used in winter (11%) (Table 2). The maximum amount of blanking (%) was observed in control treatment (13.9%) with no significant difference with fall application. Therefore, poultry manure application at over the year in four different times decreased nut blanking percent. Yield component improving demonstrat-

Table 2 - Effect of poultry manure application time on yield parameters

Parameter\treatment	lwo	lwd	lwod	lwj	Mm	lwjm	lwodjm	control
Number of fruit	27.4 a	26.9 b	22.6 c	21.2 e	21.05 e	22.1d	26.9 b	19.2 f
Nut splitting %	77.3 b	76.4 b	83.5 a	70 c	69.8 c	83.1 a	84.3 a	64.8 d
Fruit ounce	22.6 a	22.6 a	22 ab	22.6 a	22.3 a	20.6 b	20.6 b	23 a
Blanking %	13.3 ab	13.5 ab	13.1 bc	12.5 bc	12.8 bc	11 d	8.6 e	13.9 a
Half seed fruit %	1.3 c	1.2 d	0.7 e	4.4 b	3.1 c	0.9 e	0.7 e	5.0 a
Weight of 1000 nuts	1249 b	1249 b	1288 b	1249 b	1267 b	1372.1 a	1370 a	1231 b

lwo= last week of October, lwd= the last week of December, lwj= the last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid. Means followed by the same letters are not significantly different (Duncan test, P≤ 0.05).

ed by Ram and Rajput (2002) and Arancon *et al*. (2003).

Half kernel fruit percent. Half kernel nuts followed a trend similar to blanking percent, it was decreased with dividing poultry manure into four parts (0.7%), followed by manure divided into two parts and used in fall (0.7%) or winter (0.9%) (Table 2). The highest half kernel nuts percent was obtained in control (Table 2).

Weight of 1000 nuts. Weight of 1000 nuts increased and positively and significantly (P>0.05) responded to the treatments. Highest weight of 1000 nuts (1372.1 g) were observed in poultry manure divided into two parts and applied in winter followed by treatment consisting in manure divided into four parts and used in last weeks of October, December, January and mid-March (1370 g). However, other treatments exhibited lowest weight of 1000 nuts (Table 2). Previous findings indicated that poultry manure improved yield component in different crops (Ram and Rajput, 2002; Arancon *et al.,* 2003).

Effect of poultry manure application time on nut quality parameters

Fruit stening. Fruit stening was influenced by application of poultry manure in different times (Table 3). Maximum amount for fruit stening (7.23%) was noted at mid-March application (7.23%) and control (7.16%) and the lowest value for this parameter was obtained with treatment divided into two parts and used in fall treatment (Table 3). It has been demonstrated earlier that poultry manure improved yield and quality in different crops (Ram and Rajput, 2002; Arancon *et al.,* 2003; Ingle *et al.,* 2003).

Nut protein. Nut proteins percent was also enhanced by the application of poultry manure in all treatment versus control (Table 3), but maximum value for nut protein percent was observed at mid-March (Mm) application (19.63%) followed by the last week of January (19.5%) and in poultry manures divided into four parts and used in last weeks of October, December, January and mid-March (19.2%) with no significant differences (Table 3). The minimum value for nut protein percent was observed in control treatment (17.4%). Increasing amount of total carbohydrates, proteins and ascorbic acid content reported by Abusaleha and Dutta (1988) in a similar work that well described poultry manure application on increasing secondary metabolites. Enhancing crude protein in *D. bulbifera* was reported by Ezeocha *et al*. (2014). The results reported by Adekiya and Agbede (2017) showed that poultry application produced higher percent protein content, more growth and yield in tomato.

Effect of poultry manure application time on vegetative and reproductive parameters

Leaf fresh weight. According to obtained results, poultry manure application time had significant effects on leaf fresh weight (P≤ 0.05) (Fig. 1). The highest amount of leaf fresh weight was obtained at poultry application in mid-March (2.1 g), followed by

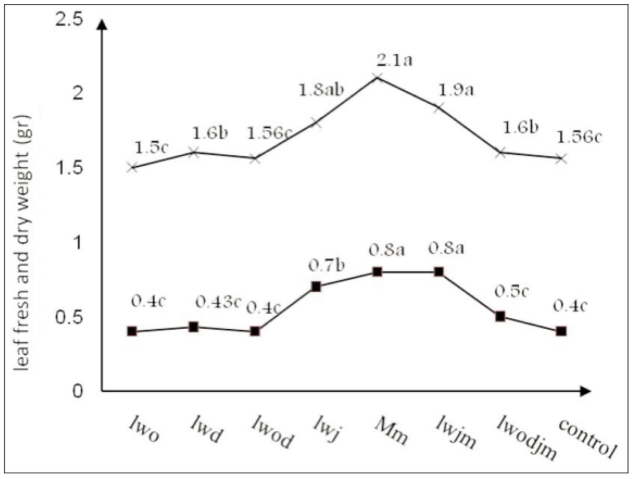

Fig. 1 - Changes of leaf fresh (x) and dry weight (■) (gr) of pistachio (*P. vera* cv. Akbari) grown in Khatam, Yazd, Iran. Duncan was calculated at P≤0.05. lwo= last week of October, lwd= last week of December, lwj= last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid.

Table 3 - Effect of poultry manure application time on nut quality parameters

Parameter\treatment	lwo	lwd	lwod	lwj	Mm	lwjm	lwodjm	control
Fruit stening (%)	5.6 d	6.56 b	4.2 f	6.8 b	7.23 a	5.9 c	4.96 e	7.16 a
Nut protein (%)	19.1 bc	19 c	18.9 c	19.5 ab	19.63 a	19.2 ab	19.2 ab	17.4 d

lwo= last week of October, lwd= the last week of December, lwj= the last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid. Means followed by the same letters are not significantly different (Duncan test, P≤ 0.05).

application divided into two parts and used in winter (1.9 g) (Fig. 1). The lowest rate of leaf fresh weight was occurred in control (1.5 g) and last week of October (1.5 g), respectively (Fig. 1). Based on our result, poultry manure application in mid-March at once increased leaf fresh weight. Increasing photosynthesis and plant biomass of *Stevia* was also demonstrated before (Xiangyang *et al*., 2010).

Leaf dry weight. Dry weight of leaves was significantly affected by different times of manure application (P≤ 0.05). Maximum leaf dry weight was found in poultry application in mid-March (0.8 g) and divided into two parts and used in winter (0.8 g), whereas the minimum was observed in control (0.4 g) and last week of October (0.4 g) (Fig. 1). This increase in leaf dry and fresh weights with application of poultry manures at the end of dormant season can be explained as following, this time of application cannot make the expected effect and causes to increase vegetative parameters, similar to reports published by Xiangyang *et al*. (2010).

Leaf nitrogen content. Results revealed that the leaf nitrogen content of pistachio trees fertilized with poultry manure in different times varied significantly (P≤ 0.05). Leaf nitrogen content was the highest (3.1%) at poultry application in mid-March once at any time compared to the treatments divided into two parts and applied in winter (3.03%) and in last week of January (2.9%) (Fig. 2). The higher leaf nitrogen content in mid-March application of poultry manures could be in relation with releasing more nitrogen NH_4^+ form which is useful for vegetative parameters and causes nitrogen accumulation in the leaves. Previous findings emphasis on high level of NH_4^+ form in poultry manure. The application of poultry manure with high level of N, have influenced the growth and vegetative factors of tree and production of fruits (Reddy and Reddy, 1995). Adekiya and Agbede (2017) indicated that application of poultry manure increased percent of leaf N, P, K, Ca, Mg content, growth parameters and yield in tomato. Poultry manure usage at 3 weeks before transplanting caused higher leaf nutrients concentrations and more growth and yield in tomato (Adekiya and Agbede, 2017).

Shoot length. Based on our findings, shoot length was significantly affected by different application times of manure (P≤0.05). Maximum shoot length was obtained when manure was divided into two parts and used in winter (44 cm) and mid-March application (43.3 cm). Our results revealed that minimum value for this parameter was observed in control (26.6 cm) and in manure divided into two parts and used in fall (32.6 cm) (Fig. 3). Similar to the dry and fresh weights, application of poultry manures at the end of dormant season caused to increase shoot length. Our results demonstrated that application of poultry manures during fall stimulates a least vegetative growth with no significant differences between various times in fall (Fig. 3). Increasing vegetative growth and similar findings by using poultry manure

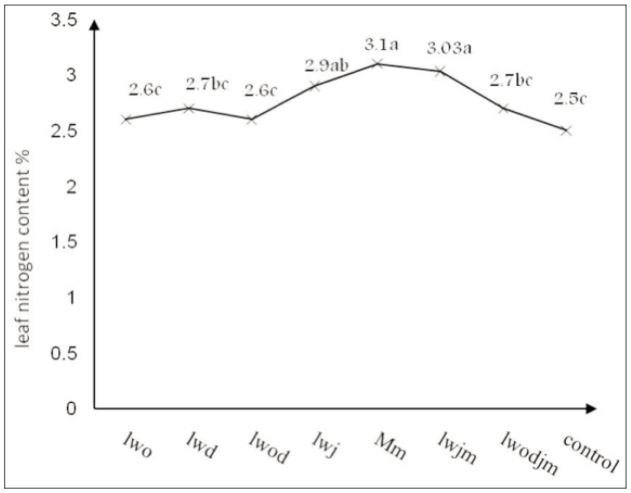

Fig. 2 - Changes of leaf nitrogen content of pistachio (*P. vera* cv. Akbari) grown in Khatam, Yazd, Iran. Duncan was calculated at P≤0.05. lwo= last week of October, lwd= last week of December, lwj= last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid.

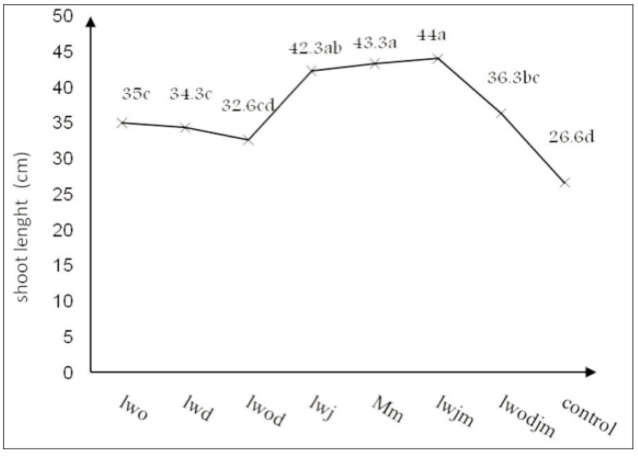

Fig. 3 - Changes of shoot length of pistachio (*P. vera* cv. Akbari) grown in Khatam, Yazd, Iran. Duncan was calculated at P≤0.05. lwo= last week of October, lwd= last week of December, lwj= last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid.

have been reported before (Xiangyang *et al.,* 2010).

Leafy bud number, floral bud number and leafy to flower bud ratio. Results indicated that leafy and floral buds and leafy to flower bud ratio of pistachio trees fertilized with poultry manure was positively affected by treatments (P≤ 0.05). Number of floral buds in the next growing season was the highest in last week of October and fall (in last weeks of October and December) (7.33) (Fig. 4). Minimum value for floral bud number was observed in control (Fig. 5). Results for leafy bud numbers showed that maximum value was obtained in treatment divided into two parts and applied in winter (5.6) and mid-March (5.3) (the letter two treatments have not any significant difference) (Fig. 4).

Floral bud abscission ratio. It was revealed that floral bud abscission during the grow season by poultry manure usage was significantly less than control, which it might be due to the role of poultry manure in decreasing floral bud abscission totally. Any treatment could not show significant effect on floral bud abscission, but minimum amount for this parameter was observed in treatment that was conducted in last week of December (0.32) (Fig. 5). Previous reports showed that poultry manure improves the yield component (Ram and Rajput, 2002; Arancon *et al.,* 2003), although there are no exact results about reproductive phase and parameters affected by poultry manure treatment, but flower bud abscission can be directly related to nutrition management (Mahmoudi Meimand and Ghanbari Odivi, 2013).

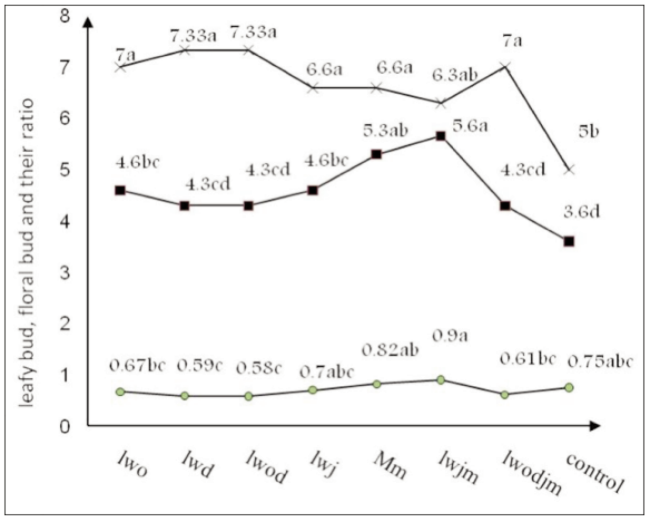

Fig. 4 - Changes of leafy bud number (■), floral bud number (×) and leafy to flower bud ratio (○) of pistachio (*P. vera* cv. Akbari) grown in Khatam, Yazd, Iran. Duncan was calculated at P≤0.05. lwo= last week of October, lwd= last week of December, lwj= last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid.

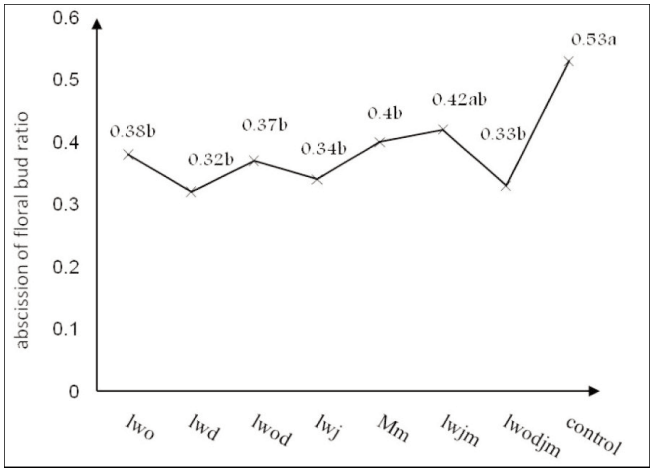

Fig. 5 - Changes of floral bud abscission ratio of pistachio (*P. vera* cv. Akbari) grown in Khatam, Yazd, Iran. Duncan was calculated at P≤0.05. lwo= last week of October, lwd= last week of December, lwj= last week of January, Mm= March mid, lwjm= divided into two parts and used in the winter, lwod= divided into two parts and used in the fall, lwodjm= poultry manures divided into four parts and used in the last week of October, the last week of December, the last week of January and March mid.

4. Conclusions

It is clear that application of organic fertilizers will improve the nut yield and quality, but application of poultry manure through dormant season can be effective on reproductive and vegetative parameters in pistachio. Based on our findings, dividing poultry manures into four parts and applying in last weeks of October, December, January and Mid-March, or using a single application in fall showed the best results for optimum nut yield, quality and floral bud emergence in pistachio.

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Effects of nano-silver pulsing, calcium sulfate and gibberellin on an antioxidant molecule and vase life of cut gerbera flowers

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Key words: antioxidant, flavonoid, GA_{4+7}, *Gerbera jamesonii*.

Abbreviations: nano-silver= NS; deionized water= DI; calcium sulfate= CS; gibberellin$_{4+7}$ = GA; anthocyanin leakage= AL; total soluble solids= TSS.

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Abstract: The aim of this study was to evaluate interactions between NS coupled with CS and GA on flavonoid, cell membrane behavior and extending the vase life of cut gerbera. Pulse treatments of flowers were conducted in NS at concentrations of 0 (DI), 3 or 9 mg/l for 24 h. Then, flowers were treated with preservative solutions containing calcium sulfate (0, 10 or 20 mM) and GA_{4+7} (0 or 20 mg/l), plus 1.5% sucrose in all preservative solutions. Pulse treatments with 3 or 9 mg NS/l and holding in solution containing 20 mM CS compared to the control treatment (holding in the solution of sucrose following pulse treatment in DI) significantly extended vase life by 8 days. According to the antioxidant role of flavonoids, and lower amounts of flavonoid in the flowers that pretreated with NS, therefore, it may be said that NS prevented from microbial attack.

1. Introduction

Gerberas (*Gerbera jamesonii*) are well-known flowers for the variety of their colors, and are popular in the world flower trade (Liu *et al.*, 2009 a; Solgi *et al.*, 2009). However, often the growers and florists suffer further loss from short vase life of gerberas. For example, the mean of vase life in some cultivars of *Gerbera* ('Bayadere' and 'Sunway') are reported only between 6 to 10 days when water tap is used (Shabanian *et al.*, 2018). Gerberas are ethylene insensitive, but bacterial plugging of the xylem is a main cause of early and rapid senescence in their cut flowers (Liu *et al.*, 2009 a). The decrease of water uptake and consequently the increase of the ratio between transpiration and water uptake (i.e., high value of

water balance) will be caused as a result of xylem obstruction, and will end the cut flower vase life. Nano-silver (NS) pulse and continuous treatments for cut flowers are newly used and are known as novel agents of anti-microbial (Liu *et al.*, 2009 a; Solgi *et al.*, 2009; Lü *et al.*, 2010). As a novel antiseptic, NS is used in the medical industry, silver embedded fabrics, water purification and vegetable disinfection. Due to their high surface area to volume ratio, among other unique chemical and physical properties, NS formulations provide full contact with microorganisms and are highly effective as germicides. NS particles can connect the cell membranes and penetrate into bacteria. Then, NS can disrupt the respiration and cell division and cause the cell death. NS releases silver ions (Ag^+) within bacterial cells, silver ions have bactericidal activity (Liu *et al.*, 2009 a; Solgi *et al.*, 2009; Nair *et al.*, 2010; Sharon *et al*., 2010; Lü *et al.*, 2010; Liu *et al.*, 2012). Naghsh (2010) described the inhibited meiosis in *Aspergillus niger* due to NS activity. Alavi and Dehpour (2010) reported that the nano-silver solution is effective on greenhouse cucumber downy mildew disease. Liu *et al.* (2009 a) and Lü *et al.* (2010) observed that NS pulse treatments extended vase life of the cut gerbera and the rose flowers. Also, Solgi *et al.* (2009) reported that NS continuous treatments inhibited the growth of bacteria in the solution and xylem vessels and increased vase life of cut gerbera flowers.

Calcium increase postharvest longevity of fresh cut flowers (Gerasopoulos and Chebli, 1999; De Capdeville *et al*., 2005; Sosa Nan, 2007). This increased postharvest longevity may be due to a delay of physiological events related to senescence, such as a decrease in water uptake, increased water transpiration loss, decreased fresh weight, stem bending (Sosa Nan, 2007).

Since the level of soluble carbohydrates will be maintained by the treatment of gibberellin (GA) (Ranwala and Miller, 2000; Whitman *et al.*, 2001; Hatamzadeh *et al.*, 2010), therefore, GA can have a positive effect on the water balance. Furthermore, sucrose in the preservative solutions maintains water balance, in addition to act as a food source (Solgi *et al.*, 2009).

The objective of this research was to evaluate the interactions of calcium sulfate and gibberellin continuous treatments by NS pulse treatments on flavonoid as an antioxidant component, and vase life of cut gerberas. Flavonoids have antioxidant effects and can be effective on the vase life. Antioxidant molecules can be efficient systems to protect cells against pathogen and water deficit-induced oxidative stress, and this prevents the senescence and cell death (Shabanian *et al.*, 2018).

2. Materials and Methods

Plant material

Cut gerbera (*Gerbera jamesonii* cv. Pink Elegance) flowers that were grown in standard hydroponic greenhouse conditions were purchased from a flower and plant growing company (Pakdasht, Tehran, Iran). Flowers were harvested by pulling the stems off in the plants when 2-3 rows of stamens of the bisexual disc florets were mature (Gerasopoulos and Chebli, 1999; Solgi *et al.*, 2009) in the morning. Stem bottom of harvested flowers was put in the flower capsule containing deionized water (DI). Flowers were packed and transported within 8 h to the laboratory. In the laboratory, stems were re-cut to a length of 45 cm into the DI to remove air emboli (Liu *et al*., 2009 a; Solgi *et al.*, 2009). Flowers were re-cut 2-3 times, when it was necessary.

The flowers were placed in a controlled environment room at 20±2°C with 60±10% R.H. and 12 µmol/m.s. light intensity (cool white fluorescent lamps; 12 h/day).

Experimental design and treatments

Solutions of pulse treatment were prepared in two concentrations of NS (3 or 9 mg/ l), and DI was used as a control treatment. Flowers were treated for 24 h with the two concentrations of NS (Nanonasb-Pars Company, Iran) or DI. Each pulse treatment contained 18 flowers. Following pulse treatment, the flowers were individually kept into 1000 ml glass vases containing 500 ml of fresh solutions (as continuous treatment) that were prepared at second day of the experiment and were not renewed. In the continuous treatments, three concentrations (0, 10 or 20 mM) of calcium sulfate, CS, ($CaSO_4 2H_2O$, Merck Company), and two concentrations (0 or 20 mg/ l) of GA_{4+7} (Serva Company, USA) were used. In the all continuous treatments, sucrose 1.5% was used.

There were three replications and three samples per treatment in a completely randomized design as factorial experiment. Each sample was one flower per bottle. Data were analyzed using three-way analysis of variance (PROC GLM), and the means

were compared by Tukey's Test (HSD) at p≤0.05 using SAS (9.1) statistical software. Correlation coefficients between vase life and cell conditions and flavonoid were conducted by SPSS (version 11.5). Regression analysis (path analysis) was taken to determine the major factors that affect vase life (dependent variable). The independent explanatory variables were anthocyanin leakage, tissue pH, TSS and flavonoid. The software used for path analysis was SPSS/PC+ "Stepwise" (version 11.5).

Vase life

Vase life was recorded from harvest time by the time the flowers showed symptoms of petal wilting or curling, stem bending (≥90°) or breaking, therefore, the flowers were visited daily.

Total flavonoid assay

In termination of the vase life, to extract total flavonoid, 20 ml of acidic methanol (1% HCl) was added to 0.2 g fresh weight of petals, and the mixture was stirred for 48 h in the dark. The extract was used to measure total flavonoid content immediately (Chang *et al.*, 2002). Total flavonoid content was measured by aluminum chloride colorimetric assay (Chang *et al.*, 2002; Kumar *et al.*, 2008). An amount of 200 µl of plant extract was added to 600 µl of methanol, 40 µl $AlCl_3$ (10%), 40 µl of potassium acetate (1 M) and was made to 2000 µl by distilled water. The solution was vigorously mixed and after keeping at room temperature in the dark for 30 min, the absorbance was measured against reagent blank at 510 nm with a spectrophotometer (T80+ UV/VIS Spectrometer, PG Instruments Ltd). The calibration curve of standard solutions of catechin (5-40 µg/ ml of 1% HCl in methanol) was drew (y= 0.0003x + 0.1654, R^2= 0.9989). Total flavonoid content of flower was expressed as mg catechin equivalent per 100 g of fresh weight.

Anthocyanin leakage

To evaluate the effects of treatments on the cell membrane structure, anthocyanin leakage was measured to observe the stability of plasma membrane. At tenth day of vase life, 0.5 g of petals was sliced to pieces of 1×1 cm, these pieces were washed in DI water two times and within a period of 2 h. Then 10 ml of DI water was added to samples. After 12 h in 25°C, the absorbance was recorded with a spectrophotometer (T80+ UV/VIS Spectrometer, PG Instruments Ltd) at 525 nm (Poovaiah, 1979).

Tissue pH measurement

Tissue pH was measured according to the method of Hill (1999) to consider the treatment influences on the conditions of cell reactions. In termination of vase life, 2 g of petals was crushed in liquid N_2, and then was placed at -80°C for 48 h. The frozen tissues were removed from -80°C, thawed at 20°C, then were frozen in liquid N_2 again and placed at -80°C for a further 36 h. After thawing at 20°C again, 25 ml distilled water was added to tissues in the test tube, then were frozen at -20°C for 24 h. The pH of filtered fluid was recorded after thawing with a pH meter.

Total soluble solid

Total soluble solid (TSS) of petal juice was measured with a refractometer (CETI, Belgium) as °Brix (Roein *et al.*, 2009).

3. Results and Discussion

Interactions between NS and CS significantly extended the vase life (Fig. 1). NS pulse treatments and then holding in preservative solutions (10 or 20 mM CS) extended the vase life compared to DI pulsing and preservative solution containing sucrose (control treatment) (Fig. 1). However, no significant (p<0.05) difference was found among various concentrations of NS and CS to extend the vase life. Also, interactions between CS and GA_{4+7} had significant effect on the extension of the vase life (p<0.05) (Fig. 2). The longest vase life was found in the pulse treatment with 3 mg NS/l and preservative solution containing 20 mM CS. It was 7.5 days (i.e., 62.5%) added to vase life compared to the control (Fig. 1). Gerbera is insensitive to ethylene (Liu *et al*., 2009 a); there-

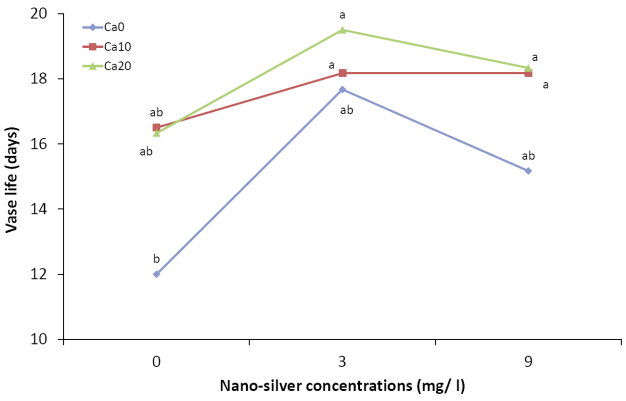

Fig. 1 - Interactions between nano-silver (0, 3 or 9 mg/l) and calcium sulfate concentrations (◆= 0 mM; ■= 10 mM; ▲= 20 mM) on the vase life (days) in cut gerbera flowers. The means with same letters have no significant differences (MS_{ANOVA} = 6.27; Type 1 Error, $HSD_{0.05}$), N=3.

fore, NS effects for extending the vase life of gerbera are not related to anti-ethylene effects of NS. Therefore, it must be explained that the basic role of NS is to prevent from bacterial plugging of the xylem (Liu *et al*., 2009 a, b; Solgi *et al*., 2009; Chaloupka *et al*., 2010; Lü *et al*., 2010); then increasing water uptake and calcium. According to Gerasopoulos and Chebli (1999), post-calcium uptake prevents appearing the symptoms of the end of vase life in gerbera [wilting, petal curling, stem bending (in this cultivar was not observed) and breaking (in this cultivar was observed partially)]. The longest vase life was obtained in preservative solutions containing 10 mM CS without GA_{4+7} [approximately 19 days, i.e., 32.3% more than treatment with 20 mg/l GA, without CS, which had significant ($p<0.05$) differences with solutions containing 20 mg L^{-1} GA_{4+7} without CS] (Fig. 2). Gibberellin can enhance hydrolization of starch to glucose, and during enzymatic process sucrose will be produced. More production of sucrose causes strength of cell walls. Having more sugar in tissues preserves them of early disruption and increases their longevity (Halevy and Mayak, 1981). Also, Whitman *et al*. (2001) determined that GA_{4+7} sprayed to *Lilium longiflorum* had a positive effect to decrease foliar chlorosis and increased vase life contrary to our results; moreover, it decreased the effect of CS.

There were significant interactions ($p<0.05$) between GA, CS and NS on total flavonoid at the end of vase life (Table 1). The highest flavonoid content

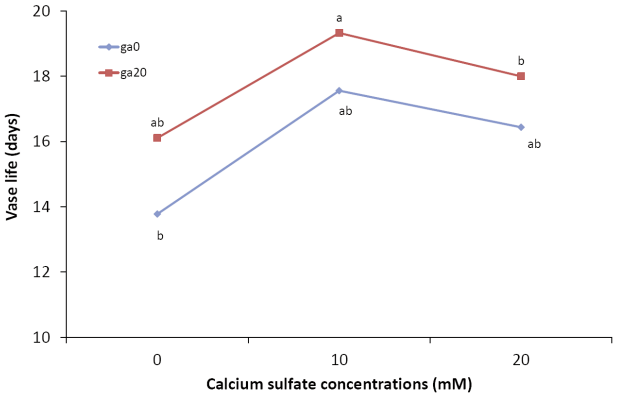

Fig. 2 - Interactions between calcium sulfate (0, 10 or 20 mM) and GA_{4+7} concentrations (◆= 0 mg GA_{4+7}/l; ■= 20 mg GA_{4+7}/l) on the vase life (days) in cut gerbera flowers. The means with same letters have no significant differences (MS_{ANOVA} = 1.41; Type 1 Error, $HSD_{0.05}$), N=3.

Table 1 - The effect of nano-silver pulsing and continuous treatments by calcium sulfate and gibberellin on the biochemistry factors of cut gerbera flowers

Treatments			Flavonoid (mg equivalent catechin per 100 g F.W.)	Anthocyanin leakage (Absorbance in 525 nm)	Tissue pH	TSS (° Brix)
Pulse treatment	Continuous treatment					
NS (mg/ l)	CS[z] (mM)	GA (mg/ l)				
0	0	0	3933.3 bcd	0.165 a	4.98 abcd	11.2 a
		20	2350.0 d	0.160 abc	5.15 a	10.8 ab
	10	0	4516.7 abcd	0.161 abc	4.94 bcd	11.1 a
		20	3877.8 bcd	0.161 abc	4.98 abcd	11.3 a
	20	0	8183.3 ab	0.160 abc	4.91 bcd	11.2 a
		20	8933.3 a	0.157 bc	4.94 bcd	9.9 ab
3	0	0	5711.1 abcd	0.159 abc	4.98 abcd	9.4 ab
		20	2988.9 cd	0.163 ab	5.01 abc	10.0 ab
	10	0	5655.6 abcd	0.155 c	4.88 bcd	9.0 ab
		20	3433.3 bcd	0.157 bc	4.83 cd	8.9 ab
	20	0	7544.4 abc	0.156 bc	4.86 cd	8.8 ab
		20	5377.8 abcd	0.155 c	4.83 cd	7.9 b
9	0	0	2933.3 cd	0.156 bc	4.96 abcd	9.8 ab
		20	3100.0 cd	0.157 bc	5.05 ab	10.4 ab
	10	0	2766.7 cd	0.155 c	4.85 cd	8.3 ab
		20	5322.2 abcd	0.156 bc	4.89 bcd	8.5 ab
	20	0	6488.9 abcd	0.156 bc	4.82 d	9.5 ab
		20	3711.1 bcd	0.157 bc	4.86 cd	8.9 ab
ANOVA (Mean of Square)						
NS			815234.8 NS	0.0001 **	0.04 **	18.97 **
CS			50446502.1 **	0.0001 **	0.12 **	4.47 *
GA			12438400.2 *	0.00000002 NS	0.02 *	0.74 NS
NS x CS			6853137.9 y	0.00002 *	0.0008 y	1.56 y
NS x GA			696546.46 y	0.00002 *	0.01 y	0.47 y
CS x GA			2485082.3 y	0.000003 y	0.01 y	1.52 y
NS x CS x GA			5213477.41 y	0.00001 y	0.0009 y	0.1 y

The means with different letters are significant ($HSD_{0.05}$). N= 3.** = significant at p≤0.01; * = significant at p≤0.05; NS = not significant. y= Type I Error.

was measured in the petals of flowers that were treated with DI and kept in the solution containing 20 mM CS and 20 mg/l GA. Whereas treated flowers with DI and kept in the solution containing 20 mg/l GA showed the lowest total flavonoid. The antioxidant role of flavonoids was revealed for the flowers that were not pulsed by NS, because, the microbial attack might be a signal to synthesize the flavonoids (Khatiwora *et al*., 2010). When calcium (Meyer *et al*., 1973) and gibberellin (Ranwala and Miller, 2000; Hatamzadeh *et al*., 2010) were made available, they activated the reducing of nitrate to produce phenylalanin, and to form simple carbohydrates, respectively. Therefore, the pathway of flavonoid synthesis was completed and total flavonoid was increased. Phenylalanie transforms into 4-coumaroyl-CoA in the phenylpropanoid pathway, and finally enters the flavonoid synthesis pathway (Falcone Ferreyra *et al.*, 2012).

The most significant interactions ($p<0.05$) were observed between GA, CS and NS on the anthocyanin leakage. In the flowers which were not treated with NS and/or CS, anthocyanin leakage was highest (Table 1). The stability of cell membrane will have been decreased by factors as senescence, microbial (attacking by micro-organism) or no microbial (deficit of calcium) diseases. The measurement of anthocyanin leakage at half of vase life could be a gauge to evaluate the stability of cell membrane. The accumulation of calcium in middle lamella of cell wall increases the stability of cell membrane and decreases anthocyanin leakage (Nikbakht *et al*., 2008). NS prevents microbial attack and decreases senescence and keeps stability (Liu *et al*., 2009 a; Solgi *et al*., 2009; Lü *et al*., 2010).

The most tissue pH was recorded at pre-treatment with DI and keeping in solution containing 20 mg GA/l, and the least tissue pH was at pulsing with 9 mg NS/l and keeping in calcium sulfate solution (20 Mm). Generally, significant differences were observed between treatments ($p<0.05$) (Table 1). Schmitzer *et al.* (2010) explained that increasing the cell sap pH causes the developing flowers from the bud to senescence stage.

The most TSS was measured in the flowers of control treatment, and the least TSS was recorded in the flowers which were pulsed with 3 mg NS/l and kept in solution containing 20 mM CS and 20 mg GA/l. Significant differences ($p<0.05$) were observed between two mentioned treatments. However, no one has the significant differences with other treatments. Gebremedhin *et al.* (2013) interpreted that TSS will be increased by more water uptaking to provide the required substrate for respiration.

The analysis of correlation coefficients (Table 2) shows the negative significant correlation ($p\leq0.01$) between vase life and anthocyanin leakage and TSS. Furthermore, there is positive significant correlation between anthocyanin leakage and TSS (Table 2). For the last parameter, the coefficient of multiple determinations (R^2) was 0.479 in linear model for the vase life (Table 3). This coefficient gives the proportion of the total variation in the dependent variable (vase

Table 2 - Correlation coefficients between vase life (days), anthocyanin leakage (AL), tissue pH, total soluble solids (TSS) and flavonoid (mg equivalent catechin per 100 g F.W.), N=3

Parameters	Vase life	AL	Tissue pH	TSS	Flavonoid
Vase life	1				
AL	-0.656 **	1			
Tissue pH	-0.353	0.412	1		
TSS	-0.692 **	0.757 **	0.375	1	
Flavonoid	-0.338	0.253	-0.373	0.206	1

** Significant in $p<0.01$ (two-tailed correlations), N=18.

Table 3 - Vase life of gerbera flowers regressed (stepwise regression) against anthocyanin leakage, tissue pH, TSS and flavonoid

Vase life			Linear model		
Variable	B	SE B	Standard *β*	*t*	Significance
Constant	32.265	4.04		7.987	0
TSS	-1.586	0.414	-0.692	-3.832	0.001
Multiple *R*	0.692				
R^2	0.479				
Adjusted R^2	0.446				
Standard error	1.82				
ANOVA					
	Sum of squares	df	Mean squares	*F*	Significance
Regression	48.834	1	48.834	14.688	0.001
Residual	53.197	16	3.325		
Total	102.031	17			

life) explained by the predictors included in the model. Thus, from among four independent variables, total soluble solids explained 47.9% of the observed total variation in the vase life, and other independent variable (anthocyanin leakage, tissue pH and flavonoid) had a lesser role in the vase life. Furthermore, the test statistic in linear model showed that coefficient of TSS negatively and significantly ($p \leq 0.01$) influenced vase life (Table 3, Fig 3). Therefore, the factors that cause the increased TSS and anthocyanin leakage lead to decrease vase life. This is also confirmed by other researchers (Gebremedhin *et al.*, 2013).

According to the results, we recommend pulse treatment with 3 mg NS/l and then, continuous treatment with 20 mM CS for increasing the vase life of cut gerbera by 8 days.

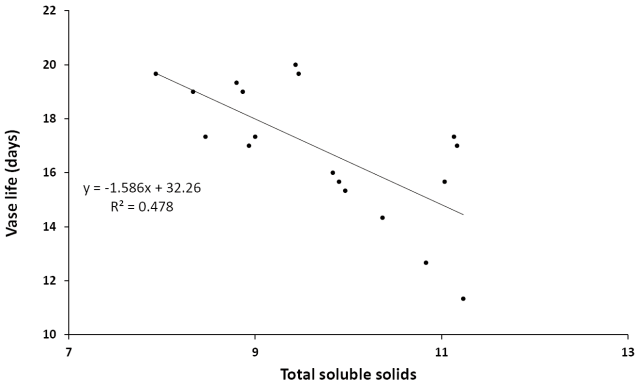

Fig. 3 - Relationship between vase life and total soluble solids, N=3.

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Yield and yield components of coriander under different sowing dates and seed rates in tropical environment

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Key words: biomass yield, coriander, fruit yield, seed rate, sowing date.

Abstract: Coriander makes use of favorable environmental conditions when it is sown at optimum time and rate. However, this information is very limited in the southeastern mid-highlands of Ethiopia. Field experiments were, therefore, conducted between 2011 and 2014 at three different research stations to determine optimum sowing dates and rates. The experiment had split plot design in randomized complete block with three replications, in which sowing dates and seed rates were the main and sub-plot treatments, respectively. The four sowing date treatments were June 20, July 10, July 30 and August 20 while the four seed rate treatments were 30, 40, 50 and 60 kg ha^{-1}. Coriander sowed in the third decade of July at Arsi Robe and from the first to the third decades of July at Kulumsa and Sagure gave the highest fruit and biomass yields. Earlier sowing in the second decade of June, and delayed sowing in the second decade of August brought fruit yield reductions of 37-66 and 37% at Arsi Robe, 27-45 and 58-66% at Kulumsa, and 24-40 and 26% at Sagure, respectively. However, coriander did not respond to seed rates. Owing to the enhanced yields of coriander, intermediate cultivation at a seed rate of 30 kg ha^{-1} was found optimum.

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1. Introduction

Coriander (*Coriandrum sativum L.*), which belongs to the family of Umbelliferae (Apiaceae) is one of the most important annual spice and medicinal herb. It is grown in Ethiopia and throughout the world for its seeds as well as leaves and has immense uses (Diederichsen, 1996; Hedburg and Hedburg, 2003; Parthasarathy *et al.*, 2008; Nowak and Szemplinski, 2014). Coriander originated from the Mediterranean and Western Asian regions (Burdock and Carabin, 2009). Along with central Asia and near east countries, Vavilov (1992) mentioned Ethiopia in the lists of centers of origin for coriander. Ivanova and Stoletova (1990) also reported that India, Northern Africa, Central Asia and Ethiopia are centers of formation and cradles for different types of coriander. There is a long-standing tradition of cultivation of coriander in Ethiopia (Diederichsen, 1996; Geremew *et al.*, 2014).

The immense uses of coriander depend on the choice of fruits or green herbs, which are linked to their chemical compositions. The most important constituents are the essential and fatty oils (Diederichsen, 1996). Coriander has got significant importance as a spice in culinary, food, beverage, medicine, perfumery, pharmaceuticals and sanitary industries (Jansen, 1981; Diederichsen, 1996; Delaquis *et al.*, 2002; Kubo *et al.*, 2004). On the other hand, its green foliage is used in vegetables owing to its richness in vitamins and other minerals (Singh *et al.*, 2005). In Ethiopia, coriander is widely used for domestic culinary. The seeds are used for flavoring the powder of hot red pepper locally called "*berbere*" and used for numerous meat and vegetarian dishes, leavened flat Ethiopian bread locally called "*injera*", cakes and bread. The leaves are added as an aromatic herb to tea and stew locally called "*wot*" (Jansen, 1981; Geremew *et al.*, 2014).

Coriander is also a good melliferous plant since it produces a considerable quantity of nectar and thereby attracts many different insects for pollination. Studies indicated that one hectare of coriander allows honeybees to collect about 500 kg of honey (Diederichesen, 1996). The residues left after extraction of the essential oils are used as best ruminant feed since they still contain as nearly the same digestible fat and protein content as the whole fruits (Diederichesen, 1996).

The success of coriander production is influenced by genetic, weather and agronomic factors (Nowak and Szemplinski, 2014). The maximum fruit and essential oil yields are attained only when an appropriate combination of these factors are provided for the plant (Rangappa *et al*., 1997; Gil *et al*., 2002). Coriander is among the tropical crops and generally sown in winter season if the objective is seed for production (Sharangi and Roychowdhury, 2014). As a temperature-sensitive crop, it generally requires a relatively cool, comparatively dry and frost-free weather during its early stage for good vegetative growth and relatively warm temperature during flowering and reproductive stage for high yields and good quality (Peter, 2004; Kalra, 2008; Sharangi and Roychowdhury, 2014). The ideal temperature for germination and growth of coriander is 20-25°C (Singhania *et al.*, 2006).

Coriander exploits the environment most favorably when it is sown at optimum time (Kuri *et al*., 2015) since sowing date significantly affects the photoperiodic response of plants and determines yields and qualities (Rasam *et al.*, 2007). Time of sowing controls the crop phenological development along with efficient conversion of biomass into economic yield (Khichar and Niwas, 2006). Earliness in sowing leads to untimely flowering; however, it may also pose susceptibility to the damage of extreme cold and frost. On the other hand, delay in sowing hampers growth, yield and quality of the crop due to deficiency of soil moisture at latter stages (Sharangi and Roychowdhury, 2014; Rashed and Darwesh, 2015).

Determination of optimum seed rate is also a basic element for successful coriander production (Rasam *et al.*, 2007). Many agronomic studies conducted in the world revealed that seed rate had a highly significant effect on the productivity and quality of coriander (Diederichsen, 1996; Kumar *et al*., 2007; Ghobadi and Ghobadi, 2010). Both low and high seed rates resulted in reduced yield and oil concentrations.

Rapid life cycle of coriander allows it to fit into different growing seasons, making it possible to grow the crop under a wide range of conditions (Lopez *et al.*, 2007). Cultivation of coriander in Ethiopia; however, is limited to the mid to highlands (1500-2500 m a.s.l.), where sufficient soil moisture can be provided from rainfall. It can also be cultivated in the lowlands if the rainfall is sufficiently supplemented by irrigation (Jansen, 1981; Geremew *et al.*, 2014).

Although coriander has got diverse uses, economic importance and one of the several plant species for which Ethiopia is known as a center of origin and diversity (Jansen, 1981; Diederichsen, 1996), it is one of the most neglected or under-utilized aromatic and spice crop (Beemnet and Getinet, 2010). The wealth of coriander is not yet exploited in Ethiopia. Compared to other crops, there is no or very limited information available on the agronomic packages. This study was, therefore, carried out to determine optimum sowing date and seed rate for increased yield of coriander in the southeastern mid-highlands of Ethiopia.

2. Materials and Methods

Description of the study sites

The experiment was conducted at three locations, namely Arsi Robe, Kulumsa and Sagure in the southeastern mid-highlands of Ethiopia. The sites are representatives of the region, where coriander cultivation can potentially be carried out, and optimum

sowing dates and seed rate studied. It was conducted for two seasons in 2011 and 2012 at Arsi Robe, and 2011 and 2014 at Kulumsa and Sagure. Due to infestation by unknown disease, the crop could not perform well in 2012 and 2013 at Kulumsa and Sagure, and harvesting could not be done. Arsi Robe, Sagure and Kulumsa are located from 8.4 to 8.6 N and 40.1 to 40.4 E, 8.01 to 8.15 N and 39.2 to 39.3 E and from 7.77 to 8.03 N and 38.94 to 39.31 E, respectively. The altitudes of the locations vary from 2200 m a.s.l. at Kulumsa to about 2500 m a.s.l. at Arsi Robe and Sagure. The dominant soil type of the three locations is characterized as vertisol (IUSS Working Group WRB, 2014).

Climate

Long-term mean annual rainfall at Arsi Robe, Kulumsa and Sagure were 937, 812 and 653 mm, respectively. Hence, Arsi Robe and Sagure had the highest and lowest, respectively rainfall with intermediate values at Kulumsa (Fig. 1). 56, 55 and 67% of the annual rainfall concentrated in the months of July and August at Arsi Robe; June, July and August at Sagure and July, August and September at Kulumsa. The major crop production activities are conducted between June to November; therefore, the rainfall amount and distribution during these months have significant influence on the yield and yield attributes. The highest rainfall was recorded in August at all study sites (Fig. 1). From August, the rainfall amount and distribution reduce sharply and reach the lowest in the month of December at all locations. November, December, January and February are dry months with the lowest records of rainfall amounts.

Long-term mean maximum temperature records of Arsi Robe, Kulumsa and Sagure were 22.3, 23.2 and 22.5°C, respectively. The corresponding values for mean minimum temperatures were 8.3, 10.5 and 8.8°C, respectively (Fig. 2). February was the hottest month at Arsi Robe and Sagure while the corresponding month at Kulumsa was March. December was the coldest month at all locations with the lowest records of 4.9, 8.0 and 5.1°C at Arsi Robe, Kulumsa and Sagure, respectively.

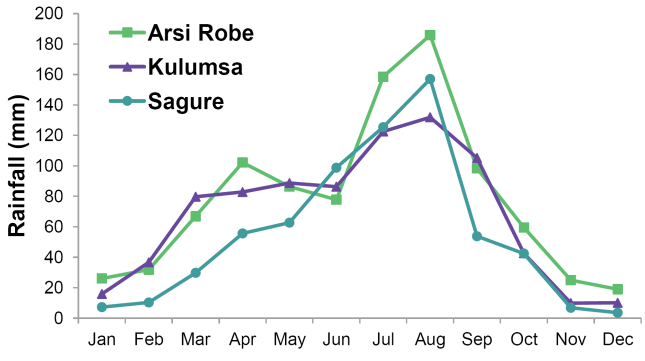

Fig. 1 - Total mean monthly rainfall at Arsi Robe, Kulumsa and Sagure.

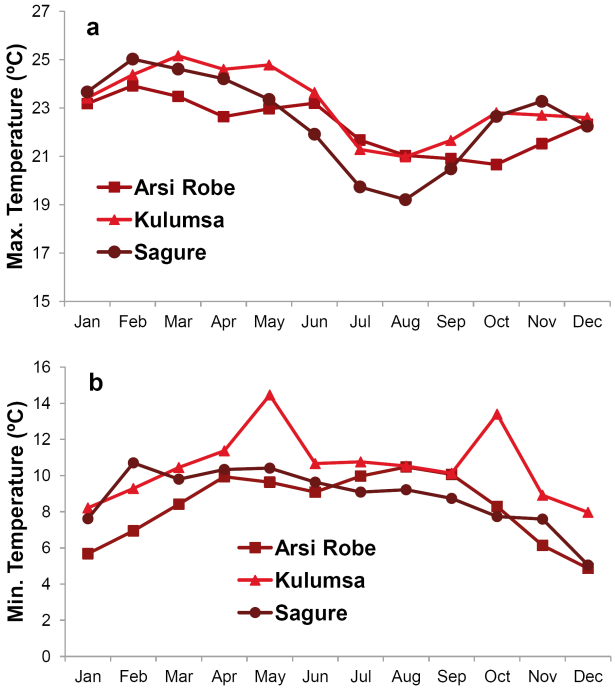

Fig. 2 - Mean annual maximum (a) and minimum (b) temperatures at Arsi Robe, Kulumsa and Sagure.

Experimental set-up and procedure

The experiment had split plot design in randomized complete block with three replications, in which the sowing dates and seed rates were the main and sub-plot treatments, respectively. The four sowing date treatments were June 20, July 10, July 30 and August 20 whereas the four seed rate treatments were 30, 40, 50 and 60 kg ha^{-1}. The sowing dates were set to choose the optimum time by allowing coriander to make maximum benefit from the suitable environmental parameters, especially rainfall and temperature for its successful establishment, survival and performance. On the hand, the seed rates were set by making reference to the existing recommendation of 40 kg ha^{-1}.

The seedbed preparation started in early April and totally plowed four times prior to planting. All experimental plots at each location and season were planted with coriander (cv. *Keteba*). The seedbeds were prepared in ridge and furrow, and seeds were drilled on raised beds by hand at 0.30m spacing between rows on the aforementioned sowing dates for all

sites in plot sizes of 1.8 m by 6 m. The spacing between plots and replications were 0.5 m and 1 m, respectively. The recommended phosphorus (20 kg P ha^{-1}) and nitrogen (18 kg N ha^{-1}) nutrients were uniformly applied to all plots close to the seed rows as basal dose at the time of sowing from di-ammonium phosphate (20-18 P-N). Weeds were controlled by manual cleaning.

Data collection

Twenty-five randomly selected plant samples were manually cut at the ground level from the inner four rows by excluding the outer two to avoid any border effect, air-dried, the moisture content adjusted to constant level and used for measuring above ground total biomass. The harvest index was calculated by dividing the dry mass of seeds collected from the 25 plant samples and threshed manually by the dry mass of biomass, and multiplying the ratio by 100. For the measurement of fruit yield, the whole crop was harvested from a net plot area of 6 m^2 (1.2 m by 5 m), subjected to air-drying and threshed manually. The fruits were detached from the biomass, cleaned and weighed. The seed moisture content was determined by placing samples from each plot in an oven at 105°C for 24 hours. The above ground total biomass and seed mass from each plot were then adjusted to 0 g kg^{-1} moisture content (dry weight) and expressed in kg ha^{-1} for statistical analysis purpose. Plant height data were taken from each plot at physiological maturity from ten plant samples.

Data analysis

All yield and yield components data were combined across sites and seasons and subjected to analysis of variance using the general linear model procedure (Proc GLM) of SAS statistical package version 9.2 (SAS Institute, 2002). Least significance difference (LSD) tests were employed to evaluate the means of the main and interaction effects of the treatments for each parameters measured (determined). Mean separation for the interaction effects were conducted using Minitab®18 statistical package (Minitab Inc.). When P<0.05, means values of treatments were declared as significantly different.

3. Results

The analysis of variance over two seasons indicated that sowing date and season as well as their interaction had very significant (p<0.001) effect on most of the traits measured at all locations (Tables 1-3).

Table 1 - Effects of sowing dates and rates by year, and their interaction on yield and yield components of coriander at Arsi Robe in 2011 and 2012

Sources of variation	Yield and yield components of coriander			
	Plant height (cm)	Harvest index (%)	Fruit yield (kg ha^{-1})	Biomass yield (kg ha^{-1})
Rep	NS	NS	NS	NS
Sowing Date (SD)	***	***	***	***
Error (a)	45.64	11.12	19566.89	317296
Year (Y)	***	NS	***	***
Seed rate (SR)	***	NS	NS	NS
SD x SR	NS	NS	*	NS
Y x SD	***	***	***	***
Y x SR	NS	NS	NS	NS
Y x SD x SR	NS	NS	NS	NS
Error (b)	32.67	13.5	16423.2	202979
CV	6.63	10.02	13.21	16.75

*** and NS means significant at P<0.001 and not significant at P<0.05, respectively.

Table 2 - Effects of sowing dates and rates by year, and their interaction on yield and yield components of coriander at Kulumsa in 2011 and 2014

Sources of variation	Yield and yield components of coriander			
	Plant height (cm)	Harvest index (%)	Fruit yield (kg ha^{-1})	Biomass yield (kg ha^{-1})
Rep	NS	NS	*	NS
Sowing Date (SD)	***	***	***	***
Error (a)	5.86	1.83	14609.46	870629
Year (Y)	***	***	***	NS
Seed rate (SR)	*	NS	NS	NS
SD x SR	NS	***	***	NS
Y x SD	***	***	***	NS
Y x SR	***	***	NS	NS
Y x SD x SR	*	***	NS	NS
Error (b)	7.09	8.84	46135.74	2447414
CV	3.02	10.06	13.2	25.05

*** and NS means significant at P<0.001 and not significant at P<0.05, respectively.

Table 3 - Effects of sowing dates and rates by year, and their interaction on yield and yield components of coriander at Sagure in 2011 and 2014

Sources of variation	Yield and yield components of coriander			
	Plant height (cm)	Harvest index (%)	Fruit yield (kg ha^{-1})	Biomass yield (kg ha^{-1})
Rep	NS	NS	NS	NS
Sowing Date (SD)	***	***	***	***
Error (a)	11.04	4.24	295392.79	2299583
Year (Y)	***	***	***	NS
Seed rate (SR)	***	NS	NS	NS
SD x SR	***	***	***	***
Y x SD	***	***	***	***
Y x SR	NS	***	NS	***
Y x SD x SR	*	***	NS	*
Error (b)	19.36	11.21	69124.93	928593
CV	4.93	10.71	18.52	20.75

*** and NS means significant at P<0.001 and not significant at P<0.05, respectively.

The effects of seed rate on fruit and biomass yields were not significant; however, its interaction with sowing date and season brought significant improvement on some of the variables measured. Results further showed that the interaction effects among sowing date, seed rate and season were not significant on most of the yield and yield components. The amount of variance associated with the sowing date x season interaction was the most important for this study.

Effect of sowing date

Sowing dates and their interaction with season significantly (P<0.001) affected the fruit and biomass yields of coriander at all locations except for the biomass yield at Kulumsa. The highest fruit yields of coriander were attained from coriander sowed on July 30 in 2012 and 2014 at Arsi Robe and Kulumsa, respectively; and July 30 and July 10 in 2014 and 2011 at Sagure, respectively (Tables 4-6). The coriander sown on July 30 and June 20 in 2012 and 2011, respectively at Ari Robe and July 10 in 2014 at Sagure gave the highest biomass yields. Fruit yields of 2713, 2028 and 2006 kg ha^{-1} were obtained at Kulumsa in 2014, Sagure in 2014 and Arsi Robe in 2012, respectively from coriander sowed on July 30. July 10 sown coriander at Sagure gave a fruit yield of 1928 kg ha^{-1}, which was statistically equivalent to the July 30 sown coriander. Similarly, biomass yields of 4732 and 4573 kg ha^{-1} were harvested at Arsi Robe in 2012 and 2011 from July 30 and June 20 sown coriander, respectively. The highest biomass yield at Sagure, 7217 kg ha^{-1}, was found from the coriander sowed on July 10 in 2011. The result further revealed that early (June 20) and late (August 20) sown coriander produced inferior fruit and biomass yields compared to the intermediate sowing dates.

The effects of sowing date and its interaction with season on the harvest index were also very significant (P < 0.001) at all locations (Table 1-3). The highest harvest indexes were obtained from the August 20 sown coriander at Arsi Robe and Kulumsa in both years (Tables 4-6). The values of harvest index from the August 20 sown coriander at Arsi Robe were 42.7 and 40.9% in 2012 and 2011, respectively. The corresponding values from the same sowing date at Kulumsa were 42.1 and 41% in 2014 and 2011,

Table 4 - Influences of sowing date and season on yield and yield components of coriander at Arsi Robe in 2011 and 2012

Sowing date	Year							
	Plant height (cm)		Harvest index (%)		Fruit yield (kg ha^{-1})		Biomass yield (kg ha^{-1})	
	2011	2012	2011	2012	2011	2012	2011	2012
June 20	72 de	112 a	34 cd	28 e	239 f	1272 b	721 e	4573 a
July 10	79 c	108 a	34.8 c	31.1 d	502 e	1147 c	1446 d	3702 b
July 30	68 e	96 b	38.6 b	42.9 a	683 d	2028 a	1809 d	4732 a
August 20	76 cd	80 c	40.9 ab	42.7 a	696 d	1193 bc	1736 d	2828 c

Table 5 - Influences of sowing date and season on yield and yield components of coriander at Kulumsa in 2011 and 2014

Sowing date	Year							
	Plant height (cm)		Harvest index (%)		Fruit yield (kg ha^{-1})		Biomass yield (kg ha^{-1})	
	2011	2014	2011	2014	2011	2014	2011	2014
June 20	106 a	105 a	18.9 e	20.5 de	1366 c	1493 c	7841 ab	8013 ab
July 10	100 b	101 b	29.2 c	27.5 c	1835 b	1991 b	6326 c	7206 bc
July 30	88 c	105 a	22 d	35 b	1876 b	2713 a	8559 a	7859 ab
August 20	48 d	50 d	41 a	42.1 a	793 d	934 d	1942 d	2220 d

Table 6 - Influences of sowing date and season on yield and yield components of coriander at Sagure in 2011 and 2014

Sowing date	Year							
	Plant height (cm)		Harvest index (%)		Fruit yield (kg ha^{-1})		Biomass yield (kg ha^{-1})	
	2011	2014	2011	2014	2011	2014	2011	2014
June 20	96 c	108 a	33.4 b	26.5 e	1523 b	1162 c	4638 cd	4507 cd
July 10	96 c	103 b	28.2 cde	29.1 cd	2006 a	1435 b	7217 a	4947 bc
July 30	78 d	102 b	40.4 a	35 b	1557 b	1928 a	3846 de	5495 b
August 20	64 e	66 e	30.2 c	27.4 de	888 d	845 d	3204 e	3320 e

respectively. Statistically equivalent harvest index (42.9%) was also provided at Arsi Robe from the coriander sowed on July 30 in 2012. The highest harvest index at Sagure (40.4%) was obtained from the July 30 sown coriander in 2011.

The influences of sowing date and its interaction on plant height was also very significant (P <0.001). Generally, early and late sown coriander resulted in the tallest and shortest plant heights, respectively, which were consistent over years and locations. Early sown coriander resulted in the tallest plant at Arsi Robe in 2012 (112 cm), Kulumsa in 2011 (106 cm) and 2014 (105 cm), and Sagure in 2014 (108 cm). The tallest plant heights at Kulumsa in 2014 (105 cm) and Arsi Robe in 2012 (108 cm) were also attained from coriander sowed on July 30 and July 10, respectively. Delay in sowing of coriander (August 20) brought the shortest plant height at Kulumsa (48 and 50 cm in 2011 and 2014, respectively) and Sagure (64 and 66 cm in 2011 and 2014, respectively).

Temporal variabilities significantly affected both yield and yield components of coriander at all locations (Tables 1-3). Most of the variables measured at Arsi Robe, Kulumsa and Sagure gained better advantage owing to sowing of coriander in 2012 than 2011, 2014 than 2011 and 2011 than 2014, respectively implied the influences of seasonal variabilities on yield and yield attributes. The effects of temporal variations on yield and yield attributes were more pronounced at Arsi Robe than Kulumsa and Sagure areas, which could be justified by the magnitudes of differences in the fruit yields recorded during the study period. The variances in the highest fruit yields between 2012 (2028 kg ha^{-1}) and 2011 (696 kg ha^{-1}) at Arsi Robe, 2014 (2713 kg ha^{-1}) and 2011 (1876 kg ha^{-1}) at Kulumsa, and 2011 (2006 kg ha^{-1}) and 2014 (1928 kg ha^{-1}) at Sagure were 1331, 837 and 78 kg ha^{-1}, respectively (Tables 4-6). This implied that the magnitude of the variabilities associated with the fruit yields between the two years were so large at Arsi Robe compared to Kulumsa and Sagure.

Spatial variabilities were also accountable for large disparities in yield and yield components of coriander among the testing locations. Fruit and biomass yields of coriander were superior at Kulumsa compared to Sagure and Arsi Robe. Mean fruit yields of coriander combined over season at Arsi Robe, Kulumsa and Sagure were 970, 1625 and 1418 kg ha^{-1}, respectively. The corresponding values for biomass yields were 2693, 6246 and 4647 kg ha^{-1}, respectively (Tables 4-6). Compared to Arsi Robe, Kulumsa and Sagure produced 68 and 46% more fruit yields and 132 and 73% more biomass yields of coriander, respectively.

Effect of seed rate

Seed rate did not bring significant effect on most of the yield and yield components of coriander measured at all locations (Tables 1-3), which were consistent over years and locations. However, its interaction with sowing date and year had significant effect on some of the yield and yield attributes of coriander at all locations (Tables 1-3). The fruit yields obtained from the July 30 sown coriander at a seed rate of 30 kg ha^{-1} at Arsi Robe and any one of the four seed rates at Kulumsa were found to be statistically superior over all other possible sowing date x seed rate interactions. This implied that the lowest seed rate, 30 kg ha^{-1}, could be sufficient for optimum yield of coriander in the study areas. Sowing of coriander on July 30 at a seed rate of 30 kg ha^{-1} gave fruit yield of 1526 kg ha^{-1}. Similarly, coriander sowed on July 30 at seed rates of 30, 40, 50 and 60 kg ha^{-1} at Kulumsa gave fruit yields of 2239, 2306, 2427 and 2204 kg ha^{-1}, respectively, which were statistically equivalent to each other but significantly different from the other treatments (Table 7). The seed rate x sowing date interactions at Sagure were not significant for the fruit yields of coriander.

Seed rate interaction with year brought significant effect on the fruit and biomass yields of coriander at Sagure only (Tables 1-3). Except for the biomass yield at Sagure, the sowing date x seed rate x year interac-

Table 7 - Influence of sowing date x seed rate interactions on the fruit yield of coriander at Arsi Robe, Kulumsa and Sagure from 2011 to 2014

Sowing date	Seed rate (kg ha^{-1})											
	Arsi Robe				Kulumsa				Sagure			
	30	40	50	60	30	40	50	60	30	40	50	60
June 20	907 de	746 f	695 f	674 f	1378 de	1293 e	1506 cde	1541 cd	1508 bcd	1272 def	1456 cde	1133 efg
July 10	781 ef	909 de	822 def	786 ef	2176 b	2086 b	1702 c	1688 c	1846 ab	1708 abc	1677 abc	1650 abc
July 30	1526 a	1356 b	1355 b	1184 c	2239 ab	2306 ab	2427 a	2204 ab	1544 bcd	1955 a	1683 abc	1787 abc
August 20	936 d	958 d	958 d	926 de	969 f	834 f	850 f	802 f	783 h	942 fgh	917 gh	826 gh

tions were also not significant at all locations for most of the attributes measured (Tables 1-3).

4. Discussion and Conclusions

The highest fruit and biomass yields from the July 30 sown coriander at Arsi Robe and Kulumsa, and July 10 to 30 sown coriander at Sagure attributed to the fulfillment of optimum soil moisture and thermal conditions from vegetative to reproductive stages (Figs. 3-5). Rainfall and its expected consequent soil moisture had significant impact on the performance of coriander at all locations. Coriander seeded to the sowing dates within or after the highest precedent rainfall events provided the highest fruit and biomass yields. The third decade of July in 2011 and 2012 at Arsi Robe, and the first decade of July in 2011 and 2014 at Sagure fell within the highest precedent rainfall amounts and linked with the highest fruit and biomass yields (Figs. 3-5). The highest amount of precedent rainfall could lead to the retention of optimum amount of water in the soil, which soak the seeds, soften its cover and assist embryonic stem to be emerged very easily. Compared to the other three sowing dates, the third sowing date (July 30) at Arsi Robe and the second sowing date (July 10) at Sagure in both years were linked with the highest rainfall events of the previous 10 days, and the highest fruit and biomass yields. The highest rainfall events of the previous 10 days at Kulumsa occurred in the second decade of August. Though the performance of crop at its initial stages was greater, it faced acute moisture stress at its latter development stage and resulted in inferior fruit and biomass yields. Compared to the remaining two sowing dates, the highest rainfall event at Kulumsa was recorded in the third decade of July, which was associated with superior fruit and biomass yields of coriander. Katar et al. (2016) reported increase in the fruit yield of coriander with increase in precedent rainfall.

The variations in the responses of coriander to sowing time were also accounted for temperature. The superior yield and yield attributes of coriander were found to be linked to the sowing dates, when their maximum temperatures were the lowest. The maximum temperature records of the periods were the lowest in the third decade of July at Arsi Robe, Kulumsa and Sagure (Figs. 3-5), which were associated with the highest fruit and biomass yields. The minimum temperatures at each location were not too low enough to affect the germination of seeds; rather it was modest for coriander. For successful germination, coriander requires low temperature since it favors germination by promoting the breakdown of reserve proteins in seeds to particular amino acids, which are necessary for growth of embryo (Robinson, 1954; Guha *et al.*, 2014). Temperature above optimum value hampers germination of seeds owing to the encouraged activities of several microorganisms such as bacteria and fungi. Activation of microorganisms, in turn, adversely affects the embryo and endosperm of coriander seeds (Naeem et al*.,* 2002; Ali et al., 2015). Generally, winter crops like coriander are vulnerable to high temperature particularly during reproductive stages (Kalra, 2008).

Earlier sowing (June 20) resulted in the fruit yield reductions of 37 and 66% in 2012 and 2011, respectively at Arsi Robe, 45 and 27% in 2014 and 2011, respectively at Kulumsa, and 24 and 40% in 2011 and 2014, respectively at Sagure. The corresponding declines in biomass yield were 60% in 2011 at Arsi Robe, and 36 and 18% in 2011 and 2014, respectively at Sagure (Tables 4-6). Those reductions in yields could be because of the storage of excess soil moisture in the root zone during seeding and the adverse effect of intensive rainfall particularly in August on the leaves and flower of coriander. The relatively older leaves and flowers of coriander sown earlier (June 20 and July 10) at Arsi Robe and Kulumsa, and (June 20) at Sagure fell due to heavy rainfall in August (Figs. 3-5). The premature shed of leaves and flowers reduced the performance of photosynthesis, fruit formation and ultimately the yield of coriander. The July 30 and August 20 sown coriander were not adversely affected due to the intensive rain fell in August as their leaves were still so young enough to recover soon, and the flowers were not yet blossomed.

Delayed sowing (August 20) of coriander also produced inferior fruit and biomass yields. With delay in sowing to August 20, fruit yield reductions of 37% in 2012 at Arsi Robe, 66 and 58% in 2014 and 2011, respectively at Kulumsa, and 26% in 2011 at Sagure were recorded. Similarly, delayed sowing resulted in biomass yield reductions of 40% in 2012 at Arsi Robe, 77 and 72% in 2011 and 2014 at Kulumsa, and 26% in 2011 at Sagure (Tables 4-6). The inferior yields of coriander attributed to the acute scarcities of rainfall in November at all locations (Figs. 3-5). Owing to delayed sowing and its consequential soil moisture stress, coriander had inadequate time to complete its vegetative growth since it entered to the reproduc-

tive phase at quicker rates and generally the whole crop-growing period was shortened (Carrubba *et al.*, 2006; Sharangi and Roychowdhury, 2014). Consequently, inferior development of shoots and reduced yield attributes occurred (Tables 4-6), which were the coriander's response to the acute shortage of rainfall and high temperatures that encountered at latter stages in the growing season (Carrubba *et al.*, 2006; Nowak and Szemplinski, 2014). Shortage of rainfall and its resulting soil moisture stress during the growing season brought physiological disorders such as a reduction in transpiration and photosynthesis (Sarker *et al.*, 2005; Carrubba *et al.*, 2006). Besides, reduced vegetative as well as reproductive growth of coriander and their consequent decrease in seed yield could be accounted for the above optimum temperatures. The optimum temperature might have been surpassed for the late sown coriander, and adversely influenced its physiological processes including photosynthesis and respiration (Sharma *et al.*, 2003).

The influences of precipitation and air temperatures on yield and yield components of coriander were reported in the works of Nowak and Szemplinski (2014). Bhadkariya *et al.* (2007) found 30.6 and 76.4% declines in the fruit yield of coriander owing to delay in sowing from March 30 to April 14 and April 29, respectively. Moosavi *et al.* (2012) reported that with delay in sowing, coriander fruit and biomass yields reduced by 76.4 and 74.7%, respectively. Moniruzzaman *et al.* (2015), Carrubba *et al.* (2006) and Zheljazkov *et al.* (2008) also reported that productivity declined as sowing postponed to latest dates.

Though earlier sowing resulted in the tallest plant heights, it could not increase the yields of coriander. The inferior yields attributed to logging of the crop, shed of leaves and flowers during the cropping season in response to heavy rain fell particularly in August. This result is in agreement with Pan *et al.* (2003), Carrubba *et. al.* (2006), Bhadkariya *et al.* (2007), Ghobadi and Gobadi (2010), and Moosavi *et al.* (2012) who reported that earlier sowing of coriander led to the highest plant height. Delay in sowing resulted in stunted growth and finally led to reduced yields of coriander. The significant decrease in plant height because of delay in sowing could be correlated with higher temperatures that the plants experienced at their latter stages. This incidence shortened their growing period and assimilate-building process owing to earlier maturity. Thus, plants could not maintain adequate opportunity for conducting photosynthesis; hence, their height and branch-bearing capacity could be decreased (Moosavi *et al.*, 2012). Sharangi and Roychowdhury (2014) found that with delay in sowing from October to December, plant height of coriander decreased significantly. Moosavi

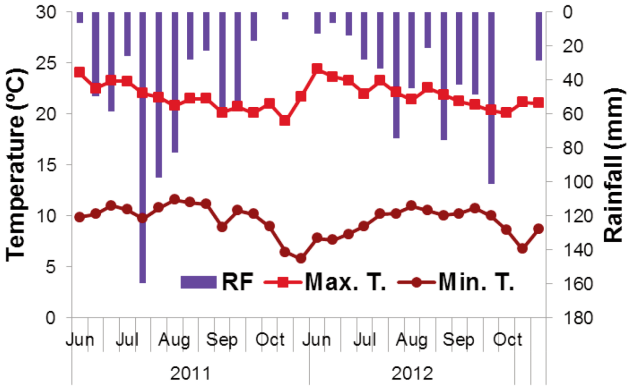

Fig. 3 - Ten-day values of rainfall, maximum and minimum daily air temperature records in Arsi Robe from June to October in 2011 and 2012 cropping seasons.

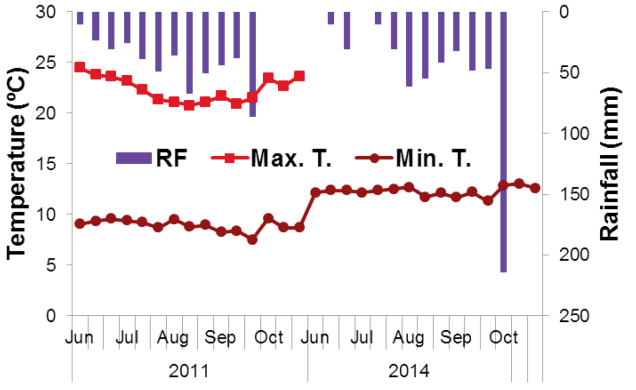

Fig. 4 - Ten-day values of rainfall, maximum and minimum daily air temperature records in Kulumsa from June to October in 2011 and 2014 cropping seasons.

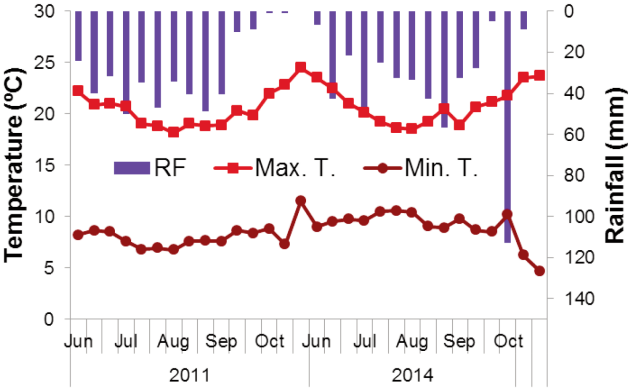

Fig. 5 - Ten-day values of rainfall, maximum and minimum daily air temperature records in Sagure from June to October in 2011 and 2014 cropping seasons.

(2012) also reported significant decrease in plant height with delay in sowing from March 30 to April 29.

The major factors that attributed to the variations in yields of coriander among locations and seasons were related to climate particularly rainfall and soil. In their studies, Rashed and Darwesh (2015) found that microclimate significantly affected sowing dates implied the need for determination optimum site-specific sowing date. Rainfall had contrasting effect at Arsi Robe and Kulumsa in 2011; it was excess at Arsi Robe, but optimum at Kulumsa. Compared to 2011, the rainfall amount and distribution in 2012 at Arsi Robe and 2014 at Kulumsa were optimum; hence, fruit and biomass yields obtained could be superior. These results corroborate the findings of Carrubba *et al.* (2006), who reported a highly significant dependence of coriander yields on precipitation during its growing period with linear correlation coefficient of 0.93. Nowak and Szemplinski (2014) also reported the significant influence of temporal variations on coriander yield and yield attributes.

The rainfall amount and its influence need to be interpreted in relation to the soil types of the study areas. Compared to Arsi Robe and Sagure, where the soils are heavy vertisols, the soil type of Kulumsa is relatively light vertisol. Hence, the occurrence of water logging at Kulumsa particularly during the months with heavy rainfalls was lower as compared to Arsi Robe and Sagure. Coriander gave the highest fruit and biomass yields in areas, where rainfall was modest and the soil was light vertisol. During the cropping season, rainfall was lower at Kulumsa than Sagure and Arsi Robe, and Sagure was lower than Arsi Robe. For example, cumulative rainfall from the first decade of June to the end of the third decade of August at Arsi Robe, Kulumsa and Sagure were 508, 282 and 302 mm, respectively indicating rainfall at Kulumsa was robust for the development of coriander. This further implied that rainfall amount had strong effect on fruit and biomass yields of coriander at the studied sites. As rainfall amount increased, fruit and biomass yields of coriander decreased; hence, fruit and biomass yield obtained from Kulumsa was superior followed by Sagure.

Among the tested seed rates, the lowest, 30 kg ha^{-1}, has been found optimum because under lower seed rate, plants tended to compensate the reduced sowing density by producing new branches and result in optimum fruit and biomass yields (Diederichsen, 1996). Okut and Yidirum (2005) reported that seed rate had no effect on harvest index of coriander while Ghobadi and Ghobadi (2010) observed no significant effect on 1000 fruit weights of coriander. The superior yield and yield attributes in response to the seed rate of 30 kg ha^{-1} signified the need for investigation under further reduced rates. Diederichsen (1996) reported that based on the weight of 1000 fruits, 3 to 20 kg ha^{-1} fruits of coriander could be sufficient for optimum yield.

The results of the current study indicated that sowing date significantly influenced the yield and yield attributes of coriander. However, the effects of seed rate and its interaction with sowing date were not significant on most of the variables measured. The optimum sowing dates that gave the highest fruit and biomass yields of coriander fall on third decade of July at Arsi Robe, and from first-third decades of July at Kulumsa and Sagure. Earliness and delay in sowing resulted in inferior yields. Though coriander did not respond to seed rate in this study, the lowest rate, 30 kg ha^{-1}, was found sufficient to give optimum yield. However, further low seed rate studies need to be conducted. Considering the demand of low input, fruit and biomass yields obtained throughout experimental periods, cultivation of coriander was satisfactory provided the crop was sown at appropriate sowing time. Owing to its economic benefits, soil improvement, nutritional qualities and reproductive roles, wider cultivation of coriander in the southeastern mid-highlands of Ethiopia and other areas with similar agro ecologies is encouraged.

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AHS
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Micropropagation of dwarf schefflera [*Schefflera arboricola* (Hayata) Merr.] via direct shoot regeneration

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Key words: *In vitro* propagation, NAA, *Schefflera arboricola*, shoot induction, TDZ, variegated leaves.

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Abstract: Schefflera [*Schefflera arboricola* (Hayata) Merr.] is one of the most popular ornamental house plants conventionally propagated by seeds. Rapid multiplication of elite clones is an important driving force for the pot plant's market. In this regard, *In vitro* clonal propagation of three schefflera cultivars, 'Luseane', 'Charlotte' and 'Gold Capella', was examined. Sterilization was done by 70% ethanol for 2 min and 1% sodium hypochlorite solution for 15 min. Shoot proliferation of the nodal segments was dependent on cytokinin supply. The greatest number of shoots was obtained when nodal explants were cultured on the MS medium with 0.5 mg l^{-1} TDZ for 'Luseane', or 8 mg l^{-1} BA for 'Charlotte' and 'Gold Capella'. Subculture of nodal segments harvested from the *in vitro* derived axillary shoots on the multiplication medium enabled continuous production of healthy shoots with similar frequency. Plantlets of 'Luseane' and 'Gold Capella' demonstrated 100% rooting using 2 mgl^{-1} NAA, while 'Charlotte' showed 93.75% root induction by 1 mgl^{-1} NAA. Plantlets were acclimatized successfully using peat moss and sand mixture ('Luseane'), loam soil, sand and leaf compost ('Charlotte') or peat moss and perlite mixture ('Gold Capella').

1. Introduction

Dwarf Schefflera [*Schefflera arboricola* (Hayata) Merr.] is an evergreen ornamental plant of the Araliaceae family, native to China and Taiwan (Ohashi, 1993). It is mostly used indoors as a foliage pot plant because of the attractiveness of the umbrella-like palmately compound leaves and variegated cultivars (Gilman and Watson, 1994; Chen *et al*., 2002). Schefflera's ability to clean the air and its tolerance to harsh interior environments has further increased its worldwide popularity (Yang *et al*., 2009; Dela Cruz *et al*., 2014). To satisfy grower's calls for potted plants of schefflera, methods for rapid propagation of selected cultivars are crucial. Multiplication of this house plant is mainly by seeds which results with the segregation of the progeny traits and limited to its native plantation in the tropics (Griffith, 1998; Chen *at al.*, 2002) and only to cultivars of

schefflera which have non-variegated green leaves (Marcotrigiano, 1997). Other propagation means are leaf-bud cuttings (Hansen, 1986), air layering (Gilman and Watson, 1994) and stem cuttings (Hansen, 1986). These practices are hindered by difficulties such as low number of propagules per plant, increased time of production and the risk of disease spread from several pathogens such as *Pseudomonas cichorii*, *Xanthomonas campestris*, *Phytophthora parasitica*, *Pythium splendens* and *Alternaria panax* (Chase and Poole, 1986).

Micropropagation is a reasonably more efficient way for schefflera production as a greater number of plants can be produced faster compared to the traditional cuttings. Moreover, *in vitro* culture of tropical ornamental plants has been recommended as a tool to eradicate the diseases that are frequently widespread in the mother plants (Hartmann and Kester, 2011). Tissue culture has furthermore resulted in enhanced features compared to common propagations. It was revealed that micropropagated foliage pot plants such as syngonium, spathiphyllum, dieffenbachia (Conover, 1992) and philodendron (Chen *et al*., 2012) had a packed and denser plant forms in comparison to the conventional stem cuttings. Consequently, small tissue culture-grown scheffleras may be readily suitable for the limited space available in the terrariums or bonsai pots.

Despite the fact that micropropagation is extensively exploited by the floriculture industry, their formulas are not released to the general public. To our knowledge, there is no tissue culture protocol available in the literature for *S. arboricola*, though a few number of articles have been published so far for other Araliaceae species with horticultural importance such as *Cussonia paniculata* (Tetyana and van Staden, 2001), *Fatsia japonica* (Choi *et al*., 2005), *Eleutherococcus senticosus* (Amin *et al*., 2003; You *et al*., 2005), *Panax quinquefolius* (Uchendu *et al*., 2011) *Hedera helix* (Sivanesan *et al*., 2011) *Polyscias balfouriana* (Ilyas *et al*., 2013) and *Polyscias fruticosa* (Sakr *et al*., 2014).

The current research developed, for the first time, a micropropagation procedure for three cultivars of shefflera with green, white and yellow variegated leaves. In order to reach a high propagation rate and get well plantlets, the effects of various sterilization treatments, plant growth regulators' type and concentration were studied on the survival rate, shoot proliferation and rooting of scheffleras. Subsequent acclimatization of the micropropagated plantlets was also investigated using different potting media.

2. Materials and Methods

Plant material and culture medium

Three marketable cultivars with green, white and yellow variegated leaves (i.e. 'Luseane', 'Charlotte' and 'Gold Capella', respectively) were used for micropropapagation. The explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) of sucrose, and 0.65% (w/v) of agar. The pH of the medium was set to 5.7 before autoclave sterilization (i.e., 20 min, at 121°C). The plant growth regulators were added to the medium later by filter sterilization. The sterile nodal segments were established in culture medium containing 2 mg l^{-1} benzyl adenine (BA). In order to proliferate the shoots, the emerging buds were removed after 30 days and subcultured to media containing different cytokinin treatments.

Sterilization of three Schefflera cultivars

The stock plants were sprayed with Ridomil fungicide (0.3%) one week before explant excision. Stem cuttings were soaked in 0.1% detergent solution for 10 min, then washed under running tap water for 30 min. The explants were moved to laminar flow cabinet and 1.5-cm long nodal segments were excised from the stem. For surface sterilization of the explants, 70% EtOH was tested for 0, 30, 60, 90, 120, 150 and 180 seconds. Explants were then disinfected using 0, 0.5, 0.75, 1 or 1.25% (v/v) sodium hypochlorite containing 0.01% Tween-20 for 5, 10 or 15 min on a shaker. After four rinses with sterile water and excision of the damaged edges, the explants were cultured on PDA medium (300 g potato extract, 30 g l^{-1} of dextrose and 8 g l^{-1} of agar) for the evaluation of the contamination. Five replicates were examined for each treatment with three explants in each flask. Contaminations and survival rates were recorded after 1 month and those having noticeable infection signs were instantly discarded.

Effect of cytokinin type and concentration on shoot proliferation

Inducing new shoots was carried out using BA and Kinetin (Kin) at 0, 1, 2, 4 and 8 mg l^{-1} concentrations while thidiazuron (TDZ) was tested at 0, 0.125, 0.25, 0.5 and 1 mg l^{-1} concentrations. Five replicate flasks were used for each treatment with three explants in each flask. The number and length of the proliferated shoots, and number of leaves produced per explant were recorded after 30 days. The regenerated shoots were later subcultured to the best cytokinin-containing MS medium.

Effect of auxin type and concentration on root induction

Shoots from the finest proliferation treatment were put in the glass flasks of MS medium containing 0, 0.1, 0.5, 1, 2 or 4 mg l^{-1} of indole-3-butyric acid (IBA) or naphthaleneacedic acid (NAA). After 15 days the shoots were transferred to half-strength MS medium devoid of auxins. Five replicate flasks were tested in each treatment with three shoots in each flask. The number and length of the induced roots and rooting percentage of the shoots were recorded 15 days later.

Effect of potting media on plant acclimatization

Rooted shoots were carefully taken out from the glass flasks and washed under distilled water to get rid of attaching agar from the roots. The plantlets were then transferred to 10-cm plastic pots filled with different potting media: peat moss, peat moss and perlite (1:1), peat moss and sand (1:1), loam soil, sand and leaf compost (1:1:1). Ten replications were tested for each of the treatments. Potted plants were grown in a greenhouse with temperatures ranging from 24 to 28°C, relative humidity of between 70 and 90%, and light intensity of 35 µmol m^{-2} s^{-1} under a 12 h photoperiod. After 45 days the survival rate and plant height were analyzed.

Culture conditions, experimental design and data analysis

The *in vitro* researches were carried out with an environment temperature of 25±2°C and a 16/8 h light/dark photoperiod delivered by cool fluorescent lamps at 35 µmol m^{-2} s^{-1}. All the tests were arranged in a completely randomized design. Analysis of the variances was done by SAS software (SAS Institute Inc., 2002) and means were compared by LSD test at 5% probability level.

3. Results

Sterilization of three Schefflera cultivars

For the determination of the most effective surface sterilization, explants were soaked in 70% EtOH solution for 0-180 sec. As the soaking time was increased to 180 sec, surface sterilization was increased gradually and eventually reached 100% in all three cultivars, however viability was rapidly decreased after 120 sec (Table 1). Thus, 120 sec, providing the highest disinfection (72.8%, 79.6% and 72.8% for 'Luseane', 'Charlotte' and 'Gold Capella', respectively) and viability (72.8%, 74.6% and 72.8% for Luseane', 'Charlotte' and 'Gold Capella', respectively) altogether, was selected. The results were not significant between the cultivars.

The explants of 'Luseane' were effectively disinfected when 1.25% sodium hypochlorite was applied for 15 min, however this application resulted with a significant decrease of the viability to 13% (Table 2). The most effective combination of disinfection (93.2%) and viability (86.4%) was obtained using 1% sodium hypochlorite for 15 min. 'Charlotte' demonstrated a similar pattern of sterilization. The utmost control of contamination (93.2%) and viability (86.4%) was observed using 1% sodium hypochlorite for 15 min or 1.25% sodium hypochlorite for 10 min. Similarly, also for 'Gold Capella', the best percentages of sterilization (93.2%) and viability (86.4%) were obtained by using 1% sodium hypochlorite for 15 min.

Effects of cytokinin type and concentration on shoot proliferation

Effects of benzyl adenine and kinetin treatments on each schefflera cultivars. The increase of BA concentration to 8 mg l^{-1} significantly increased the num-

Table 1 - Effects of ethanol treatments on surface sterilization and viability of three schefflera cultivars

70% Ethanol (sec)	'Luseane'		'Charlotte'		'Gold Capella'	
	Bacterial sterilization (%)	Viability (%)	Bacterial sterilization (%)	Viability (%)	Bacterial sterilization (%)	Viability (%)
0	0±0 e	0±0 e	0±0 d	0±0 d	0±0 e	0±0 d
30	19.8±8.08 d	19.8±0 d	13.2±8.08 d	13.2±0 d	13.2±8.08 de	13.2±0 cd
60	33±0 d	33±0 cd	46.2±8.08 c	46.2±0 b	26.4±6.60 d	26.4±0 c
90	52.8±8.08 c	52.8±0 b	53.2±8.25 c	53.2±0 b	59.6±6.65 c	59.6±0 a
120	72.8±6.80 b	72.8± 0 a	79.6±8.33 b	74.6±6.6 a	72.8±6.80 bc	72.8±0 a
150	93.2±6.80 a	39.6±6.60 c	100±0 a	33±0 c	86.4±8.33 ab	39.6±6.6 b
180	100±0 a	19.8±8.08 d	100±0 a	13.2±8.08 d	100±0 a	19.8±8.08 c

Data (±SE) are the mean values of five replicates with three explants in each.
For each cultivar, different lowercase letters indicate significant differences among treatments (LSD multiple range test, P≤0.05).

ber of shoots per explant of 'Luseane' to 3.75 (Table 3). This concentration of BA also produced 12.5 leaves per explant. However, the largest shoot length of 0.88 cm was obtained using 4 mg l^{-1} BA. On the contrary, increasing Kin concentration to 4 mg l^{-1} and above resulted with the decrease in the shoots number. Kin also produced significantly lesser leaves at all the concentrations tested, and did not significantly increase the shoot length.

Treatment of 'Charlotte' with cytokinins revealed a similar pattern as 'Luseane'. Indeed, the greatest shoot proliferation of 3.75 shoots per explant was obtained using 8 mg l^{-1} BA, which also produced 15.3 leaves per explant and maximum shoot length of 0.90 cm. Also Kin increased the shoots number at the same concentration, though it was significantly lower than what was obtained with BA. The maximum shoot length and number of leaves observed with 8 mg l^{-1} Kin were 0.73 cm and 5.67, respectively, and both were significantly lower than what was observed with BA.

Proliferation of the 'Gold Capella' with 8 mg l^{-1} BA resulted with maximum of 1.75 shoots per explant, the largest shoot length of 0.83 cm and 7.58 leaves per explant, which were all significantly higher than Kin treatments (Fig. 1). Adding 2 or 4 mg l^{-1} Kin to the medium slightly increased the shoot length compared to the control.

Fig. 1 - Effect of different cytokinin treatments on shoot proliferation of schefflera cvs. Luseane (a), Charlotte (b) and Gold Capella (c). Shoots were produced using MS medium supplemented with 0.5 mg l^{-1} TDZ for 'Luseane' and 8 mg l^{-1} BA for the others.

Table 2 - Effects of sodium hypochlorite treatments on sterilization and viability of three schefflera cultivars

Cultivar	Minutes	Sodium hypochlorite (%)									
		Sterilization (%)					Viability (%)				
		0	0.5	0.75	1	1.25	0	0.5	0.75	1	1.25
'Luseane'	5	13.2±8.08 g	24.75±6.39 fg	33±0 e-g	41.25±6.39 d-f	49.5±7.38 de	13.2±0 f	24.75±0 ef	33±0 d-f	41.25±0 c-e	49.5±0 cd
	10	13.2±8.08 g	33±0 e-g	52.8±8.08 de	74.5±6.58 bc	86.4±8.33 ab	13.2±0 f	33±0 d-f	52.8±0 cd	74.5±0 ab	79.6±6.80 ab
	15	13.2±8.08 g	49.5±7.38 de	59.6±12.51 cd	93.2±6.80 ab	100±0 a	13.2±0 f	49.5±0 cd	59.6±0 cd	86.4±6.80 a	13.2±8.08 f
'Charlotte'	5	19.8±8.08 f	33±10.44 ef	33±0 ef	41.25±6.39 de	49.5±7.38 de	19.8±0 ef	33±0 d-f	33±0 d-f	41.25±0 c-e	49.5±0 cd
	10	19.8±8.08 f	41.25±6.39 de	52.8±8.08 de	74.5±6.58 bc	93.2±6.80 ab	19.8±0 ef	41.25±0 c-e	52.8±0 b-d	74.5±0 ab	86.4±6.80 a
	15	19.8±8.08 f	49.5±7.38 de	59.6±12.51 cd	93.2±6.80 ab	100±0 a	19.8±0 ef	49.5±0 cd	59.6±0 bc	86.4±6.80 a	13.2±8.08 f
'Gold Capella'	5	13.2±8.08 g	19.8±8.08 fg	26.4±6.60 e-g	39.6±6.60 d-f	52.8±8.08 cd	13.2 0 f	19.8±0 ef	26.4±0 d-f	39.6±0 c-e	52.8±0 bc
	10	13.2±8.08 g	26.4±6.60 e-g	52.8±8.08 cd	66±0 bc	86.4±8.33 ab	13.2±0 f	26.4±0 d-f	52.8±0 bc	66±0 ab	86.4±6.80 a
	15	13.2±8.08 g	46.2±8.08 c-e	59.6±12.51 cd	93.2±6.8 0ab	100±0 a	13.2±0 f	46.2±0 b-d	59.6±0 bc	86.4±6.80 a	13.2±8.08 f

Data (±SE) are the mean values of five replicates with three explants in each.
For each cultivar, different lowercase letters indicate significant differences among treatments (LSD multiple range test, P≤0.05).

Table 3 - Effects of Benzyl adenine and Kinetin on shoot proliferation of each schefflera cultivars

Cultivar	Control	BA (mgl^{-1})				Kin (mgl^{-1})			
		1	2	4	8	1	2	4	8
'Luseane'									
No. of shoots	0.83±0.1 f	1.25±0.05 de	1.5±0.07 c	2.75±0.08 b	3.75±0.25 a	1.42±0.03 cd	1.42±0.05 cd	1.17±0.06 e	0.92±0.08 f
Shoot length (cm)	0.24±0.03 f	0.50±0.02 c	0.39±0.01 d	0.88±0.01 a	0.73±0.02 b	0.27±0.03 ef	0.27±0.02 ef	0.31±0.005 e	0.31±0.01 e
No. of leaves	0.66±0.36 f	8±0.64 c	8.5±0.4 c	10.42±0.81 b	12.5±2.06 a	1.42±0.32 e	2±0.49 e	2.75±0.52 d	1.67±0.27 e
'Charlotte'									
No. of shoots	0.45±0.21 g	1.67±0.14 f	1.92±0.36 e	2.5±0.48 c	3.75±0.55 a	2.25±0.28 d	1.67±0.41 f	1.67±0.14 f	3±0.36 b
Shoot length (cm)	0.36±0.11 f	0.36±0.12 f	0.45±0.16 de	0.72±0.11 b	0.90±0.15 a	0.47±0.05 d	0.40±0.08 ef	0.38±0.05 f	0.73±0.13 b
No. of leaves	1.17±0.4 g	10.5±0.74 c	10.83±0.1 c	11.83±0.74 b	15.3±1.6 a	2.25±0.31 f	3.67±1.03 e	3.33±0.36 e	5.67±0.62 d
'Gold Capella'									
No. of shoots	0.42±0 g	0.75±0.04 de	0.83±0.1 cd	1.08±0.08 b	1.75±0.08 a	0.67±0.08 ef	0.75±0.08 de	0.92±0.08 c	0.58±0.08 f
Shoot length (cm)	0.19±0.05 e	0.59±0.04 b	0.47±0.04 c	0.76±0.06 a	0.83±0.03 a	0.25±0.02 e	0.39±0.02 cd	0.33±0.01 d	0.23±0.01 e
No. of leaves	0.58±0.08 e	4.41±0.64 c	5.66±0.56 b	5.83±0.52 b	7.58±0.75 a	0.75±0.21 e	0.83±0.22 e	2±0.38 d	1.66±0.14 d

Data (±SE) are the mean values of five replicates with three explants in each.
For each cultivar, different lowercase letters indicate significant differences among treatments (LSD multiple range test, P≤0.05).

Comparison of thidiazuron treatments on shoot proliferation of schefflera cultivars. The highest shoot proliferation (6.25 shoots per explant) was observed in 'Luseane', using 0.5 mg l^{-1} TDZ (Table 4) (Fig. 1). The maximum shoot proliferation of 'Charlotte' and 'Gold Capella' significantly lower than 'Luseane', were 3.33 and 1.33 shoots per explant, using 0.5 and 1 mg l^{-1} TDZ, respectively. The largest shoot length of 2.33 cm was observed in the 'Charlotte' when 0.5 mg l^{-1} TDZ was added to the medium. The other cultivars' shoot length were significantly lower. Furthermore, 'Charlotte' produced the maximum leaves per explant (i.e. 13.5 leaves) using 0.5 mg l^{-1} TDZ, while other cultivars yielded significantly fewer leaves per explant (8.75 for 'Luseane', 2.42 for 'Gold Capella') at 0.25 mg l^{-1} TDZ and 1 mg l^{-1} TDZ, respectively.

Effect of auxin type and concentration on root induction

Rooting of 'Luseane' was 100% when 2 or 4 mg l^{-1} IBA as well as 0.5, 2 or 4 mg l^{-1} NAA treatments were applied (Table 5). The maximum number of roots per explant (i.e. 18.67 roots) was observed using 2 mg l^{-1} NAA (Fig. 2). Moreover, the largest root length of 2.74 cm was produced by this treatment. However, 0.5 or 2 mg l^{-1} NAA appeared to have no significant differences with the former treatment. Also, the highest shoot length of 2.96 cm was recorded using 1 mg l^{-1} NAA, albeit no significant differences were found with 0.5, 2 and 4 mg l^{-1} NAA treatments.

The rooting of 'Charlotte' raised to the maximum 93.75% using 2 mg l^{-1} IBA or 1 mg l^{-1} NAA. Other con-

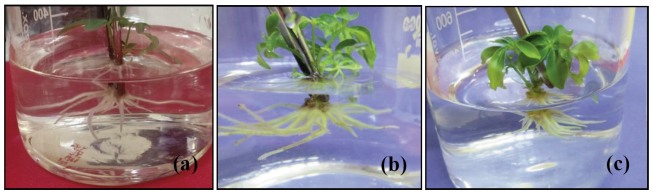

Fig. 2 - Root induction of schefflera cvs. Luseane (a), Charlotte (b) and Gold Capella (c) after using 2, 1 and 2 mg l^{-1} of NAA, respectively. Note: the roots were submerged in distilled water for better visibility.

Table 4 - Effects of thidiazuron on shoot proliferation of schefflera cultivars

TDZ (mgl^{-1})	Luseane'			Charlotte'			'Gold Capella'		
	No. of shoots	Shoot length (cm)	No. of leaves	No. of shoots	Shoot length (cm)	No. of leaves	No. of shoots	Shoot length (cm)	No. of leaves
0	0.83±0.1 gh	0.24±0.03 l	0.66±0 36 j	0.45±0.21 l	0.36±0.11 h	1.17±0.4 l	0.42±0 l	0.19±0.05 l	0.58±0.08 j
0.125	1.65±0.02 e	0.71±0.01 ef	5.40±0.48 f	1.75±0.08 e	1.28±0.19 c	5.75±0.67 f	0.67±0h l	1.02±0.01 d	1.92±0.16 h
0.25	5.58±0.08 b	1.05±0.05 d	8.75±0.76 c	1.83±0.1 e	1.34±0.13 c	7.33±0.79 d	0.75±0.08 h	0.58±0.04 g	1.75±0.21 h
0.5	6.25±0.08 a	0.66±0.04 fg	6.25±0.82 e	3.33±0.47 c	2.33±0.25 a	13.5±2.58 a	1.08±0.08f g	0.36±0.02 h	1.75±0.16 h
1	5.75±0.25 b	0.80±0.02 e	5.75±0.61 f	2.33±0.24 d	1.76±0.15 b	9.5±1.31 b	1.33±0.02 f	0.41±0.02 h	2.42±0.08 g

Data (±SE) are the mean values of five replicates with three explants in each.
For each cultivar, different lowercase letters indicate significant differences among treatments (LSD multiple range test, P≤0.05).

Table 5 - Effects of auxins on rooting and young shoot growth of three schefflera cultivars

Cultivar	Control	IBA (mgl^{-1})					NAA (mgl^{-1})				
		0.25	0.5	1	2	4	0.25	0.5	1	2	4
'Luseane'											
Rooting (%)	8.25±6.39 d	33±0 c	66.25±10.59 b	74.5±6.58 b	100±0 a	100±0 a	33±10.44 c	100±0 a	91.75±6.39 a	100±0 a	100±0 a
No. of roots	0.25±0.11 h	2.58±0.26 g	7.43±0.24 f	11.24±0.35 d	13.83±0.56 c	10.38±0.37 de	2.75±0.12 g	9.75±0.54 e	15.08±0.34 b	18.67±0.33 a	13.83±0.44 c
Root length (cm)	0.31±0.24 c	0.58±0.14 c	1.78±0.16 b	1.78±0.2	1.94±0.1 b	1.49±0.08 b	0.66±0.2 c	2.46±0.17 a	2.67±0.23 a	2.74±0.08 a	1.95±0.11 b
Shoot length (cm)	1.85±0.1 c	1.5±0.05 d	1.95±0.08 c	2.03±0.14 bc	1.94±0.06 c	1.89±0.06 c	2.27±0.08 b	2.87±0.12 a	2.96±0.19 a	2.77±0.12 a	2.82±0.14 a
'Charlotte'											
Rooting (%)	58.25±12.44 c	62.5±7.76 bc	83.5±7.38 ab	87.5±5.59 a	93.75±4.84 a	85.5±6.61 a	62.5±7.76 bc	91.75±6.39 a	93.75±4.84 a	91.75±6.39 a	83.5±7.38 ab
No. of roots	3.75±0.58 e	5.02±0.33 d	9.49±0.36 c	12.25±0.45 b	12.28±0.33 b	10.18±0.56 c	6.33±0.86 d	15.06±0.46 a	16.46±0.69 a	13.33±1.21 b	9±0.32 c
Root length (cm)	1.13±0.12 c	1.04±0.14 c	1.88±0.04 b	1.86±0.1 b	1.88±0.24 b	1.17±0.06 c	1.32±0.19 c	2.49±0.06 a	2.47±0.15 a	2.49±0.33 a	1.49±0.09 b
Shoot length (cm)	2.41±0.26 b	1.53±0.07 c	1.99±0.07 b	2.28±0.11 b	2.19±0.13 b	1.52±0.1 c	2.28±0.1 b	2.92±0.1 a	3.34±0.16 a	3.21±0.18 a	2.26±0.14 b
'Gold Capella'											
Rooting (%)	0±0	41.75±12.44 b	85.5±6.61 a	100±0 a	100±0 a	100±0 a	50±16.70 b	100±0 a	100±0 a	100±0 a	100±0 a
No. of roots	0±0	0.72±0.14 d	6.99±0.3 c	6.86±0.25 c	9.39±0.46 b	7.14±0.35 c	1±0 d	8.69±0.62 b	8.88±0.26 b	11.83±0.76 a	9.13±0.32 b
Root length (cm)	0±0	0.75±0.06 f	1.63±0.03 e	1.91±0.1 d	2.09±0.07 cd	1.92±0.02 c	0.90±0.08 f	2.15±0.04 c	2.53±0.14 b	2.78±0.09 a	2.55±0.03 b
Shoot length (cm)	1.29±0.01 g	1.3±0.03 g	1.66±0.13 d-f **	1.45±0.07 fg	1.92±0.14 cd	1.52±0.06 e-g	1.77±0.11 de	2.47±0.18 b	2.17±0.09 bc	2.83±0.19 a	2.26±0.08 b

Data (±SE) are the mean values of five replicates with three explants in each.
For each cultivar, different lowercase letters indicate significant differences among treatments (LSD multiple range test, P≤0.05).

centrations of IBA and NAA did not show significant discrepancies. Supplementing the medium with 1 mg l^{-1} NAA produced the greatest number of roots per explant with an average of 16.46 roots (Fig. 2). On the other hand, 0.5 mg l^{-1} NAA resulted with 15.06 roots which had no significant differences with the former treatment. Furthermore, using 0.5 and 2 mg l^{-1} NAA produced the largest root length of 2.49 cm, though 1 mg l^{-1} NAA produced similar root length of 2.47 cm with no significant differences. The maximum shoot length of 3.34 cm was measured using 1 mg l^{-1} NAA in the medium, although 0.5, and 2 mg l^{-1} NAA treatments did not yield significant differences with the former.

One hundred percent rooting was observed with 'Gold Capella' shoots when IBA was applied at 1, 2 or 4 mg l^{-1}, and NAA at 0.5, 1, 2 or 4 mg l^{-1}. The greatest number of roots per plantlet (i.e. 16.46 roots) was obtained using 2 mg l^{-1} NAA treatment (Fig. 2). Moreover, the highest shoot and root length (2.78 and 2.83 cm respectively), were measured by addition of 2 mg l^{-1} NAA, which showed significant differences compared to the other auxin treatments.

Effect of potting media on plant acclimatization

Schefflera's survival rate was not significantly affected by different potting media. The highest survival rate of 100% was obtained for 'Luseane' and 'Charlotte' using peat moss and sand (1:1) and loam soil, sand and leaf compost (1:1:1), respectively (Table 6). 'Gold Capella' showed a survival rate of 90% using peat moss or peat moss and perlite medium (1:1).

4. Discussion and Conclusions

Infection of tissue cultures of ornamental pot plants is a common phenomenon and an important barrier for their mass production. Contaminations in *Aglaonema* (Chen and Yeh, 2007), *Anthurium* (Kunisaki, 1980), *Dieffenbachia* (Brunner *et al*., 1995), *Spathyphyllum* and *Syngonium* (Kneifel and Leonhardt, 1992), *Zantesdeschia* (Kritzinger *et al*., 1998), as well as *Philodendron* (Fisse *et al*., 1987, Chen *et al*., 2012) are commonly reported. Thus, the current research was designed by setting up a disease-free shoot stock culture. Following surface sterilization with 70% EtOH for 120 sec and disinfection with 1% sodium hypochlorite for 15 min, more than 93% of the cultures were devoid of visible contaminations and they demonstrated 86.4% of viability for all tested cultivars. These results are consistent with the findings on *Fatsia japonica* Decne. (Choi *et al.*, 2005), *Polyscias balfouriana* (Ilyas *et al.*, 2013) and *Polyscias fruticosa* (Sakr *et al.*, 2014) from the Araliaceae family.

The green schefflera cultivar 'Luseane', showed a significantly greater number of shoot induction (6.25 shoots per explant) compared to the white- and yellow-variegated cultivars (Tables 3, 4). This phenomenon could be ascribed to the fact that variegated plants have a decreased propagation rate (Marcotrigiano, 1997). Vitrification problem and further necrosis of the new shoots was observed in 'Charlotte' and 'Gold Capella' when applying maximum concentration of 8 mg l^{-1} BA (Fig. 3). Thus, a comparable 0.5 mg l^{-1} TDZ was suggested for *in vitro* propagation of these cultivars instead of the former treatment. Sivanesan *et al*. (2011) reported increased number of shoots in *Hedera helix* 'Mini' by using a combination of 0.5 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA. The mode of action of TDZ may be through alteration in energy levels, nutrient uptake, nutrient assimilation, or cell membranes of plants (Murthy *et al*., 1998). However, Tetyana and van Staden (2001) demonstrated that 2.5 mg l^{-1} BA supplement to the media in

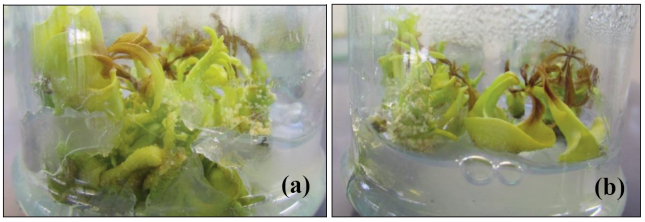

Fig. 3 - Vitrification and necrosis of schefflera plantlets using 8 mg l^{-1} of BA in 'Charlotte' (a) and 'Gold Capella' (b).

Table 6 - Effect of potting media on acclimatization of three schefflera cultivars

Mixture	Survival (%)			Shoots length (cm)		
	'Luseane'	'Charlotte'	'Gold Capella'	'Luseane'	'Charlotte'	'Gold Capella'
Peat moss	90±10 ab	90±10 ab *	90±10 ab	3.86±0.07 b	3.66±0.25 b	3.48±0.18 bc
Peat moss and perlite	90±10 ab	90±10 ab *	90±10 ab	4.81±0.22 a	4.49±0.2 a	4.92±0.17 a
Peat moss and sand	100±0 a	90±10 ab *	80±13.33 ab	3.60±0.1 bc	3.23±0.18 cd	2.94±0.23 d
Loam soil, sand and leaf compost	90±10 ab	100±0 a *	70±15.28 b	3.65±0.08 bc	3.72±0.24 bc	3.79±0.17 b

For each cultivar, different lowercase letters indicate significant differences among treatments (LSD multiple range test, P≤0.05).

Cussonia paniculata of the Araliaceae family, resulted with the highest shoot induction of 3.5 shoots per explant. On the other hand, the outcomings of You *et al*. (2005) in *Eleutherococcus senticosus*, the other member of the Araliaceae family, were different than our findings. It was revealed that 2 mg l^{-1} BA proliferated shoots more effectively than TDZ, Kin and 2iP. Various cytokinin types and concentrations affect shoot proliferation of plant species, though all belong to the same family. Therefore, for each plant species, the shoot induction has to be newly investigated.

It was revealed that after the TDZ treatment, shoots' length of all cultivars were comparatively longer than those of the Kin and BA treatments. The white variegated schefflera 'Charlotte' showed the greatest length (2.33 cm) using 0.5 mg l^{-1} TDZ. This effect could be related to further increase of auxin production by TDZ treatment (Murthy *et al*., 1998). It is assumed that TDZ has the ability to affect the amount of internal plant hormones (Murch and Saxena, 2001). Zaytseva *et al*. (2016) reported a similar high activity for low concentrations of TDZ in *Rhododendron*.

Although the highest number of leaves was recorded by using 8 mg l^{-1} BA in all of the cultivars, other treatments are suggested due to the aforementioned vitrification problem. For 'Charlotte', 0.5 mg l^{-1} TDZ treatment and for the two other cultivars 4 mg l^{-1} BA produced maximum leaves without necrosis. Generally, cytokinins enhance the photosynthesis and help translocate the nutrients to the leaves (Taiz and Zeiger, 2006).

A sought-after plant tissue culture protocol essentially depends on sufficient rooting along with a successful acclimatization of the young plants. The results of the current study show that in all of the cultivars NAA is more effective in rooting than IBA. There are inconsistent reports on the rooting efficacy of different auxins. The research of You *et al*. (2005) indicated that 0.5 mg l^{-1} NAA is more effective than IBA for rooting of *E. senticosus* shoots, while a concentration of 0.75 mg l^{-1} IBA or 1 mg l^{-1} NAA have proven useful for *C. paniculata* (Tetyana and van Staden, 2001). Auxin treatment of 'Charlotte', 'Luseane' and 'Gold Capella' increased the rooting percentage of the shoots, compared to the control by 35%, 92% and 100%, respectively. All shoots of 'Charlotte' and 'Luseane' were well acclimatized with a survival rate of 100% using media of loam soil, sand and leaf compost (1:1:1) and a mixture of peat moss and sand (1:1), respectively, while 'Gold Capella' demonstrated a none-significant lower survival frequency of 90% in the peat moss or peat moss and sand media (1:1).

To our knowledge, the current research is the first report on micropropagation of different cultivars of schefflera. Results demonstrated that green cultivar 'Luseane' had a greater shoot proliferation than the other two cultivars with average shoot number of 6.25 shoots per explant using 0.5 mg l^{-1} TDZ. The multiplicated shoots could lengthen on PGR-free MS medium and later showed effective rooting on NAA-contained media. Although the yellow variegated cultivar 'Gold Capella' revealed a smaller proliferation rate of shoots compared to the control, its rooting was 100% improved. Subsequent relocation of the 'Luseane' and 'Charlotte' plantlets to the acclimatization greenhouse resulted with 100% survival rate of the rooted shoots. Generally, peat moss and perlite media (1:1) had a more positive effect on the young acclimatized plants of schefflera. The protocol developed in this research can be used for the aseptic production of schefflera.

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The effect of polyamines and SICS on the compatibility, fertility and yield indices of apple cv. Golden Delicious

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Abstract: The most critical problems of temperate fruit trees are poor fruit set and low yield. To solve the problem, some major chemical compounds such as polyamines and SICS (self-incompatibility control substance, Mn+B) can be used. Popular polyamines including Putrescine (0.1 and 0.25 mM), both Spermine and Spermidine (0.05 and 0.25 mM), and SICS (1 and 2 mg L^{-1}) were used alone or with cotton coverage bags to cover branches in order to investigate self-incompatibility in *Malus domestica*. Results showed that Spermidine (0.25 mM) led to higher yields in comparison with that of the control. SICS (2 mg L^{-1}), also, demonstrated the highest yield compared with that of the control. At June fruit set, treatment with Spermidine (0.25 mM) led to the highest percentage of fruit set and also the highest index of self-incompatibility and percentage of final fruit set among treatments.

1. Introduction

Alternate bearing and self-incompatibility are the main issues in apple. Alternate bearing is vigorous in some apple cultivars like 'Golden Delicious'. Most apple cultivars are self-incompatible and self-unfruitful; therefore, they need another cultivar for pollination and fertilization. Gametophytic self-incompatibility (GSI) occurs when the S allele of the pollen grain matches either of the S alleles of the stigma. In such a case, the pollen tube begins to develop but stops before reaching the micropyle (Asatryan and Tel-Zur, 2013). Gametophytic self-incompatibility is controlled by glycoprotein with RNase (S-RNase) activity expressed in the pistil. S-RNase (Qing-qing *et al.*, 2009; Duca *et al*., 2010; Uchida *et al.*, 2012) encoded by the *S*-locus gene is named *SFB* (*S*-haplotype-specific F-box gene) in the Rosaceae. To date, in Rosaceae family, SI has mainly been studied in Japanese pear (*Pyrus pyrifolia*) and almond (*Prunus dulcis*) (Qing-qing *et al.*, 2009; Uchida *et al.*, 2012). However, some requirements are necessary for suitable cross pollination: having (1) compatible pollen grains with enough quantity and high quality (in fact, pollination with

semi-compatible pollen resulted in lower fruit-set than that with fully compatible pollen) (Sapir *et al.*, 2008), (2) an overlap period of pollination between pollinizer and pollinator (pollinizer trees must be cultivated along with the pollinator trees) (3) proper time of flowering and blooming, and finally, (4) having attractive flowers in order to reduce the number of bees visiting weeds on the orchard floor (the pollen grain of fruit trees is sticky and heavy causing not to be carried by the wind) (Bekey and Burgett, 1981).

Hand pollination is a type of cross pollination that can be effective on producing crop in adverse weather conditions. When the king flower opens, it is necessary to place bee colonies in order to do cross pollination by bees and then remove them at petal fall in fruit orchards: however, this method is also time-consuming and needs a big number of workers (Bekey and Burgett, 1981; Maib *et al.*, 1996). Despite the presence of hives, pollen transfer limitation and subsequent seed set reductionwas observed in orchards (Quinet *et al.*, 2016). The other suitable technique applied to do pollination involves chemical methods; in fact, some chemical compounds can be used in order to achieve enough fruit set and high yield. Plant bio regulators (PBRs) had significant effects on increasing pollen germination and pollen tube length in almond pollen: the action of these PBRs significantly increased the percentage of fruit set at both the bud pink and petal fall phenological stages (Maita and Sotomayor, 2015). As an alternative, the use of polyamines (putrescine, spermine, spermidine), that are natural compounds involved in plant growth and development process, has been suggested reduce flowers and fruits drop; polyamines competes with ethylene synthesis with whose they share the same precursor called s-adenosyle methionine (Crisosto *et al.*, 1988; Khezri *et al.,* 2010). Polyamines as plant bio regulator revealed to increase pollen tube growth and fruit set by stimulating pollen germination (Crisosto *et al.*, 1988; Liu *et al.,* 2006) and were effective on pollen tube elongation (Aloisi *et al.*, 2015). Also, self-incompatibility control substance (SICS), which is a mixture of manganese and boron (Son *et al.*, 2009), could be useful for increasing fruit set. Polyamines and SICS were effective on increasing yield and fruit set in crops such as pear (Crisosto *et al.*, 1988; Son *et al.*, 2009), apple and apricot (Asadi *et al.*, 2013), olive (Costa *et al.*, 1986), sweet cherry (Grant Sheard, 2008), sweet orange (Saleem *et al.*, 2008), pistachio (Khezri *et al.*, 2010), mango (Malik *et al.*, 2005), and date palm (Tavakoli and Rahemi, 2014). With regard to the effect of these compounds on pollen tube growth, Spermidine (Spd) and Spermine (Spm) influence the promotion of the pollen tube elongation at Polyamines concentrations up to 50 mM in Rosaceae family, whereas higher concentration of Spd and Spm resulted inhibitory for pollen tube elongation in Rosaceae family and correlate with male sterility in *Actinidia deliciosa* (Aloisi *et al.,* 2015). They are compounds useful in enhancing ovule longevity (Crisosto *et al.*, 1988; Liu *et al.*, 2006), without having any deleterious and toxic effects on human life (Azh *et al.*, 2014). In the initial stage of fruit development, an active cell division takes place, which possibly needs sufficient polyamines. At the later stage of fruit development, polyamine synthesis is reduced. As biosynthesis of polyamines takes place before pollen tube emergence, low level of free polyamines in cytoplasmic male sterile plants influences cell division and its enlargement, leading to abnormal development and low viability of pollens (Liu *et al.*, 2006). Polyamines increase pollen tube growth and fruit set by stimulating pollen germination. As well, they play a role in carbohydrates and nitrogen regeneration, followed by increasing chlorophyll content and leaf area (Baninasab and Rahemi, 2008). Use of polyamines (Put, Spm, Spd) at bloom improves ovule longevity in fruit crops such as apricot and pear. Higher endogenous polyamine contents have been correlated with improved ovule viability in apricot and sour cherry (Grant Sheard, 2008).

Boron increased pollination activity (Nyomora *et al.*, 1997). In boron deficiency, phenolic compounds aggregates on stigma. The accumulation of these compounds due to the activation of dehydrogenase enzyme led to the pollen grain not to be germinated. Boron increases pollen grain viability by increasing the flavenoids content of pollen grain (Marschener, 1995).

The percentage of fruit set as a vital factor is as important as other quantitative and qualitative traits to achieve an acceptable yield in apple (*Malus domestica*). It seems the contemporary application of polyamine and SICS together can enhance their effects. Although there are numerous researches on the individual application of polyamine and SICS, there was not found any literature comparing the effect of polyamines and SICS on yield indices, especially along with cotton coverage bags on the index of fertility (IF) and the index of self-incompatibility (ISI). The aim of this study was to increase yield indices in apple cultivar 'Golden Delicious', accompanying the decrease of fruit drop using polyamines and SICS.

2. Materials and Methods

The study was carried out on thirteen year-old apple trees cv. Golden Delicious in an orchard located in Mashhad (latitude of 36°20'and altitude 59° 34'). It is an area with arid and semiarid climate and annual average precipitation of 255 mm. The foliar application was done with 5 l sprayer on four selected branches between bud swollen and flower opening pheological stages at early morning. The compounds included polyamines [putrescine (Put) 0.1 or 0.25 mM; spermine (Spm) 0.05 or 0.25 mM; spermidine (Spd) 0.05 or 0.25 mM] and SICS [1 mg/L (3.5 mg boric acid, 6.8 mg manganase sulphate) and 2 mg/L (6.8 mg boric acid, 13.6 mg manganese sulphate)] (Son *et al.*, 2009) with (+) or without (-) cotton coverage bags (ccb), and control (untreated) plants. Cotton coverage bags were used in order to prevent cross and open pollination.

In this study, the following traits were measured: yield, percentage of fruit set [initial (2 weeks after petal fall); June (fruit drop in June); final (at harvest)] and fruit drop. Index of fertility (IF) was measured based on the percentage of initial fruit set ratio in each treatment compared to control. Index of self-incompatibility (ISI) was evaluated based on the percentage of final fruit set ratio in each treatment in comparison with control; in this regard, the ratios of 0.2, between 0.2-1, and higher than 1 represent incompatibility, semi-compatibility and full self-compatibility, respectively (Zeinanlo *et al.*, 2001; Azimi *et al.*, 2008; Seifi 2008; Taslimpour and Aslmoshtaghi, 2013).

The randomized complete block design with four replicates was applied in this study. At final, the data were analyzed by SAS software ver. 9.1 (SAS Institute, 2004) and the means were compared using LSD test at 0.05.

Fruit diameter and length were measured with non destructive method during the fruit growth period until the harvest time. This method was better than the destructive technique. Non destructive method are less time consuming and no need to laboratory space, and without harvesting. According to this method, fruit length and diameter were measured every 2 weeks without harvest, whereas in destructive method, these parameters are measured in fruits harvested every 2 weeks and their length and diameter were measured. Therefore, non destructive method was used in the present study (Arzani *et al.*, 1999; Dehghani *et al.*, 2012).

3. Results

The effects of polyamines and SICS on yield and fruit set

Without cotton coverage bags. Data showed that Spd treatment (0.25 mM) led to a higher yield in comparison to the control ($P<0.05$). SICS (2 mg L^{-1}) demonstrated the highest yield among all of the treatments ($P<0.05$). It was also found that Put (0.1 mM) and Spm (0.05 mM) showed increase in yield compared with the control, but it was statistically non-significant at 0.05 level.

SICS (1 mg L^{-1}) increased initial fruit set when compared to control.

The highest June and fruit set related to Spd (0.25 mM) among all of the treatments ($P<0.05$).

With cotton coverage bags. There was significant increase in percentage of initial fruit set by application of SICS (2 mg L^{-1}), and Spm (0.25 mM) (Table 1) in comparison to the control. The results also demonstrated the higher percentage of initial fruit set under treatment with Spd (0.05 mM) than the control, but it was statistically non-significant at 0.05 level.

Percentage of final fruit set showed statistically significant difference ($P <0.05$) in most of the treatments in comparison with the control, except for Put (0.1 mM), SICS (2 mg L^{-1}), Spm (0.25 mM), Spm (0.05 mM) and Spd (0.25 mM).

The effects of polyamines and SICS on IF and ISI

The index of fertility increased in treatments with Spm (0.25 mM + ccb) and SICS (2 mg L^{-1} + ccb).

All of the treatments could led to semi or full fertility and compatibility, due to the percentage of fruit set ratio in each treatment compared to the control was larger than 0.2.

The effects of polyamines and SICS on fruit drop

In general, final fruit drop decreased significantly in treatments with Put (0.1 mM) and Spd (0.05, 0.25 mM).

Without coverage. SICS (1, 2 mgL) decreased percentage of initial fruit drop in comparison with the control of 12 and 10%, respectively ($P <0.05$). Final fruit drop decreased significantly in treatments with Put (0.1 mM) and Spd (0.05, 0.25 mM).

With cotton coverage. Spm (0.25 mM) demonstrated the significantly ($P<0.05$) decrease of 14% in percentage of initial fruit drop in comparison with the control.

Table 1 - The effect of polyamines (putrescine, spermine and spermidine) and SICS on yield, fruit set and first drop

Treatments	yield (g)	Initial fruit set (%)	June fruit set (%)	final fruit set (%)	IF	ISI	first drop (%)	final drop (%)
Control	1230 cde	55.893 d-g	40.395 b-e	18.02 f	1 b-e	1 bcd	30.7 cde	68.25 a-e
Without cotton coverage bag								
Put (0.1 mM)	1416.7 c	50.179 e-h	39.046 c-f	28.636 bc	0.8625 def	1.4872 b	34.091 cd	42.424 g
Put (0.25 mM)	1101 def	15.152 j	37.231 c-f	27.778 bc	0.5565 f	0.3715 e	31.548 cde	55.357 efg
Spd (0.05 mM)	728.5 ghi	34.953 hi	32.749 c-g	22.281 de	0.6352 ef	1.2683 bcd	25.417 d-g	51.25 fg
Spd (0.25 mM)	1990.8 b	41.692 ghi	61.722 a	34.722 a	0.7621 def	2.4166 a	18.75 gh	20.313 h
Spm (0.05 mM)	1274.5 cd	74.605 abc	43.367 bcd	29.592 bc	1.3588 ab	1.0409 bcd	24.107 efg	76.259 ab
Spm (0.25 mM)	1130 def	69.86 bcd	23.077 g	19.268 f	1.2552 abc	1.529 b	64.245 a	68.593 a-e
SICS (1 mg L^{-1})	452 ij	75.63 abc	52.241 ab	20.26 f	1.3693 ab	1.2772 bcd	19.063 fg	56.818 d-g
SICS (2 mg L^{-1})	2349 a	59.127 c-e	40.857 b-e	29.241 b	1.0773 bcd	1.4317 bc	9.43 h	58.772 c-f
With cotton coverage bag								
Put (0.1 mM)	556 ij	42.361 f-i	28.03 fg	18.374 f	0.8869 c-f	1.4954 b	35.714 c	64.249 b-e
Put (0.25 mM)	1116 def	33.145 i	27.083 fg	22.917 de	0.7276 def	1.2771 bcd	48.889 b	72.222 a-d
Spd (0.05 mM)	696 hi	60.348 cde	30.925 efg	20.049 c	1.0839 bcd	1.4104 bc	50.379 b	63.258 b-f
Spd (0.25 mM)	984 efg	56.25 d-g	43.75 bc	18.5 f	1.015 b-e	0.7663 de	16.193 gh	77.399 ab
Spm (0.05 mM)	355 j	51.667 e-h	43.089 bcd	20.365 f	0.9567 cde	0.8757 cde	56.034 ab	74.353 abc
Spm (0.25 mM)	599 ij	84.858 ab	31.579 efg	17.526 f	1.5371 a	1.2116 bcd	22.5 efg	55 efg
SICS (1 mg L^{-1})	900.7 fgh	57.143 defg	32.712 c-g	25.173 cd	1.054 bcd	1.3247 bcd	28.333 c-f	56.667 d-g
SICS (2 mg L^{-1})	720 ghi	87.326 a	44.355 bc	19.903 f	1.5973 a	1.1082 bcd	49.958 b	83.953 a

Means with the same letters were not significantly different according to LSD (0.05).
Spm= Spermine, Spd= Spermidine, Put= Putrescine, IF=index of fertility, ISI=index of incompatibility.

The effects of polyamines and SICS on fruit growth habit

Based on figure 1, fruit growth totally increased in all of the treatments; in fact, fruit length and diameter increased gradually from July 9 to harvest (Fig. 1 b and d) whereas Spm (0.25 mM + ccb) showed a different trend.

4. Discussion and Conclusions

The effects of polyamines and SICS on yield and fruit set

In the present study, application of polyamines [Put (0.1, 0.25 mM), Put (0.25 mM + ccb), Spd (0.05, 0.25 mM) and Spd (0.05 mM + ccb)] between periods of swollen buds and the start of flowering phenological stages could increase fruit set at final fruit set stage. Several researchers studied about the effects of polyamines on yield indices. Polyamines (Put, Spm, Spd) enhanced ovule longevity at bloom in fruit crops such as apricot, pear and sour cherry (Grant Sheard, 2008). Polyamine synthesis had also positive influence on development and viability of pollen and it occurred before pollen tube emergence. Therefore, lower content of free polyamines caused male sterility in flowers (Liu *et al.*, 2006). Exogenous application of different polyamines at full bloom had influence on increasing fruit set and total yield in apples, olive, litchi and mango. Increase in fruit set and yield by polyamines was due to raising pollination, fertilization and fruit retention (Costa *et al.*, 1986). Saleem *et al*. (2008) stated that polyamines significantly increased initial fruit set and yield and maximum fruit set was observed in Spd, Spm and Put, respectively. Put had positive effect on increasing ovule longevity, EPP (Effective pollination Period), N and B and might raise the pollen tube growth rate in the styles of pears (Crisosto *et al.*, 1988), but their role on raising ovule longevity might be due to the improved nutritional status of the flower (Grant Sheard, 2008). Application of Put raised fruit set and yield of 'Comice' pear at the start of flowering (Crisosto *et al.*, 1988). Fruit set, crop density and yield efficiency under low fruit set conditions were improved by Put application at flower opening stage in pear (Crisosto *et al.*, 1988).

According to our results, the effects of polyamines on fruit set and yield were in agreement with Crisosto *et al.* (1988) on pear, Malik *et al.* (2005) on mango, Grant Sheard (2008) on sweet cherry, Saleem *et al.* (2008), Khezri *et al.* (2010) and Asadi *et al.* (2013) on pistachio.

In present study, SICS (2 mg L^{-1}) had also a positive influence on fruit set and yield. In boron deficiency, phenolic compounds aggregate on stigma. Accumulation of these compounds due to activation of dehydrogenase enzyme did not lead to germination of pollen grain. Boron increased pollen grain viability by increasing the flavenoids content of pollen grain (Marschener, 1995). Boron increased pollination activity (Nyomora *et al.*, 1997). Foliar application

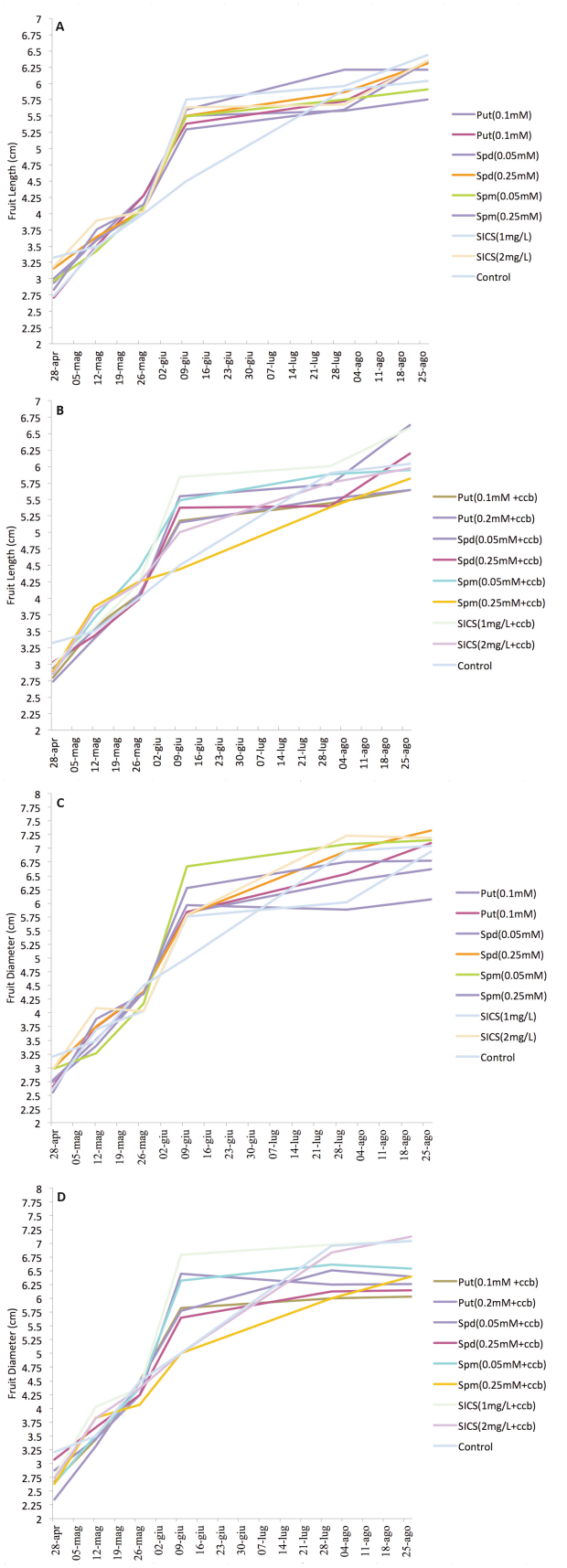

Fig. 1 - Effects of foliar application of polyamines and SICS on fruit length without ccb - (a), with ccb (b) and fruit diameter without ccb (c) and with ccb (d) of apple (cv. 'Golden Delicious'). Put= Putrescine, Spm= Spermidine, Spr= Spermine, ccb= cotton coverage bags.

of boron increased yield (Mashayekhi and Atashi, 2008) and fruit set in comparison with control and fruit abscission was lower than control (Khoshghalb *et al.*, 2011). Maz Ardalan and Savaghebi Firoozabadi (1997) reported that foliar application of Mn increased fruit yield. High levels of boron in floral organs such as the stigma and style, may aid pollen germination and make faster pollen tube growth down the style and into the ovary. Application of SICS a day before full bloom at 1 or 2 mg L^{-1} on three pear cultivars increased fruit set especially at 1 mg L^{-1} SICS. Furthermore, SICS at 2 mg L^{-1} ascended the number of seeds (Son *et al.*, 2009). In addition, regarding fruit yield, foliar application of Mn alone showed significant increase in fruit yield of sweet oranges due to its effect on increasing the number of fruit/tree as well as fruit average weight (Hasani *et al.*, 2012).

Our results for SICS were in accordance with Maz Ardalan and Savaghebi Firoozabadi (1997), Khoshghalb *et al.* (2011) on pear, Mashayekhi and Atashi (2008) on strawberry, Son *et al.* (2009) on pear, Hasani *et al*. (2012) on pomegranate.

The effects of polyamines and SICS on If and ISI

In our experiment, all of the treatments could led to semi or full fertility and compatibilty. So, this result was in line with Duca *et al.* (2010) on pear. If the ISI is lower than 0.2 means (self or cross) incompatibility, if it is between 0.2 to 1 interpret as semi (self or cross) compatibility, and if it is higher than 1, reveals full (self or cross) compatibility (Zeinanlo *et al.*, 2001; Azimi *et al.*, 2008.; Seifi, 2008).

Duca *et al.* (2010) reported that, in compatible pollinated styles, the levels of Put and Spm were similar and higher than Spd, whereas in self-incompatible pollinated styles, Put was the highest. In the compatible pollinated styles, these three polyamines showed higher content when compared to self-incompatible pollinated styles (Duca *et al.*, 2010).

Pollen germination and pollen tube development are important for fertilization (Kuruki *et al.*, 2017). Extremely low pollen germination rates may cause fruit setting failure because of ovule degradation before the pollen tube reaches the ovary (Kuruki *et al.*, 2017). Full compatibility is superior to semi-compatibility for ensuring high fruit set, even when environmental conditions are favorable for growth and pollination (Sapir *et al.*, 2008). Among the natural polyamines, Spm showed strongest effects on tube growth (Aloisi *et al.*, 2015).

The effects of polyamines and SICS on fruit drop

Put treatments significantly reduced secondary

fruit drop on date palm, apple, pear, mango, sweet orange and avocado (Asadi *et al.*, 2013). A reduction in secondary fruit drop was observed in date palm, apple, pear, mango, sweet orange and avocado (Tavakoli and Rahemi, 2014).

Due to preventing enzymatic conversion of 1-aminocyclopropane-l-carboxylic acid (ACC) to ethylene by polyamines, the ethylene production reduced and is followed by fruit drop (Tavakoli and Rahemi, 2014). Decreased fruit drop one week before full bloom in the "on" year and increased yield per shoot two weeks before full bloom in the "off" year were observed by application of Spm (0.1 and 1 mM), but Spd (1 mM) just lowered fruit abscission one week before full bloom in the "on" year (Khezri *et al.*, 2010).

In our study, Spd (0.25 mM) was the best treatment on fruit set (June and final), yield, ISI and fruit drop. Asadi *et al*. (2013) reported that the most effective treatment on raising fruit set were Spm and Spd in apricot, respectively (Asadi *et al.*, 2013).

The content of Spd was the highest at four development stages, followed by Put and Spm, respectively (Valero, 2010). Tavakoli and Rahemi (2014) stated that treatment with Spd 1 mM led to the highest fruit yield.

The effects of polyamines and SICS on fruit growth habit

Regarding the effects of polyamines on fruit length and diameter, fruit growth totally increased because of increasing fruit length and diameter in all of the treatments in our study. In the case of fruit growth, Malik *et al.* (2005) demonstrated that the amount of polyamines increased during initial fruit growth period of apple, pear, apricot and strawberry followed by gradually decrease near maturity. Polyamine content of pericarp declined from fruit set to maturity. Spd and Spm were higher than Put during initial fruit growth compared to later during fruit development (Malik *et al.*, 2005). Put application had a positive effect on fruit size and weight, which might due to its role in cell division leading to improved weight and diameter of fruit (Saleem *et al.*, 2008).

Mn application in SICS increased fruit diameter and length but only the 0.6% rate of manganese was significant on fruit diameter (Hasani *et al.*, 2012) and influenced fruit growth habit.

Control trees produced significantly lower fruit yield, so naturally the fruit size was greater compared to fruit from polyamines-treated trees (Saleem *et al.*, 2008).

Spermidine in concentration of 0.25 mM, with/without cotton coverage bags, was the best polyamine treatment to increase yield, fruit set and to decrease fruit drop. Put in concentration of 0.1 mM, with/without cotton coverage bags, was effective on improving percentage of final fruit set and IS. Although, SICS in two applied concentrations in this study had suitable influence on raising percentage of fruit set and lowering percentage of fruit drop. Also, SICS in concentration of 2 mg L^{-1} was the best on traits such as yield, index of fertility (IF) and index of compatibility (IS). In general, all treatments were useful to induce semi and full fertility and compatibility.

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Growth analysis of lettuce under different substrate compositions

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Abstract: The objective of this work was to evaluate lettuce growth in greenhouse under different types of substrates. The experiment was conducted in a greenhouse, under randomized block design, with six treatments and three replicates. The compositions of the substrates were: T1= 100% organic compound; T2= 75% organic compound plus 25% substrate Plantmax®; T3= 50% organic compound plus 50% substrate Plantmax®; T4= 25% organic compound plus 75% substrate Plantmax®; T5= 100% substrate Plantmax®; T6= vermiculite. The number of leaves, dry mass, leaf area index, culture growth rate, relative growth rate, net assimilation rate, specific foliar area, foliar area ratio and foliar weight ratio were evaluated. Higher growth of lettuce plants are produced by mixture of organic compound and substrate Plantmax®. The isolated use of vermiculite does not give good results for the growth of lettuce plants, but is an alternative for mixing with other substrates.

1. Introduction

Lettuce (*Lactuca sativa* L.) is one of the most consumed vegetables in the world (Gomes *et al*., 2008), due to its taste, nutritional quality and low price (Teodoro *et al*., 2016). Its adaptability to different climatic conditions that allows successive crops during the year, low cost of production and safe marketing, makes it a crop preferred by small producers, adding economic and social value to its cultivation (Medeiros *et al*., 2007).

Lettuce is responsive to organic fertilization, varying according to the cultivar and source of nutrients used (Teodoro *et al*., 2016). Thus, the use of substrates must ensure that lettuce presents characteristics appropriate to plant growth, with adequate physical and chemical compositions (Lima *et al*., 2006). It should be well structured and with good texture, adequate pH, good fertility and pathogen free (Araújo *et al*., 2013).

The different substrates compositions have an effect on the biomass production of the plants, since they are able to comply with the species'

requirements (Afonso *et al*., 2012). There is a trend toward using organic compounds because they provide nutrients and contribute to good development of the root system (Lima *et al*., 2006). A lettuce crop has high production potential with organic fertilizers (Santos *et al*., 2001). The organic compounds, resulting from composting, vermicomposting or other sources, have good aeration, structure, water retention capacity, ability to regulate the temperature of the substrate, and are sources of several nutrients that may be readily available (Trindade *et al*., 2001). Organic compounds are also stabilized products, rich in nutrients and derived from vegetable and animal waste (Souza and Alcântara, 2008).

Moreover, the use of organic substrates reduces cultivation time and consumption of chemical inputs (Medeiros *et al*., 2015), modifying the microbial populations that improve substrate quality and plant production. These microorganisms decompose the residues, releasing nutrients and substances that stimulate plant growth (Medeiros *et al*., 2015).

Growth analysis is still the most accessible and accurate way to evaluate plant growth and the contribution of physiological processes to plant behavior. It allows differentiating the behavior of the same cultivar under different cultivation conditions (Benincasa, 2003).

Due to the influence of factors on plant production and the importance of knowing the growth and development of the plants, the objective of this work was to evaluate lettuce growth in a greenhouse under different types of substrates.

2. Materials and Methods

Location of experiment, plant material and cultivation

The experiment was conducted in a greenhouse located on the experimental area of the Federal University of Santa Maria, Campus of Frederico Westphalen, RS, Brazil, with geographic location 27° 23’ S, 53° 25’ O and altitude of 490 m. According to Köppen’s classification, the climate of the region is humid Cfa-temperate with hot summer, with maximum air temperatures in warmer months over 22°C (Alvares *et al*., 2013). The average temperature inside the greenhouse were 22±3 °C during the day and 18±3°C in the night.

The lettuce seedlings, cultivar *Pira Verde*, were transplanted to the wooden benches on 3 days in October 2013, arranged in spacing of 20 cm between plants and 30 cm between rows. The plants were cultivated for 49 days until November 21, when all had reached the harvest point. A 150-micron double-sided canvas was placed on the substrate to reduce water loss through soil evaporation. Before transplantation, small holes were opened to introduce the seedlings into the substrates. The irrigation method used was drip irrigation, allowing a distribution of the same volume of water for all plants.

Water was supplied depending on environmental conditions, taking into consideration temperature, relative humidity, and other factors. Along with water, nutrients were supplied through nutrient irrigation. The nutrients were chosen according the recommendations of Furlani (2009), obtaining electrical conductivity around 1.2 µS cm^{-2}, by use of hidrogoodfert, calcinit and chelated iron, which have soluble nutrients in water. Hidrogoodfert is composed of the macronutrients nitrogen, phosphorus, potassium, magnesium, sulfur and the micronutrients boron, copper, molybdenum and zinc. Calcinit is composed of calcium nitrate. The solution was replaced according to the evaporation and absorption of the plants.

Experimental design and treatments

The experiment was conducted on wooden benches inside the greenhouse, with a randomized block design, with six treatments and three replications per treatment. Each replication have had 40 plants. The compositions of the substrates were T1= 100% organic compound; T2= 75% organic compound plus 25% substrate Plantmax®; T3= 50% organic compound plus 50% substrate Plantmax®; T4= 25% organic compound plus 75% substrate Plantmax®; T5= 100% substrate Plantmax®; and T6= vermiculite. The organic substrates were obtained by Mechanized and Automated Composting Unit (UMAC), a project of the company LPC-Ambiental Technology, located in the municipality of Concórdia-SC. This residue is generated by mixing pig slurry with compost shavings and storing it in retention rails.

The commercial substrate Plantmax® has excellent physical properties and high water retention capacity. This substrate is composed mainly of pine bark and vermiculite, and presents chemical properties constituted of macronutrients and micronutrients. Vermiculite was used for application of nutrients by means of irrigation, since it is a non-nutritive substrate with high water retention capacity.

Evaluations and analysis

The evaluations of the plants were conducted every week, when 3 plants of each treatment were sampled by the destructive method, from the transplant to the beginning of the stem elongation for posterior emission of the floral tassel. The evaluations were made by counting the number of leaves and separating the morphological parts of the plant, which was put into the drying oven at 60°C until it reached constant weight for determination of the dry mass. The analyzed variables were number of leaves (NL), dry mass (DM) and leaf area index (LAI). The number of leaves was determined by counting all the leaves of the plants, and dry mass was obtained by measuring the mass of the aerial part of the plant. Leaf area (LA) was determined by the disc method, in which 10 discs were removed from the leaves, obtaining its dry mass, and estimating the leaf area of the plant through the equation:

$$LA = \frac{\text{disc number x nozzle number x total leaf dry mass}}{\text{dry mass of the discs}}$$

From the leaf area, the leaf area index was calculated by the following equation:

$$LAI = \frac{LA}{SA}$$

where SA is the area of soil occupied by a plant.

In addition, culture growth rate (CGR), relative growth rate (RGR), net assimilation rate (NAR), specific foliar area (SFA), foliar area ratio (FAR) and foliar weight ratio (FWR) were calculated according to the methodology presented by Barbero *et al*. (2013).

The CGR was determined by:

$$CGR = \frac{DM}{SA \times \Delta t}$$

where *Δt* is the time interval of the evaluations. This variable is an indicator of productivity, since it represents the increase of dry matter in a time interval, considering the area of soil occupied by the plant.

The equation to obtain the RGR is:

$$RGR = \frac{\log \Delta DMA}{\Delta t}$$

where log ΔDM is the logarithm of the increment of DM, so RGR is an increment of productivity of dry mass in a time interval.

NAR was calculated as:

$$NAR = \frac{CGR}{LAI}$$

This variable indicates the rate of increase in dry matter per unit of time and per unit of leaf area. According to Benincasa (2003), NAR demonstrates the photosynthetic efficiency of the leaves.

FAR is the ratio between leaf area and dry mass and expresses the foliar area useful for photosynthesis; it is the measure of the size of the assimilating apparatus. It is obtained by:

$$FAR = \frac{LA}{DM}$$

FWR relates to how much of the dry matter accumulated by the plant is composed by leaves, which, besides being the organ responsible for photosynthesis, is the organ of commercial interest. It is given by:

$$FWR = \frac{\text{DM leaves}}{\text{DM plant}}$$

SFA were determined by the equation:

$$SFA = \frac{LA}{\text{DM leaves}}$$

This variable indicates the accumulation of photoassimilates in the leaves or the translocation to the other organs, that is, differences in leaf thickening (Taiz and Zeiger, 2013).

The data were submitted to analysis of variance and comparison of means by the Tukey's test, with a 5% probability of error.

The meteorological data, like air temperature and global solar radiation, were collected at the mobile weather station installed inside the greenhouse.

3. Results and Discussion

Weather conditions

During the experiment, minimum and maximum average temperatures were 15-27±3°C, respectively. These values are in agreement with the values considered optimal for the culture between 15-24°C (Knott, 1962) and 15-20°C (Santana *et al*., 2009).

The global solar radiation presented variations between 653 and 1.090 kJ m^{-2}. These values are considered adequate for the growth of the culture. A reduction of solar radiation at the beginning of the crop cycle can be observed, but it did not have any influence, since the plants were in establishment period and had little leaf area (Fig. 1).

Thus, air temperature and incident solar radiation were the main environmental factors that affected lettuce growth, development and yield.

Growth variables

During the growth and development of the plants,

small burns were observed in the border of the new leaves. These were symptoms of calcium deficiency, because it is a little mobile element in the plant and then wasn't available to the plants. However, by harvest, the plants had recovered with no apparent symptoms.

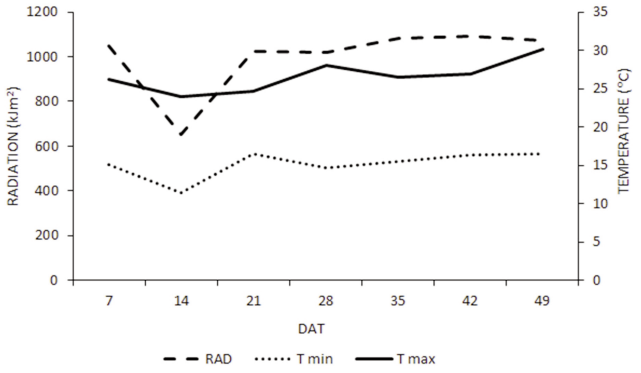

Fig. 1 - Solar global radiation (kJ m^{-2}), maximum temperature and minimum temperature (°C) during the lettuce crop cycle. Frederico Westphalen, RS, Brazil, 2013.

The leaves are the main component of interest of lettuce. Thus, it is important that the plant produces the maximum number of leaves, dry matter and leaf area for good production. Figure 2 shows that the number of lettuce leaves increased during the crop cycle for the different substrate compositions. Therefore, it can be observed that the T1 treatment (100% organic compound) showed a smaller number of leaves at 28 days after transplantation. However, at 35 days after transplant, the treatment composed of 100% substrate Plantmax® (T5) was less effective than the others, with approximately 12 leaves. At the end of the cycle of the culture, the substrate with the lowest number of leaves was vermiculite, with approximately 20 leaves, but that did not differ statistically from the others. This demonstrates that the combination of different substrates such as organic compost and commercial substrate present better conditions for the development of plants than when used alone.

The results obtained show that the analyzed growth variables presented different behaviors in relationship to the types of substrates used in the lettuce crop. A decline was also observed on the growth variables toward the end of the crop cycle (Fig. 3), which is considered normal because they are relational to growth of this culture.

Figure 3A shows the dry mass accumulated by the plants during the cycle. Slow initial growth due to the small size of the plants and small leaf area makes a lower nutrient absorption and a low solar radiation,

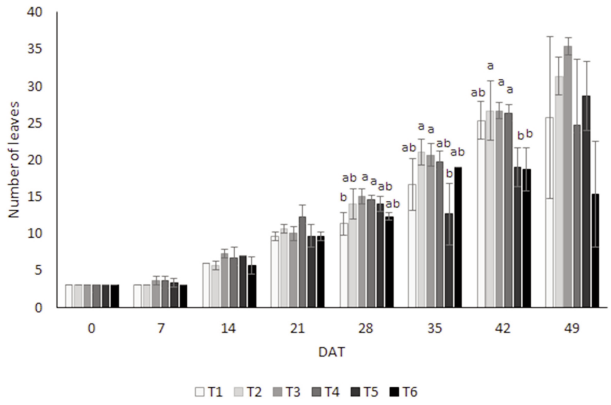

Fig. 2 - Number of leaves of lettuce growth under different substrates. Frederico Westphalen, RS, Brazil, 2013.

causing little growth of these plants. From 21 days after transplant, an increase in the DM for all the substrates was observed, with emphasis on mixtures of substrates. The substrate composed of 50% organic compound plus 50% substrate Plantmax® (T3), was outstanding for presenting higher values in most of the crop cycle, reaching maximum increment at 49 days after the transplant, with approximately 26 g. The worst results were obtained with vermiculite, reaching approximately 11 g (Fig. 3A).

The leaf area index presented a similar behavior to DM, with the most accentuated and perceptible increase at 21 days after transplant, when the plant presented an increase in dry matter (Fig. 3B). LAI falls at certain points in the crop cycle may be related to the abscission of older leaves. LAI is important for studying crop growth, development and productivity. The leaf area will depend in addition to the number and size of the leaves of the plant and the period in which the leaves remain on the plant (Monteiro *et al*., 2005). This demonstrates that the increase of leaf area provides greater interception and absorption of the available solar radiation, and, consequently, higher photo assimilates production, which results in higher growth and dry matter of the plants. The best results for these variables were obtained by substrate mixtures.

Since CGR represents the increment of dry matter per unit of soil area occupied over a given period of time, the pattern of the graph was very similar to that for dry matter. At 49 days after transplant, the substrate, composed of 50% organic compound plus 50% substrate Plantmax®, was higher than the others, differing statistically just from vermiculite (T6). At this point, T3 reached approximately 60 g m^{-2} day^{-1} (Fig. 3C). According to Beckmann-Cavalcante *et al*. (2009), growth rate of the crop is mainly determined

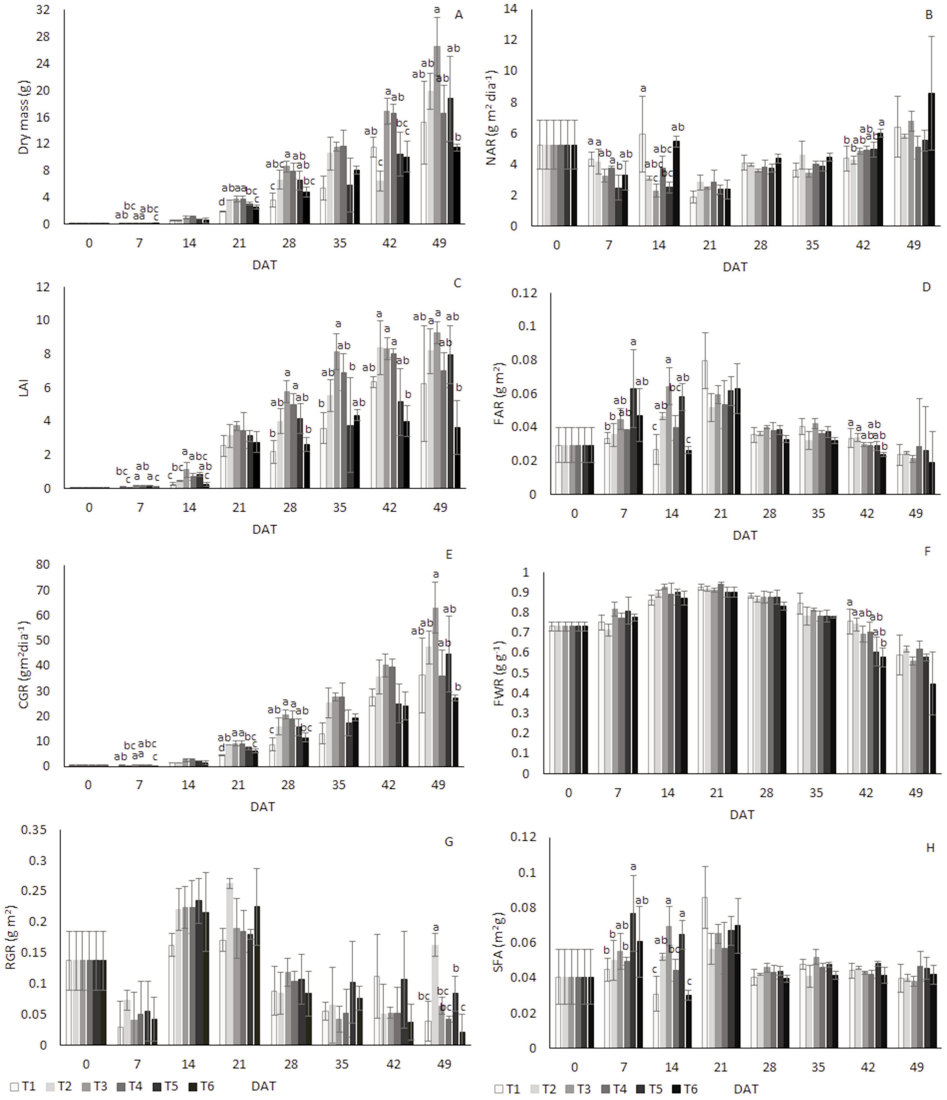

Fig. 3 - Dry mass (A), Leaf area index (B), Culture growth rate (C), Relative growth rate (D), Net assimilation rate (E), Foliar area ratio (F), Foliar weight ratio (G) Specific foliar area (H) for lettuce culture growth under different substrates. Frederico Westphalen - RS, UFSM , 2013. Lowercase letters differ by Tukey means test at 5% error probability.

by the air temperature. Thus, from 21 day after transplant (DAT), CGR also increased as a function of changes in air temperature.

The highest rates of RGR were observed at 14 and 21 days after transplant (Fig. 3D). Substrate with 75% organic compound plus 25% substrate Plantmax® (T2) reached maximum RGR-approximately 0.25 g m^{-2}. This may be because the plants were in a phase of high photosynthetic rate due to the elevation of LAI and high growth. This same treatment has achieved the highest RGR at 49 DAT, statistically differing from the others. At 28 days after transplant, the growth rate tended to decrease. According to Zuffo *et al*. (2016), this is mainly due to the shading between the plants and the increase in the respiratory rate. This shows that with shading and high respiration of the plants, the growth promotes a reduction of the RGR of the crop.

The photosynthetic efficiency of the leaves presented variations along the cycle and is represented by NAR. This rate tends to be higher at the beginning of the development of the crop due to lower self-shading (Gondim *et al*., 2008). However, in this study the NAR values increased at the end of the cycle because NAR depends on available leaf area, leaf distribution, leaf angle, and translocation and assimilation partition (Pedó *et al*., 2010; Aumonde *et al*., 2011). Some statistical differences were observed between the treatments on 7, 14 and 42 DAT (Fig. 3E).

The highest rates of FAR were obtained at 7, 14 and 21 DAT (Fig. 3F). After this period, the ratio steadily decreased for all treatments, corroborating the results of Pedó *et al*. (2013) for pepper. However,

these authors obtained results in the higher FAR, providing a greater area useful for photosynthesis, which produced higher rates of NAR. As for Caron *et al.* (2007), the decrease indicates that at this stage, most of the photosynthesized material is accumulated in the aerial biomass of the lettuce to increase available solar radiation. This shows that the decrease may be due to self-shading and leaf fall resulting from the age of plants, or to energy demand for the development of other organs, such as flowers. Statistical differences were found only at 7, 14 and 42 DAT.

Foliar weight ratio remained similar between the treatments, with values ranging from 0.7 to 0.9 g g^{-1} during a large part of the crop cycle, with decreases at 42 and 49 DAT (Fig. 3G). This may be due to the appearance of preferential metabolic sinks by the formation of the reproductive organs. T1 and T2 were superior and differed statistically to T6 at 42 DAT.

Specific foliar area reached differences between the treatments at 7 and 14 DAT. At 7 DAT, the major SFA were obtained by T5, which was statistically equal to T3 and T6 treatments. At 14 DAT, the higher SFA was observed by T2, T3 and T5. At 21 DAT, the values were similar to those obtained at 7 and 14 days. In the rest of the cycle, SFA remained around 0.04 m^2 g^{-1}. After the development of the plants, there were an increase in leaf area and dry mass of the leaves, causing decreases in SFA (Benincasa, 2003).

Table 1 shows the means of fresh mass at 42 and 49 DAT. The values show that substrates mixtures obtained the better results. Although they had not shown a significant difference from the others at 49 DAT - with the exception of vermiculite - there were differences that could bring greater profits. However, from the values observed at 42 DAT, it can be concluded that the treatments with mixtures of substrates were superior to the others. In addition, the use of mixtures such as T2 (75% organic compound plus 25% substrate Plantmax®) and T4 (25% organic compound plus 75% substrate Plantmax®) anticipate the harvest, because the fresh mass was higher at 42 DAT compared to 49 DAT.

Table 1- Lettuce fresh mass at different substrates mixtures

Treatment	Fresh mass (g)	
	42 DAT	49 DAT
100% Organic compounds	153.20 b	160.67 ab
75% Organic compounds + 25% Plantmax ®	220.03 a	198.40 a
50% Organic compounds + 50% Plantmax ®	199.03 a	270.00 a
25% Organic compounds + 75% Plantmax ®	199.70 a	163.43 ab
100% substrate Plantmax ®	110.40 bc	161.43 ab
Vermiculite	73.07 c	71.17 b

DAT = Day after transplant.

It is important to emphasize that vermiculite was used like a control treatment, since it is a inert substrate used in hydroponics cultivation or mixed with soil; it led to worse results for all variables evaluated and reduced the growth of plants cultivated under this substrate.

4. Conclusions

Higher growth of lettuce plants are produced by mixture of organic compound and substrate Plantmax®. The isolated use of vermiculite does not give good results for the growth of lettuce plants, but is an alternative for mixing with other substrates.

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Colchicine-induced autotetraploidy and altered plant cytogenetic and morpho-physiological traits in *Catharanthus roseus* (L.) G. Don

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Abstract: Artificially induced polyploidy is often used to alter plant growth pattern and genetic makeup of certain plant species. This experiment was conducted to induce autotetraploidy in *Catharanthus roseus* ('Alba') which contains diploid chromosomes. Application of four levels (0, 100, 200 and 400 mg/l) of colchicine concentrations were utilized at the two true leaf stages of *C. roseus*. It has been observed that 200 mg/l colchicine treatment had the most striking effect on producing polyploid plants. This concentration was able to boost yield performance and survival of tetraploids to 35% and 79% respectively. Increasing of ploidy level was confirmed by flow cytometry and chromosome number. But, plant survival significantly decreased with increased of colchicine concentration. Chromosome number, length and diameter of stomata and chloroplast number in stomata of guard cells increased with increased ploidy level, whereas the numbers of stomata decreased from 390 to 177 mm^2 in tetraploid plants. The overall consequence of colchicines treatment appeared to be a beneficial approach. It elucidated that the chlorophyll content, diameter of the lateral branches, leaf length and width, petal length and width, duration length of flowering, durability of flowering, root diameter, fresh and dry weight of roots, seed length and seed diameter significantly increased in tetraploid as compared to diploid plants.

1. Introduction

Morpho-physiological characters of ornamental and flower plants can be promoted via duplicating their chromosome numbers. Plants with polyploidy chromosomes are usually capable of producing larger organs than diploid chromosomes. It has been proved that the chromosome

numbers in certain plant species can be naturally doubled due to autoploid and alloploid mechanisms that caused to generate plant with polyploidy chromosome (Dhawan and Lavania, 1996). Polyploidy not only occur naturally, it also can be induced artificially using mutation agents. Colchicine is one of these agents (Blakeslee and Avery, 1937). Application of mutation agents on plant is not only able to multiple the chromosomes, but also to induce variations due to changes in chromosome numbers, used to manipulate morphological and biochemical production of plants (Yasuda *et al.,* 2008). A research report indicated that the results have shown colchicine and LAECV treatments to induce different types of mitotic abnormalities including: c-metaphase, vagrant chromosomes, sticky chromosomes, anaphase bridges and increased frequency of micronuclei, along with a reduction in mitotic index in *Allium cepa* root apical meristem cells (Kundu and Ray, 2016). Induction of polyploidy tends to expand nucleus and cell size of organs which causes to have positive impact on enlargement of leaves, branches and flowers of plant. So, a suitable procedure capable of altering chromosomes numbers can become an important approach for improvement of traits such as plant size, flowers size and duration of flowering in horticultural plants (Shao *et al.,* 2003). In overall, polyploidy induction is a beneficial trend for those plant tissues that contain effective compounds in such way that these tissues become enlarge, thus able to sustain more chemical substances than diploid plants (Adaniya and Shirai, 2001).

Catharanthus roseus (L.) G. Don (Apocynaceae) is an ornamental plant that grows up to 30-100 cm in height. It was previously known as *Vinca rosea* (L.) and commonly known as Madagascar periwinkle. Although, it is originated from Madagascar but, widely distributed throughout the world due to survival ability in various habitats and recognized as an ornamental plant (Van Bergen and Snoeijer, 1996). It is a perennial plant with diploid (2n=2x=16) chromosomes (Verma *et al.,* 2011). The Apocynaceae family contains 114 genera and 4650 species. *Catharanthus roseus* is a tropical plant and very sensitive to cold climate. If it grew under a favorite condition, it would produce flowers and its growth development remain over long period of times (Jaleel *et al.,* 2007). It has been reported that polyploidy induction using colchicine and sodium azid on clustered bean plants (*Cyamopsis tetregonoloba*) caused to promote germination, flowering time, plant height, leaf number, cluster numbers of pods, pod length, pod and dry weight (Velu *et al.,* 2008). Similar study of colchicine treatment on lace plant seedling indicated that morphological growth and cytogenetic components significantly changes. These alterations were included: increase in leaf area index, length, thickness and dark leaves, stomata size increases, reduction in the number of stomata and leaf epidermis, increases of chloroplast number in stomata guard cells, increase in the diameter of the flower, pollen diameter, petal length, size of capsules and seeds, and doubling of the chromosomes (Ye *et al.,* 2009; Niu *et al.,* 2016). It has also been observed that application of 0.1% colchicine concentration on *Jatropha curcas* L. plants had the most striking effect on producing polyploidy with yielding improvement of 15% in tetraploids but showed that there was no significant difference between tetraploid and diploid plants in considering plant height. On the other hand, increasing the level of chromosomes in the plant, the stomata numbers and pollen grains became abundant, while stomata density decreased and the leaves became thicker. In general, the tetraploid plants had larger leaves, flowers and seeds as compared to diploid plants (Niu *et al.,* 2016). When the apical meristems and seedlings of hyssop (*Agastache foeniculum* L.) plants received 17.500 µM colchicines and 50 µM trifluralin, a maximum growth of 16% tetraploid induction was obtained. Size of stomata, chloroplast number, morphological traits, leaf length and width, distance between the nodes, leaf area, plant height, fresh and dry weight, and spikes length significantly increased in polyploidy plants (Talebi *et al.,* 2017). An investigation on tissue of Dendrobium plant *in vitro* showed that, when culture medium received 0.075 % colchicines concentration for 14 hours, significant alterations occurred with polyploidy plant. It has revealed that tetraploid plants contained wider and dark leaves, reduce leaf angle and increase the diameter of the roots and stems as compared to diploid plants (Sarathum *et al.,* 2010). The aim of the present investigation is to evaluate the effect of colchicine treatment on *C. roseus* 'Alba' and to study the impact of cytogenetically and morph-physiological alterations in both polyploidy and diploid plants.

2. Materials and Methods

Plant material and autotetraploidy induction

This experiment was conducted as a completed randomized design. *C. roseus* 'Alba' seeds were purchased from Seed-Pakan Company and olchicines

was obtained from Sigma Company. The seeds were planted into culture trays containing cocopeat medium (with EC 0.2 ds/m) and kept in greenhouse with 26±2°C day temperature and 19±2°C night temperature, relative humidity of 70±5% in both dark as well as light (16/8 h photoperiod) conditions, with irrigation round 2 days. The seeds were germinated after 14 days. After seeds germination, 300 apical meristems of the seedlings were treated with different concentrations of colchicine (0, 100, 200 and 400 mg/l with pH=6) at the two true leaf stages using micro spray. Tween 20 (500 µm/l) was also added to colchicine solution in order to increase the surface contact of colchicine with plant's leaves. Colchicine treatments were repeated during seven consecutive days. The treated seedling then kept in greenhouse condition as mentioned above. When the plants reached the sixth-leaf growth stages, the treated plants were transferred into individual pot (22 diameter × 28 cm length) containing sand: clay: rotten manure (1:1:1) and they remained in the pots till the end of the experiment. All the plants within the pots received similar completed Hoagland nutrient solution at flowering stage. The irrigation was applied with intervals of 3 days and same day and night temperatures and photoperiod as mentioned above, except relative humidity of 40±5% in greenhouse condition.

Flow cytometry analysis

Flow cytometry apparatus (Model PA, Partec, Germany D-48161) was used to detect ploidy levels in plant tissues according to Ju *et al.* (2005). The ploidy level of plants was determined at full blooming stage, exactly 15 weeks after transplanting. Chemical agents such as nuclear extraction buffer solution and 4, 6 Diamid-2-phenyl indol (DAPI), under common name of CyStain UV persices, were provided from Partec companies. Parsley (*Petroselinum crispum* Mill.) which has nuclear weight of 4.46 pg was used as a standard plant for calibration of the apparatus. Nuclei suspensions were obtained when approximately 100 mg of fully developed fresh leaf tissue from different parts of the plant was chopped by a sharp razor blade in a specific buffer on ice, according to Gao *et al*. (1996). Nuclear suspensions were filtered through a 50 µm nylon filter and Rnase A (Sigma Aldrich Co.) at a concentration of 2 µg/mL was added to each sample (Gao *et al.,* 1996). Prior to running the experiment all the prepared samples were kept in ice till analyzes via flow cytometry initiated. The internal software of the FCM (BD FAC Station data processing system) was used to analyze histograms for determination of peak position and the relative ploidy index of the samples for each individual plant (Gao *et al.,* 1996). The ploidy level analysis (DNA content) was performed using ratio (peak 1= unknown plants and peak 2= index plant) (Valente *et al.,* 1998). Determination of the ploidy levels of each of the samples were performed in three replicates.

Measurement of stomata

Three matured and developed leaves were cut off from different parts of each tetraploid and diploid plant. Nail varnish technique was used with some modifications to isolate samplings from surface epidermises in order to observe stomata (Smith *et al.,* 1989). The epidermis were mounted on glass slides and a light microscopy "Olympus U-DA" with a digital camera "DPI 12" was used to photograph and measure stomata density. Light microscope was used to assess stomata (numbers/mm^2) and size of stomata (length and diameter) (Fig. 1) with magnification of 100x and 400x respectively (Smith *et al.,* 1989). Since feature of stomata density on the leaf was not uniform at the surrounding nervure, photographs from seven sections of stomata where obtained by rotating each individual sample under the microscope. Then the mean was calculated for each measurement.

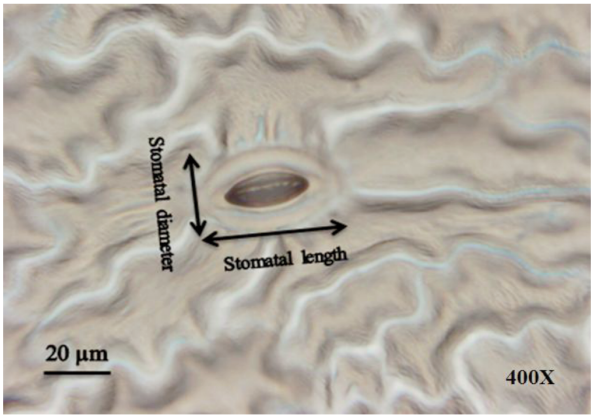

Fig. 1 - Measurement of stomata (length and diameter) with light microscope magnification 400x.

Observation and measure of chloroplast in stomata guard cells

Forceps was used to isolate epidermis from leaf surface in order to observe and count chloroplast in stomata guard cells in the detached leaf. The isolated epidermis was then placed in logol solution (1%) for 5 min. After coloring, each individual sample mounted on glass slides and light microscopy "Olympus U-DA"

with a digital camera "DPI 12" at the 400x magnification was used to count chloroplasts and simultaneously take photograph for the following counting of the number of chloroplasts in the stomata guard cells.

Comparison of morphological traits of diploid and tetraploid plants

After identification of tetraploid plants, morphological and physiological characteristics, as well as growth behavior of both tetraploid and diploid plants were recorded in order to characterize the differences. Number of lateral branches of each sample was counted accurately. A rule (with accuracy 1 mm) and a digital caliper (with accuracy 0.1 mm) were used to measure branch and root diameters. Length of leaf and petal were measured by using the Caliper digital (with accuracy 0.1 mm). As phenological characteristics, it was measured flowering period, which was considered from initial time of flowering to seeds formation, flowers durability on plants was evaluated according to the number of days. Number of seeds per follicle was counted accurately. Fresh root and dry weight was measured using a digital scale (accurately 0.01 g). Oven (48 hours at 70°C) dry weight of root was measured. Seed dimensions including length and diameter were measured using a dial binocular microscope magnification of 40x.

Evaluation of chlorophyll content

Fresh samples of apical leaves were collected from both tetraploid and diploid plants and washed thoroughly with distilled water. Approximately, 0.1 g of leaves was weighed and placed in a mortar then 2 ml of 80% acetone added to the samples and gently crushed the leaves till the mixture was formed in a uniform state. The samples were centrifuged at 6000 rpm for 15 min. A spectrophotometer device with wavelength of 663 and 645 nm was set to read the absorption of chlorophyll. Chlorophyll a and b then were calculated using the following formulas (Arnon, 1949).

1) Chlorophyll a=12.25(A663)-2.55(A645) × V/W
2) Chlorophyll b=20.31(A645)-4.91(A663) ×V/W

where V = volume of extract (ml) and W = weight of tissue (mg)

Observation of chromosome numbers

Study of cytogenetic event was implemented based on counting the set of chromosomes in individual plant cells of diploid and tetraploid plants. The cells of root tip tissue from germinated seeds were used to observe chromosomes numbers. The seeds of each individual plants (diploid and tetraploid) were sterilized separately with Sodium hypocolorid solution (5%) for 5 min (24°C) and rinsed completely with distilled water for 10 min. The seeds were then placed on filter paper in petri dish to germinate at 25±1°C temperature inside the growth chamber. After germination, when the root length reached 5 mm, the roots were separated and washed with distilled water and placed in the pretreatment solution of 8-hydroxyquinoline citrate at temperature of 4°C for two hours. Ethanol and acetic acid in ratio of 1:1 (v:v) were used to stabilize the samples at temperature of 4°C for 20 hours. The samples were washed once with distilled water for 30 min, then with 40% ethanol for 15 min, and kept in 50% ethanol for 15 minutes. After this time the samples were removed and kept in 70% ethanol for 15 min. Ethanol and xylene were used to detect transparency of the samples (Chehrazi *et al.,* 2012).

Hydrolysis of the samples

Hydrolysis of the samples occurred when samples were immersed in 70% ethanol and hydrochloric acid (vol. 2:1) solution for 15 minutes. The samples were then washed with distilled water for 15 minutes and used acetocarmine solution to stain the samples for 5 hours at 25±2°C. Approximately, 2 mm of tip apex of root was removed at the end of the root tip and placed on glass slides. A light microscopy "Olympus U-DA" which capable of magnifying of 400x with a digital camera "DPI 12" was used to get photograph and observe number of chromosome (Chehrazi *et al.,* 2012).

Statistical analysis

SPSS software 16.0 was used to perform statistical analysis of the data. The T-test also was applied for the mean comparison at level of 1% of probability.

3. Results and Discussion

Effect of colchicine on the rates of survival and tetraploid plants

Application of 200 and 400 mg/l colchicine solutions on true two-leaf growth stages of *C. roseus* 'Alba' diploid plants tended to induce tetraploidy. The concentration of 200 mg/l had the highest survival (79%), whereas the highest percentages of tetraploid plants were induced at the 400 mg/l concentration colchicines. The information from results of flow cytometry analysis, cytogenetic and morphological evaluations indicated that concentration of

400 mg/l was not only able to promote autotetraploidy, but caused to generate the highest mortality among treated plants (Table 1). This observation showed the potential of *C. roseus* 'Alba' in responding to colchicines treatments reacted differently, so the certain of plants can't preserve their chromosome sets in balance within the cells, in particular at the high concentration rate of colchicines. Flow cytometry analysis elucidated that the majority of those plants tetraploid with high dose of colchicines developed defective chromosomes; mixed ploidy with abnormal structure and the plants die before reaching the maturity. An investigation implemented to induce polyploidy in *Challistephus chinensis* Nees. (Hanzelka and Kobza, 2001) and *Agastache foeniculum* L. (Talebi *et al.,* 2017) was indicated that the increase of colchicine concentration on the targeted plants decrease the rate of plant survival. Another experiment reported that there is a positive correlation between various concentrations of colchicine applications and mortality in seedlings of Chamomile (*Tanacetum parthenium*) (Saharkhiz, 2007). It has been documented that when high dose of colchicines used as a mutation agent for plant, toxic contamination, phytotoxicity and abnormality became main cause of death in the plants (Han *et al.,* 1999).

Table 1 - Percentage of plant survival and tetraploid plants of *C. roseus* 'Alba' seedlings treated with colchicine

Colchicine concentration (mg/l)	Plant survival (%)	Tetraploid plants (%)
0	98±2 a	0 c
100	96±4 b	0 c
200	79±3 c	35±2 b
400	55±3 d	44±3 a

± Standard error (SE).

Identification of tetraploid plants using flow cytometry

Results on analysis of ploidy levels by flow cytometry are shown in figures 2 and 3. The ploidy level was detected by placing plant tissue from tetraploid and diploid plants into Flow cytometry, and then the DNA content was recorded (Sari *et al.,* 1999). The amount of DNA content was calculated according to formula: which is described by (Bharathan *et al.,* 1994). The rate of DNA contents was equal to 0.35-0.45 and 0.7-0.9 for diploid and tetraploid plants respectively. These findings were similar to the results which were reported by Talebi *et al.* (2017), Niu *et al.* (2016).

Stomata size and density and chloroplast number of stomata guard cells

The results of comparison of stomata at the different locations on the dorsal surface of fully developed leaves have shown that there were significant (1% probability level) differences between diploid and tetraploid plants in relation to density and size of stomata. The results also indicated that density of stomata in diploid and tetraploid plants were 390 and 177 numbers in mm^2 (Table 2 and Fig. 4 b and f). While, the length of stomata and diameter were 17 and 25 µm in diploid and 22.5 and 35.5 µm in tetraploid plants respectively (Table 2 and Fig. 4 a and e). Numbers of chloroplasts of stomata per guard cells were equal to 10 and 20 in diploid and tetraploid plants respectively (Table 2 and Fig. 4 c

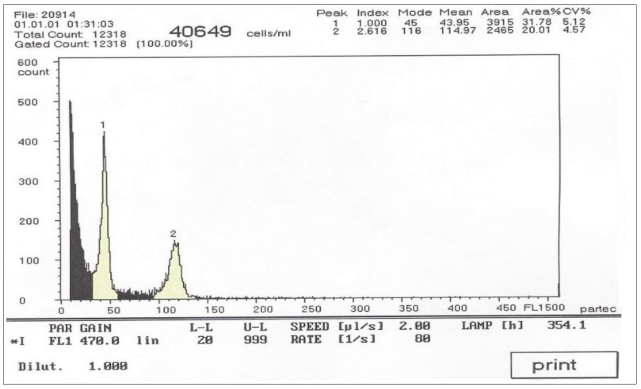

Fig. 2 - Flow cytometry analysis of *C. roseus* 'Alba' cell nuclei in diploid (peak 1) and index plant of parsley in diploid status (peak 2).

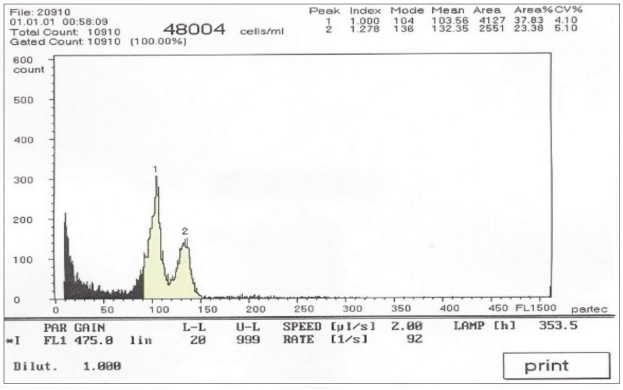

Fig. 3 - Flow cytometry analysis of *C. roseus* 'Alba' cell nuclei in tetraploid (peak 1) and index plant of parsley in diploid status (peak 2).

Table 2 - Mean of stomata size, density and chloroplast numbers in diploid and tetraploid plants of *C. roseus* 'Alba'

Genotype	Stomata length (µm)	Stomata diameter (µm)	Stomata density (n/mm^2)	Chloroplasts number
Diploid	22.5±0.6 b	17±0.3 b	390±2.4 a	10±0.2 b
Tetraploid	35.5±0.4 a	25±0.3 a	177±2.3 b	20±0.3 a

± Standard error (SE).

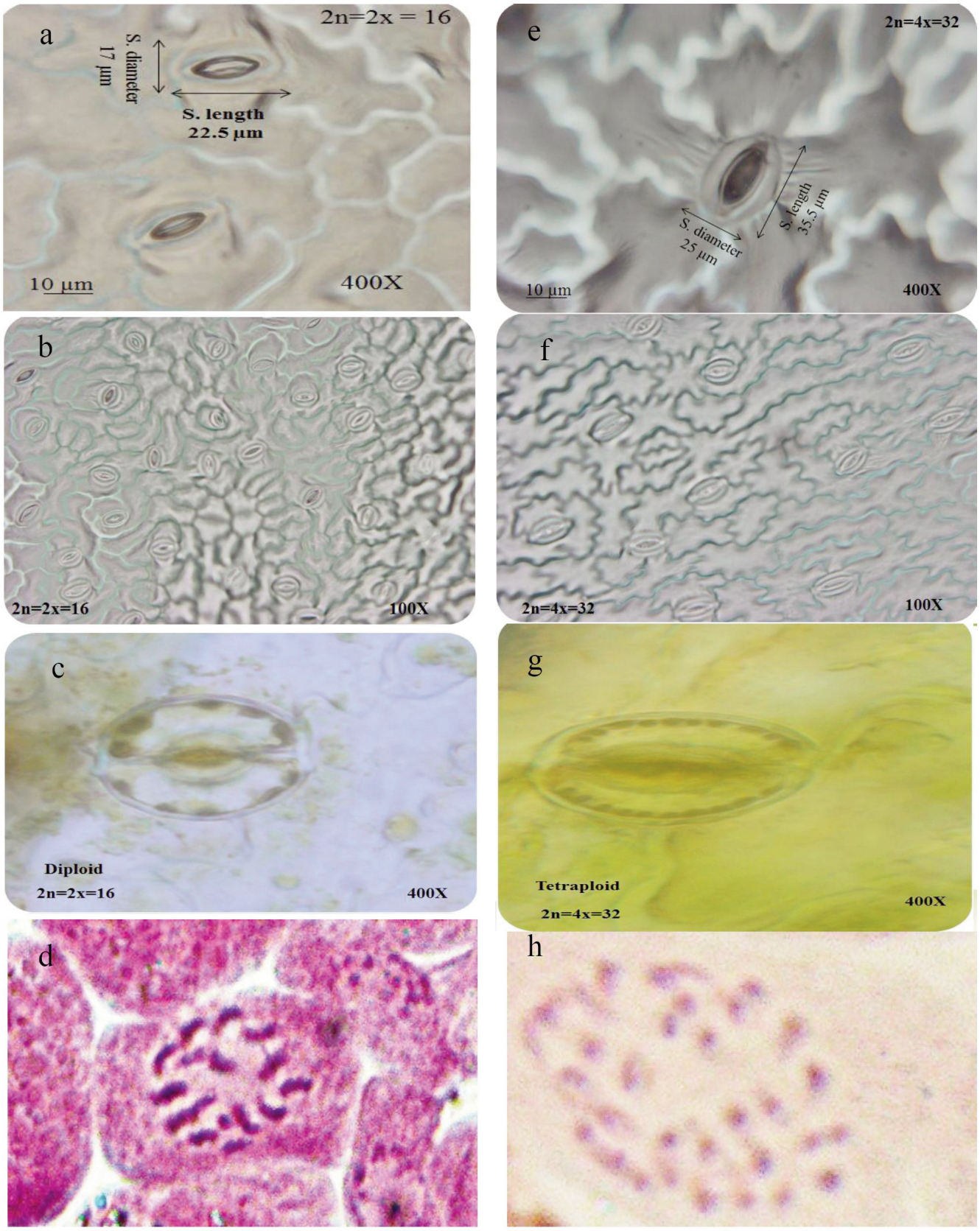

Fig. 4 - Stomata size (a), stomata density (b), chloroplast numbers (c), chromosome numbers (d) in diploid and stomata size (e), stomata density (f), chloroplast numbers (g) and chromosome numbers in tetraploid (h) of *C. roseus* 'Alba' plants.

and g). Early research reports on the size of stomata in the guard cells indicated that these cells are more dependent on genetic than environmental factors as compared to other cells in plant (Yasuda *et al.,* 2008). Another study on *Jatropha curcas* L. indicated that with increase ploidy, the stomata and pollen grains became larger, but stomata density decreased (Niu *et al*., 2016). A study on the increase polyploidy level of Japanese persimmon *(Diospyrus kaki* L.) has shown that with increasing in ploidy level, plant's stomata guard cells have become enlarge but with less density (Tamura *et al.,* 1996). In a report by Roy *et al.* (2001) it has been shown that the length and diameter of the stomata were set to be standard parameters for identification of tetraploid plants. Similar work has been done to identify autotetraploid using colchicines treatments in *Humulus lupulus* plant (Roy *et al.,* 2001). Several other studies showed that application of colchicines treatments tend to increase of ploidy level in plant, the length and diameter of stomata and chloroplast numbers increased (Gu *et al.,* 2005; Talebi *et al.,* 2017).

Observation and chromosomes counting

In order to determine the ploidy level in the treated plants, the cells of root tip were isolated from both diploid and tetraploid plants after the series of preparation, stained and then light microscope used to count chromosomes sets. Chromosome numbers of root tip cells of diploid and tetraploid were 16 and 32 respectively (Fig. 3). Application of colchicine tends to prevent the activity of the subunits join of microtubule (tubulin protein) and/or keep them apart that impedes the formation of the spindle fibers during the cell division and stops chromosomes movement in metaphase stage (Kundu and Ray, 2016). Thus, cell division occurs without cell wall formation which leads to double the number of chromosomes in plant cells. Colchicine treatment could induce different types of mitotic abnormalities including c-metaphase, vagrant chromosomes, sticky chromosomes, anaphase bridges and increased frequency of micronuclei (Gupta, 2002; Kundu and Ray, 2016).

Phenotypic variation between diploid and tetraploid plants

The t-test at the 1% probability level showed significant differences between diploid and tetraploid plants. As chromosome numbers duplicated, stem number, stem diameter, leaf area, leaf number, flower diameter, diameter of flower ovary, total chlorophyll content, fresh and dry weight of roots, duration of flowering length, durability of flowering, root diameter, length and diameter of seeds were significantly increased in the tetraploid as compared to diploid plants while. On the other hand, increases ploidy level from diploid to tetraploid decreased length of lateral branches and root and number of seed in follicle (Table 3). In the present experiment, promotion of ploidy level caused to reduce lateral branch length, but tended to increase diameter and number of branches (Table 3). In fact, the formation of polyploidy in plant is simply due to reduction of the frequency of cell division during initiation of growth and development which caused to lowering growth rate in tetraploid when compared to diploid plants. In an experimental study that was conducted to induce polyploidy in Cumin plant it has been reported that autotetraploid plants have lower growth at the early growth stages and shorter time to flowering stage than diploid plants (Dijkestra and Speckmann, 1980). While, at the same time, the diameter of lateral branches and its numbers significantly enhanced in tetraploid plants (Table 3). Rubuluza *et al.* (2007) in their research proved that colchinice treatments are capable of producing similar out puts on seedling of *Colophospermum mopane* L. plants.

Table 3 - Comparison of morphological and physiological traits in diploid and tetraploid plants of *C. roseus* 'Alba'

Characteristics	Diploid	Tetraploid
Length of lateral branches (cm)	24.5±0.5 a*	13.3±0.6 b*
Diameter of lateral branches (mm)	3.2±0.2 b	5.3±0.2 a
Lateral branch number	3.3±0.2 b	8.4±0.3 a
Leaf length (mm)	60±1 b	97±3 a
Leaf width (mm)	20±0.04 b	47±0.2 a
Petal length (mm)	22±0.25 b	27±0.4 a
Petal width (mm)	15±0.05 b	25.3±0.03 a
A chlorophyll (mg/g)	0.6±0.015 b	0.9±0.01 a
B chlorophyll (mg/g)	0.21±0.01 b	0.37±0.01 a
Duration length of flowering (day)	148±2 b	181±2 a
Durability of flowering (day)	3.6±0.24 b	7±0.15 a
Root length (cm)	30.8±1.8 a	17.5±1.7 b
Root diameter (mm)	3.5±0.2 b	6±0.3 a
Fresh weight of roots (g)	8.5±0.2 b	14.2±0.3 a
Dry weight of root(g)	1.4±0.03 b	2.3±0.05 a
Number of seeds in follicle	17.5±0.4 a	7.7±0.4 b
Seed length (mm)	2±0.04 b	3±0.06 a
Seed diameter (mm)	1.1±0.02 b	1.5±0.04 a

± Standard error (SE).

* In each row, means with similar and dissimilar letters are no significant and significant respectively according to t-test.

The length and width of leaves were larger in tetraploid than diploid plants (Table 3 and Fig. 5). So, a plant with such characteristic is being able to sustain more chemical substances in vegetative organs. Artificially Induction of polyploidy in many plant species caused to increase cell size and consequently enhance flower size, inflorescence, leaves, vegetative and generative organs (Watrous and Wimber, 1988; Omidbaigi, 2009; Niu *et al.,* 2016; Talebi *et al.,* 2017). Other researchers reported the beneficial effect of artificially inducing polyploidy in the *Melaleuca alternifolia* (Zhang, 2000), *Tanacetum parthenium* L. (Saharkhiz, 2007), *Jatropha curcas* (Niu *et al.,* 2016) *and Agastache foeniculum* L. plants (Talebi *et al.,* 2017) which caused to enlarge size of leaves and branches. The amount of chlorophyll a and b were significantly increased in the leaves of tetraploid (Table 3). The accumulation of high chlorophyll contents in the leaves of tetraploid plants may be relevant to increase number of chloroplasts in the stomata guard cells (Fig. 4 c and g). As shown above, the number of chloroplasts in stomata guard cells of tetraploid plants was two folds greater than those in diploid plants. In a similar experiment conducted on Acacia (*Acacia mearnsii*), Mathura *et al.* (2006) have shown that chlorophyll content was significantly higher in tetraploid than diploid plants. Polyploidy tended to have positive effect on size of reproductive organs. The growth of length and width of petals developed significantly higher in tetraploid than diploid plants (Table 3 and Fig. 5). A considerable growth increases was occurred in petal and flower size of tetraploid when carnation and *Jatropha curcas* plants received leaf foliar application of colchicines (Roy Chowdhury and Tah, 2011; Niu *et al.,* 2016).

In the present study, colchicine treatments caused to make the length of flower duration and durability of flowers longer in tetraploid than diploid plants (Table 3). Generally, in tetraploid plants, the beginning of flowering is delayed and the flowering period is longer than the diploid plants (Blakeslee and Avery, 1937; Lavania and Srivastava., 1991). The roots length in tetraploid (17.5 cm) is less than diploid plants (30.8 cm) (Table 3). This reduction may be due to reduction of cell division in the longitudinal direction, but it has been reported that with reduction of root length, root diameter increased in these plants (Sarathum *et al.,* 2010). It has been found that when ploidy level was increased in salvia plant, root diameter significantly increased as compared to control plants (Gao *et al*., 1996). Increase in fresh and dry weight of root in tetraploid can be due to production of lateral roots and enhance of root diameter (Table 3). A series of experimental studies which were carried on *Hyoscyamus niger* L. and *Agastache foeniculum* L. plants, elucidated that doubling level of chromosome from diploid to tetraploid caused to increase fresh and dry weight of root and shoot (Lavania and Srivastava, 1991; Talebi *et al.,* 2017).

In the present experiment, colchicines treated plants were able to produce seeds with a bigger size than untreated plants. Lengths of seeds in diploid and tetraploid plants were 2 and 3 mm, respectively. And seed diameter measurement indicated that tetraploid had thicker seed in diameter than diploid, but the number of seeds per follicle has decreased from 17.7 to 7.7 numbers in tetraploid plants (Table 3). The implication behind the reduction of the seed number per follicle may be attributed to increasing the seed size in the plant. It has been reported that artificially induced polyploidy plant usually caused to produce seeds with bigger size as compared to diploid plants, whereas the seed numbers simply reduced. Niu *et al.* (2016) have shown when chromosomes of *Jatropha curcas* plant duplicated due to colchicines treatment the seeds of the tetraploid plants grown bigger than those of the diploid. Study the application of colchicines on other plants such as Chickpea, Crape myrtle and Dendrobium could promote the seeds with bigger size and weight in autotetraploidy plant (Pundir *et al.,* 1983; Ye *et al.,* 2009; Sarathum *et al.,* 2010). Niu *et al.* (2016), Dijkestra and Speckmann (1980) reported that low seed formation in autotetraploid may be relevant to meiotic abnormalities during cell division in which caused to produce the bigger size of seed in the plant.

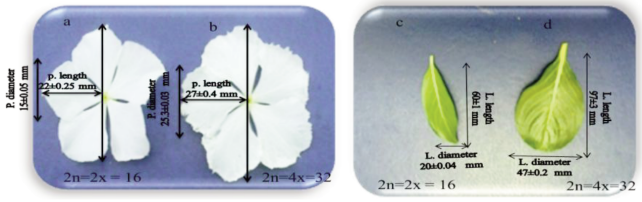

Fig. 5 - Size of diploid petal (a), size of tetraploid petal (b), size of diploid leaf (c) and size of tetraploid leaf (d) in diploid and tetraploid genotypes of *C. roseus* 'Alba'.

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Somatic embryogenesis, biochemical alterations and synthetic seed development in two varieties of coriander (*Coriandrum sativum* L.)

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Key words: biochemical attributes, conversion frequency, *Coriandrum sativum* L., somatic embryogenesis, synthetic seeds.

Abstract: Somatic embryogenesis (SE), biochemical alterations and synthetic seed formation were carried out in two *Coriandrum sativum* L. varieties (Rajendra Swathi 'RS' and Co-1). Callus was induced profusely in 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) added MS medium but Co-1 had more callus induction frequency (96.0%) compared to RS (89.3%). The callus turned into embryogenic tissue and variable embryogenic frequency (77.6% in RS and 72.8% in Co-1) was noted. Somatic embryos started to differentiate on the same 2,4-D added medium but the numbers of somatic embryos were more in RS (63.0 embryos per culture) compared to Co-1 (51.0 embryos per culture). These somatic embryos progressed well and showed maximum maturation in RS (78.7%) in 0.25 mg/l 6-benzyladenine (BA) + 0.5 mg/l α-naphthalene acetic acid (NAA) added medium. The biochemical analyses of non-embryogenic-, embryogenic-callus and different stages of embryos were conducted in order to know the changes of physiology in different tissues. Sugar and proline content were noted to be high at embryo induction stage while protein level was higher at embryo maturation stage. Biochemical analysis also revealed that the catalase (CAT) and superoxide dismutase (SOD) activities were higher at maturation stage of embryos compared to other embryogenic stages. Matured somatic embryos were germinated in MS added with 1.0 mg/l BA + 0.5 mg/l gibberellic acid (GA_3) in which 83.3% and 76.7% plantlet regeneration were noticed in RS and Co-1 respectively. Somatic embryos were encapsulated in various alginate and calcium chloride ($CaCl_2$) solutions and were kept in different temperature regimes for varied periods. On regeneration medium, the encapsulated embryos germinated into plantlets; in 3% sodium alginate + 100 mM $CaCl_2$, maximum plant regeneration (74.0% in RS and 70.6% in Co-1) was noted. The influence of low temperature on storage of synthetic seeds and their conversion into plantlets were also studied and we noted that the 4°C was the optimum temperature for synthetic seed conservation and plantlet regeneration compared to -20°C and 25°C temperature conditions.

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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

Coriandrum sativum L. is an annual herb and belongs to the family Apiaceae. The plant is used as a spice and flavouring compound (Burdock and Carabin, 2009). The plant has potential in pharmaceutical industry as it exhibits antimicrobial (Cao *et al*., 2012), antioxidant (Hashim *et al*., 2005), antidiabetic (Eidi *et al.*, 2009), hepatoprotective (Samojlik *et al.*, 2010) and antiarthritic properties (Rajeshwari *et al.*, 2012). Plant tissue culture technology is widely used for large scale plant propagation, beside being used as an *in vitro* experimental model for studying cellular processes like cell division, differentiation and morphogenesis, all play important key role in somatic embryogenesis (SE) and plant development (Zimmerman, 1993). SE is a process in which somatic cells undergo morphological and metabolic changes under *in vitro* cultural conditions, acquire embryogenic potency and later develop into somatic embryos (Feher *et al.*, 2003). Somatic embryos mimic zygotic embryos in various ways and hence it has proved to be a good model for studying morpho-physiological, biochemical and molecular events during the course of embryogenesis (Dodeman *et al.,* 1997). The fast identification of embryogenic tissue is very important as it plays a central role in marker-assisted selection (Dodeman *et al*., 1997). Beside morphology, the embryogenic tissues are often differentiated from non-embryogenic callus by various biochemical markers and these markers based selection would be of immense value of SE based micropropagation (Samar *et al*., 2011). Embryogenic tissues have the ability of producing embryos for an extended period of time without any genetic alteration, which requires efficient conservation protocol for long-term preservation of coriander (Murthy *et al.,* 2008). Somatic embryos also have an important application in the formation of synthetic seeds and this artificial seed (an alternative to natural seed) technology may be exploited as a complementary method for *in vitro* production of plantlets (Reddy *et al*., 2012). It also promotes fast plantlet regeneration, facilitates germplasm exchange and conservation (Palanyandy *et al.*, 2015). The synthetic seed technology with advantages like easy handling, conservation with germplasm exchange possibility is being widely used between national and international laboratories (Rai *et al.,* 2009). Beside bipolar somatic embryos, other explants like unipolar micro-bulbs, rhizomes, protocorms, nodal cuttings and shoot buds are used for synthetic seed (Rihan *et al*., 2011; Sharma and Shahzad, 2012). Among the various natural and synthetic polymers available for encapsulation, sodium alginate is used more frequently because of its easy gelling properties, non-toxicity and low cost (Saiprasad, 2001). Different concentrations of sodium alginate ranging from 1.5 to 6.0% are used in encapsulation in different plant species *Cacicus carota* (Latif *et al.*, 2007), *Spartina alterniflora* (Utomo *et al.*, 2008), *Catharanthus roreus* (Maqsood *et al.,* 2012), *Rinacanthus nasutus (*Meena *et al*., 2013). In the present study, an efficient synthetic seed formation protocol was optimized in *C. sativum* and their regeneration potential was described after *in vitro* storage at different storage conditions. We also discussed SE and associated biochemical alterations in two varieties of *C. sativum*.

2. Materials and Methods

Seed germination and cultural conditions

Seeds of two varieties of *C. sativum*, Rajenda swathi (RS) and Co-1 were obtained from National Research Centre for Seed Spices (NRCSS) Ajmer, Rajasthan, India for this experimental study. The seeds were washed thoroughly under running tap water using cetrimide as detergent and surface disinfection was made with 0.1% (w/v) $HgCl_2$ for 2 min. The seeds were rinsed three times with sterilized double-distilled water before allowed to germinate on half strength MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). The basal medium was solidified by adding 8.0 g l^{-1} agar. The pH of the medium was adjusted to 5.7 using 1.0 M NaOH and 0.1 N HCl and was sterilised in an autoclave for 15 min at 121°C. All the reagents were prepared using water supplied by a Milli-Q system (Billerica, Massachusetts, USA). The cultures were incubated at 25±2°C under 12-h photoperiod provided by cool white fluorescent lamps (100 µmol m^{-2} s^{-1} PFD).

Callus induction and somatic embryogenesis (SE)

Callus was induced from hypocotyl explants of 10-day old germinated seedlings. Different concentrations (0.5-2.0 mg/l) of 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5-1.0 mg/l α- naphthalene acetic acid (NAA) were used for callus induction. After 4 weeks in induction medium, the callus was transferred in fresh medium for induction of somatic

embryos. Somatic embryos were induced on same 2,4-D added callus-induction medium. For embryo maturation, MS was supplemented with NAA (0.5-1.5 mg/l) and 6-benzyladenine, BA (0.25-0.5 mg/l). The germination of somatic embryos was made by transferring matured green embryos into MS, added with 0.5-2.0 mg/l BA and 0.2, 0.5 mg/l gibberellic acid (GA_3).

Biochemical analysis

Proline, protein and sugar assay. Proline estimation was made according to Bates *et al.* (1973). About 0.05 g of callus was homogenized in 2.0 ml of 3.0% aqueous sulphosalicylic acid under cold conditions; the homogenate was filtered with Whatman filter paper (No. 1). The filtrate (1.0 ml) was added with 1.0 ml ninhydrin and 1.0 ml glacial acetic acid; the reaction mixture was incubated for 1 h at 100°C. The reaction was terminated in ice bath and 2.0 ml toluene was added to it. Proline content was measured by spectrophotometric assay at 520 nm. The concentration of proline is expressed in mg per 1 g fresh weight.

Protein estimation was made following Bradford *et al*. (1976). The homogenate of 0.25 g callus in 1.5 ml phosphate buffer (0.1 M, pH 7.0) under pre-cold condition was centrifuged at 10^4 rpm for 10 min and 1.0 ml supernatant was added with 0.5 ml trichloroacetic acid (10%). After centrifugation, the pellets were washed with acetone and dissolved in 1.0 ml of NaOH (0.1 N). To 1.0 ml of aliquot, 1.0 ml of Bradford reagent was added and optical density was measured at 595 nm The protein concentration was expressed in mg per 1 g fresh weight.

Dey (1990) method was used for sugar estimation. Callus tissue 0.1 g was extracted twice with 90% alcohol at 60°C. Final volume of extract was made up to 10 ml by adding DDW. After mixing 0.5 ml of aliquot with 0.5 ml of 5% phenol, 1.0 ml of concentrated sulphuric acid was added and cooled in air. The optical density was measured at 485 nm. The sugar concentration was expressed mg per 1 g fresh weight.

Analysis of catalase (CAT), ascorbate peroxidise (APX) and superoxide dismutase (SOD) activity. Calli at different stages such as non-embryogenic, embryogenic tissue and different embryo stages (induction, proliferation and maturation) were homogenized in 2.0 ml of 0.1 M extraction buffer (0.1 M K-phosphate, 0.5 mM EDTA, 1.0 mM ascorbic acid, pH 7.5). After centrifugation at 10^4 rpm for 20 min, the supernatant was used for enzyme analysis.

CAT activity was determined according to Aebi (1984) method by measuring a decrease in the absorbance at 240 nm of reaction mixture containing 1.0 ml of 0.5 M reaction phosphate buffer (Na-phosphates, pH 7.5), 0.1 ml EDTA, 0.2 ml enzyme extract and 0.1 ml H_2O_2. The reaction was run for 3 min. One unit of enzyme determines the amount necessary to decompose 1.0 µM of H_2O_2 per min. CAT activity was calculated by using the co-efficient of absorbance at 0.036 mM^{-1} cm^{-1}. The activity of CAT was expressed in EU mg^{-1} protein.

The method developed by Nakano and Asada (1981) was used for APX activity. To the mixture of 1.0 ml sodium buffer (0.1 M, pH 7.2), 0.1 ml of EDTA and 0.1 ml of enzyme extract, 1.0 ml of 0.5 mM ascorbate were added and the reaction was run for 3 min at 25°C. The APX activity was estimated by monitoring the decrease in absorbance due to the breakdown of ascorbate by APX and was calculated by using the coefficient of absorbance 2.81 mM^{-1} cm^{-1}. The activity of APX was expressed in EU mg^{-1} protein.

The SOD activity was estimated following Dhindsa *et al*. (1981) method with slight modifications. Callus tissue (0.1 g) was homogenised in 2.0 ml of extraction mixture (0.5 M phosphate buffer (pH 7.3), 3.0 mM EDTA, 1.0% (w/v) polyvinylpyrollidone (PVP), 1.0% (v/v) Triton X100) and centrifuged at 10^4 for 10 min. The SOD activity in the supernatant was assayed by adding 0.1 ml enzyme extract with 1.5 ml reaction buffer, 0.2 ml methionine, 0.1 ml each of 1.0 M $NaCO_3$, 2.25 mM Nitro Blue Tetrazolium (NBT) solution, 3.0 mM EDTA, riboflavin and 1.0 ml of Millipore H_2O was taken in test tubes and was incubated under light for 10 min at 25°C. The absorbance at 560 nm, 50% reduction in colour is 1.0 unit and the enzyme activity was expressed in EU mg^{-1} protein.

Synthetic seed preparation

Mature green somatic embryos at the cotyledonary stage were collected and suspended in a solution of MS, added with different concentrations of sodium alginate (2%, 3% and 4%) for a few seconds and then dropped into the sterile aqueous solution of calcium chloride ($CaCl_2$:75 mM, 10 mM and 125 mM) in order to encapsulate the embryo. Encapsulated embryos in $CaCl_2$ solution were shaken in an orbital shaker at 60 rpm. $CaCl_2$ solution was poured off and the beads were washed twice with sterilized water and placed on sterilized filter paper to remove excess water.

Storage and conversion of synthetic seeds

Encapsulated somatic embryos were stored at 4°C (in refrigerator), 25°C (incubation room temperature) and at -20°C for varied periods (weeks) in order to evaluate the viability and regeneration/conversion potential of stored synthetic seeds. The synthetic seeds were stored using airtight dark 100 ml conical flasks. The synthetic seeds were periodically removed from the respective storage temperatures and cultured in MS added with various plant growth regulators (PGRs). The conversion rate was recorded by observing the development of shoots in 1.0 mg/l BA and 0.5 mg/l GA_3 added MS after 2 and 4 weeks of culture.

Statistical analysis

For callus induction, hypocotyls explants were cultured in test tubes with a minimum of 30 explants per experiment. In case of somatic embryo, induction each experiment was replicated three times. Data on frequency of response and numbers of somatic embryos induced per 500 mg of callus were recorded after 4 weeks of culture. The data presented as mean and its standard deviation (mean ± SD). The significance of differences among means was carried out using Duncan's Multiple Range Test, DMRT (Duncan, 1995) at P= 0.05.

3. Results

Callus induction and somatic embryogenesis

On 2,4-D (0.5-2.0 mg/l) supplemented MS medium, prolific callus was induced from hypocotyl explants. The optimum callus induction frequency of 96.0% and 89.3% was noticed in Co-1 and RS respectively with 1.0 mg/l 2,4-D added medium. The callus induction frequency decreased as the concentration of 2,4-D above 1.0 mg/l was used (Table 1). Callus induction was also observed on NAA at 0.5 mg/l added MS but the induced callus was non-embryogenic in nature. The 2,4-D induced embryogenic callus was white and friable, which produced globular embryos on the same induction medium (Fig. 1 a). Of the different 2,4-D concentrations used, 1.0 mg/l exhibited higher embryogenic ability (77.6%) in RS with 63.3 somatic embryos; in Co-1, the embryogenic ability was also equally high (72.8%) with relatively lower numbers of embryos (51.0) (Fig. 1 b). Beside 2,4-D, other PGR combinations were also tested for embryo differentiation. In 0.5 mg/l NAA + 0.25 mg/l BA amended medium, maximum differentiation of embryos (78.7% in RS and 74.0% in Co-1) was noticed (Fig. 1 c-d) and in increasing NAA concentrations the embryo differentiation decreased. The somatic

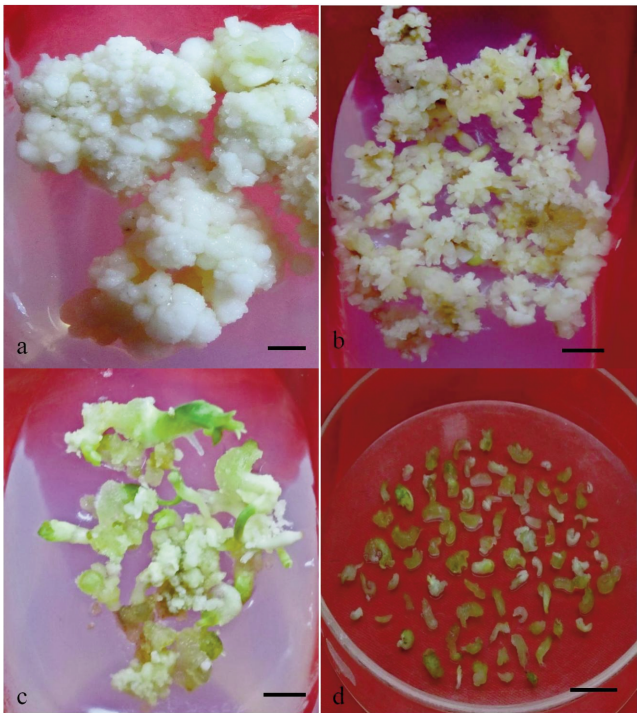

Fig. 1 - Somatic embryo formation in *Coriandrum sativum* (RS). a) Globular embryos at induction stage; b) Somatic embryos at proliferation stage; c and d) Somatic embryos at maturation stage (scale bar, a-2 mm; b-c= 3 mm; d=1 cm).

Table 1 - Effect of different concentrations of 2,4-D on callus induction and somatic embryogenesis from hypocotyl explants of 'Rajendra Swathi' and 'Co-1' varieties of *Coriandrum sativum*

2,4-D (mg/l)	Rajendra Swathi			Co-1		
	Callus induction (%)	Embryogenic callus induction frequency	No. of embryos formed/culture (0.5 g)	Callus induction (%)	Embryogenic callus induction frequency	No. of embryos formed/culture (0.5 g)
0.5	86.0±4.0 a	55.3±2.5 b	39.3±2.1 b	89.3±3.0 b	52.0±2.64 b	36.3±2.08 c
1	89.3±4.2 a	77.6±3.2 a	63.0±4.5 a	96.0±2.3 a	72.8±3.0 a	51.0±2.64 d
1.5	74.0±4.0 b	58.0±2.6 b	44.5±2.5 b	80.7±4.1 c	54.2±2.51 b	41.5±2.0 b
2	47.3±1.1 c	45.2±2.3 c	31.0±3.0 c	64.0±2.0 d	42.0±3.0 c	27.0±3.0 d

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at p= 0.05) according Duncan's multiple range test.

embryo germination was also influenced by PGRs combination; 1.0 mg/l BA + 0.5 mg/l GA_3 showed maximum germination of embryos in both varieties (83.3% in RS and 76.7% in Co-1). The increased levels of BA reduced plantlet conversion rate (Table 2).

Biochemical analysis

The embryogenic callus is often biochemically different from non-embryogenic tissues which could be used in marker assisted early selection. Protein, proline and sugar level were noted to be high in early embryogenic tissue compared to non-embryogenic callus (Fig. 2 a-c). The sugar content was also noted high at induction stage of embryogenesis (37.2 mg g m^{-1} fresh weight in 'RS' and 33.4 mg g m^{-1} fw in 'Co-1') as compared to other stages of embryo (matured) and non-embryogenic callus. The same was the case with proline where induction stage had more levels of proline in both the varieties (2.78 mg gm^{-1} fw in RS and 2.54 mg g m^{-1} fresh weight in Co-1). Protein content was higher at maturation stage of embryos showing 7.3 mg gm^{-1} fw in 'RS' and 7.0 mg gm^{-1}fw in Co-1compared to other stages of tissues.

Antioxidant enzymes activity behaved differently in different cultivating tissues (Fig. 2 d-f). The highest CAT activity was observed in RS with 5.4 EU mg^{-1} protein min^{-1} compared to Co-1 with 4.7 EU mg^{-1} protein min^{-1} at matured stage of somatic embryos; so was SOD activity (4.8 EUmg^{-1}protein min^{-1} and 4.3 EU mg^{-1} protein min^{-1} in RS and Co-1 respectively). The APX activity was highest at induction stage of embryos with 2.1 EU mg^{-1} protein min^{-1} in RS and 1.8 EU mg^{-1} protein min^{-1} in Co-1 compared to other tissues.

Synthetic seed preparation and plantlet conversion

Green and matured somatic embryos were encapsulated in different gelling conditions. Good synthetic seeds were produced in combination of sodium alginate and calcium chloride mix, which later responded differently in germination medium (Fig. 3 a-c). The study revealed that 3% sodium alginate + 100 mM $CaCl_2$ was the ideal mixture condition that had fairly good conversion rate (74.0% in RS and 70.6% in Co-1) compared to 2% (48.6% in RS and 43.3% in Co-1) and 4% (30.0% in RS and 26.6% in Co-1) sodium alginate solution (Table 3). The higher sodium alginate level (4%) reduced synthetic seed conversion ability. Similarly, the level of $CaCl_2$ was also equally important in making synthetic seeds and later in obtaining

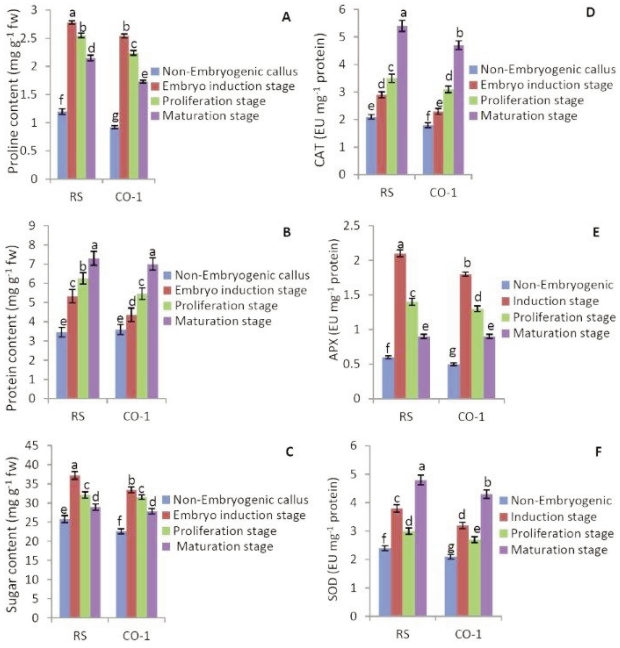

Fig. 2 - Biochemical and enzyme activities in two varieties of *Coriandrum sativum* (RS and CO-1). a) Proline content; b) Protein content; c) Sugar content; d) Catalase activity; e) Ascorbate peroxidase activity and f) Superoxide dismutase activity.

Table 2 - Somatic embryo differentiation and germination frequency in 'RS' and 'Co-1' on different concentrations of NAA, BA and GA_3 supplemented MS medium

NAA (mg/l)	BA (mg/l)	GA_3 (mg/l)	Rajendra Swathi		Co-1	
			Embryo differentiation	Conversion rate	Embryo differentiation	Conversion rate
0	0.5	0.25	0	54.6±3.0 c	0	51.3±3.0 c
0	1	0.25	0	68.0±3.4 b	0	64.0±3.4 b
0	1	0.5	0	83.3±4.6 a	0	76.7±4.1 a
0	1.5	0.5	0	63.6±3.0 b	0	54.0±2.0 c
0.5	0.25	0	78.7±4.1 a	0	74.0±4.0 a	0
1	0.25	0	61.3±3.0 b	0	55.3±3.0 b	0
1	0.5	0	56.0±3.4 b	0	47.2±2.3 c	0
1.5	0.5	0	39.3±3.0 c	0	34.0±2.0 d	0

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at p = 0.05) according Duncan's multiple range test.

plantlets; 100 mM $CaCl_2$ had a high plantlet conversion, followed by 75 mM and 125 mM. The synthetic seeds prepared at low (2%) sodium alginate +75 mM $CaCl_2$ were soft, fragile and showed poor conversion; while high (4%) level of sodium alginate and $CaCl_2$ (125 mM) produced hard beads, reducing conversion ability. The duration of $CaCl_2$ exposure also had an influence on conversion; 20 min exposure resulted in higher rate of germination compared to 10 and 30 min exposure (data not shown).

The synthetic seeds containing somatic embryos, were kept in three different storage temperatures for varying periods in order to find the right temperature for preservation. It was observed that the storage temperature and duration had a direct influence on synthetic seed viability and final conversion rate (Table 4). The encapsulated somatic embryos stored at 4°C for a week, showed higher regeneration ability (62.0% in RS and 58.6% in Co-1) compared to incubation at room temperature (25°C) and -20°C where the conversion rate was 42.6% and 13.3% in RS and 34.6% and 9.3% in Co-1 respectively. The conversion rate decreased as the storage duration increased as was observed in RS (44.0%) and Co-1 (37.3%) after 5 weeks of 4°C storage (Table 4). The synthetic seed's viability was lost in both the varieties when stored at ultralow temperature (-20°C) for extended period (3 weeks). The regeneration ability gradually declined with increasing storage time in all tested conditions.

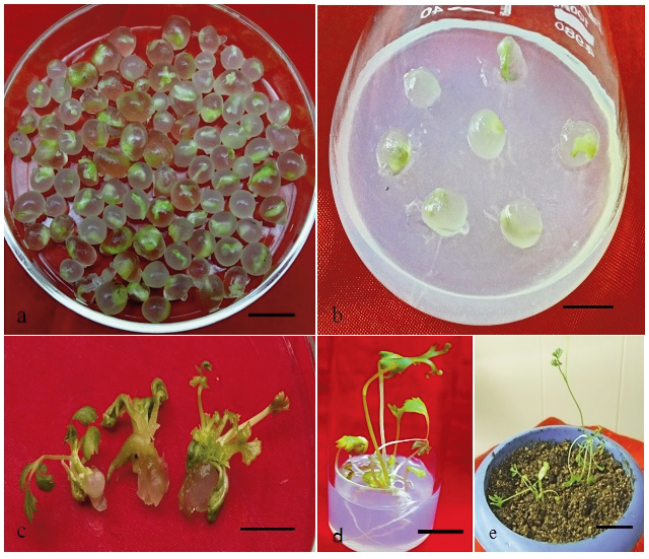

Fig. 3 - Synthetic seed development and plantlet formation in *Coriandrum sativum* (RS). a) Encapsulated somatic embryos; b) Synthetic seeds on the germination medium; c) Germinating synthetic seeds; d) Rooted plantlet and e) Synthetic seed derived plant, grown in outdoor condition (scale bar, a= 1.5 cm, b-d= 1 cm, e=2 cm).

The plantlets derived from synthetic seeds had a prominent shoot primordia without much root growth and these shoots were transferred to root induction medium, added with 0.5 mg/l IBA. Well rooted plants (Fig. 3 d) were taken out from the test tube, washed with water and transferred in paper cup (Fig. 3 e). After 2 weeks of hardening, the cups were transferred to the field. Out of 80 plants transferred to soil, 43 (55%) survived in the field. The survived plants in outdoor looked healthy and morphologically similar to mother plants.

4. Discussion and Conclusions

In these two varieties of coriander, callus was vigorously induced from hypocotyl in 1.0 mg/l 2,4-D added MS medium, consistent with previous studies in which 2,4-D played a crucial role in callusing in var-

Table 3 - Effect of different concentrations of sodium alginate and calcium chloride on the conversion rate of encapsulated somatic embryos on 1.0 mg/l BA and 0.5 mg/l GA_3 MS medium

Alginate (%)	Calcium chloride (mM)	Conversion rate (%)			
		Rajendra Swathi		Co-1	
		2 weeks	4 weeks	2 weeks	4 weeks
2	75	36.7±2.3 d	45.3±3.0 d	29.3±2.3 d	38.0±3.5 d
	100	42.6±3.0 c	48.6±2.3 cd	36.6±3.0 c	43.3±3.0 c
	125	30.0±2.0 e	37.3±3.0 e	24.0±2.0 e	32.6±2.3 e
3	75	46.0±3.4 c	52.0±3.4 c	41.3±3.0 e	51.3±3.0 b
	100	63.3±4.2 a	74.0±4.0 a	64.0±3.4 a	70.6±4.1 a
	125	52.0±3.4 b	57.3±2.3 b	45.3±2.3 b	54.7±3.0 b
4	75	21.3±2.3 f	24.6±3.0 g	18.0±2.0 f	24.0±2.0 f
	100	25.3±3.0 ef	30.0±2.0 f	21.3±2.3 e	26.6±2.5 f
	125	12.0±2.0 g	20.6±2.3 g	10.6±1.15 f	19.3±1.1 f

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at p= 0.05) according Duncan's multiple range test.

ious investigated plants (Kim *et al.,* 2011). In the present study, embryogenic callus started to differentiate somatic embryos in 2,4-D added medium but the continuous presence of 2,4-D inhibited somatic embryo development beyond globular/heart shaped stage. The formation of somatic embryos on callus induction medium was earlier reported in several other species (Kim *et al.,* 2011). The hindrance of embryo differentiation and progression in continuous presence of 2,4-D has earlier been reported in a number studied cases and the transfer of embryogenic tissues or embryos into '2,4-D-free' or less PGR concentrated medium has been suggested for embryo development (Junaid *et al*., 2006). The embryogenic tissues with developing somatic embryos were later cultured on NAA and BA added medium in which fast embryo development and maturation were observed. Somatic embryo differentiation and maturation in NAA and BA added medium was earlier reported in several investigated plants (Junaid *et al.*, 2006; Puhan and Rath, 2012). The use of sucrose, ABA and GA_3 has also been noted to be very efficient in promoting somatic embryo development and maturation (Yang *et al.*, 2013; Mujib *et al.*, 2014).

As the non-embryogenic callus transforms into embryogenic tissues, biochemical profiles also alter simultaneously. In this study, we noted differences in biochemical and enzyme activities in embryogenic and non-embryogenic tissues. Here, we noted increased protein and decreased soluble sugar levels in maturation stage of somatic embryo, which is in confirmatory with Kumar and Kumari (2010). Enhanced protein content was also observed here during maturation stage of embryo and it could be used as a good tissue- specific indicator. The increase in biochemical attributes in embryogenic callus over non-embryogenic tissue was previously reported in several plants (Nieves *et al.*, 2003). The extra proline accumulation has been noted in response to stress, which is believed to act as osmo-regulator in protecting cells against osmotic perturbation (Elmaghrabi *et al.*, 2013). Similar changes in biochemical attributes were earlier observed during transition to differentiated embryogenic state from meristematic tissue under *in vitro* cultural conditions (Samar *et al.*, 2011). Kumar *et al*. (2010) observed alteration of enzyme activities during cellular differentiation, this and similar other findings are important in understanding the metabolic changes in developmental events (Santos *et al.*, 2011). Here, the enzyme activities were lower in non-embryogenic callus compared to embryogenic one, but it was higher at specific embryo stages. In *Hevea brasiliensis* similar increased peroxidase activity was noted during embryo organization (Blanc *et al*., 2002). Increased APX and CAT activity in embryogenic tissue, designates its higher efficiency in scavenging H_2O_2 during SOD metabolism, prevents membrane lipids peroxidation process (Niknam *et al*., 2006). Beside distinguishing morphogenic events, CAT facilitates in forming resistant cell walls and helps in defense mechanism against reactive oxygen species (Gaspar *et al.*, 2002).

The induced mature somatic embryos were encapsulated in gelling mix and the conversion of synthetic seeds into plantlets was monitored. Singh *et al*. (2010) observed that the quality bead formation and germination ability of synthetic seeds are partly dependent on concentrations of sodium alginate and calcium chloride solution and on duration of exposure. Our observation indicated that the somatic embryos, encapsulated in 3% sodium alginate + 100

Table 4 - Conversion rate at temperature conditions of somatic embryos encapsulated in 3% sodium alginate and 100 mm $CaCl_2$, after storage. MS was added with 1.0 mg/l BA and 0.5 mg/l GA_3

Storage temperature	Storage duration (weeks)	Regeneration (%)			
		Rajendra Swathi		Co-1	
		2 weeks	4 weeks	2 weeks	4 weeks
-20°C	1	12.6±2.3 f	13.3±1.1 f	6.6±1.1 f	9.3±1.15 e
	3	0	0	0	0
4°C	1	57.3±4.1 a	62.0±4.0 a	54.0±3.5 a	58.6±3.0 a
	3	38.0±2.0 b	44.0±3.4 b	31.3±3.0 b	37.3±3.0 b
	5	24.0±2.0 d	32.0±2.0 c	19.3±2.3 d	27.3±3.0 c
	7	10.6±1.1 f	18.6±1.1 e	09.3±1.1 f	16.0±2.0 d
25°C	1	33.3±3.0 c	42.6±3.0 b	27.3±2.3 c	34.6±2.3 b
	3	18.0±2.0 e	27.3±2.3 d	13.3±1.15 e	19.3±1.15 d

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at p= 0.05) according Duncan's multiple range test.

mM $CaCl_2$ produced quality bead with more conversion as compared to the use of lower (2%) and higher level (4%) of sodium alginate. The earlier reports suggested that somatic embryos encapsulated with 3% sodium alginate and 100 mM $CaCl_2$ produced uniform and moderately hard beads with optimum regeneration potential (Sarmah *et al*., 2010). The maximum plantlet generation frequency with 3% sodium alginate was found in *Paulownia elongata* (Gozukirmizi, 2003), *Coelogyne breviscapa (*Mohanraj *et al.*, 2009), *Stevia rebaudiana (*Ali *et al.*, 2012). In *Stevia rebaudiana*, hard beads were formed at 4% sodium alginate, which adversely affected germination rate (Andlib *et al.*, 2011). The 20 min exposure in $CaCl_2$ solution improved conversion of plantlets, the conversion rate however, declined with increasing exposure time and this observation corroborates with similar other previous studies (Malabadi and Van Staden, 2005). Over duration of exposure affects germination ability of the synthetic seeds and this decline of conversion may be due to growth inhibition caused by over absorption and penetration of calcium chloride (Malabadi and Van Staden, 2005). The use of liquid MS for preparing gel matrix was found to be beneficial in emerging shoot and this may be attributed to the presence of nutrients in gel matrix, which apparently serves as a nutrient bed around the propagules and facilitates synthetic seed survival and germination (Ara *et al*., 1999). Matrix of synthetic seed mimics endosperm of natural seed and this nutrient rich artificial endosperm is essential for maintaining survival of germplasm (Antonietta *et al.*, 1999). The storage conditions also affect the germination potential of synthetic seeds. Our observation indicated that the viability and regeneration ability of synthetic seeds decreased with increasing storage duration. The same view i.e. the longer storage of synthetic seeds reduced conversion ability was reported in earlier few cases (Parveen and Shahzad, 2014). During long storage, carbohydrate reserves decrease gradually, which may be responsible for the reduced conversion ability of synthetic seeds (Ding *et al.*, 1998). The storage of *in vitro* grown tissues at -20°C showed a very poor regenerative ability and loss of tissue viability. It is believed that the short spell of low temperature exposure helps forming ice crystals in tissues, prevents food exchange, causes frost injury/stress and reduces tissue viability (Mittler, 2006).

The successful regeneration system requires germination of synthetic seeds/mature somatic embryos into plantlets at fast pace. The encapsulated somatic embryos were germinated into plantlets on BA and GA_3 added MS. The combination of BA and GA_3 was found to be very responsive for somatic embryo germination in *Solanum lycopersicum* (Godishala *et al.*, 2011). The well-developed plantlets were transferred to IBA added half MS for promoting root growth. The positive effect of IBA on root induction was reported in several investigated plants like *Camellia nitidissima* (Lu *et al.*, 2013), *Talinum triangulare* (Swarna and Ravindhran, 2013). The synthetic seed derived plants were healthy and morphologically very similar to parent plants. The developed plant regeneration protocol from synthetic seeds will be very important in preserving coriander germplasm for short and medium term basis.

In summary, synthetic seed development and plant regeneration after storage may provide a good alternative strategy for coriander germplasm. The 'short and medium term' storage can open up and enable conservation possibility of elite important coriander. Observed biochemical and enzyme activity differences in various *in vitro* grown tissues may play an important role in marker based selection of tissues in cellular differentiation investigation. The developed synthetic seed preparation protocol can also be used as an alternative propagation method.

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Evaluation of salinity tolerance in fourteen selected pistachio (*Pistacia vera* L.) cultivars

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Abstract: Cultivars and rootstocks tolerant to salinity are determinant to increase the salt tolerance of planted fruit trees including pistachio. In this research, the effect of salinity stress on morphological and physiological traits as well as the concentration of nutrition elements in some pistachio cultivars was investigated based on completely randomized design (CRD), with two factors cultivars and irrigation water salinity. Studied cultivars were Ghazvini, Shahpasand, Akbari, Khanjari, Jandaghi, Italiyayi, Fndoghi 48, Sabz Pesteh Tohg, Ahmad Aghaee, Rezaie Zood Res, Mousa Abadi, Ebrahimi, Kaleh Ghochi and Badami Zarand and levels of salinity were 0.5, 4.9, 9.8, 14.75 and 19.8 dS/m. Each treatment had nine replicas. The results showed that increasing salinity reduced branch height, branch diameter, number of total leaves, and percentage of green leaves, relative humidity content, chlorophyll a, chlorophyll b and total chlorophylls in all cultivars. But percentage of necrotic leaves, percentage of downfall leaves, relative ionic percentage and cell membrane injury percentage were increased. The results showed that salinity stress affected the young trees through increasing the amount of minimum fluorescence (F_0) and decreasing the maximum fluorescence (F_M) and reducing variable fluorescence (F_V) as well as the ratio of variable fluorescence to maximum fluorescence from 0.83±1 in the control plants to 0.59±0.015 in Rezaie Zood Res cultivar and 0.61±0.009 in Mousa Abadi cultivar. The results also showed that in the total cultivars studied, the highest amount of Na^+ in leaves and roots (2.09±0.04% and 3.04±0.06%), and the lowest amount of K^+ in leaves and roots (0.40±0.02% and 0.34±0.01%), were observed in treatment 19.75 dS/m. Overall, Ghazvini was found to be the most tolerant cultivar to salinity stress. This cultivar could well tolerate salinity 14.75 dS/m.

1. Introduction

Pistachio (*Pistacia vera* L.) is one of the important commercial crops in Iran. Majority of pistachio orchards are located in areas with saline soil and are irrigated with low quality and salty waters. Although pistachio trees are classified as tolerant to salinity, researches have demonstrated

that growth rates of pistachio trees decrease with increasing sodium chloride (NaCl) concentration in soil and there is a positive correlation between sodium (Na^+) as well as chloride (Cl^-) concentration in plant tissue and soil (Sepaskhah and Maftoun, 1988; Noitsakis *et al*., 1997; Munns and Tester, 2008; Zrig *et al*., 2015). Salinity stress also negatively affects photosynthesis rate, morphology of leaves, and nutrient balance in pistachio trees (Picchioni and Myamoto, 1990; Saadatmand *et al*., 2007; Karimi *et al*., 2011). Walker *et al*. (1987) and Karimi *et al*. (2009) reported that the highest chloride concentrations were observed in lamina and petiole of pistachio seedlings irrigated with salty water, whereas highest sodium concentration was observed in roots. Ferguson *et al.* (2002) suggested that the decrease of water potential in plant in higher salinity levels is one of the main reason for decrease pistachio yield.

It has been reported that salinity stress is one of the most important environmental factors limiting photosynthesis in the majority of worldwide cultivated crops, including pistachio crop (Maxwell and Johnson, 2000; Ranjbarfordoei *et al*., 2006). Chlorophyll fluorescence (CF) has been used to study plant responses to different kinds of stress (Baker and Rosenqvist, 2004). Chlorophyll (Chl) fluorescence yield (Chl FY) such as minimal Chl FY (F_0) and variable Chl FY (F_v) can be used for evidencing stress and damage of the photosynthetic apparatus, and characterizing the environment where plants grow (Herda *et al*., 1999; DeEll and Toivonen, 2003; Kodad *et al*., 2010). F_v/F_m ratio has been used in many studies related to stress in plants. In most of plants, when ratio F_v/F_m is around 0.83 means that stress has not been introduced to the plant. Values lower than this will be seen when the plant has been exposed to stress, indicating in particular the phenomenon of photo inhibition (Herda *et al*., 1999; DeEll and Toivonen, 2003; Kodad *et al*., 2010).

Selecting nutrient sources that do not add harmful ions and salinity to irrigation water to avoid compounding salinity problems would be the best option. In areas affected by soil and water salinity, nevertheless, it is more convenient to use salt-tolerant rootstocks for the species characterized by a certain degree of salt tolerance, i.e. *Pistacia* sp. An important characteristic of *Pistacia* sp. is their ability to store large quantities of Na^+ in roots, which might make pistachio tolerant to Na^+ (Picchioni and Myamoto, 1990; Karimi *et al*., 2011). Sepaskhah and Maftoun (1988) reported that 50% reduction in shoot growth was observed when the average root-zone salinity was between 7.9 and 10 dS/m (ECe). Saadatmand *et al.* (2007) postulated that salinity stress had more negative influence than drought stress on pistachio growth. They reported that Sarakhs variety showed higher sensitivity to soil salinity than Qazvini variety, but with increasing in irrigation intervals, Sarakhs was more-tolerate to salinity than Qazvini.

Although, other researchers have studied the influence of soil and water salinity on the growth indices and chemical composition of pistachio cultivars, but in before researches a low number of cultivars was investigated. Therefore, in this research, the effects of five levels of irrigation water salinity on morphological and physiological traits as well as the concentration of nutrition elements in fourteen selected pistachio (*Pistacia vera* L.) cultivars have been investigated in order to find most tolerant cultivars to salinity.

2. Materials and Methods

Plant material and natural salt treatments

In this research, the effects of salinity stress on morphological and physiological traits and on the concentration of nutrition elements in 14 pistachio cultivars such as Ghazvini, Shahpasand, Akbari, Khanjari, Jandaghi, Italiyayi, Fndoghi 48, Sabz Pesteh Tohg, Ahmad Aghaee, Rezaie Zood Res, Mousa Abadi, Ebrahimi, Kaleh Ghochi and Badami Zarand were investigated. The experiment was carried out in the research greenhouse of Temperate Fruit Research Center, Horticultural Research Institute in Karaj-Iran in years of 2013 and 2014 based on completely randomized design (CRD), with two factors; cultivars with 14 levels and irrigation water salinity by 5 levels (Control= 0.5 dS/m, A= 4.8 dS/m, B= 9.8 dS/m, C= 14.75 dS/m and D=19.8 dS/m) and with nine replications for each treatment, for a total of 630 pots. Seeds were germinated according to the method described by Karimi *et al.* (2009). Seeds were pretreated with benomyl (wettable powder-50%; DuPont, Wilmington, DE, USA) for 24 h, and then incubated at 30°C within layers of sterile moist crisped cloth. After radicle emergence, seeds were planted in Jiffy pots (Jiffy Group, Moerdijk, Netherlands) and grown in a greenhouse for three months. Seedlings with 10 to 15 cm height were transplanted to pots 2 Kg filled with soil series of fine loamy mixed, which its characteristics are listed in Table 1.

Salinity treatment was started and continued for

Table 1 - Physical and chemical characteristics of soil mixture

Title	Value
Saturation percentage (%)	39
Field capacity (%)	27.33
Permanent wiltering point (%)	14.8
dS/m (EC)	1.28
pH	7.5
N (%)	0.15
Organic carbon (%)	1.49
P (ppm)	104.9
Sand (%)	46
Silt (%)	34
Clay (%)	20
Texture	Loam
Ca (ppm)	1230
Mg (ppm)	316.2
Total neutralizing value (%)	13.8
Cu (ppm)	2.12
Zn (ppm)	4.86
Fe (ppm)	27.34
K (ppm)	690
Mn (ppm)	16.26
Na (ppm)	93.15

two and half months. For salinity treatments, salts were collected of salt lake shore in Qom-Iran. Then, salinity treatments were obtained by solving 0, 2.4, 4.8, 7.2 and 9.6 g of salt in 1 L of water (treatments composition is reported in Table 2). Also, to avoid sudden shock and plasmolysis, salt treatments were gradually added and reached to the final concentration within a week (2 stages of irrigations). Field capacity (FC) of soil in pots was determined before transferring plants to units by a pressure plate (Model F1, make USA). Irrigation schedule was organized according to pots changes in weight and leaching requirement. Electric conductivity and pH rate were regularly measured in drainage water to maintain the electric conductivity of both input and soil solutions in a stable range. At the end of the experiment, the soil of pots in each level salinity was mixed together. Then three samples of each treatment (in total 15 samples) were analyzed (Table 3).

Growth parameters

At the end of the experiment, growth characteristics including main-shoot length, trunk diameter and number of leaves, were measured as well as percentage of necrotic leaves, downfall leaves and green leaves were calculated (Papadakis *et al*., 2007). Fresh weight of leaves, main-shoots and roots were measured immediately after removing, using a digital scale. Dry weight of the samples was measured using an oven at 75°C for 48 h (Papadakis *et al*., 2007).

Physiological parameters

For determination of leaf chlorophyll, 0.2 g of leaf was extracted (in total 630 samples, means nine replicas for each cultivar and for each salinity treatment), with ethanol 80% and chlorophyll *a*, chlorophyll *b* and total chlorophyll content were calculated with the method described by Arnon (1949). Leaf greenness (chlorophyll index) was evaluated on the same leaves used for gas exchange and fluorescence using a SPAD (Minolta, 502, made in Japan) after 75 days since treatments introduction.

Leaf relative water content (RWC) was determined with nine replicas (made by four leaves each) for each treatment and for each cultivar, for a total of 630 samples. Fresh weight (Fw) was recorded and then samples were put into distilled water and kept at 4°C for 24 h in the dark. After the emission of extra humidity, samples were weighed again to obtain the Total weight (Tw). Subsequently, samples were kept in the oven at 105°C for 24 hours and Dry weight (Dw) was recorded. Finally, relative water content was calculated via formulae (Yamasaki and

Table 3 - EC and pH soil treated with different levels of salinity

Treatments	EC (dS/m)	pH
Control	1.2	7.4
A	5.7	7.65
B	10.9	7.87
C	15.95	7.96
D	21.3	8.05

Table 2 - Salt solution characteristics

Treatments	Electrical conductivity (dS/m)	(pH)	Na (mg/L)	Cl (mg/L)	Ca (mg/L)	Mg (mg/L)	HCO_3^-(mg/L)
Control	0.50	7.30	22.1	35.5	62	17.1	98
A	4.90	7.60	809	1386	79	23.01	137
B	9.80	7.78	1653	2836	99	25.7	159
C	14.75	7.87	2443	4199	123	28.5	186
D	19.80	7.95	3276	5610	151	31.9	214

Dillenburg, 1999).

$$RWC= [(Fw-Dw) / (Tw-Dw)] \times 100$$

For the determination of relative ionic content was determined with nine replicas (made by four leaves each) for each treatment and for each cultivar, for a total of 630 samples. The amount of 0.5 g of each sample was put in tubes with 25 ml of distilled water at 25°C for 24 h on a shaker with speed 120 in/min. Electrical conductivity (EC) of the medium was then read using a conductivity meter (conduct meter; Radiometer, Copenhagen). Following the initial reading (Lt), samples were autoclaved for 20 min to kill leaf tissues and then kept at 25°C for 2 h on shaker with speed 120 in/min and a final reading (Lo) was obtained. Finally, relative ionic percentage was calculated via formulae:

$$\text{Relative ionic percentage} = (Lt/Lo)/100$$

as described in Lutts *et al.* (1995).

After calculation relative ionic percentage, cell membrane injury in samples' treatment with natural salt ratio samples control was performed as follows:

$$\% \text{ Injury} = 1- [1- (T1/T2)/ 1- (C1/C2)] \times 100$$

Where T and C refer to the EC values of stress-treated and control tubes and 1 and 2 refer to the initial and final EC, respectively (Lutts *et al*., 1995).

Chlorophyll fluorescence parameters

Chlorophyll fluorescence of leaves was measured using a portable fluorometer *PAM-2000* (H. Walz, Effeltrich, Germany). Before measuring chlorophyll fluorescence parameters, three leaves on main-branch of each plant were put in dark-adapted state (DAS) for 30 min using light exclusion clips (Maxwell and Johnson, 2000). Maximum quantum efficiency of photosystem II (F_v/F_m) was determined as $F_v/F_m= (F_m-F_0)/F_m$; where F_m and F_0 were maximum and minimum fluorescence of dark-adapted leaves, respectively.

Concentration of Na^+ and K^+

Concentration of Na^+ and K^+ in leaves and roots was determined with nine replicas for each treatment and for each cultivar, for a total of 630 samples. Leaves and roots of each plant, oven-dried at 75°C for 48 h, and then milled to a fine powder to pass through a 30-mesh screen. The amount of 0.5 g of each sample was dry-ached for 6h at 550°C, dissolved in 3 mL of 6 mol L^{-1} HCl and diluted to 50 mL with deionized water. Subsequently, concentration of Na^+ and K^+ were determined using atomic absorption spectroscopy (Papadakis *et al*., 2007).

Statistical analysis

This experiment was carried out based on completely randomized design (CRD), with factors cultivars in 14 levels and irrigation water salinity in 5 levels and with nine replicas for each treatment in research greenhouse of Temperate Fruit Research Center, Horticultural Research Institute in Karaj-Iran in years 2013 and 2014. Finally, data were analyzed using analysis of variance (ANOVA) using SAS software. Means were also compared by Duncan's Multiple Range test at 1% level.

3. Results

As reported in Table 4, salinity treatments negatively affected plant height, trunk diameter and number of leaves. With increasing salinity concentration in irrigation water, final height, trunk diameter and number of leaves in all studied cultivars were decreased. The lowest branch height, trunk diameter and leaf number were observed in salinity level D. The rate of decrease branch height, trunk diameter and number of leaves among the cultivars showed a significant difference with each other. The height of Khanjari, Jandaghi, Italiyayi, Fndoghi 48, Sabz Pesteh Tohg, Ahmad Aghaee, Rezaie Zood Res, Mousa Abadi, Ebrahimi and Kaleh Ghochi cultivars were decreased in salinity level B compared to control plants. While the height of Akbari, Shahpasand and Badami Zarand cultivars in salinity level C and in Qazvini cultivar only in salinity level D was decreased significantly compared to control plants.

As reported in Table 4, as the salt concentration increases, the trunk diameter and its growth were decreased during the application of salinity stress in all cultivars. The decrease in trunk diameter in the cultivars showed a significant difference with each other. The trunk diameter of Khanjari, Fndoghi 48, Rezaie Zood Res, Mousa Abadi and Kaleh Ghochi cultivars was decreased in salinity level B compared to control plants. While the trunk diameter of Italiyayi, Jandaghi, Ebrahimi, Sabz Pesteh Tohg, Ahmad Aghaee, Akbari, Shahpasand and Badami Zarand cultivars in salinity level C and in Qazvini cultivar only in salinity level D was decreased significantly compared to control plants.

The results showed that number of leaves with increasing salinity concentrations were reduced, but the amount of reduction in the number of leaves in different cultivars had significant differences. The maximum number of leaves were observed in control

Table 4 - Effect of interaction between salinity and cultivar on some of the morphologic traits

Cultivars	Treatments	No. of green leaves	Green Leaves (%)	Trunk diameter (mm)	Branch height (cm)
Khanjari	Control	27.33±1.0 h-n	100.00±0.0 a	8.38±0.13 a	32.50±1.56 f-g
	A	25.00±1.93 n-r	100.00±0.0 a	8.38±0.09 a	31.47±0.37 g-j
	B	22.55±1.42 s-v	97.02±3.81 a-d	8.07±0.09 b	28.67±1.17 k-n
	C	20.00±1.22 t-w	76.21±5.82 m-o	7.57±0.09 cd	25.55±0.52 r-s
	D	16.33±1.32 x-z	51.41±6.87 q	6.94±0.05 f-h	21.31±0.54 w-x
Akbari	Control	24.67±1.80 o-s	100.00±0.0 a	7.53±0.10 c-d	25.05±2.78 r-s
	A	22.89±0.78 s-u	100.00±0.0 a	5.55±0.09 c-d	24.38±2.69 s-u
	B	22.11±0.70 s-v	100.00±0.0 a	7.31±0.05 d-e	23.55±1.26 s-v
	C	18.45±0.72 u-y	96.86±4.23 a-d	7.14±0.06 e-g	22.67±1.29 t-y
	D	16.45±0.88 x-z	89.43±4.92 g-i	6.65±0.08 h-j	20.18±1.79 w-y
Ghazvini	Control	27.23±0.77 h-n	100.00±0.0 a	5.82±0.07 p-u	22.57±1.08 t-w
	A	27.07±0.26 i-m	100.00±0.0 a	5.81±0.07 p-u	22.55±0.57 t-w
	B	26.52±0.67 j-o	99.55±0.0 a	5.70±0.05 s-w	21.67±0.50 u-x
	C	25.77±0.67 m-r	97.72±5.63 a-c	5.46±0.04 u-x	20.7±1.0 w-y
	D	23.81±2.24 q-u	90.88±7.07 e-h	5.02±0.06 x-y	18.11±1.21 y-z
Italiaie	Control	31.67±2.34 e	100.00±0.0 a	6.71±0.03 h-j	33.00±2.95 e-h
	A	31.06±2.0 e-f	100.00±0.0 a	6.74±0.16 h-j	32.30±1.21 f-h
	B	29.22±1.39 f-i	98.85±2.40 a-b	6.53±0.12 j-m	30.17±0.94 i-k
	C	25.22±1.71 n-r	90.72±8.49 e-h	6.15±0.29 n-q	26.43±1.19 o-r
	D	22.33±2.34 q-u	84.88±5.04 i-k	5.60±0.24 t-x	22.71±1.90 t-w
Kaleh Ghochi	Control	27.00±1.93 i-n	100.00±0.0 a	6.63±0.05 f-h	32.09±0.87 f-h
	A	26.85±0.86 i-n	10000±0.0 a	6.59±0.04 j-k	31.87±0.86 f-i
	B	23.44±0.72 q-u	97.66±3.04 a-c	6.28±0.03 l-p	28.87±0.86 k-n
	C	19.33±1.0 u-w	89.60±5.26 f-i	5.78±0.04 p-u	25.43±0.71 r-s
	D	15.33±1.0 y-z	72.92±7.64 o	5.25±0.03 w-x	20.28±0.48 x-y
Jandaghi	Control	27.89±1.05 g-j	100.00±0.0 a	6.05±1 p-r	23.56±0.73 s-v
	A	27.11±2.26 i-m	100.00±0.0 a	6.04±1 p-r	23.45± 1.05 s-w
	B	25.33±0.70 n-r	95.80±3.46 a-e	5.97±1 q-s	21.03±0.53 w-x
	C	20.67±1.93 t-w	80.96±6.21 k-m	5.33±1 u-x	18.73±0.68 y-z
	D	15.44±1.42 y-z	64.71±7.56 p	4.81±1 y-z	16.45±0.51 a
Mousa Abadi	Control	32.00±1.22 e	100.00±0.0 a	5.78±1 p-u	30.00±1.87 j-l
	A	30.44±1.74 e-f	100.00±0.0 a	5.61±1 s-w	29.48±0.87 k-m
	B	26.88±1.69 i-n	93.80±3.13 b-e	5.33±1 w-x	26.65±0.75 n-r
	C	22.11±2.02 s-v	78.71±5.28 l-n	5.12±1 x-y	21.75±0.72 u-x
	D	15.00±1.22 z	52.47±4.98 q	4.57±1 z	14.90±1.07 b
Ebrahimi	Control	29.33±1.93 f-h	100.00±0.0 a	6.07±0.09 p-r	33.67±1.58 e-f
	A	28.77±1.20 f-i	100.00±0.0 a	6.08±1.29 p-r	33.55±1.26 e-f
	B	27.44±1.33 g-k	91.85±2.60 d-h	5.75±0.66 p-u	31.26±1.29 h-j
	C	23.67±1.32 q-u	84.68±4.53 i-k	5.44±0.09 u-x	26.56±1.44 n-q
	D	19.33±1.32 u-w	62.98±8.62 p	5.17±0.09 w-y	22.15±1.34 s-v
Badami Zarand	Control	25.00±1.22 n-r	100.00±0.0 a	6.73±0.05 h-j	29.32±1.09 k-m
	A	24.67±1.32 o-s	100.00±0.0 a	6.69±0.06 h-j	29.35±1.30 k-m
	B	23.33±1.0 q-u	97.39±0.0 a-c	6.56±0.08 j-l	27.40±0.92 m-q
	C	21.44±1.23 t-v	93.00±3.37 c-h	6.25±0.06 m-p	24.51±0.81 r-t
	D	17.78±1.48 v-y	83.71±4.45 j-l	5.80±0.12 p-u	21.19±0.90 w-x
Fandoghi 48	Control	35.00±1.87 d	100.00±0.0 a	6.62±0.19 i-j	36.53±1.11 d
	A	34.33±1.58 d	100.00±0.0 a	6.53±0.11 j-m	36.23±0.92 d
	B	31.11±1.69 e-f	94.55±4.30 b-e	6.30±0.10 k-o	33.17±1.26 e-g
	C	26.77±1.85 j-o	87.96±2.29 h-j	5.90±0.15 q-s	29.34±1.65 k-m
	D	21.11±1.69 t-w	75.97±6.88 o	5.67±0.09 s-w	23.45±1.02 s-v
Sabs Pesteh Togh	Control	32.00±1.50 e	100.00±0.0 a	6.43±0.09 j-n	28.22±1.21 l-o
	A	31.67±0.86 e	100.00±0.0 a	6.44±0.05 j-n	27.73±1.10 m-p
	B	28.00±1.87 g-k	93.78±0.05 b-g	6.13±0.08 n-p	25.70±0.92 p-r
	C	24.33±1.32 p-t	83.08±3.09 h-j	5.73±0.08 s-w	22.91±0.29 s-v
	D	20.11±1.45 t-w	67.29±5.39 no	5.33±0.09 u-x	21.01±0.64 w-x
Ahmad Aghaee	Control	30.00±1.87 e-g	100.00±0.0 a	5.71±0.11 s-w	23.11±1.16 s-w
	A	27.88±1.16 g-k	100.00±0.0 a	5.67±0.08 s-w	21.33±1.32 w-x
	B	25.00±1.22 n-s	95.55±3.16 a-e	5.35±0.11 u-x	18.33±0.96 y-z
	C	22.11±1.26 s-v	82.32±4.41 kl	5.11±0.08 x-y	17.21±0.54 z
	D	19.04±0.72 u-x	72.01±2.50 o	4.73±0.08 y-z	16.22±0.66 a/*
Rezaie Zodres	Control	30.67±1.32 e-g	100.00±0.0 a	5.81±0.18 p-u	34.63±2.23 e
	A	29.22±1.30 f-h	100.00±0.0 a	5.50±0.26 u-x	32.22±1.27 e-h
	B	25.77±1.71 m-r	91.76±5.73 d-h	5.17±0.16 w	28.11±1.32 m-p
	C	20.55±1.01 t-w	75.51±6.87 no	5.05±0.33 x-y	23.46±1.48 s-v
	D	16.00±0.86 y-z	53.48±4.82 q	4.49±0.24 z-a	17.95±1.08 y-z
Shahpasand	Control	45.00±1.65 a	100.00±0.0 a	7.77±0.11 c	47.51±1.40 a
	A	43.67±1.0 a	100.00±0.0 a	7.65±0.10 c	47.07±1.29 a
	B	41.67±0.70 a-b	98.55±0.0 a-b	7.52±0.10 cd	45.01±0.98 a-b
	C	39.11±1.16 c	93.89±1.81 b-g	7.21±0.06 e-f	42.19±3.33 c
	D	35.33±1.58 d	83.01±4.73 j-l	6.90±0.09 g-i	37.01±1.06 d

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

*= a/ is less than z. Given that variety of data was very wide, Duncan's test grouped data between a to z and less than z such as a/, b/, c/, d/ and e/.

plants of Shahpasand cultivar (45±1.65 leaves), and the lowest amount of them were observed in Mousa Abadi, Kaleh Ghochi, Jandaghi and Rezaie Zood Res cultivars in salinity level D (15±1.22, 15.33±1.00, 15.44±1.42 and 16±0.86 leaves), respectively.

The results showed that with increasing salinity of irrigation water, the percentage of green leaves in all cultivars was decreased. In control plants and also plants treated with salinity level A, all leaves of plants were green and were not observed any necrotic leaves. Necrosis and fallen leaves in all cultivars were observed in salinity levels B (except for Akbari cultivar), C and D. The lowest percentage of green leaves was found in salinity level D and in Mousa Abadi (52.47±4.98%), and Rezaie Zood Res (53.48±4.82%), cultivars, respectively.

As reported in Table 5, in all the cultivars as the salinity increases, the percentage of necrosis leaves were increased and the first symptoms of necrosis except for Akbari cultivar were observed in salinity level A. In all cultivars, the highest incidence of necrosis leaves was observed in salinity level D. The percentage of fallen leaves also were increased with increasing salinity levels while in all cultivars except for Akbari and Ghazvini cultivars were observed fallen leaves in salinity levels C and D.

The results showed that leaves and shoots fresh and dry weights in all studied cultivars significantly decreased by applying salinity stress and increasing its concentration. Shoots and leaves fresh and dry weights in Kaleh Ghochi, Italiyayi, Jandaghi, Fndoghi 48, Ebrahimi, Mousa Abadi and Rezaei Zood Res cultivars in salinity levels B, C and D, and in Khanjari, Sabz Pesteh Tohg, Ahmad Aghaee and Badami Zarand cultivars in salinity levels C and D, and in Akbari, Shahpasand and Qazvini cultivars only in salinity level D, were decreased significantly compared to control plants.

Based on the results of this study, as the salinity increases, the amount of minimum chlorophyll fluorescence (F_0) was increased significantly. The highest amount of F_0 in all cultivars was observed in salinity level D. The highest amount of F_0 was observed in the leaves of Khanjari cultivar treated with salinity level D (Table 7). Also, maximum chlorophyll fluorescence (F_m) in all cultivars was decreased significantly as the salinity increased. The highest amount of F_m was observed in control plants while the lowest amount of F_m was observed in Rezaie Zood Res (365.22± 20.90), Jandaghi (380.67±11.69) and Mousa Abadi (387.67±29.06) cultivars that was treated with salinity level D, respectively (Table 7).

The results showed that in all studied cultivars, (F_v/F_m) ratio was reduced significantly by applying salinity stress and increasing its concentration. Furthermore, there was a significant difference F_v/F_m values in different levels of salinity among tested cultivars. In the leaves of the control plants F_v/F_m was 0.83±1 indicating the existence of ideal and non-stressed environmental conditions for the growth of all cultivars throughout the experimental period.

Regarding changes in (F_v/F_m) ratio the stress intensity in Rezaie Zood Res and Mousa Abadi cultivars was more severe than other cultivars, (0.59±0.015 and 0.61±0.009 respectively). Therefore, the susceptibility of these cultivars to salinity stress in levels C and D were higher than other cultivars. On the contrary, Gazvini and Akbari cultivars were less damaged, (0.76±0.003 and 0.75±0.007, respectively) (Table 7). In other words, F_v/F_m in this cultivars, showed the lowest decrease.

Results on chlorophyll a, b and total chlorophyll content of the leaves treated in different salinity levels are reported in Table 8. Chlorophyll a content was reduced significantly in all of the studied cultivars in salinity level D compared to control plants while chlorophyll b content in salinity levels C and D was reduced significantly compared to the control plants. Total chlorophyll content was decreased significantly in Ghazvini cultivar only in salinity level D, and in Akbari and Badami Zarani cultivars in salinity levels C and D while total chlorophyll content in other cultivars decreased significantly in salinity levels B, C and D (Table 8). Chlorophyll index was decreased significantly under salinity stress. The lowest chlorophyll index was observed in the leaves of the plants that were irrigated with salinity level D. The highest reduction in chlorophyll index was observed in Mousa abadi (22.60±1.50), Jandaghi (27.83±0.98) and Kaleh Ghochi (31.47±3.08) cultivars. The lowest reduction in chlorophyll index was observed in Shahpasand (55.35±1.35), Akbari (53.70±1.24) and Ghazvini (49.78±1.10) cultivars (Table 8).

According to the results reported in Table 9, the content of relative humidity of leaves decreased significantly as the salinity increased. The content of relative humidity in leaves of control plants were higher than 79.83% (of 79.83±0.24% in control plant leaves of Shahpasand cultivar to 85.25±0.64% in control plant leaves of Khanjari cultivar), while relative humidity content in leaves of Mousa Abadi, Rezaie Zood Res and Sabz Pesteh Togh plants in salinity level D, were 64.17±0.52%, 66.49±0.57% and 66.95± 0.77%, respectively. In Ghazvini and Akbari cultivars

Table 5 - Effect of interaction between salinity and cultivar on the morphologic traits measured

Cultivar	Treatments	Leaf dry weight (g)	Leaf fresh weight (g)	Downfall leaves (%)	Necrosis leaves (%)
Khanjari	Control	2.77±0.02 d-f	6.28±0.06 e-f	0.00±0.0 l	0.00±0.0 q
	A	2.77±0.01 d-f	6.30±0.02 e-f	0.00±0.0 l	0.00±0.0 q
	B	2.67±0.02 f-g	6.05±0.06 f-g	0.00±0.0 l	2.98±3.81 n-q
	C	2.54±0.01 g-i	5.71±0.03 h	11.72±3.47 d	16.07±4.47 d-e
	D	2.47±0.03 h-k	5.04±0.06 k-m	19.24±3.22 b	29.35±4.24 a
Akbari	Control	1.73±0.12 s-u	3.67±0.26 q-t	0.00±0.0 l	0.00±0.0 q
	A	1.61±0.05 t-u	3.41±0.11 r-u	0.00±0.0 l	0.00±0.0 q
	B	1.48±0.04 u-x	3.16±0.10 s-v	0.00±0.0 l	0.00±0.0 q
	C	1.43±0.06 v-x	2.95±0.14 t-w	0.00±0.0 l	3.14±4.23 m-q
	D	1.34±0.06 w-y	2.69±0.13 u-x	0.00±0.0 l	10.57±4.34 f-h
Ghazvini	Control	2.45±0.06 h-l	5.44±0.15 h-j	0.00±0.0 l	0.00±0.0 q
	A	2.44±0.02 h-l	5.41±0.05 h-j	0.00±0.0 l	0.00±0.0 q
	B	2.40±0.06 h-l	5.30±0.13 i-k	0.00±0.0 l	0.45± 0.30 q
	C	2.31±0.06 k-o	5.02±0.13 j-l	0.00±0.0 l	3.28±5.63 m-q
	D	2.25±0.21 m-p	4.76±0.44 l-n	0.00±0.0 l	9.12±3.68 g-i
Italiaie	Control	2.52±0.18 h-j	5.70±0.42 h	0.00±0.0 l	0.00±0.0 q
	A	2.54±0.15 h-j	5.75±0.35 g-h	0.00±0.0 l	0.00±0.0 q
	B	2.30±0.10 k-o	5.14±0.24 j-l	0.00±0.0 l	1.15±2.40 p-q
	C	1.98±0.13 o-s	4.38± 0.29 o-p	3.78±3.50 g-l	5.50±5.62 i-n
	D	1.73±0.18 s-u	3.77±0.39 q-t	5.13± 3.05 f-j	8.99±3.09 g-j
Kaleh Ghoch	Control	2.36±0.16 h-m	5.13±0.36 i-k	0.00±0.0 l	0.00±0.0 q
	A	2.37±0.07 h-m	5.13±0.16 i-k	0.00±0.0 l	0.00±0.0 q
	B	2.01±0.06 o-s	4.29±0.13 o-p	1.19±2.50 kl	1.15±3.04 p-q
	C	1.58±0.08 t-u	3.32±0.17 r-u	4.91±2.70 f-k	5.49±2.78 i-n
	D	1.18±0.11 y-z	2.43±0.15 v-y	17.10±7.08 b-c	9.98±6.03 f-h
Jandaghi	Control	2.92±0.23 c-d	6.41±0.24 e	0.00±0.0 l	0.00±0.0 q
	A	2.89±0.06 c-e	6.32±0.50 e-f	0.00±0.0 l	0.00±0.0 q
	B	2.55±0.18 g-h	5.53±0.14 hi	1.87± 1.90 i-l	2.33±2.95 n-q
	C	1.95±0.12 p-t	4.13±0.38 p-r	11.54± 4.80 d	7.50±4.54 g-l
	D	1.41±0.04 v-x	2.90±0.26 t-w	17.74± 6.60 b-c	17.55±6.71 cd
Mousa Abadi	Control	1.83±0.02 r-t	4.07±0.09 p-r	0.00±0.0 l	00.00±0.0 q
	A	1.91±0.09 p-t	3.99±0.06 p-s	0.00±0.0 l	00.00±0.0 q
	B	1.51±0.10 u-x	3.22±0.20 s-u	2.65±1.30 i-l	3.55±1.50 m-q
	C	1.18±0.05 y-z	2.43±0.22 v-y	8.15± 2.50 d-f	13.04±2.74 e-f
	D	0.72±0.12 z	1.42±0.11 z	27.06± 4.90 a	20.47±4.70b-c
Ebrahimi	Control	1.94±0.07 p-s	4.52 ±0.29m-o	0.00±0.0 l	00.00±0.0 q
	A	1.87±0.10 r-t	4.32±0.18 o-p	0.00±0.0 l	00.00±0.0 q
	B	1.61±0.04 t-u	3.61±0.23 q-t	3.82±2.33 g-l	5.33±2.11 j-o
	C	1.22±0.12 x-y	2.70±0.09 u-x	5.02±2.89 f-k	9.29±3.24 f-h
	D	0.71±0.12 z	1.50±0.25 y-z	15.05±2.95 c	21.96±3.11 b
Badami Zarand	Control	2.47±0.13 h-k	5.25±0.25 i-k	0.00±0.0 l	0.00±0.0 q
	A	2.43±0.09 h-k	5.15±0.27 j-k	0.00±0.0 l	0.00±0.0 q
	B	2.29±0.11 k-o	4.91±0.20 k-m	0.00±0.0 l	2.61±1.11 n-q
	C	2.08±0.14 o-r	4.28±0.24 o-p	3.50±2.70 h-l	3.50±2.51 m-q
	D	1.71±0.16 s-u	3.41±0.28 r-u	5.64±2.99 f-i	10.65±3.80 f-h
Fandoghi 48	Control	3.11±0.14 c	7.03±0.37 d	0.00±0.0 l	0.00±0.0 q
	A	3.06±0.14 c	6.87±0.31 d	0.00±0.0 l	0.00±0.0 q
	B	2.73±13 e-f	6.07±0.32 f-g	0.00±0.0 l	5.45±2.37 l-p
	C	2.29±0.15 k-m	4.98±0.34 k-m	3.74±2.50 g-l	8.28±2.30 g-k
	D	1.73±0.13 s-u	3.67±0.29 q-t	18.15±3.70 b-c	15.88±3.53 de
Sabs Pesteh Togh	Control	2.34±0.04 j-n	4.99±0.09 k-m	0.00±0.0 l	0.00±0.0 a
	A	2.35±0.05 j-n	4.99±0.12 k-m	0.00±0.0 l	0.00±0.0 a
	B	2.29±0.06 k-o	4.76±0.13 m-n	1.11±1.17 j-l	5.01±1.39 k-p
	C	2.13±0.08 o-r	4.33±0.16 o-p	6.88±2.70 e-h	10.04±2.84 f-h
	D	1.98±0.08 o-s	3.96±0.17 p-s	14.46±3.67 cd	18.25±3.53 cd
Ahmad Aghaee	Control	1.89±0.05 p-u	3.85±0.11 p-s	0.00±0.0 l	0.00±0.0 q
	A	1.87±0.03 p-t	3.68±0.06 q-t	0.00±0.0 l	0.00±0.0 q
	B	1.78±0.04 s-t	3.48±0.08 r-u	0.50±1.10 l	3.95±2.73 l-q
	C	1.66±0.04 t-v	3.18±0.08 t-v	6.56±2.12 e-h	11.12±2.64 f-g
	D	1.42±0.05 v-x	2.66±0.09 u-x	11.37±2.50 d	16.62±2.56 de
Rezaie Zodres	Control	2.27±0.05 k-m	4.52±0.10 m-o	0.00±0.0 l	0.00±0.0 q
	A	2.25±0.03 k-m	4.45±0.05 m-o	0.00±0.0 l	0.00±0.0 q
	B	2.15±0.04 m-p	4.21±0.07 p-s	1.31±0.70 j-l	6.93±3.84 h-m
	C	1.85±0.067 r-t	3.56±0.13 q-t	9.31±3.12 de	15.18±3.82 de
	D	1.58±0.05 t-u	2.97±0.09 t-w	27.41±4.45 a	29.11±4.80 a
Shahpasand	Control	4.34±0.05 a	8.36±0.10 a	0.00±0.0 l	0.00±0.0 q
	A	4.34±0.02 a	8.32±0.05 a	0.00±0.0 l	0.00±0.0 q
	B	4.29± 0.03 a	8.17±0.06 a-b	0.00±0.0 l	1.45±0.5 o-q
	C	4.23±0.05 a	7.93±0.10 b	3.06±1.50 h-l	3.05±1.62 m-q
	D	4.02±0.08 b	7.41±0.16 c	7.54±3.01 e-g	9.45±3.08 f-h

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

Table 6 - Effect of interaction between salinity and cultivar on the morphologic traits measured

Cultivar	Treatments	Branch fresh weight (g)	Root fresh weight (g)	Root dry weight ratio to aerial organ dry weight	Root fresh weight ratio to aerial organ fresh weight
Khanjari	Control	4.42±0.02 g-i	8.12±0.13 i-l	0.65±0.01 m-v	0.76±0.01 o-y
	A	4.38±0.01 g-j	8.13±0.07 h-l	0.65±0.01 m-v	0.76±0.01 o-y
	B	4.16±0.05 h-m	7.75±0.28 l-o	0.67±0.02 m-u	0.78±0.02 o-x
	C	3.69±0.05 j-s	6.69±0.11 r-s	0.69±0.01 m-u	0.81±0.01 m-u
	D	3.01±0.06 r-z	6.14± 0.06 t-u	0.73±0.01 k-s	0.88±0.01 i-r
Akbari	Control	3.25±0.36 o-x	8.58±0.51 f-i	0.99±0.08 c-j	1.24±0.10 c-f
	A	3.17±0.34 o-x	8.51±0.25 f-j	1.03±0.05 c-h	1.28±0.06 c-e
	B	2.99±0.16 s-z	8.01±0.14 j-n	1.03±0.04 c-h	1.28±0.05 c-e
	C	2.76±0.15 u-a/	7.95±0.05 j-n	1.04±0.04 c-g	1.31±0.06 c-e
	D	2.28±0.20 a/-c/	7.79±0.04 l-o	1.11±0.06 c-d	1.42±0.08 a-c
Ghazvini	Control	3.44±0.16 n-v	9.46±0.15 a-b	0.91±0.02 d-l	1.06 ±0.02e-n
	A	3.38±0.08 o-w	9.42±0.35 a-b	0.91±0.03 d-l	1.07±0.04 e-n
	B	3.18±0.07 o-x	9.35±0.03 a-b	0.94±0.01 d-k	1.10±0.01 e-k
	C	2.66±0.14 v-a/	9.29±0.03 a-c	1.03±0.01 c-h	1.21±0.03 c-g
	D	2.42±0.16 y-b/	9.15±0.04 a-e	1.09±0.07 c-e	1.28±0.08 c-e
Italiaie	Control	4.35±0.39 g-k	9.60±0.07 a	0.82±0.06 g-n	0.96±0.06 g-p
	A	4.23±0.15 h-l	9.58±0.05 a	0.83±0.03 g-n	0.96±0.03 g-p
	B	3.89±0.12 h-o	9.41±2.98 a-b	0.85±0.28 f-n	0.99 f±0.33-o
	C	3.30±0.14 o-x	9.19±0.06 a-d	1.02±0.05 c-i	1.19±0.06 c-h
	D	2.72±0.22 v-a/	8.96±0.07 b-f	1.20±0.09 b-c	1.37±0.10 b-d
Kaleh Ghochi	Control	3.21±0.08 o-x	5.46±0.07 w-y	0.56±0.01 p-w	0.65±0.01 r-y
	A	3.12±0.08 p-y	5.46±0.03 w-y	0.57±0.01 o-w	0.67±0.01 q-y
	B	2.74±0.08 v-a/	5.41±0.04 w-z	0.66±0.01 m-u	0.77±0.02 o-y
	C	2.23±0.06 a/-c/	5.20±0.05 w-a/	0.80±0.03 h-p	0.93±0.04 h-q
	D	1.62±0.03 c/	4.90±0.03 z-b/	1.03±0.04 c-h	1.21±0.05 c-g
Jandaghi	Control	3.49±0.10 m-u	5.24±0.06 w-a/	0.46±0.01 u-w	0.53±0.01 v-y
	A	3.42±0.15 n-v	5.22±0.04 w-a/	0.47±0.02 t-w	0.54±0.02 u-y
	B	2.90±0.07 t-z	5.01±0.07 y-b/	0.52±0.01 r-w	0.59±0.01 t-y
	C	2.34± 0.08 z-b/	4.78±0.05 a/-c/	0.65±0.03 m-v	0.74±0.04 o-y
	D	1.81±0.05 b/	4.28±0.03 d/	0.79±0.03 i-p	0.91±0.04 i-r
Mousa Abadi	Control	3.30±0.20 o-w	5.16±0.18 w-a/	0.63±0.03 n-w	0.70±0.03 p-y
	A	3.18±0.09 o-w	5.08±0.10 y-a/	0.63±0.01 n-w	0.71±0.01 p-y
	B	2.46±0.07 x- a/	4.97±0.07 y-b/	0.75±0.02 k-r	0.84±0.03 k-t
	C	1.96±0.06 a/- d/	4.53±0.05 b/-d/	0.95±0.03 d-k	1.08±0.04 e-l
	D	1.16±0.08 d/	4.13±0.07 d/e/	1.39±0.06 a-b	1.60±0.07 a-b
Ebrahimi	Control	5.05±0.23 e-g	7.51±0.09 n-p	0.76±0.03 j-q	0.79±0.03 n-w
	A	5.04±0.18 e-g	7.49±0.06 n-q	0.77±0.02 j-p	0.80±0.02 n-v
	B	4.41±0.18 f-j	7.32±0.04 o-q	0.87±0.02 e-m	0.91±0.02 i-r
	C	3.50±0.19 m-t	7.04±0.07 p-r	1.07±0.03 c-f	1.13±0.03 d-j
	D	2.68±0.16 w-a/	6.69±0.06 r-s	1.47±0.13 a	1.61±0.16 a
Badami Zarand	Control	5.57±0.20 d-e	8.80±0.09 c-g	0.78±0.02 j-p	0.81±0.02 m-u
	A	5.54±0.24 d-e	8.73±0.13 d-g	0.78±0.03 j-p	0.82±0.03 l-t
	B	5.10±0.17 e-f	8.65±0.13 e-h	0.84±0.02 g-n	0.87±0.03 j-s
	C	4.41±0.14 g-j	8.32±0.26 g-k	0.92±0.03 d-l	0.96±0.04 g-p
	D	3.64±0.15 k-s	8.06±0.17 i-m	1.09±0.05 c-e	1.14±0.06 d-i
Fandoghi 48	Control	7.71±0.23 a-b	7.62±0.11 l-o	0.41±0.01 w	0.51±0.01 x-y
	A	7.60±0.19 a-b	7.58±0.05 m-o	0.42±0.01 v-w	0.52±0.01 w-y
	B	6.81±0.25 c	7.30±0.09 o-q	0.46±0.01 u-w	0.57±0.01 t-v
	C	5.78±0.32 d	6.99±0.08 q-s	0.52±0.02 r-w	0.65±0.033 r-y
	D	4.36±0.19 g-j	6.50±0.14 s-t	0.65±0.03 m-v	0.82±0.04 l-t
Sabs Pesteh Togh	Control	3.81±0.16 i-p	6.02±0.11 t-v	0.69±0.02 l-u	0.68±0.02 q-y
	A	3.74±0.14 i-r	5.99±0.07 u-v	0.70±0.01 l-t	0.69±0.01 p-y
	B	3.21±0.11 o-x	5.71±0.18 u-w	0.73±0.01 k-s	0.71±0.01 p-y
	C	2.61±0.03 x-a/	5.38±0.14 w-z	0.78±0.01 j-p	0.78±0.01 o-x
	D	2.14±0.06 a/-c/	4.95±0.11 y-a/	0.82±0.02 g-n	0.81±0.02 m-u
Ahmad Aghaee	Control	3.83±0.07 i-p	5.72±0.06 u-w	0.76±0.01 j-q	0.75±0.01 o-y
	A	3.76±0.03 i-q	5.73 ±0.07u-w	0.79±0.01 j-p	0.77±0.01 o-y
	B	3.55±0.07 l-t	5.61±0.07 v-x	0.82±0.01 g-n	0.80±0.01 m-v
	C	3.35±0.07 o-w	5.47±0.08 w-y	0.86±0.01 f-n	0.84±0.01 k-t
	D	2.85±0.09 t-z	5.02±0.07 y-a/	0.91±0.01 d-l	0.91±0.01 i-r
Rezaie Zodres	Control	4.56±0.09 f-h	4.56±0.12 b/-d/	0.49±0.01 t-w	0.50±0.01 y
	A	4.46±0.12 f-i	4.41±0.11 b/-d/	0.50±0.02 s-w	0.50±0.02 y
	B	4.13±0.09 i-n	4.28±0.16 d/	0.51±0.01 s-w	0.51±0.01 x-y
	C	3.78±0.09 i-q	3.98±0.15 e/	0.53±0.02 q-w	0.54±0.02 u-y
	D	3.05±0.10 q-z	3.53±0.09 f/	0.58±0.01 o-w	0.59±0.01 t-y
Shahpasand	Control	8.01±0.07 a	9.44±0.11 a-b	0.47±0.01 t-w	0.57±0.01 t-y
	A	7.98±0.08 a	9.43±0.08 a-b	0.48±0.01 t-w	0.58±0.01 t-y
	B	7.83±0.12 a-b	9.21±0.05 a-d	0.48±0.01 t-w	0.58±0.01 t-y
	C	7.49±0.07 a-b	8.98±0.13 b-f	0.49±0.01 t-w	0.59±0.01 t-y
	D	7.18±0.10 b-c	8.53±0.16 f-i	0.50±0.01 s-w	0.60±0.01 s-y

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

*= a/ is less than z. Given that variety of data was very wide, Duncan's test grouped data between a to z and less than z such as a/, b/, c/, d/ and e/.

Table 7 - Effect of interaction between salinity and cultivar on chlorophyll fluorescence parameters

Cultivar	Treatments	(F_v/F_m)	Maximum florescence (F_m)	Minimum florescence (F_o)
Khanjari	Control	0.82±0.003 b-c	603.67±3.46 b-d	107.22±1.71 r-u
	A	0.82±0.003 b-c	606.44±3.39 b-d	110.67±1.58 o-r
	B	0.80±0.005 d-e	586.00±8.74 e-f	116.55±1.66 k-m
	C	0.74±0.015 j-k	540.33±5.50 k-m	139.88±8.52 e
	D	0.65±0.025 q	467.77±5.65 r	163.11±11.20 a
Akbari	Control	0.83±0.002 a-b	625.44±6.72 a	106.11±2.31 s-u
	A	0.82±0.004 b-c	617.67±5.85 a-c	109.77±2.16 p-s
	B	0.82±0.007 b-c	614.11±5.01 a-c	110.55±3.77 o-s
	C	0.78±0.003 f-g	607.11±7.09 b-d	134.22±2.38 f-g
	D	0.75±0.007 i-j	555.44±11.58 h-k	141.33±1.58 e
Ghazvini	Control	0.82±0.003 b-c	602.44±11.54 c-d	106.11±3.51 s-u
	A	0.82±0.003 b-c	602.55±17.00 c-d	108.67±4.09 q-t
	B	0.81±0.004 c-d	591.00±2.87 d-f	109.77±2.27 o-s
	C	0.80±0.003 c-e	578.33±5.61 d-g	118.67±1.93 j-m
	D	0.76±0.003 h-i	538.11±4.59 l-m	129.33±2.39 h
Italiaie	Control	0.83±0.004 a-b	600.77±7.41 c-e	101.22±3.89 v-w
	A	0.83±0.003 a-b	604.22±9.31 b-d	103.77±3.70 t-v
	B	0.81±0.004 c-d	576.44±3.71 f-g	112.00±2.39 n-q
	C	0.78±0.008 f-g	532.11±13.50 m	117.11±2.14 k-m
	D	0.73±0.009 l	488.67±6.61 o-q	133.55±5.12 f-g
Kaleh Ghochi	Control	0.83±0.002 a-b	538.44±9.46 l-m	93.22±1.98 y-z
	A	0.82±0.005 b-c	542.88±5.94 j-m	93.33±2.64 y-z
	B	0.80±0.005 d-e	529.88±7.18 m	106.44±2.29 s-u
	C	0.76±0.005 h-i	489.00±8.38 o-q	119.55±3.20 i-k
	D	0.69±0.009 m	444.11±12.31 s	137.00±3.46 e-f
Jandaghi	Control	0.83±0.005 a-b	580.33±18.67 f	100.67±2.64 v-w
	A	0.82±0.006 b-c	557.00±3.80 h-j	101.44±3.46 v-w
	B	0.77±0.006 g-h	479.88±6.73 p-r	110.33±2.54 p-s
	C	0.7±0.0043 l	417.33±9.04 t-u	113.89±3.25 m-p
	D	0.66±0.004 p	380.67±11.69 w	129.44±3.08 h
Mousa Abadi	Control	0.84 a±0.005	551.78±7.15 h-l	91.00±2.87 z
	A	0.82±0.007 b-c	530.00±7.29 m	94.00±3.57 x-z
	B	0.79±0.002 e-f	495.22±14.77 op	104.55±3.46 t-v
	C	0.73±0.008 kl	447.22±21.89 s	122.44±4.97 i-j
	D	0.61±0.009 s	387.67±29.06 w	151.00±10.34 b-c
Ebrahimi	Control	0.83±0.003 a-b	608.11±8.08 b-c	104.55±2.50 t-v
	A	0.83±0.004 a-b	609.67±8.17 a-c	105.44±3.77 t-v
	B	0.80±0.006 d-e	586.44±10.27 e-f	114.44±3.35 m-p
	C	0.77±0.006 g-h	539.67±14.41 k-m	123.67±4.24 i
	D	0.73±0.011 kl	512.44±12.28 n	140.67±4.44 e
Badami Zarand	Control	0.82±0.003 b-c	602.44±9.83 cd	105.55±2.12 s-u
	A	0.82±0.002 b-c	606.33±8.81 a-c	106.55±2.45 s-u
	B	0.81±0.003 c-d	597.11±10.32 d-f	108.55±1.58 q-t
	C	0.77± 0.007 g-h	542.55±13.92 j-m	124.44±4.92 i
	D	0.73±0.004 k-l	512.67±12.79 n	137.44±3.16 e-f
Fandoghi 48	Control	0.84±0.006 a	585.44±10.90 f	95.11±2.97 x-z
	A	0.83±0.004 a-b	585.48±9.48 f	98.11±2.47 w-x
	B	0.79±0.004 e-f	579.00±8.95 f	118.89±3.33 j-m
	C	0.73±0.010 k-l	491.44±10.87 o-q	131.55±4.63 g-h
	D	0.64±0.018 r	404.55±14.39 u-v	147.33±4.74 d
Sabs Pesteh Togh	Control	0.83±0.005 a-b	562.00±14.96 g-h	95.67±1.73 x-y
	A	0.82±0.007 b-c	539.00±21.68 l-m	97.77±1.78 w-x
	B	0.76±0.009 h-i	470.89±14.88 r	110.88±2.52 o-r
	C	0.72±0.007 l	427.11±15.39 t	118.55±4.15 j-l
	D	0.67±0.009 o	409.11±14.58 u	134.00±3.93 f-g
Ahmad Aghaee	Control	0.83±0.003 a-b	610.33±12.40 a-c	106.44±2.24 s-u
	A	0.82±0.004 b-c	603.22±19.07 cd	111.22±3.07 o-r
	B	0.78±0.011 f-g	543.89±20.01 i-m	117.11±2.61 k-m
	C	0.75±0.009 i-j	510.33±12.10 n	130.00±3.27 g-h
	D	0.67±0.022 o	469.89±30.25 r	153.33±4.66 b
Rezaie Zodres	Control	0.84±0.008 a	562.89±13.27 g-h	92.67±3.04 y-z
	A	0.82±0.006 b-c	538.55±14.52 k-m	95.67±2.00 x-y
	B	0.76±0.012 h-i	467.77±16.20 r	105.33±3.60 t-v
	C	0.68±0.016 n	391.00±10.14 v-u	123.55±5.41 i
	D	0.59±0.015 t	365.22±20.90 x	147.77±7.52 cd
Shahpasand	Control	0.83±0.003 a-b	531.00±9.73 m	86.67±2.00 a/
	A	0.83±0.004 a-b	530.88±8.26 m	88.00±1.58 a/
	B	0.82±0.007 b-c	527.44±17.25 m-n	95.11±2.52 x-z
	C	0.79±0.012 e-f	499.67±18.36 o-p	105.77±2.81 s-u
	D	0.75±0.011 i-j	461.00±13.79 r	115.00±3.42 l-o

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

Table 8 - Effect of interaction between salinity and cultivar on the physiologic traits measured

Cultivar	Treatments	Total chlorophyl (mg/g)	Chlorophyll b (mg/g)	Chlorophyll a (mg/g)	Chlorophyll index (SPAD)
Khanjari	Control	1.13±0.03 k-n	0.36±0.02 g-j	0.77±0.02 g	57.58±1.52 f-h
	A	1.11±0.02 m-n	0.36±0.04 g-j	0.75±0.02 g-i	56.62±1.62 g-j
	B	1.06±0.02 p-r	0.34±0.02 i-l	0.72±0.05 i-k	53.60±0.82 i-l
	C	0.88±0.01 w	0.31±0.01 l-o	0.57±0.02 s	48.55±1.50 o-q
	D	0.59±0.02 a/	0.22±0.01 t	0.37±0.04 w-x	40.00±1.73 y-z
Akbari	Control	1.45±0.007 c	0.51±0.008 a	0.95±0.003 c	61.27±0.98 c-d
	A	1.45±0.11 c	0.52±0.008 a	0.94±0.009 c	61.40±1.57 c-d
	B	1.40±0.02 c-d	0.51±0.1 a	0.89±0.03 c-d	60.15±1.55 c-e
	C	1.23±0.01 f	0.46±0.03 cd	0.77±0.01 g	56.97±1.04 g-i
	D	1.05±0.009 q-s	0.38±0.02 e-h	0.67±0.02 m-o	53.70±1.24 k-m
Ghazvini	Control	1.20±0.01 f-g	0.46±0.007 c-d	0.74±0.01 h-j	55.17±1.27 g-l
	A	1.21±.005 f-g	0.47±0.01 c-d	0.74±0.008 h-j	54.67±1.25 i-l
	B	1.17±0.006 g	0.45±0.007 c-d	0.72±0.005 j-k	54.04±1.09 j-m
	C	1.14±0.02 g-m	0.43±0.008 d	0.71±0.003 j-l	51.98±0.85 l-n
	D	0.97±0.004 t	0.35± 0.01 h-k	0.62±0.008 q-r	49.78±0.82 n-o
Italiaie	Control	1.10±0.01 m-o	0.36±0.004 g-j	0.74±0.003 h-j	49.95±1.10 n-o
	A	1.09±0.01 n-p	0.36±0.004 g-j	0.73±0.01 i-j	50.02±1.38 n-o
	B	1.04±0.007 q-s	0.32±0.01 k-n	0.72±0.003 j-k	45.17±1.36 r-u
	C	0.91±0.008 v	0.26±0.009 q-r	0.6±0.0075 op	43.95±1.43 t-w
	D	0.70±0.01 z	0.20±0.004 t	0.50±0.005 u	40.41±1.46 x-z
Kaleh Ghochi	Control	1.07±0.007 o-q	0.38±0.008 e-h	0.69±0.005 l-m	45.17±0.60 r-u
	A	1.06±0.008 p-r	0.37±0.004 f-i	0.69±0.003 l-m	43.95±0.16 t-w
	B	1.03±0.01 q-s	0.37±0.004 f-i	0.66±0.009 m-o	41.50±0.28 u-z
	C	0.80±0.009 x	0.31±0.008 l-o	0.49±0.02 u-v	36.24±0.40 a/
	D	0.60±0.003 a/	0.22±0.01 t	0.38±0.01 w-x	31.47±3.08 b/
Jandaghi	Control	1.04±0.007 q-s	0.36±0.04 g-j	0.69±0.002 l-m	42.77±0.82 u-x
	A	1.04±0.008 q-s	0.36±0.04 g-j	0.68±0.004 l-n	42.77±0.76 u-x
	B	0.95±0.01 t-u	0.32±0.03 k-n	0.63±0.006 p-q	38.54±1.37 y-z
	C	0.74±0.004 y	0.26±0.03 q-r	0.48±0.010 u-v	35.45±0.85 a/
	D	0.53±0.004 b/	0.17±0.004 u	0.36±0.01 x	27.83±0.98 c/
Mousa Abadi	Control	1.05±0.007 q-s	0.36±0.008 g-j	0.69±0.002 l-m	42.40±0.95 v-y
	A	1.04±0.008 q-s	0.36±0.004 g-j	0.68±0.003 l-n	42.65±0.51 u-y
	B	0.95±0.01 t-u	0.30±0.005 m-p	0.63±0.005 p-q	38.11±1.00 z-a/
	C	0.72 ±0.01y-z	0.23±0.01 s-t	0.49±0.009 u-v	32.33±1.10 b/
	D	0.48±0.03 c/	0.15±0.004 u	0.33±0.008 y	22.60±1.50 d/
Ebrahimi	Control	1.20±0.04 f-h	0.45±0.03 d	0.74±0.01 h-j	55.21±1.22 g-l
	A	1.19±0.008 g-i	0.45±0.02 cd	0.74±0.01 h-j	54.97±1.65 h-l
	B	1.12±0.02 l-n	0.42±0.01 e-g	0.70±0.008 j-l	49.97±1.98 no
	C	0.95±0.01 t-u	0.35±0.01 h-k	0.60±0.01 r	45.51±1.88 r-t
	D	0.74±0.01 y	0.27±0.01 p-r	0.47±0.01 v	40.27±1.54 x-z
Badami Zarand	Control	1.15±0.02 j-l	0.38±0.008 e-h	0.77±0.008 g	54.86±1.32 h-l
	A	1.15±0.02 j-l	0.39±0.01 e-g	0.76±0.01 g-h	54.80±1.47 i-l
	B	1.10±0.02 l-p	0.37±0.01 f-i	0.73±0.01 g-l	52.89±0.70 l-m
	C	0.93±0.01 u-v	0.30±0.01 m-p	0.63±0.01 p-q	49.39±1.53 n-p
	D	0.73±0.02 y-z	0.20±0.01 t	0.53±0.01 t	45.55±1.19 r-t
Fandoghi 48	Control	1.23±0.02 f	0.41±0.03 e	0.83±0.01 e	56.88-i±1.16 g
	A	1.23±0.02 f	0.40±0.02 e-f	0.83±0.01 e	56.00±1.22 g-k
	B	1.16 ±0.009h-j	0.36±0.01 f-i	0.80±0.01 e-f	51.67±1.32 m-n
	C	1.02±0.03 s	0.32±0.01 k-n	0.70±0.01 j-l	47.22±1.48 p-r
	D	0.73±0.02 y-z	0.25±0.01 r-s	0.49±0.01 u-v	40.00±1.87 y-z
Sabs Pesteh Togh	Control	1.19±0.03 g-i	0.44±0.05 d	0.74±0.02 h-j	50.36±1.14 n-o
	A	1.16±0.02 i-k	0.44±0.02 d	0.73±0.007 i-j	46.93±7.47 o-r
	B	1.05±0.02 q-s	0.35±0.01 h-k	0.70±0.009 j-l	46.77±1.28 q-s
	C	0.81±0.008 x	0.28±0.02 o-q	0.53±0.01 t	42.47±0.90 v-y
	D	0.58±0.01 a/	0.21±0.01 t	0.36±0.005 x	36.92±1.03 a/
Ahmad Aghaee	Control	1.04±0.01 q-s	0.37±0.01 f-i	0.67±0.007 m-o	46.48±3.78 q-t
	A	1.02±0.02 s	0.35±0.01 h-k	0.67±0.007 m-o	44.15±0.22 s-v
	B	0.97±0.01 t	0.32±0.009 k-n	0.64±0.006 o-q	41.37±0.44 w-z
	C	0.83±0.01 x	0.29±0.01 n-q	0.53±0.01 t	36.60±0.30 a/
	D	0.62±0.01 a/	0.23±0.01 s-t	0.39±0.01 w	31.23±0.75 b/
Rezaie Zodres	Control	1.12±0.02 l-n	0.36±0.008 g-j	0.76±0.02 g-h	57.72±1.04 e-g
	A	1.11±0.02 m-n	0.37±0.01 f-i	0.75±0.01 g-i	56.01±0.84 g-k
	B	1.02±0.02 s	0.32±0.01 k-m	0.70±0.01 j-l	53.25±1.63 lm
	C	0.86±0.02 w	0.29±0.01 n-q	0.57±0.01 s	48.56±1.41 o-q
	D	0.58±0.03 a/	0.21±0.005 t	0.37±0.02 w-x	40.24±1.61 x-z
Shahpasand	Control	1.54±0.007 a	0.50±0.008 a-b	1.04±0.007 a	65.52±1.16 a
	A	1.54±0.01 a	0.50±0.01 a-b	1.03±0.009 a-b	64.52±1.19 a-b
	B	1.50±0.03 a-b	0.48±0.02 b-c	1.02±0.008 a-b	62.57±0.99 a-c
	C	1.33±0.03 e	0.40±0.02 e-f	0.93±0.01 c	59.98±0.79 d-f
	D	1.10±0.02 m-o	0.29±0.02 n-q	0.81±0.01 e-f	55.35±1.35 g-l

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

Table 9 - Effect of interaction between salinity and cultivar on the physiologic traits measured

Cultivar	Treatments	Cell membrane injury (%)	Relative ionic leakage (%)	Relative water content (%)
Khanjari	Control	-	37.70±0.010 m-o	85.25±0.64 a
	A	2.98±1.28 w-y	39.14±0.008 k-o	84.16±0.46 a-b
	B	8.09±0.91 t-u	44.34±0.005 i-o	81.31±0.51 b
	C	23.09±3.10 k-m	51.75±0.019 d-k	77.60±0.62 c
	D	46.47±0.70 b	64.42±0.004 a-c	70.36±1.04 f
Akbari	Control	-	38.05±0.007 l-o	85.08±0.40 a
	A	0.83±1.21 x-y	38.41±0.016 l-o	84.52±0.60 a-b
	B	5.27±1.07 u-w	42.30±0.006 j-o	82.90±0.60 a-b
	C	16.37±1.78 o-q	45.96±0.011 g-o	82.08±0.68 a-b
	D	27.83±2.18 h-i	50.09±0.013 d-k	80.22±0.56 b
Ghazvini	Control	-	35.62±0.013 o	83.19±0.57 a-b
	A	0.42±0.19 y	36.59±0.006 m-o	82.98±0.64 a-b
	B	2.67±2.28 x-y	38.37±0.014 l-o	81.58±0.42 b
	C	5.89±2.81 u-w	39.73±0.018 k-o	80.88±0.36 b
	D	16.17±0.62 o-q	45.50±0.16 g-o	78.95±0.45 b-c
Italiaie	Control	-	40.85±0.012 j-o	84.43±0.35 a-b
	A	0.75±0.79 x-y	40.88±0.005 j-o	84.21±0.38 a-b
	B	12.16±3.06 r-s	47.75±0.018 e-n	81.51±0.36 b
	C	22.24±2.62 l-m	52.75±0.015 c-i	78.40±0.37 c
	D	33.65±3.51 j-l	60.54±0.020 a-d	74.06±0.50 d-e
Kaleh Ghochi	Control	-	39.43±0.015 k-o	83.41±0.32 a-b
	A	3.89±2.81 v-y	40.81±0.017 k-o	82.41±0.29 a-b
	B	11.97±0.59 r-s	43.67±0.003 i-o	81.45±0.32 b
	C	21.88±1.09 l-n	48.89±0.006 d-l	78.35±0.33 c
	D	38.43±2.46 de	59.29±0.015 b-f	73.59±0.30 e
Jandaghi	Control	-	37.68±0.015 m-o	82.57±0.32 a-b
	A	3.06±1.36 w-y	38.85±0.008 l-o	81.45±0.34 b
	B	10.47±1.29 s-t	45.53±0.008 h-o	79.35±0.25 b-c
	C	18.68±2.28 n-p	55.70±0.014 b-i	74.67±0.29 d-e
	D	31.21±3.47 g-h	66.61±0.021 a-b	67.38±0.41 g
Mousa Abadi	Control	-	37.59±0.007 l-o	80.42±0.27 b
	A	4.47±1.83 u-x	39.39±0.011 k-o	79.53±0.28 b-c
	B	18.68±2.77 n-p	49.41±0.017 d-k	75.55±0.35 d
	C	33.47±2.27 f-g	57.80±0.014 b-g	70.52±0.66 f
	D	54.83±4.50 a	71.34±0.028 a	64.17±0.52 i
Ebrahimi	Control	-	40.04±0.010 k-o	81.51±0.25 b
	A	4.88±4.12 u-w	42.77±0.027 j-o	80.40±0.45 b-c
	B	12.92±4.01 q-s	46.57±0.024 f-o	78.49±0.31 b-c
	C	18.34±2.62 n-p	49.90±0.016 d-l	76.34±0.34 c-d
	D	27.70±4.05 h-i	56.65±0.024 b-h	69.95±0.45 f-g
Badami Zarand	Control	-	35.90±0.018 n-o	80.56±0.26 b-c
	A	2.36±3.55 w-y	37.60±0.028 l-o	79.41±0.24 b-c
	B	6.96±2.50 t-v	41.66±0.016 j-o	78.13±0.30 c
	C	14.18±2.66 q-r	45.00±0.017 h-o	76.38±0.32 c-d
	D	21.21±2.04 l-n	49.51±0.013 d-l	73.21±0.47 e
Fandoghi 48	Control	-	41.44±0.012 j-o	82.51±0.33 a-b
	A	4.18±3.07 v-y	43.52±0.022 j-o	81.64±0.30 b
	B	10.24±2.12 s-t	47.33±0.012 f-o	79.22±0.47 b-c
	C	26.48±4.68 i-k	56.86±0.027 b-h	75.25±0.54 d
	D	41.04±3.79 c-d	65.40±0.022 a-b	69.12±0.47 f-g
Sabs Pesteh Togh	Control	-	41.06±0.014 j-o	81.34±0.31 b
	A	5.35±3.95 u-w	44.64±0.029 h-o	79.60±0.41 b-c
	B	14.57±6.87 q-r	50.94±0.039 d-k	77.18±0.32 c-d
	C	28.48±4.47 h-i	57.93±0.025 b-g	73.67±0.59 e
	D	42.89±6.21 c	66.20±0.035 a-b	66.49±0.77 g-i
Ahmad Aghaee	Control	-	39.50±0.013 k-o	85.02±0.32 a
	A	3.27±1.44 v-y	41.35±0.008 j-o	84.39±0.36 a-b
	B	9.63±0.93 s-t	44.05±0.005 i-o	83.26±0.42 a-b
	C	19.71±1.64 m-o	50.30±0.010 d-j	80.1±0.38 b
	D	35.57±2.85 e-f	60.11±0.017 a-e	75.38±0.38 d
Rezaie Zodres	Control	-	40.12±0.006 k-o	81.05±0.42 b
	A	2.18±1.61 w-y	40.97±0.010 j-o	80.50±0.25 b-c
	B	10.48±1.51 s-t	48.81±0.009 d-l	77.17±0.51 c-d
	C	26.78±3.43 i-j	55.85±0.020 b-i	74.34±0.45 d-e
	D	47.45±2.95 b	68.31±0.017 a-b	66.95±0.57 g-i
Shahpasand	Control	-	39.92±0.006 k-o	79.83±0.47 b-c
	A	3.61±3.10 v-y	40.43±0.021 k-o	79.49±0.22 b-c
	B	5.98±1.87 u-w	42.63±0.011 j-o	78.89±0.51 b-c
	C	15.32±3.43 p-r	46.68±0.020 f-o	77.15±0.53 c
	D	27.77±2.98 h-i	53.23±0.018 c-j	74.41±0.39 d-e

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

was observed the least decrease in the relative humidity content of the leaves.

Relative ion leakage percentage in all studied cultivars was increased by increasing salinity concentration. The increase in the relative ion leakage percentage was significant between the studied cultivars. The highest relative ion leakage percentage was observed in Mousa Abadi cultivar in salinity level D. After this cultivar, Rezai Zod Res, Jandaghi, Sabz Pesteh Togh, Fndoghi 48, Kanjari and Italiyayi cultivars had the highest relative ion leakage percentage. The increase in relative ion leakage percentage was not significant in Ghazvini cultivar compared to the control plants (Table 9). The results showed that the cultivars had a significant difference in cell membrane injury percentage. The highest cell membrane injury percentage was observed in the leaves of Mousa Abadi (54.83±4.50%), and the lowest cell membrane injury percentage was observed in the leaves of Ghazvini (16.17±0.62%).

Results reported in Table 10 assessed that with increasing salinity concentration in irrigation water, the sodium concentration in the leaves and roots of total cultivars increased. The increase in sodium concentration in the leaves of Ghazvini cultivar was only significant when plants were treated with salinity level D, while in Akbari, Badami Zarand and Shahpasand cultivars was observed a significant increased when treated with salinity levels C and D, compared to the control plants. While the increase of sodium concentration in the leaves of other cultivars was significant different salinity levels B, C and D, compared to the control plants (Table 10). The highest sodium concentration in leaves was observed in the salinity level D and in Mousa Abadi (2.09± 0.045%), Rezaie Zood Res (2.05±0.030%), Khanjari (2.03±0.115%) and Jandaghi (1.90±0.035%) cultivars treated. Also the highest sodium concentration in roots was also observed in salinity level D, and in Mousa Abadi (3.04±0.06%) and Rezaie Zood Res (2.99±0.05%) cultivars.

With increasing salinity levels (to 14.75 dS/m), potassium concentration increased in leaves and roots of Akbari, Ghazvini, Shahpasand, Badami Zarand and Ebrahimi cultivars while potassium content in the leaves and roots of other cultivars except Mousa Abadi and Rezaie Zood Res increased to salinity level C. Potassium content in the leaves and roots of Mousa Abadi and Rezaie Zood Res cultivars was increased only in salinity level B. Overall, the highest potassium content in leaves and roots was observed in salinity level C and in Ghazvini (1.81±0.02%) and Akbari (1.38±0.02%) cultivars.

4. Discussion and Conclusions

Based on the results of this study, with increasing salinity concentration in irrigation water, final height, trunk diameter and number of leaves in all studied cultivars decreased. Plant height is heavily dependent on growth environment. Since the growth phenomenon gained vital activities in which condition the plant must be in possession of enough water, reduction in the height occurs in case of failure to provide the required water due to the reduction of cell turgor pressure and length of the cells would be negatively affected (Munns, 2002; Munns and Tester, 2008). The osmotic effects of salinity stress can be observed immediately after salt application and are believed to continue for the duration of exposure, resulting in inhibited cell expansion and cell division (Munns 2002; Munns and Tester, 2008). In this research, trunk diameter and its growth were decreased during the application of salinity stress in all cultivars. These results are consistent with other results (Sepaskhah and Maftoun, 1988; Munns and Tester, 2008; Zrig *et al*., 2015). It has been reported that growth rates of pistachio trees decrease with increasing sodium chloride (NaCl) concentration in soil. It has been also reported that there is a positive correlation between sodium (Na^+) as well as chloride (Cl^-) concentration in plant tissue and soil (Sepaskhah and Maftoun, 1988; Munns and Tester, 2008; Zrig *et al*., 2015). Based on the results of this study, number of leaves with increasing salinity concentrations reduced. Our results are consistent with studies reporting that increasing salinity levels negatively affect morphology and number of leaves in pistachio trees (Picchioni and Myamoto, 1990; Saadatmand *et al*., 2007; Karimi *et al*., 2011). The results of this research showed that with increasing salinity, percentage of green leaves, leaves and shoots fresh and dry weights in all cultivars decreased but the percentage of necrotic leaves and percentage of downfall leaves increades. The cultivars showed different responses to salinity levels. These results are consistent with the results of Karimi *et al.* (2009 and 2011). In these studies, effect of salinity levels on pistachio cultivars was investigated and was reported that pistachio cultivars showed different responses to salinity levels. Although pistachio trees are classified as tolerant to salinity, but amount of their tolerance to salinity is differently (Sepaskhah and Maftoun, 1988;

Table 10 - Effect of interaction between salinity and cultivar on root and leaf K^+ and Na^+ contents

Cultivar	Treatments	Root Na^+ (%)	Leaf Na^+ (%)	Root K^+ (%)	Leaf K^+ (%)
Khanjari	Control	0.55±0.03 s-y	0.43±0.027 q-v	0.60±0.03 z-a/	1.35±0.03 k-m
	A	0.59±0.01 q-y	0.46±0.023 q-v	0.76±0.05 p-w	1.44±0.11 d-i
	B	0.70±0.06 o-x	0.64±0.023 m-r	0.73±0.05 q-y	1.56±0.03 c-f
	C	1.57±0.11 g-i	1.25±0.055 g-h	0.50±0.04 b/	1.30±0.06 i-o
	D	2.59±0.06 c-d	2.03±0.116 a	0.37±0.04 d/	1.01±0.04 u-x
Akbari	Control	0.42±0.01 y	0.37±0.013 t-v	0.80±0.03 m-u	1.08±0.03 r-x
	A	0.44±0.008 x-y	0.39±0.005 t-v	0.99±0.03 f-i	1.21±0.05 m-r
	B	0.48±0.03 v-y	0.42±0.007 r-v	1.37±0.03 a	1.58±0.04 c-e
	C	0.97±0.03 l-n	0.73±0.035 k-n	1.38±0.02 a	1.59±0.02 b-d
	D	1.86±0.04 f	1.40±0.066 e-g	0.77±0.02 o-v	1.11±0.03 q-v
Ghazvini	Control	0.46±0.003 w-y	0.41±0.014 s-v	0.79±0.02 n-v	1.31±0.02 i-n
	A	0.47±0.004 w-y	0.41±0.007 s-v	0.84±0.02 k-r	1.48±0.02 d-h
	B	0.49±0.006 v-y	0.43±0.004 q-v	0.95±0.02 g-k	1.55±0.03 c-g
	C	0.52±0.006 u-y	0.46±0.007 q-v	1.08±0.02 d-f	1.81±0.02 a
	D	1.05±0.02 k-m	0.82±0.023 k-m	0.85±0.02 j-q	1.35±0.02 h-m
Italiaie	Control	0.57±0.02 r-y	0.34±0.006 v	0.93±0.02 h-l	1.08±0.02 r-x
	A	0.61±0.01 q-y	0.36±0.007 t-v	0.98±0.03 f-i	1.19±0.02 n-t
	B	0.78±0.03 n-t	0.43±0.007 q-v	1.13±0.02 cd	1.23±0.02 m-p
	C	1.21±0.02 j-k	0.82±0.027 k-m	0.88±0.02 i-p	0.99±0.02 v-x
	D	1.81±0.04 f	1.55±0.035 c-e	0.60±0.02 z-a/	0.67±0.02 a/
Kaleh Ghochi	Control	0.67±0.03 p-y	0.45±0.005 q-v	0.89±0.02 h-o	1.10±0.02 r-w
	A	0.70±0.02 o-x	0.47±0.003 p-v	0.95±0.02 g-k	1.18±0.04 n-t
	B	0.75±0.009 n-u	0.49± 0.003 p-v	1.14±0.02 b-d	1.57±0.02 c-e
	C	1.38±0.02 i-j	0.76±0.029 k-n	1.11±0.02 de	1.55±0.02 c-g
	D	2.55±0.04 d	1.50±0.044 d-f	0.82±0.02 l-t	1.17±0.02 n-t
Jandaghi	Control	0.49±0.006 u-y	0.41±0.005 s-v	0.68±0.02 u-z	0.82±0.02 y-z
	A	0.60±0.005 q-y	0.46±0.004 q-v	0.72±0.03 r-z	0.93±0.02 x-y
	B	0.82±0.01 m-q	0.57±0.017 n-u	0.75±0.05 q-x	1.08±0.02 r-w
	C	1.52±0.02 g-i	1.22±0.035 g-h	0.69±0.02 u-z	0.71±0.02 z-a/
	D	2.73±0.03 cd	1.90±0.035 a-b	0.35±0.03 d/	0.46±0.02 b/
Mousa Abadi	Control	0.53±0.007 t-y	0.44±0.002 q-v	0.60/±0.01 z-a	1.05±0.02 s-x
	A	0.65±0.01 q-y	0.47±0.005 p-v	0.85±0.02 j-q	1.13±0.02 p-v
	B	0.83±0.03 m-p	0.71±0.034 l-o	0.63±0.02 a/	1.07±0.02 r-x
	C	1.76±0.02 f-g	1.33±0.027 f-h	0.45±0.02 c/	0.95±0.02 w-y
	D	3.04±0.06 a	2.09±0.045 a	0.34±0.01 d/	0.40±0.02 c/
Ebrahimi	Control	0.46±0.005 w-y	0.39±0.005 t-v	0.68±0.02 u-z	1.26±0.03 k-q
	A	0.48±0.005 v-y	0.41±0.004 s-v	0.77±0.02 o-v	1.37±0.02 h-l
	B	0.53±0.007 t-y	0.45±0.006 q-v	0.82±0.02 l-t	1.48±0.02 d-h
	C	1.14±0.04 k-l	0.87±0.020 j-l	0.83±0.02 k-s	1.50±0.02 d-h
	D	2.11±0.03 e	1.62±0.027 c-d	0.60±0.02 z-a/	1.15±0.01 o-u
Badami Zarand	Control	0.51±0.007 u-y	0.48±0.005 p-v	0.71±0.02 s-z	1.04±0.01 t-x
	A	0.53±0.003 t-y	0.49±0.004 p-v	0.74±0.02 q-y	1.16±0.02 n-u
	B	0.56±0.008 r-y	0.51±0.019 o-v	0.97±0.02 f-j	1.43±0.01 e-j
	C	0.83±0.02 m-q	0.64±0.027 m-r	1.01±0.01 e-h	1.47±0.01 d-h
	D	1.73±0.04 f-g	1.23±0.027 g-h	0.70±0.02 t-z	1.05±0.01 s-x
Fandoghi 48	Control	0.60±0.006 q-y	0.42±0.002 r-v	0.82±0.02 l-t	1.19±0.02 n-t
	A	0.64±0.008 q-y	0.44±0.005 q-v	0.91±0.02 h-n	1.28±0.03 j-p
	B	0.99±0.01 l-n	0.79±1.21 k-m	0.92±0.02 h-m	1.35±0.10 h-m
	C	1.43±0.02 h-j	0.93±0.01 j-k	0.90±0.02 h-n	1.30±0.02 i-o
	D	2.74±0.04 c-d	1.73±0.04 b-c	0.64±0.02 x-z	1.01±0.02 u-x
Sabs Pesteh Togh	Control	0.60±0.007 q-y	0.48±0.005 p-v	0.62±0.09 y-z	1.07±0.02 r-x
	A	0.64±0.01 q-y	0.51±0.005 o-v	0.88±0.01 i-p	1.28±0.02 j-p
	B	0.79±0.04 n-s	0.57±0.02 n-u	1.01±0.02 e-h	1.44±0.02 d-i
	C	1.55±0.03 g-i	1.02±0.05 i-j	0.67±0.009 v-z	1.19±0.01 n-t
	D	2.81±0.07 b-c	1.83±0.06 b	0.44±0.01 c/	0.83±0.01 y-z
Ahmad Aghaee	Control	0.62±0.006 q-y	0.55±0.004 n-v	0.95±0.02 g-k	1.37±0.02 h-l
	A	0.65±0.006 q-y	0.58±0.005 n-t	1.08±0.02 d-f	1.54±0.03 c-g
	B	0.81±0.02 n-p	0.63±0.02 m-s	1.39±0.02 a	1.65±0.03 b-c
	C	1.64±0.02 f-h	1.17±0.02 h-i	1.35±0.01 a	1.50±0.05 d-h
	D	2.60±0.03 c-d	1.83±0.04 b	0.64±0.01 w-z	1.17±0.02 n-t
Rezaie Zodres	Control	0.55±1.41 s-y	0.42±0.005 r-v	0.82±0.02 l-t	1.22±0.02 l-r
	A	0.60±0.008 q-y	0.45±0.005 q-v	1.08±0.02 d-f	1.41±0.02 f-k
	B	0.93±0.02 l-o	0.68±0.03 l-p	0.85±0.01 j-q	1.21±0.02 m-r
	C	1.74±0.03 f-g	1.31±0.02 f-h	0.62±0.03 y-z	0.98±0.03 v-x
	D	2.99±0.05 a-b	2.05±0.03 a	0.39±0.02 d/	0.64±0.02 a/
Shahpasand	Control	0.43±0.004 y	0.35±0.005 u-v	0.97±0.02 f-j	1.40±0.03 g-k
	A	0.45±0.003 x-y	0.36±0.004 t-v	1.05±0.01 d-g	1.48±0.02 d-h
	B	0.48±0.006 v-y	0.38±0.006 t-v	1.23±0.01 b-c	1.68±0.03 a-c
	C	0.91±0.02 l-p	0.65±0.02 m-q	1.24±0.02 b	1.72±0.03 a-b
	D	1.76±0.02 f-g	1.28±0.07 g-h	0.90±0.03 h-n	1.37±0.03 h-l

Means in each column and for each factor, followed by similar letter(s) are not significantly different at the 1% probability level, using Duncan's Multiple Range Test.

Munns and Tester, 2008).

Based on the results of this study, F_v/F_m ratio was 0.83±1 in the leaves of the control plants indicating the existence of ideal and non-stressed environmental conditions for the growth of all cultivars throughout the experimental period. In many plant species, when F_v/F_m ratio is about 0.83, it means that stress hasn't been introduced to the plant and, lower levels indicate stress condition in plants (Maxwell and Johnson, 2000). Regarding changes in F_v/F_m values the stress intensity in Rezaie Zood Res and Mousa Abadi cultivars were more severe than other cultivars (0.59±0.015 and 0.61±0.09, respectively). On the contrary, Gazvini and Akbari cultivars were less damaged (0.76±0.003 and 0.75±0.007, respectively). These results are consistent with the results of (Herda *et al*., 1999; Starck *et al*., 2000; DeEll and Toivonen, 2003; Kodad *et al*., 2010). It has been reported that salinity stress is one of the most important environmental factors limiting photosynthesis. Symptoms of salinity stress are expressed at both stomatal and non-stomatal levels. At stomatal level, the plant closes its stomata to prevent injuries (Maxwell and Johnson, 2000, Ranjbarfordoei *et al*., 2006). As a result, net photosynthesis is unavoidably reduced due to a decrease in CO_2 availability, which potentially damages the photosynthetic apparatus (Lawlor and Cornic, 2002). Most of the decrease in photon flux energy used for photochemistry can be explained as an increase in non-photochemical dissipation of excitation energy (Lawlor and Cornic, 2002).

The results of this research indicated that under salinity stress amount of chlorophyll b was reduced more than amount of chlorophyll a. These results are consistent with the results of Dejampour *et al.* (2012). These researchers investigated the effect of NaCl on the amount of chlorophyll a, b and total chlorophyll in some of the *Prunus* genus, and they reported that amount of chlorophyll b and total chlorophyll significantly decreased under salinity stress. However, reduction in amount of chlorophyll a in these plants was not significant. Also, total chlorophyll content was decreased significantly in all studied cultivars with increasing salinity that are consistent with the results of Karimi *et al.* (2009 and 2011). Researcher reported that salinity stress leads to reduction chlorophyll content and photosynthesis capacity in plants which are the major reasons of decreases growth and yield in plants (Levitt, 1980; Munns, 2002; Munns and Tester, 2008).

The results showed that content of relative humidity were decreased significantly as the salinity increased. The highest reduction in relative humidity content was observed in leaves Mousa Abadi, Rezaie Zood Res and Sabz Pesteh Togh cultivars under salinity level of 19.8 dS/m. The results are consistent with the data reported by Shibli *et al*. (2000) and Massai *et al*. (2004). Salinity, through the gradual accumulation of sodium ions, reduces the relative water content and osmotic potential of the leaf in full turgor state. Relative ion leakage percentage and cell membrane injury percentage in all studied cultivars were increased by increasing salinity concentration. The highest relative ion leakage percentage and cell membrane injury percentage were observed in Mousa Abadi cultivar under treatment 19.8 dS/m. These results are consistent with the results of other studies. It has been reported that using a relative ionic leak test is one way to find out the extent to which cell membranes are damaged. Recording the relative ion leakage rate allow for tissue damage estimation. This method was used for the first time by Dexter *et al*. (1930 and 1932) to investigate the resistance to cold in plants and, over time, was used to measure cell membrane damage in relation to other environmental stresses, including salinity stress (Chen *et al*., 1999).

With increasing salinity concentration in irrigation water, the sodium concentration in the leaves and roots of total cultivars studied increased. The highest sodium concentration in leaves and roots were observed in salinity level 19.8 dS/m and in Mousa Abadi and Rezaie Zood Res cultivars which had the highest percentage of leaves necrosis and loss, and at the end of the experiment, only 52.47±4.98% and 53.48±4.82% of leaves were greens. In researches on various plants under salt stress, it has been reported that the loss of water availability, toxicity of Na^+ and ion imbalance leads to growth limitation in plants (Mahajan and Tuteja, 2005; Szczerba *et al*., 2009). It is repeatedly reported that K^+ deficiency and Na^+ toxicity are major restrictors of crop production worldwide (Mahajan and Tuteja, 2005; Szczerba *et al*., 2008, 2009). The results indicated that the type of cultivar is effective in potassium absorption and its transmission to the aerial part. In this research, Ghazvini and Akbari cultivars with increasing the amount of potassium in its leaves and roots could reduce the negative and destructive effects of sodium better than other cultivars. Potassium plays an important role in vital metabolites in salinity stress conditions, so that the K^+ can counteract Na^+ stresses, thus the potential of plants to tolerate salinity is strongly dependent on their potassium nutrition

(Aleman *et al*., 2011; Nieves *et al*., 2016).

Generally, the results of this study showed that by applying salinity stress and increasing its concentration, growth indices including branch height, branch diameter, number of total leaves, percentage of green leaves, fresh and dry weight of leaves, shoots and roots, relative humidity content, chlorophyll a, chlorophyll b and total chlorophyll content, have been reduced in the all cultivars studied. But the percentage of necrotic leaves, percentage of downfall leaves, relative ionic percentage and cell membrane injury percentage were increased. However, the reduction and increase of measured traits were significantly different among studied cultivars. The results also showed that salinity stress affected the young trees through increasing the amount of minimum fluorescence (F_0) and decreasing the maximum fluorescence (F_m) and reducing variable fluorescence (F_v) as well as F_v/F_m ratio from 0.83±1 in the control plants to 0.59±0.015 in Rezaie Zood Res and 0.61±0.009 in Mousa Abadi cultivar. Based on the results mentioned above, reducing F_v/F_m ratio was symptoms of the damaging stress in plants. The results of method chlorophyll fluorescence in this research are consistent with the results of morphological and physiological traits and therefore, it can be said that chlorophyll fluorescence technique (F_v/F_m indicator) is a rapid, sensitive and non-destructive method to check the intensity of stress that induced to plants. Overall, the result showed that type of cultivar and level of salinity was affected on concentration of Na^+ and K^+ in leaves and roots. Ghazvini cultivar was recognized as the most tolerant cultivar to salinity. This cultivar could tolerate salinity 14.75 dS/m. After this cultivar, Akbari, Badami Zarand and Shahpasand cultivars had more tolerance to salinity, respectively. In contrast, Rezaie Zood Res and Mousa Abadi cultivars were recognized as the most sensitive cultivars to salinity stress. After these cultivars, Khanjari, Jandaghi and Fndoghi 48 cultivars had more sensitive to salinity.

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Beneficial effects of foliar application of organic chelate fertilizers on French bean production under field conditions in a calcareous soil

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Key words: Aminochelate, amino acid, calcareous soil, nutrient uptake, pod yield, quality.

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Abstract: Aminochelate are organic-based chelate fertilizers with higher efficiency for agricultural applications. In the present study, foliar application of three organic-based chelate fertilizers, a macro-micro mixture and soil applied NPK were evaluated on French bean growth characteristics under open field in a calcareous soil. The results showed that plant growth, pod yield (79%) and pod quality were improved by application of chelate fertilizers. Growth parameters as plant height, number of leaves and lateral shoots, shoot dry weight, pod number and pod length were significantly increased by foliar application of the chelate fertilizers. The concentrations of nitrogen, potassium and iron in pods and above all in leaves were increased by foliar application of chelate fertilizers compared to control and soil applied NPK. Pod pH and TSS were not influenced by treatments; however, foliar application of the chelate fertilizers resulted in higher titratable acidity (40%), vitamin C (112%) and protein (35%) content of pods. The results indicate that organic-based chelate fertilizers can be effective safer alternatives for simple chemical salts in calcareous soils.

1. Introduction

Calcareous or lime soils are the dominant type of soil in many parts of the world, and are characterized by high pH as well as high levels of carbonates and bicarbonates. Nutrients and particularly iron uptake in such soil conditions is restricted. In many cases lime-induced chlorosis refers to iron deficiency (Souri, 2015). The leaf chlorosis is mainly due to high pH of soil solution and sap solution induced by high concentrations of carbonates and bicarbonates, which results in precipitation of nutrients in soil and cell apoplast (Mengel, 1994; Nikolic and Römheld, 2002). Plant cultivation in calcareous soils requires especial management techniques and strategies particularly regarding micronutrients supplementation (Souri, 2015). Various fertilizers, as well as different forms of nutrient elements do not have the same uptake efficiency (Jeppsen, 1991; Fernández and

Ebert, 2005). Since decades chelate fertilizers have been used due to their better suitability and efficiency to meet plant's need of nutrient elements particularly under calcareous soil conditions. Simple nutrient salts are in inorganic form, which for utilization by plant roots must be restructured to ionic form in soil solution. This makes them very vulnerable to various inactivation processes, resulting in their low efficiency rate (Mengel, 1994; Nikolic and Römheld, 2002; Souri, 2016). On the other hand, high price of commercial synthetic chelate fertilizers such as EDTA or EDDHA restricts their application by many farmers. In addition, there is also great doubt on their impacts on plant, environment and ecosystem health issues (Souri, 2015). Aminochelate fertilizers are claimed as suitable alternatives for simple salt fertilizers. For nutritional purpose the safety of fertilizer products is very important. Organic chelates such as aminochelate fertilizers are formulated mainly for foliar application, even if in various studies their soil application also resulted in higher growth compared to soil applied NPK (Souri and Yarahmadi, 2016) or a more complete fertilizer (Salwa, 2011; Garcia *et al*., 2011). The method of fertilizer application also plays an important role in uptake efficiency by plant roots (Fernández and Ebert, 2005). There are generally two methods of soil and foliar application for most fertilizers. Most of micronutrients and nitrogen fertilizers including amino acids can simply be applied to leaves with acceptable uptake efficiency (Jeppsen, 1991; Marschner, 2011). Nevertheless, foliar application cannot fully replace soil application of fertilizers in agriculture (Souri, 2015; Dehnavard *et al*., 2017).

Aminochelates are composed of amino acids and a single or several nutrient elements (metals) and represent a more suitable form of fertilizers for sustainable production than routine fertilizers (Souri, 2015). In cultivation systems, application of nitrogen and micronutrients needs a precise and intelligent management, in which various organic chelates can play important role. However, general chemical properties of various aminochelate fertilizers have not been yet studied in soil or within the plant tissues. In recent studies, it was shown that application of amino acid chelates of nutrients in nutrient solution or on plant foliage, significantly increases plant growth and biomass production (Zeid, 2009; Ghasemi *et al*., 2014; Souri *et al*., 2017), leaf number, leaf area, leaf SPAD index (Garcia *et al*., 2011; Souri and Yarahmadi, 2016), fruit yield (Naseri *et al*., 2013; Pourebrahimi, *et al*., 2013; Fahimi *et al*., 2016), fruit quality (Machado *et al*., 2008; Souri *et al*., 2017) and composition of plants (Zhou *et al*., 2007; Garcia *et al*., 2011; Ghasemi *et al*., 2014).

Moreover, application of organic-based fertilizers can also result in higher soil microbial activity and fertility, whereas chemical forms of fertilizers with higher salt effects generally reduce soil microbial activity and soil fertility (Salwa, 2011; Souri, 2015). It was shown that soil application of amino acids has suppressive effect on some soil pathogens such as *Meloidogyne incognita* (Saeed *et al*., 2005). Green bean is a relatively sensitive plant to lime soil, showing dwarf growth and low yield due to restriction of flower development and fruit set. In the present study effects of foliar application of three organic-based chelate fertilizers were investigated on French bean growth characteristics and pod quality factors under calcareous soil conditions.

2. Materials and Methods

Experimental site

This study was performed at Faculty of Agriculture, Tarbiat Modares Uni., Tehran-Iran during 2013. The field in which the experiment was conducted had a calcareous soil. The soil sample was collected from three parts of the field (0-30 cm depth), mixed together and analyzed for some physicochemical characteristics that are presented in Table 1.

Application of treatments

The experiment was arranged in complete randomized blocks using six treatments and four replications. Treatments were: 1) Control (without any fertilizer application), 2) Soil application of NPK, 3) foliar application of Biomin (amino acid based fertilizer; Arbico-Co, Texas, USA), 4) foliar application of Humifolin (humic acid based fertilizer; Tradecrop Co., Spain), 5) foliar application of DelfanPlus (only amino acid; Tradecrop Co., Spain) and 6) foliar application of a mixture of macro-micro solution. Each replicate

Table 1 - Some properties of the soil used in the experiment

Physico-chemical caracteristics	Data
Texture	Loam-clay
pH	7.8
EC dS/m	2.6
Total C (%)	0.77
Total N (%)	0.09
$CaCO_3$ (%)	5.5
Extractable P (mg kg^{-1})	15.2
Exchangeable K (mg kg^{-1})	250

was a plot of 0.7×1m consisting of 12 plants. Green bean seeds (*Phaseolus vulgaris* L) were directly sown in the soil, and after germination they were thinned to 12 plants per plot. Throughout the growing period all plots and plants were treated the same regarding irrigation, and pest-disease control.

In NPK treatment, a final amount of 6 g per plant from a 20:10:20 formulation was used in three split applications that were incorporated into the soil just near the root systems using 200 ml water. The first application was before sowing and the rest was applied in one week interval after second week of emergence. Foliar application of organic-based and mix fertilizers were done five times during growth period, in a constant concentration of 0.2%. Plants were foliar sprayed at 6-7 o'clock in the morning using a portable sprayer, by which the upper and the lower surface of leaves were treated. The first spray was done at four leaf stage, and the remaining foliar sprays were applied at one-week intervals. All the organic-based commercial fertilizers used in present study were in liquid form, consisting of one or several nutrient elements.

Composition of fertilizers

The composition of various fertilizers is presented as follows: BIOMIN: a liquid fertilizer containing 2% N, 2.5% Zn, 1.5% Mn, 1% Fe, 0.4% Mg, 0.4% Cu, and 0.02% Mo; HUMIFOLIN, a liquid fertilizer containing 42% organic compounds including 37% fulvic and humic acid, 5% various vitamins, 0.5% phosphorus, 0.28% Fe, 0.041% Zn, 0.0035% Mn, 0.0023% Cu, 0.0012% Mg, and 0.0012% B; DELFANPLUS, a liquid fertilizer consisting of 24% free amino acids, 9% total nitrogen, 5% N-protein and 43% organic carbon. MACRO-MICRO mixture consisted of 5% N, 2.5% Zn, 2.5% Fe, 2% Mn, 0.5% Mg, and 0.5% Cu.

Plant sampling and measurements

French bean plants were grown for ten weeks, and various growth traits were measured during growth period, as well as at harvest time. Plant pods were harvested several times during their growth period. Cumulated yield of plants during 2-3 harvests (in each replicate) was recorded and the average was presented as plant final yield of fresh pods. Accordingly, total number of pods per plant was recorded. The average number of leaves and lateral shoots of plants were counted per replicate (plot) and calculated per plant. Chlorophyll index of leaves was recorded using a portable SPAD meter (model 502 Plus, Illinois, USA) with 10 reading per plant and at least 120 readings for each replicate, by which the average was recorded in the results. Plant stem diameter was measured by caliper (model Mitutoyo Japan). Average shoot dry weight per plant was calculated after drying four randomly chosen plants from each replicate at 65°C for 24 hours.

Total Soluble Solid (TSS) as Brix index was determined with a refractometer using a drop of pod extracted juice. For determination of pod pH and titratable acidity, 10 g of fresh pods (from each replicate) was cut, crushed and centrifuged at 9000 rpm. Ten ml of supernatant was titrated using 0.1 M NaOH until a final pH of 8.2, and the pod acidity based on citric acid in 100 g fresh pods was calculated using the following formula:

$$TA= 100 \times M \times N \times V/S \times n$$

where TA is the amount of pod acidity (mg/100 g FW), M the molecular weight of dominant pod acid (citric acid= 64 g) n: valence of dominant acid, N: normality of NaOH, S: weight of pod sample (g) and V the volume of consumed NaOH.

For measurement of pod vitamin C, 10 g of fresh pods from final harvest was gently washed, cut in small pieces and then crushed in a mortar in presence of 10 ml of 2% metaphosphoric acid. The mixture was immediately centrifugated at 9000 rpm (Eppendorf Centrifuge 5810R, Hamburg, Germany) for 5 minutes at 4°C. The supernatant was used for titration by 2,6 dichloro indophenols, and the amount of vitamin C for 100 g fresh pod was calculated in relation to records of a standard curve of L-ascorbic acid concentration of 0, 25, 50, 100 and 200 mg L^{-1}. Determination of pod protein content was done using Coomassie Brilliant blue G250 dye according to Bradford's method (1976). The nutrient concentration of N, K and Fe were determined in plant leaves and green pods using Kjeldahl, flame photometer and atomic absorption spectrophotometer methods.

Statistical analysis

Data were analyzed using SPSS 16 and differences among treatments were determined at 5% level by Duncan's test. Graphs were prepared using EXCEL Microsoft.

3. Results

Plant vegetative growth

Vegetative growth characteristics of plants are presented in Table 2. Application of various fertilizers had significantly improved plant growth. All the most important growth factors were improved especially

by foliar application of organic-based chelate fertilizers (Table 2). In particular, plant height was increased by application of three organic chelates, while there was no difference among control plants and those plants treated with soil applied NPK or by foliar application of macro-micro mixture. Stem diameter was significantly higher in plants that received foliar application of organic chelates (Table 2). Number of leaves was maximum in plants treated with Biomin and Humifolin; however they had no significant difference with DelfanPlus treated plants. The least number of leaves was in control plants. Number of lateral shoots was increased by foliar application of Biomin and Humifoline; however, there was no significant difference among all organic fertilizers. There was no significant improvement of lateral shoots by soil applied NPK or foliar application of macro-micro mixture (Table 2). Plants treated with DelfanPlus produced longest internodes that showed no difference with Biomin or Humifolin treated plants (Table 2). Application of all fertilizer treatments, except foliar application of macro-micro mixture, resulted in significantly higher SPAD values compared to control plants (Table 2). Determination of shoot dry weight revealed that plant growth and biomass production was significantly improved by foliar application of the organic fertilizers, as well as by soil applied NPK treatment. On the other hand, the maximum shoot dry weight was recorded for Biomin treatment, which showed significant difference with control and all other fertilization treatments (Table 2).

Plant yield and nutrient status

Application of all fertilizers increased the pod yield compared to control plants (Table 3). Plants produced significantly higher yield when they received foliar application of organic chelate fertilizers (Table 3). Number of seeds per pod and number of pods per plant had a similar trend, in which foliar application of three organic chelate fertilizers recorded higher values. Regarding pod length, plants treated with foliar application of three organic chelates had the longest pods compared to all other treatments. All fertilizer treatments increased the pod dry weight. Plants treated with Biomin produced significantly higher pod dry weight (Table 3), although application of all the organic chelates significantly increased pod dry weight compared to soil applied NPK or foliar application of macro-micro mixture.

Nutrient profile of plant leaves and pods were significantly increased by foliar application of organic fertilizers, only for N and K, and also by NPK soil

Table 2 - Effects of various fertilization treatments on vegetative growth traits of French bean plants in a calcareous soil under field conditions

Treatments	Plant height (cm)	Stem diameter (cm)	Number of leaves	Number of lateral shoots	Length of internode (cm)	SPAD index	Shoot dry weight (g)
Control	26 b	0.62 b	14 c	4 b	5.6 b	36 b	7.2 d
NPK	31 ab	0.79 b	19 b	5 b	5.5 b	40 a	11.3 c
Biomin	39 a	1:05	24 a	8:00	6.7 ab	41 a	22.1 a
Humifolin	39 a	0.96 a	24 a	7:00	6.1 ab	41 a	15.6 b
DelfanPlus	38 a	0.98 a	22 ab	6 ab	7:02	42 a	17.5 b
Macro-micro mixture	29ab	0.67 b	17 bc	4 b	5.1 b	38 ab	8.5 d

Data are mean of four replicates. Comparison of means was done using Duncan's test at 5% level.

Table 3 - Effects of various fertilization treatments on yield and fruit characteristics of French bean plants grown in a calcareous soil under field conditions

Treatments	Plant pod yield (g)	Number of seeds/pod	Number of pods per plant	Pod length (cm)	Pod dry weight (g)	Pod pH	Pod TSS
Control	31.4 c	2.9 c	17 c	5.5 b	8.3 d	6.20 a	1.7 a
NPK	38.3 b	3.8 b	28 b	6.5 b	10.8 c	6.30 a	1.8 a
Biomin	59.1 a	5.1 a	49 a	9.1 a	21.1 a	6.21 a	2.4 a
Humifolin	53.5 a	4.7 a	43 a	9.3 a	16.2 b	6.22 a	2.1 a
Delfan plus	56.6 a	4.8 a	45 a	9.6 a	16.6 b	6.23 a	2.0 a
Macro-micro mixture	36.7 b	3.9 b	27 b	6.5 ab	11.4 c	6.24 a	2.1 a

Data are mean of four replicates. Comparison of means was done using Duncan's test at 5% level.

application (Table 4). Foliar application of Biomin and DelfanPlus resulted in significantly higher K and Fe concentration in leaves, and K concentration of pods, compared to Humifolin. The lowest nutrient concentrations were recorded in control and macro-micro mixture treatments.

Determination of nutrients in green pods (Table 4) showed that the significant highest amount of N was in plants sprayed with Biomin aminochelate, and then by DelfanPlus, Humifolin and soil applied NPK, respectively. Potassium concentration was significantly higher in foliar spray of Biomin and DelfanPlus, while the lowest concentration was in pods treated with macro-micro mixture and in control plants. Iron concentration of green pods was significantly increased by organic fertilizers.

Pod quality

There was no significant difference in pod pH and pod total soluble solids (TSS) among treatments (Table 3). However, determination of titratable acidity (Fig. 1) revealed that plants treated with Humifolin resulted in significantly higher pod acidity (but not different from Biomin and DelfanPlus), while the least titratable acidity was measured in soil applied NPK, control and macro-micro treatments, that showed no difference with Biomin and DelfanPlus treatments (Fig. 1). Foliar application of three organic fertilizers resulted in significantly higher vitamin C content of pods compared to control plants (Fig. 2). Pod protein content was higher in plants treated with Humifolin and Biomin, and lower in control and plants treated by foliar application of macro-micro mixture. Pod protein content was intermediate for soil applied NPK and foliar application of DelfanPlus (Fig. 3).

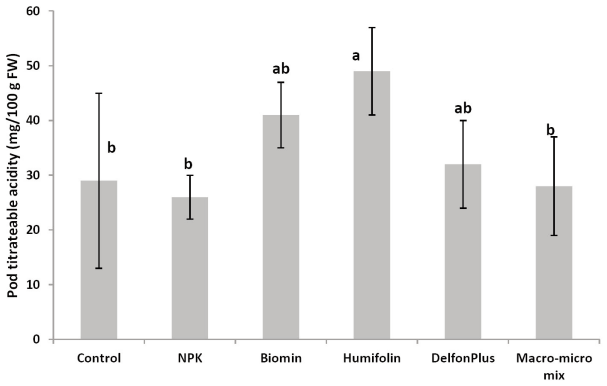

Fig. 1 - Effects of various fertilization treatments on titratable acidity of French bean pods. Data are mean of four replicates ± SD. Comparison of means was done using Duncan's test at 5% level.

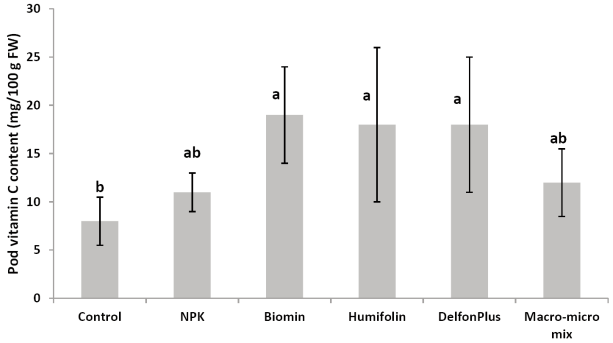

Fig. 2 - Effects of various fertilization treatments on vitamin C content of French bean pods. Data are mean of four replicates ± SD. Comparison of means was done using Duncan's test at 5% level.

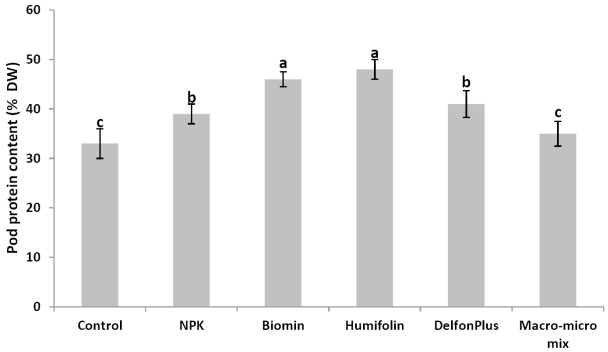

Fig. 3 - Effects of various fertilization treatments on protein content of French bean pods. Data are mean of four replicates ± SD. Comparison of means was done using Duncan's test at 5% level.

4. Discussion and Conclusions

In the present study plant growth was improved by foliar application of three organic chelate fertilizers. Many parameters of vegetative growth as well as plant yield, nutrient content and fruit quality were improved by application of these three commercial

Table 4 - Effects of various fertilization treatments on some nutrients in leaves and pods of French bean grown in field with calcareous soil conditions

Treatments	Leaf			Pod		
	N (%)	K (%)	Fe (mg kg^{-1} DW)	N (%)	K (%)	Fe (mg kg^{-1} DW)
Control	2.1 c	1.7 c	55.3 c	3.7 c	2.8 c	77.2 b
NPK	2.7 b	2.6 a	64.1 c	5.2 b	4.6 b	85.1 b
Biomin	3.5 a	2.8 a	126.3 a	6.5 a	5.3 a	108.3 a
Humifolin	2.9 b	2.3 b	97.7 b	5.6 b	4.7 b	96.6 a
DelfanPlus	3.2 ab	2.6 a	118.5 a	5.7 b	5.1 a	99.0 a
Macro-micro mixture	2.3 c	1.8 c	66.1 c	3.7 c	2.7 c	82.4 b

Data are mean of four replicates. Comparison of means was done using Duncan's test at 5% level.

organic fertilizers. Increasing in plant growth and various yield traits were also reported in other studies (Machado *et al*., 2008; Garcia *et al*., 2011; Naseri *et al*., 2013; Salwa, 2011; Ghasemi *et al*., 2014; Sadak *et al*., 2015; Souri and Yarahmadi, 2016). In a recent study it was shown that foliar application and to lesser extent soil application of a commercial aminochelate fertilizer increased the growth, yield and quality of tomato, cucumber and bean plants (Souri *et al*., 2017). Foliar application of a mixture of amino acid on bean plants significantly improved the tolerance to seawater salinity stress (Sadak *et al*., 2015). Similarly, application of amino acids significantly improved growth parameters of shoots and fresh weight as well as pod yield of soybean plants under pathogen infection (Saeed *et al*., 2005). Vegetative growth of plant height and dry weight of potato plants were increased by foliar application of amino acids (El-Zohiri and Asfour, 2009). Three foliar application at 6-7 leaves stage and two more sprays in two weeks intervals using Fe and Zn aminochelates with different concentrations up to 0.3%, resulted in 15-35% higher potato tuber yield per hectare (Pourebrahimi *et al*., 2013).

Stimulatory effects of amino acids on plant growth characteristics have been well documented, particularly under adverse climatic conditions such as salt and drought stresses (Rai, 2002; Zhou *et al*., 2007; Garcia *et al*., 2011; Salwa, 2011; Sadak *et al*., 2015). Amino acids are key important player in plant metabolism. They are the intermediate compounds in nitrogen assimilation, and represent the main form by which nitrogen is translocated within the plant through phloem (Marschner, 2011). Various amino acids and peptides are precursor of physiologically important phytohormones (Cobbett and Goldsbrough, 2002; Marschner, 2011) or they are involved in detoxification of different toxins within the plant cells (Cobbett and Goldsbrough, 2002; Souri, 2015). In addition to the amino and carboxyl groups, amino acids have a side chain or R group that is attached to the α-carbon. Each amino acid has unique characteristics arising from the size, shape, solubility and ionization properties of its R group. Nevertheless, the side chain of amino acids exerts a deep effect on their biological activity as well as on the structure and activity of proteins. By far, glycine is the main and widespread used amino acid in manufacturing aminochelate fertilizers, despite frequently a mixture of amino acids may be included (Souri, 2015). Similarly, the stimulating effect of foliar or soil application of humic acid on increased plant growth and nutrient uptake has been shown (David *et al*., 1994; El-Ghamry *et al*., 2009; Salwa, 2011; Canellas *et al*., 2015). The biostimulant effects of humic substances are characterized by both structural and physiological changes in roots and shoots related to nutrient uptake, assimilation and distribution (nutrient use efficiency traits). In addition, they can induce shifts in plant primary and secondary metabolism related to abiotic stress tolerance which collectively modulate plant growth as well as promoting fitness (Canellas *et al*., 2015).

In the present study, improved growth and plant performance of French bean might be also due to the higher nitrogen and micronutrients content of plant leaves. The N, K and Fe concentrations in plant leaves were significantly improved by foliar applications of organic chelate fertilizers. Metal ions such as Fe, Zn, Mn and Cu are essential for healthy plant growth, being required for various metabolism reactions (Marschner, 2011). They have direct and distinct effects on plant performance, as well as on yield and quality parameters. However, uptake of micronutrients such as iron by roots from soil could be limited due to low chemical stability and precipitation of these elements particularly in calcareous soil (Fernández and Ebert, 2005; Souri, 2015). In calcareous soils, with high pH and carbonate-bicarbonates levels, plants are prone to iron and other micronutrient deficiencies. Lime-induced chlorosis is one of the most important nutritional disorders, affecting many plant yield and quality traits.

Aminochelate fertilizers represent an excellent N source for plant, in both foliar and soil applications. In present study, N concentration of leaves and pods were significantly improved by organic fertilizers. Higher nitrogen content of plant was also reported for radishes (Liu *et al*., 2008) and marigold (Souri and Yarahmadi, 2016), when plants were treated by foliar application of aminochelate fertilizers. Improvement in nutrient elements profile of tomato was observed when amino acids were applied in nutrient solution, which finally improved plant growth and nutrient concentrations, particularly N status of plants (Garcia *et al*., 2011).

Nitrogen has an important role in growth, yield and quality of crops and must be usually applied to meet the plant needs. French bean can fix atmospheric N2, so it may need less N fertilization; however, in some parts of Iran farmers use also high rates of urea to enhance plant growth. On the other hand, application of high amount of N fertilizers could lead to significant reduction in yield and quality, as well as

various pollutions. Leaf greenness, as the best health indicator of plants, depends on chlorophyll biosynthesis and concentration, which in part is affected by N and micronutrients. Aminochelates generally contain all these effective nutrient elements. Application of aminochelates, as a source of N fertilizer, can also result in lower nitrate accumulation in plant tissues. High nitrate content of plant tissues is a negative factor, particularly in leafy vegetables that are fresh consumed. Application of reduced form of nitrogen (such as ammonium or amino acids) instead of oxidized form (nitrate) can lead to less nitrate accumulation in plant tissues (Marschner, 2011; Souri *et al*., 2017). On the other hand, plant might have different response to various fertilizers of a given nutrient (Souri, 2015). However, there are always distinct clear responses of vegetative and reproductive growth, as well as quality parameters to nitrogen fertilizers (Marschner, 2011; Souri, 2016).

The higher efficiency of aminochelate fertilizers can be due to the chelating effects of amino acids or organic acids on nutrient elements. The chelating effects of amino acids on nutrients has been commercially used for improving nutritional status of animals and human for more than 6 decades, and for plants in recent years (Souri, 2016). As it is well known, amino acids are “zwitterions” in biological systems, and have distinct different behavior in acidic and basic solutions, to maintain the pH of the system. The structure of an amino acid allows it to act both as an acid and a base, depending on solution pH. This behavior is quite important in plant nutrition, as pH is one of the main factors responsible for nutrient use efficiency, and frequently high pH (in calcareous soils) and low pH (in acidic soils) restrict nutrients solubility and bioavailability (Souri, 2015). Nevertheless, in present study organic chelate fertilizers had various composition of one or several components of amino acid or nutrients. Conducting scientific research with such amino or organic chelates due to their mix nature of various components is quite difficult (Souri, 2016).

In conclusion, foliar application of organic chelate fertilizers resulted in higher plant growth under calcareous soil conditions. Aminochelates are composite fertilizers of amino acids and various nutrient elements particularly iron and zinc and separating the effects of each component is relatively difficult. The effect of organic chelate fertilizers particularly aminochelates on many physiological and molecular responses of plants has not been well studied. Application of organic chelate fertilizers can avoid all negative effects of routine chemical salt fertilizers such as urea or ammonium nitrate, including leaching, volatilization and nitrate accumulation in vegetable tissues. Therefore, they represent modern and suitable alternatives to simple salts, and even to commercial synthetic chelates such as EDTA due to their cheaper price. Nevertheless, these claims need to be evaluated in deep in future studies.

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Direct and indirect *in vitro* plant regeneration of two commercial cultivars of perennial ryegrass

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Key words: Abscisic acid (ABA), 'Grassland', *Lolium perenne* L., maltose, meristem tip, 'Numan'.

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Abstract: Experiments were conducted on direct and indirect regeneration from the meristem tip and mature caryopsis explants of *Lolium perenne* L. 'Numan' and 'Grassland'. De-husked caryopses were cultured both intact and longitudinally sliced on MS media supplemented with 2,4-D alone, and in combinations with BA. The highest percentage of callus induction obtained from intact-sliced caryopses were 71 and 87% for 'Grassland' on MS basal medium supplemented with 6 mg L^{-1} 2,4-D+ 0.02 mg L^{-1} BA, and 5 mg L^{-1} 2,4-D. While, for 'Numan', the highest callus induction was achieved by the same explants as 55% and 72% with 5 mg L^{-1} 2,4-D + 0.02 mg L^{-1} BA, and 4 mg L^{-1} 2,4-D + 0.02 mg L^{-1} BA, respectively. The best regeneration medium for 'Grassland' was MS medium supplemented with 10 g L^{-1} maltose and 2 mg L^{-1} ABA. In a separate experiment, meristem tip cultures were incubated on two type combination of plants growth regulators along with control treatment. The best regeneration rate was obtained in both cultivars on MS medium supplemented with 0.1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin. Plantlets with well-developed roots were transferred to greenhouse condition. Four weeks later, all acclimatized plants were survived.

1. Introduction

Turfgrasses control the soil erosion, carpet lawns, cover athletic fields, and beautify the environment. Perennial ryegrass (*Lolium perenne* L.) is one of the most important turfgrass species for sports fields, golf course fairways, as well as urban landscapes in the areas with temperate climate. That is the fast establishing component of lawn seed mixtures comprising slow growing species such as Kentucky bluegrass (*Poa pratensis* L.), and is used for winter overseeding on warm-season turfgrasses as well. However, the poor ability to survive in drought regions have limited its distribution. Therefore, improving drought tolerance is an important goal in perennial ryegrass breeding programs via classic or modern (genetic transformation) techniques. Callus production with good quality and efficient plant regeneration of various cultivars of perennial ryegrass is a requisite to the grass transformation techniques. The formation of embryo-

genic calli and plant regeneration were affected by several factors including genotype, explant tissue, culture medium and its supplements (Bhaskaran and Smith, 1990). Many studies have been done on improving tissue culture of perennial ryegrass. In perennial ryegrass, various explants including immature inflorescence, mature seed, leaf base, meristem tip and axillary bud have been used for callus induction, and direct and indirect plant regeneration (Dale, 1977; Dale and Dalton, 1983; Torello and Symington, 1984; Creemers-Molenaar *et al*., 1989; Altpeter *et al*., 2000; Bradley *et al*., 2001; Can *et al*., 2004; Salehi and Khosh-Khui, 2005; Newell and Gray, 2005; Altpeter, 2006). Among these explants, mature seeds are most commonly used for callus induction and related subsequent studies.

Perennial ryegrass is an outcrossing, wind-pollinated and highly self-incompatible species with abundant genetic variation in each cultivar (Mohr *et al*., 1998; Smith *et al*., 2001; Wang *et al*., 2003; Bolaric *et al*., 2005). Replacing the seed explants with other vegetative explants would omit this variation. *In vitro* meristem tip culture is an efficient method for obtaining virus free and identical to mother plant materials. In addition, meristem tip culture is an appropriate method for genetic transformation. In this method, the exposed meristem could directly be used for transformation; then after, will multiplied *in vitro*, or can form multiple shoot clumps that can be bombarded with the gene(s) of interest, and then each meristem will regenerated the mature plants. Therefore, development of an optimized and short time tissue culture protocol plays a critical role in successfully transformation of the grass species. To the best of our knowledge, there is no report on meristem tip culture of 'Grassland' and 'Numan' cultivars of perennial ryegrass.

In addition to, it has been shown stimulating effect of ABA on somatic embryogenesis of some grass species calli such as wheat (Qureshi *et al*., 1989), maize (Close and Ludeman, 1987), Kentucky bluegrass (Van Ark *et al*., 1991) and bermudagrass (Li and Qu, 2002).

The objectives of the present investigation were comparison of the callus induction on intact and longitudinally sliced caryopses, and evaluation of the effects of ABA and maltose combinations on plant regeneration of two *Lolium* sp. cultivars to optimize their tissue culture condition. Furthermore, a separate experiment was conducted to find a direct regeneration and fast tissue culture method for these cultivars.

2. Materials and Methods

Seed surface sterilization

Seeds of the two commercial cultivars of perennial ryegrass, 'Grassland' and 'Numan', were purchased from Iran Bazr Seed Company (Karaj, Iran). Seeds were socked in 50% H_2SO_4 for 15-20 min to remove the husks (Salehi and Khosh-Khui, 2003). The de-husked seeds were surface sterilized with 70% ethanol for 1 min and immediately were treated with a house-hold bleach (5.25% available chlorine) for 20 min, then rinsed 6 times with sterile distilled water.

Callus induction

The surface sterilized seeds were used both intact and longitudinally sliced. For callus induction, MS basal medium (Murashige and Skoog, 1962) plus 3% sucrose supplemented with 4, 5, 6, 7, 8, 9 and 10 mg L^{-1} 2,4-D alone and with 0.02 mg L^{-1} BA. Intact and longitudinally sliced seeds were cultured on 80 mm petri dishes containing callus induction media.

After 4 weeks, the calli were separated, transferred to the same fresh media and maintained for 3 weeks. Then, the calli were transferred to optimum MS media, supplemented with 4 mg L^{-1} 2,4-D + 0.02 mg L^{-1} BA and 5 mg L^{-1} for 'Numan' and 'Grassland', respectively. The number of seeds that induced callus was recorded according to seed viability. The callus induction rate was calculated as the number of caryopses which induced callus over the total number of explants plated × 100. All cultures were kept at 24±1°C in dark.

Plant regeneration

To determine the best regeneration media for 'Numan' and 'Grassland', the embryogenic calli were transferred to MS media containing 3% sucrose and 0, 10, 15 and 20 g L^{-1} maltose with 0, 1.7, 2 and 2.3 mg L^{-1} ABA. Filter-sterilized ABA was added after autoclaving when the media temperature dropped to 50°C.

Plant regeneration was scored 7-10 days after transferring the calli to the regeneration medium. The criterion used to determine regeneration was the formation of a distinguishable shoot at least 1 cm in length. Plant regeneration rate was defined as the percentage of callus that had regenerated shoots. The regeneration cultures were maintained at 24±1°C with a 16 h light/8 h dark cycle under a light intensity of 20 µmol m^{-2} s^{-1}.

Meristem tip culture

In a second experiment, surface sterilized intact caryopses were incubated on 80 mm diameter petri dishes containing MS media, 3% sucrose, solidified

with 0.8% plant agar and were kept at 24±1°C in dark.

After emerging radicles, the petri dishes were transferred to 16h/8h light/dark photoperiodic condition. Meristem tips of 7-10 days old seedlings were excised by a Stereo microscope (C121506 Zeiss Stemi 1000 Binocular Stereo Zoom 0.7-3.5x Microscope 10x Eyepieces, Germany) and cultured on three different MS media including hormone-free MS, MS supplemented with 0.01 mg L^{-1} 2,4-D + 0.2 mg L^{-1} Kinetin, MS supplemented with 0.1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kinetin. All media were supplemented with 3% sucrose and pH was adjusted to 5.8 prior to autoclaving. The length of the meristem tips cultured ranged from 0.1-1 mm and consisted of the meristem dome plus one or several leaf primordia. After 2 weeks, the number of direct regenerated plantlets was recorded in each treatment based on green and healthy plantlets produced and then the percentage of direct regeneration of meristem tips was calculated. One hundred meristem tips were cultured in each petri dish. All media were placed in dark for 3 days at 24±1°C and then were transferred to 16h/8h light/dark photoperiodic condition. Light intensity was provided by cool-white fluorescent lamps at a photon flux density of 20 µmol m^{-2} s^{-1}.

Statistical analysis

Complete randomized design with factorial arrangements was used for all experiments. For callus induction, each treatment had four replications, each replicate consisted of 100 seed. Experiments on direct and indirect regeneration were done with three replications. All data were analyzed with SAS 9.1 software and means were compared using LSD test at 5% level.

3. Results and Discussion

Effects of cultivars, explants and hormonal treatments and their interaction on callus induction

Plant growth regulators (PGRs) are specific molecules used in plants and supplemented at relatively low concentrations to work as signaling compounds for plant growth and development (Sauer *et al.*, 2013). The most extensively used and studied class of PGRs in plant tissue cultures are auxin and cytokinin (Ikeuchi *et al*., 2013). Among auxin sources, 2,4-D is known as the most effective PGR to induce callus formation with stimulating cell elongation and enlargement in many plant species especially turfgrasses. In addition, low levels of cytokinins such as BA and BAP enhanced callus regeneration ability in several grass species (Zhong *et al*., 1991; Van der Valk *et al*., 1995; Chaudhury and Qu, 2000; Bradley *et al*., 2001).

Calli were observed in 7 to 15 days after placing the explants on callus induction media. Their morphological appearance was hyperhydrated and white. After 4 weeks, three types of calli were observed including: i) white and hyperhydrated; ii) yellowish and friable; iii) compact, nodular, yellowish to opaque (Fig. 1 B-D). This variability is probably due to different tissues origin. Results showed that callus induction was notably increased by 28% in sliced caryopses (61.37) compared to intact caryopses (48.04). In addition, there was a significant difference

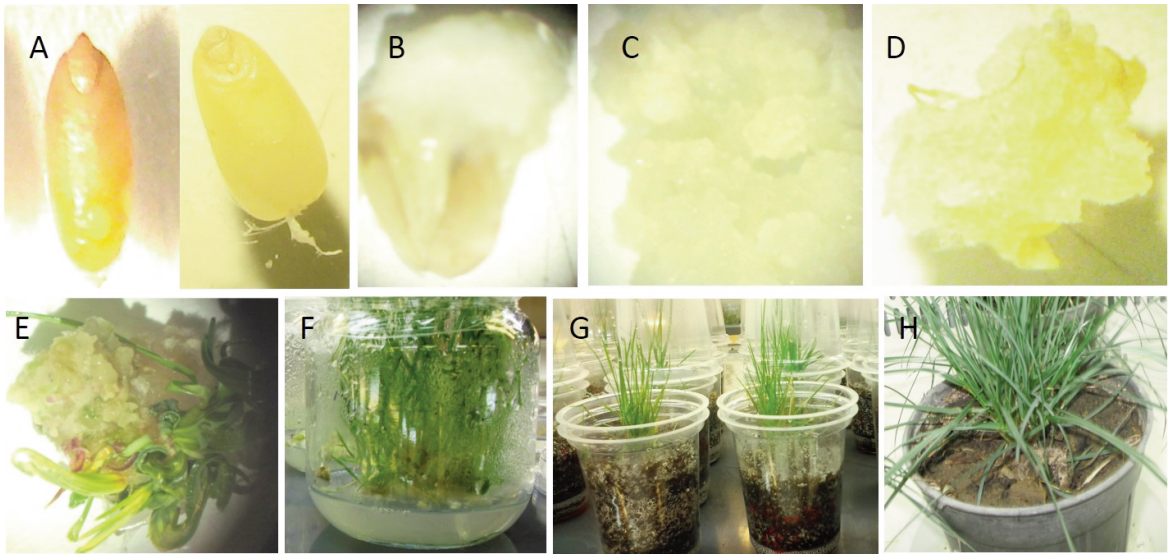

Fig. 1 - Steps of caryopses culture of two perennial ryegrass cultivars. A) The de-husked caryopses and surface sterilization of caryopses, right 'Grassland', left 'Numan'. B) Calli derived from longitudinally sliced caryopsis tissue, C) Embryogenic callus type ii. D) Embryogenic callus type iii. E) Green shoots produced on embryogenic callus. F) Shoot proliferation on the regeneration medium after two weeks. G) Plants acclimatized in soil mixture after one week. H) Healthy and green plants maintained in greenhouse after one month.

between two cultivars. The callus induction raised by 24% in 'Grassland' (60.57) compared to 'Numan' (48.83) (Data not shown).

In 'Grassland', the highest good quality callus induction was obtained at 5 mg L^{-1} 2,4-D in sliced caryopses, while 'Numan' had the highest callus induction at 4 mg L^{-1} 2,4-D with 0.02 mg L^{-1} BA treatment in sliced caryopses (Table 1).

In 'Grassland', higher concentrations of 2,4-D declined callus induction with explants sliced and entire caryopses. However, more calli showed white color with increasing 2,4-D concentration in mentioned media. The effectiveness of 2,4-D in inducing the formation of callus is attributed to its main characteristic which can stimulate cell division of plant tissues and strongly suppress their organogenesis.

In a similar study, Liu *et al*. (2006) used 2,4-D and BAP for callus induction in perennial ryegrass. They found that the highest callus induction rate with the best callus quality was obtained on MS medium containing 5 mg L^{-1} 2,4-D and 0.05 mg L^{-1} BAP. Preliminary studies on the effectiveness of BAP on somatic embryogenesis in callus culture medium in several grass species were reported (Zhong *et al*., 1991; Chaudhury and Qu, 2000; Bradley *et al*., 2001). According to previous reports, the optimal BAP concentration in the induction medium should be 0.02-0.05 mg L^{-1} for perennial ryegrass.

In this experiment, no significant difference was found on media supplemented with 0.0 or 0.02 mg L^{-1} BA. However, this amount of BA had positive effect on morphology and cellular structure of calli and their capacity to regenerate plantlets.

As previously stated, faster and more callus induction of longitudinally sliced caryopses in comparison with intact caryopses were detected. Increase in contact surface of tissue could be an important reason for callus induction. These findings are similar to other studies which have been conducted by Altpeter *et al*. (2000), Altpeter and Xu (2000), Bai and Qu (2001) and Bradley *et al*. (2001) on *Lolium* sp. and *Festuca* sp. Bai and Qu (2001) improved both callus production and regeneration rate of tall fescue, *Festuca arundinacea* Schreb. with slicing seeds in callus culture medium. Also, similar inclusion on chopping the mature seeds of *Lolium* sp. and *Festuca* sp. to suppress germination and stimulation of callus production were reported (Altpeter *et al*., 2000; Altpeter and Xu, 2000). Although, Salehi and Khosh-Khui (2005) obtained contrary results by using horizontally sliced caryopses of *Lolium* sp. cultured on callus induction medium. These results might be related to genotypic differences and callus origin. These results might be used as a general application for other grass species.

Table 1 - Interaction of different concentrations of 2,4-D and BA on callus induction of intact and sliced caryopses in two cultivars of *Lolium perenne* L.

2,4-D (mg L^{-1})	BA (mg L^{-1})	'Grassland'		'Numan'	
		Intact seeds	Sliced seeds	Intact seeds	Sliced seeds
4	0	49± 3.9 de	69± 6.0 bc	49±2.4 de	54±3.5d
5	0	61±2.7 c	84±3.2 a	46±1.9 de	61±3.8 c
	0.02	59±5.2 c	73±2.0 ab	55±1.8 d	41±2.9 e
6	0	51±3.4 de	77±3.8 a	30±2.9 g	62±3.6 c
	0.02	62±5.4 c	71±2.3 b	53±6.7 d	54±4.7 d
7	0	71±0.6 ab	60±4.8 c	41±3.2 e	58±1.7 cd
	0.02	55±3.5 d	75±5.5 ab	53±2.7 d	65±4.9 bc
8	0	53±1.1 d	73±6.3 ab	54±1.2 d	67±3.5 b
	0.02	46±5.0 e	69±3.4 bc	45±6.6 e	53±1.7 d
9	0	46±5.0 e	53±6.4 d	35±1.6 f	38±1.9 f
	0.02	38±6.6 f	57±2.5 c	29±2.4 g	53±1.9 de
10	0	43±7.9 e	54±4.1 de	38±2.8 f	41±3.2 e
	0.02	54±7.4 d	56±4.1 d	34±4.9 f	54±2.5 d

Values represent mean ± SE. Means with the same letter are not significantly different at $p \leq 0.05$.

Interaction effect of ABA and maltose on plant regeneration

The highest regeneration rates were recorded for both cultivars on MS medium supplemented with 30 g L^{-1} sucrose, 10 g L^{-1} maltose and 2 mg L^{-1} ABA. As shown in figure 2, 'Grassland' has higher regeneration rate (~13%) than 'Numan'. Incidentally, maltose had not notably effect on plant regeneration percentage in both cultivars lonely but ABA concentrations showed significant effects on plant regeneration rate (Fig. 2). This result can be referred to the role of ABA in maturation of embryos (Finkelstein *et al*., 1985).

The most important source of energy for plant growth in the culture medium is carbon. Several

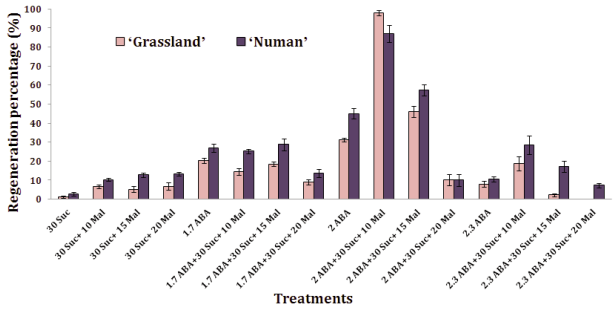

Fig. 2 - Regeneration rates of two cultivars of *Lolium perenne* L. in combination concentrations of ABA (0, 1.7, 2 and 2.3 mg L^{-1}), and maltose (0, 10, 15 and 20 g L^{-1} Mal) under same concentration of sucrose (30 g^{-1} Suc).

studies have reported the effects of carbohydrates such as sucrose and maltose on different plant species like wild cherry, rubber tree, oilseed rape and sugarcane (Reidiboym-Talleux *et al*., 1998; Blanc *et al*., 1999; Slesak and Przywara 2003; Gill *et al*., 2004). Maltose is the best carbon source for callus induction and regeneration medium. Furthermore, maltose, as an osmoticum, plays a major role in the optimization of callus cellular environment and protect calli by reducing ethylene (Darachai *et al*., 2004; Zaidi *et al*., 2006).

The regeneration rate was affected by using maltose, to almost five-fold increase at 20 g L^{-1} maltose compared to 30 g L^{-1} sucrose in both cultivars studied (Fig. 2). In addition, ABA increased the regeneration rate ~25 and 16 fold at 2 mg L^{-1} in comparison with 0 mg L^{-1} in 'Grassland' and 'Numan', respectively.

The combination of maltose and sucrose and ABA at high concentrations had no promoting effect on plant regeneration in both cultivars. In 'Grassland', significant declines was observed at 2.3 mg L^{-1} ABA + 20 g L^{-1} maltose (71.3%) and 1.7 mg L^{-1} ABA + 10 g L^{-1} maltose (100%). According to the results, both cultivars showed different responses at high concentrations of maltose and ABA combination. Generally, the regeneration rate dramatically reduced by 100% at 2.3 mg L^{-1} ABA + 20 g L^{-1} maltose in 'Numan' compared to 'Grassland' (Fig. 2).

A probable reason for reducing the regeneration percentage is related to sucrose in regeneration media that stimulates the ethylene production in plant tissues which can cause browning of calli. On the contrary, maltose might protect the calli from browning. However, beneficial effect of maltose was observed on embryogenesis and regeneration of cereals such as rice, wheat and perennial ryegrass, but the mechanisms of maltose role in tissue and cell culture media is not yet completely known.

There were significant differences between two cultivars according to plant regeneration rates. Our results are in accordance with previous studies on Kentucky bluegrass (Van Ark *et al*., 1991), Zoysiagrass (Dhandapani *et al*., 2008), and bermudagrass (Li and Qu, 2002). According to the results of Van Ark *et al*. (1991), by adding ABA to regeneration medium, the percentage of calli with somatic embryos or embryo-like structures increased (up to 29.6%) as compared to the control (16.4%). In addition, Dhandapani *et al*. (2008) found that ABA significantly increased embryogenic callus formation from stem nodes, but not from young inflorescences. ABA not only promotes the transition of somatic embryos from the proliferation to the maturation phase (Langhansova *et al*., 2004), but it also enhances embryo quality by increasing desiccation tolerance and preventing precocious germination (Li *et al*., 1997; Robichaud *et al*., 2004; Vahdati *et al*., 2006; Rai *et al*., 2008). Ultimately, based on mentioned authors results, the role of ABA on increasing somatic embryogenesis and regeneration were affected by several factors including origin of explant, physiological status and environmental condition.

Direct regeneration in meristem tip culture

An efficient and rapid method was developed for direct regeneration of two cultivars of perennial ryegrass. The results of meristem tip culture provided good information regarding changings in PGRs and their effects on plantlets growth and development. There was significant difference between two cultivars in plantlet regeneration rate. In 'Grassland', the overall regeneration rate of meristem tips was about 63.5%, while this parameter was approximately 55.7% in 'Numan'.

Meristem elongation and development as a single plantlet on MS medium without PGRs was observed (Fig. 3C). More plantlets were obtained from 'Numan' meristem tips in comparison to 'Grassland' on hormone-free MS medium (Fig. 3D). The same results were previously reported on *Curculigo latifolia* (Babaei *et al*., 2014). In the present study, overall regeneration percentage of both cultivars on MS media was obtained approximately 41%, while in

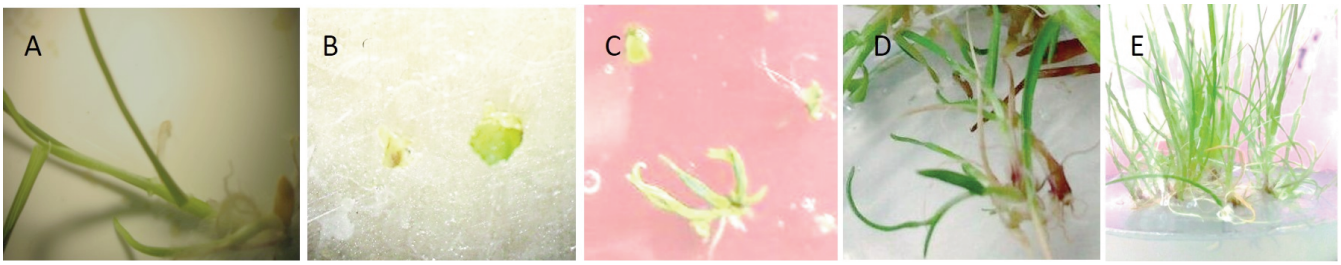

Fig. 3 - Steps of meristem tip culture. A) Ten days old seedling. B) Isolated meristem tips. C) Meristem tip cultured on MS medium. D) Meristem tips cultured on MS medium supplemented with 0.01 mg L^{-1} 2,4-D + 0.2 mg L^{-1} Kin. E) Meristem tips on MS medium supplemented with 0.1 mg L^{-1} 2,4-D +0.5 mg L^{-1} Kin.

Dale (1977) investigation, overall regeneration percentage of nine species of grasses was reported about 11% on hormone free MS medium.

By adding plant growth regulators to the media, more plantlets and tillers grew in both cultivars. As shown in Table 2, an increased regeneration percentage of 35% and 37% by adding 0.01 mg L^{-1} 2,4-D + 0.2 mg L^{-1} Kin and 53% and 48% by increasing Kin from 0.2 to 0.5 mg L^{-1} was observed in 'Grassland' and 'Numan', respectively. These results show that an exogenous supply of growth regulators is required or can be beneficial for the regeneration of whole plants from stem apices as stated in several previous studies (Dale, 1977).

Table 2 - The regeneration percentage from meristem culture of two cultivars of perennial ryegrass on MS hormone-free and with PGRs media

Medium	'Grassland'	'Numan'
MS	43±3.3 de	38±1.15 e
0.01 2, 4-D + 0.2 Kin	58±1.15 c	52±3.06 c
0.1 2, 4-D + 0.5 Kin	89±2.40 a	77±3.71 b

Values represent mean ± SE. Means with the same letter are not significantly different at $p \leq 0.05$.

In his first study on meristem tip culture of *Lolium* species, Dale (1977) stated that the best regeneration rate was obtained on MS medium compared to the other media. The higher regeneration rate was obtained for *L. multiflorum* (92%) on the medium containing 0.01 mg L^{-1} 2,4-D + 0.2 mg L^{-1} Kin and for *L. perenne* (54%) on medium containing 0.5 mg L^{-1} 2,4-D + 0.02 mg L^{-1} Kin. According to our results, the size of the meristem tips cultured usually affects their response in culture. In 'Numan' large meristem tips survived at a higher rate than small ones and generally grew more rapidly. As shown in figure 4, there was a positive relation between survival rate of meristem and meristem size in both cultivar. Differences in survival rate between cultivars also seemed to be related to the meristem tip size. This is in accordance with previous results reported by Dale (1977) in four genera of grasses including: *Lolium*, *Festuca*, *Phleum* and *Dactylis*.

4. Conclusions

In summary, for both cultivars, the best callus induction media with using longitudinally sliced caryopses at 5 mg L^{-1} 2,4-D and 4 mg L^{-1} 2,4-D + 0.02 mg L^{-1} BA was selected. In sliced caryopses more callus induction was observed in both cultivars. The best regeneration medium for them was also recommended. Generally, low concentrations of maltose and ABA was more effective on regeneration rate of two cultivars. In addition, an efficient and rapid procedure for direct *in vitro* regeneration of two perennial ryegrass cultivars has been established. Despites, MS hormone-free medium was an acceptable medium for regeneration of meristem tips but the highest regeneration rate of meristem tips was observed with 0.1 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} Kin in both cultivars. There was a positive relation between length of meristem tips and their survival percentage. It is now possible to test the effectiveness of this technique to produce virus-free plants. Moreover, the results can definitely improve the transformation efficiency of these cultivars.

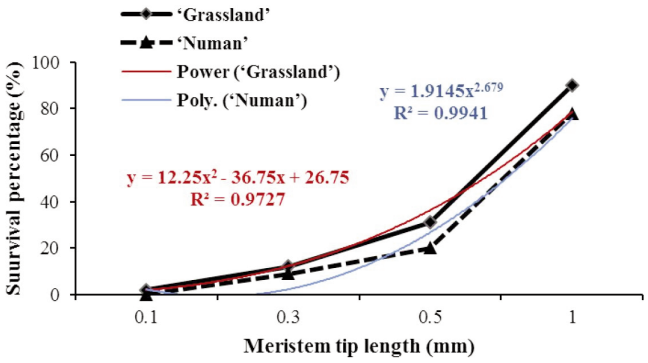

Fig. 4 - Survival percentage as a function of meristem tip length. The red and blue lines are the best fitness for 'Grassland' and 'Numan', respectively.

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The monitoring program of grapevine phytoplasmas in Tuscany (Italy): results of a four year survey

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Key words: bois noir, flavescence dorée, grapevine yellows, phytoplasma.

Abstract: Quantitative PCR protocols for phytoplasma detection were used to monitor grapevine yellows (GY) in 373 vineyards located in nine Tuscan districts. Among more than 70,000 plants visually monitored, 1.867 plants were sampled and "flavescence dorée" phytoplasmas (FD) were detected in 122 plants and mainly identified as trains belonging to 16SrV-C subgroup. The "bois noir" (BN) phytoplasma was found in 734 samples, with prevalence of *tufB* type-b strains. The 2013–2015 monitoring program was strongly influenced by the first survey (2012) in which FD was found consistently in the North West (15 samples), whereas only a few cases were observed in the East territory (2 samples). Both areas were thoroughly monitored in the following years: few foci were found in the East (2 in 2014, 1 in 2015), while several infected areas were found in the North West (6, 10 and 22 foci in 2013, 2014 and 2015, respectively). Definitely, the novel FD foci detected in the survey (17, 6, 12 and 23 in each year of survey) and the widespread of BN, suggest a dangerous distribution of GY in Tuscany.

1. Introduction

"Flavescence dorée" (FD), the most harmful grapevine yellows (GY) in Europe, is present in the northern part of Italy and in some winegrowing areas it occurs simultaneously with "bois noir" (BN) (Bianco *et al.*, 2002; Baric and Dalla Via, 2007). Both diseaases are associated with the presence of phytoplasmas enclosed in 16SrV group, subgroups C and D (Martini *et al.*, 1999) and '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013), respectively. Disease control programs for FD are very expen-

sive in Italy because, due to mandatory uprooting of infected plants, growers must be refunded for yield losses and replanting (Belli *et al*., 2010), while BN control depend on management of wild plants Indeed, grapevines become dead-end hosts for this phytoplasma, therefore the spatial spread of BN most likely does not rely on transmission of the phytoplasma from vine to vine but on other plant species frequently observed in vineyards (Maixner, 1994; Marchi *et al*., 2015). Other phytoplasmas have also been found sporadically in grapevines in Italy, such as strains belonging to ribosomal subgroups 16SrI-B (Alma *et al*., 1996) and 16SrI-C (Landi *et al.*, 2013) of '*Ca*. P. asteris' (aster yellows, AY).

As reported by Belli *et al*. (2010), control measures against GY are being implemented in Italy, targeted mainly to FD because the economic importance of BN infections has emerged only recently, following the extensive use of molecular diagnostic assays. Moreover, the present knowledge of the epidemiology and control of BN (Mori *et al*., 2015; Chuche *et al*., 2016) and FD (Rashidi *et al*., 2014; Casati *et al*., 2017) is not completely defined. However, compulsory control measures involve uprooting and destruction of any vine with GY type symptoms in the area, even before confirmation of FD infection by laboratory tests.

In Tuscany, FD was not known since 2003 (Bertaccini *et al*., 2003) when it was detected in the North-western areas of the region. Thereafter, compulsory control measures against FD were enforced. But successful eradication of FD relies on accurate diagnosis, which should be considered when the monitoring is planned. A strategic role is played by effective sampling of plants to reduce the risk of false negatives, and disease recognition is easier in grapevines affected by single infections; conversely, discrimination between diseases is more difficult in abandoned vineyards or when plants are affected by mixed infections such as mixed virus infections, frequently observed in cv. Sangiovese (Rizzo *et al*., 2012, 2015). The major risks of FD spread may derive from vineyards that are poorly protected against vectors. Thus, badly managed vineyards may represent a good target for FD detection, however such vineyards are frequently characterized by symptomatic or poorly cultivated plants, which result in increasing difficulties in recognizing FD.

In this paper the identification of novel foci of FD in Tuscany during four years of monitoring is reported together with the estimation of the monitoring activity effectiveness.

2. Materials and Methods

Districts sampled

In 2012-2015, 373 vineyards were selected in nine Tuscan districts in the most important grape for wine production areas. Where available, small vineyards (<1 ha) were included in the monitoring, as well as poorly managed vineyards. GY symptomatic samples from 200 plants in each vineyard (20 plants in 10 rows, randomly selected) were collected. Thus, more than 70,000 plants were included in this symptoms survey. Districts were grouped in five areas: North-West (Massa-Carrara, Lucca, and Pistoia), North-East (Prato, Firenze), West (Livorno, Pisa), East (Arezzo, Siena), and South (Grosseto) (Fig. 1).

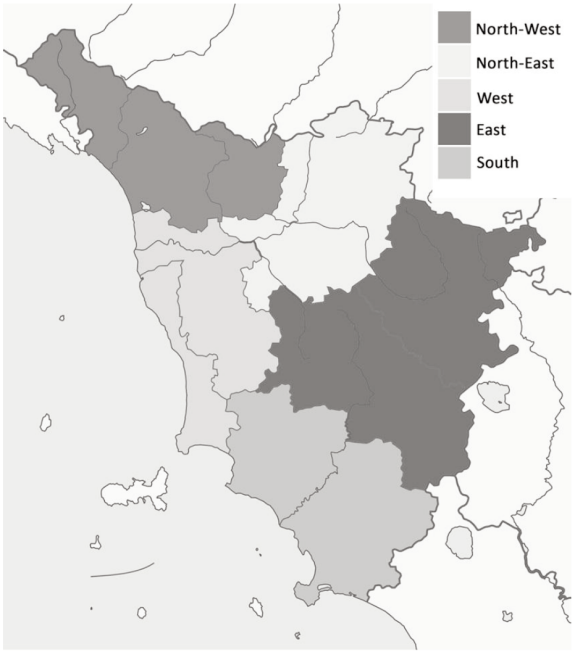

Fig. 1 - Tuscan districts were grouped in five territories: North-West (Massa-Carrara, Lucca, Pistoia), North-East (Prato, Firenze), West (Livorno, Pisa), East (Arezzo, Siena), South (Grosseto).

Sampling procedures

The overall number of vineyards and samples included in the GY monitoring was reported in Table 1. In view of the pre-2012 surveys (Bertaccini *et al*., 2013) the North West areas were considered the most susceptible to FD. In the following years, the number of samples and their distribution was determined based on the results of the previous year. Pathogen findings in North West in 2012 (Fig. 2 a), and first sporadic evidence of FD in Eastern areas (Fig. 2 b) led to increased monitoring activities in these areas, while monitoring in North East and East was drastically increased in 2013 (Table 1). Indeed, in

2013 North East sampling was increased by +574.1% in order to locate and eradicate any further infection site within a previously FD-free territory. Sampling in West was also increased (+125.0%) on the supposition that the disease would spread towards the Southern territories. In 2014, due to the results of the previous year, sample collection was globally similar (+2.3%). In 2015, sampling was concentrated in North West (+43.1%), where the presence of FD was alarming after three years of limited but constant findings (Fig. 2 a). Conversely, sampling in Western territory was reduced (-88.8%) (Fig. 3 a).

Sangiovese was the predominant cultivar sampled (more than 80% of samples). Sampling was never redone in vineyards in which FD was found. Leaf samples were collected from symptomatic grapevine plants during September and October of each year. Each sample, consisted of 10-15 leaves showing typical sectorial reddening of the laminae processed independently (Fig. 4).

Detection methods

Total nucleic acid was extracted from grapevine leaf veins tissues using a variant of the CTAB method (Angelini *et al*., 2001) and a MM400 steel bead mixer

Table 1 - Tuscan vineyards (VY) and samples included in grapevine yellows monitoring

VY position	2012		2013		2014		2015	
	No. VY	No. Sample	No. VY	No. Sample	No. VY	No. Sample	No. VY	No. Sample
NorthWest	40	202	37	186	43	216	62	309
North East	5	26	21	106	20	98	6	32
Weast	5	27	36	182	29	145	17	83
East	6	32	14	72	23	116	3	13
South	0	1	4	18	0	2	0	1
Total	58	288	113	564	115	577	88	438

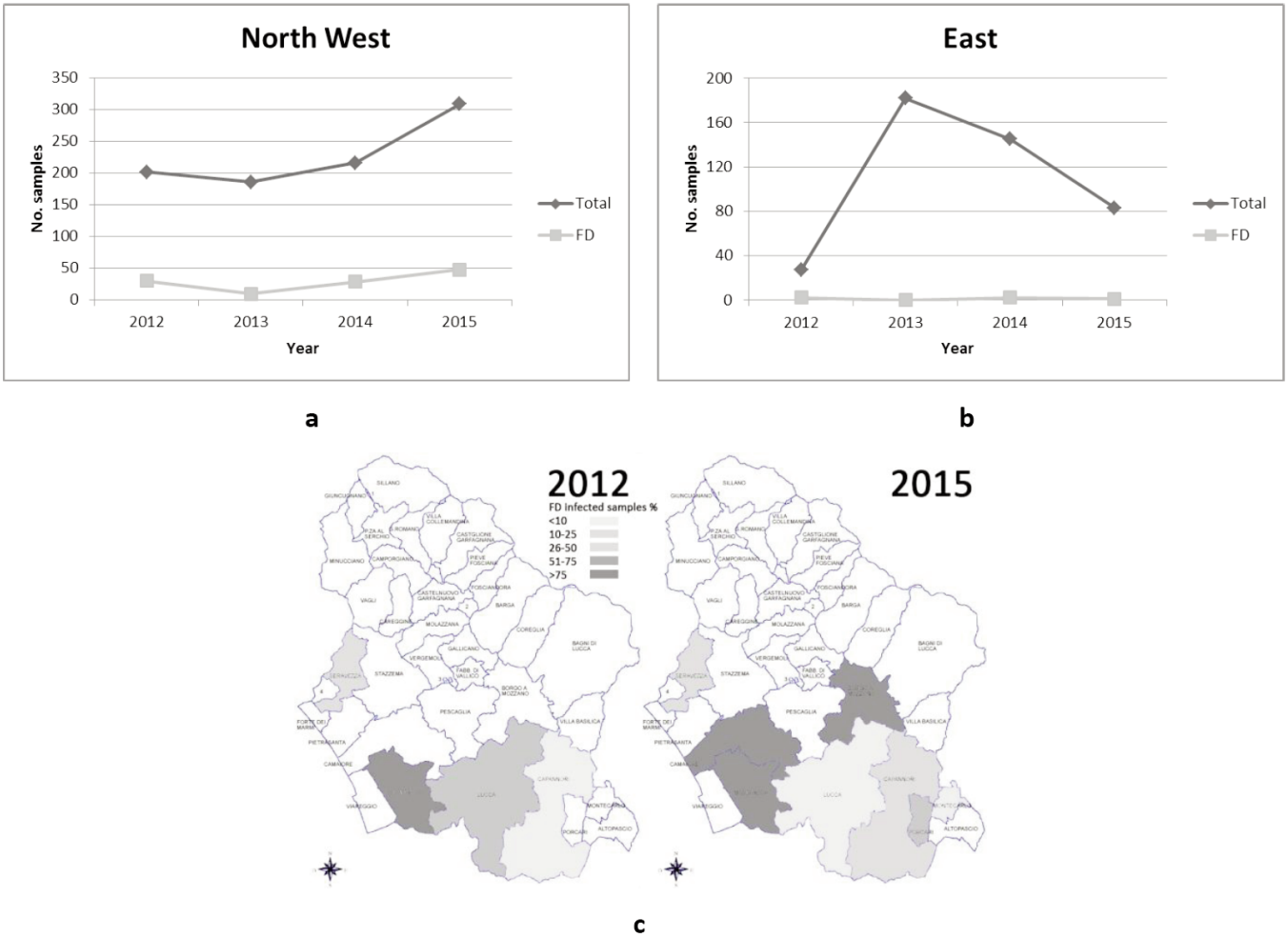

Fig. 2 - Number of samples positive to "flavescence dorée" (FD) out of total samples tested in 2012-2015 in (a) North West and (b) East areas of Tuscany; (c) comparison of incidence of FD in Lucca district in North Western Tuscany in 2012 and 2015.

mill (Retsch, Bergamo, Italy). TNA was re-suspended in TE solution (10 mM Tris; 0.1 mM EDTA; pH 8.0) and aliquots stored at -18°C until further use.

Quantitative PCR protocols targeting the 16S ribosomal RNA (16Sr rRNA) gene were used to determine the presence of the phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII-A and 16SrV (Angelini *et al*., 2007).

Leaves collected by *V. vinifera* plants, previously found infected by 'Ca. P. solani' (subgroup 16SrXII-A), Flavescence dorée phytoplasmas (subgroups 16SrV-C or -D) and '*Ca*. P. asteris' (subgroups 16SrI-B or -C) were used as infected controls (ICs). The infected controls were characterized following Angelini *et al*. (2007) or Berger *et al*. (2009) and conserved by Phytosanitary Service of the Tuscany Region.

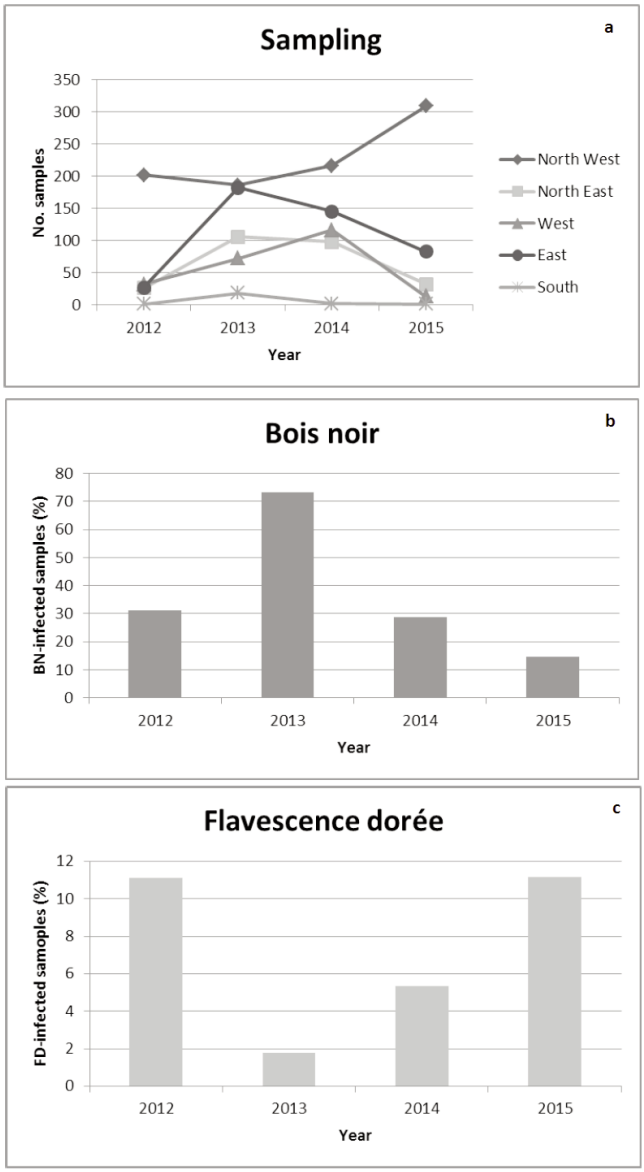

Fig. 3 - Distribution of (a) sampling among Tuscan provinces in 2012-2015. Samples infected by (b) "bois noir" and (c) "flavescence dorée".

A set of ribosomal primer pairs for universal detection of phytoplasma associated to FD was used in nested-PCR: the direct was performed with P1/P7 (Smart *et al.,* 1996) followed by the nested PCR with 16r758f/M23Sr primers (Gibb *et al.,* 1995; Padovan *et al.,* 1995). The nested amplicons obtained were digested with *Taq*I (New England Biolabs, USA), according to the manufacturer's instructions, and digestion fragments were separated through electrophoresis on 3% agarose gel in Tris-borate-EDTA (TBE) buffer. PCR conditions and protocols were as described by Angelini *et al*., 2007.

TaqMan allelic discrimination assay were performed following the protocol as described by Berger et al 2009, using *tufB* type-specific probes carrying different fluorescent dyes. The concentrations of the reagents in the PCR mix, as well as the cycling conditions, were as originally described. Reactions were performed in a CFX96 Real-Time thermocycler (Biorad, USA). Data were analyzed by measuring the threshold cycles (Ct).

Once FD was detected in a vineyard, that vineyard was not further included in the monitoring and PPS started the plant uprooting program.

3. Results

GY monitoring activity in 2012

Among collected samples, 14.9% and 7.4% were positive for FD in North West and Eastern samples respectively (Fig. 2 a, b). A total of 17 novel FD foci were detected, 15 in the North West (mainly in Massa-Carrara and Lucca districts) and 2 in both of the districts in the East (Table 2). No FD was detected in samples from the North East, West or South. In 2012, the highest level of BN presence was found in the Eastern samples (62.9%), while the lowest level of infection was observed in the North West (22.8%) (Fig. 5 a-e). At the regional level, FD was found in 11.1% of the samples, while BN was found in 31.3% (Fig. 3 b, c). AY was found at 0.3%.

GY monitoring activity in 2013

In 2013, sampling was increased in the North East, East and West (+95.8%, globally) (Fig. 3 a). North Eastern vineyards were thoroughly investigated (+307.7% of sampling) because they are situated between the historically infected territory (North West) and the newly infected one (East) (Fig. 2 a, b). No FD-infected sample was found in North East territory, while FD was found only in North West (5.4%).

Globally, 6 novel FD foci were identified, 5 of them in the Lucca district (Table 2). In 2013, infection rate of BN was generally high, reaching more than 80% of collected samples in North East and East territories, while the rate was lower in West (43.1%) (Fig. 5 a-e). At regional level, FD was set at 1.8% and while BN was found in 73.4% of samples (Fig. 3 b, c).

GY monitoring activity in 2014

Sample distribution was also similar to 2013, but more sampling was carried out in West (+61.1%) (Fig. 3 a). FD was still found in North West (13.4%) and few samples was infected in East (1.4%) (Fig. 2 a, b), confirming widespread infection sites in North West and sporadic (but difficult to eradicate) FD presence in Eastern Tuscany. In fact, 12 new foci were detected, 10 in Lucca district and 2 in Siena district (Table 2). With regard to BN, disease rates were lower in 2014 compared to the previous year. More than 50% of samples were positive only in North East or South, while very low infection rate was observed in North West (15.2%) and West (10.3%) (Fig. 5 a-e). At regional level, BN infection rate was quite low (28.8%), while FD apparently (5.4%) increased (Fig. 3 b, c).

GY monitoring activity in 2015

In 2015, the largest number of FD-infected samples was detected since this survey was started, with 15.5% of positive samples in North West and 1.2% in East territory (Fig. 2 a, b). Unfortunately, infected samples were found in many different vineyards, thus 23 novel foci were detected, most of them in Lucca (15), but a consistent number (6) in Pistoia, the eastern district of North West territory (Table 2). Further decrease in BN detection was observed in North East, East and West, where about 25% of samples were BN-positive, while a lower level was recorded elsewhere (Fig. 5 a-e). At regional level, a further increase in FD-positive samples was observed (11.1%), while BN infection was very low (14.6%) (Fig. 3 b, c).

Additional observations on FD monitoring

A comparison between FD findings in 2012 and 2015 in Lucca district of North West territory (where FD findings were numerous) were reported (Fig. 2 c) and pathogen spread seems to be directed towards the South Eastern territories. Moreover, the FD eradication was not achieved in the North West and East territories, despite application of intense monitoring

Fig. 4 - Symptoms of GY on cv. Sangiovese.

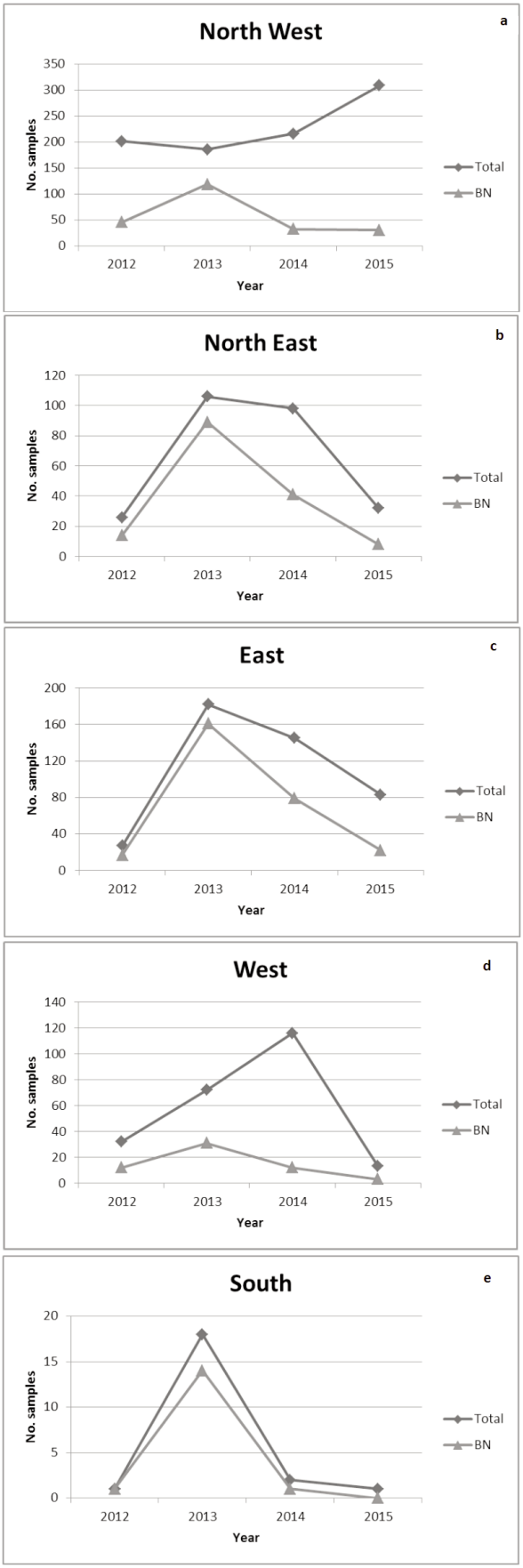

Fig. 5 - Samples positive to "bois noir" in Tuscan areas in 2012-2015.

Table 2 - Findings of novel flavescence dorée foci

Territory/districts	Novel flavescence dorée foci			
	2012	2013	2014	2015
North West	15	6	10	22
Massa-Carrara	*8*	*0*	*0*	*1*
Lucca	*6*	*5*	*10*	*15*
Pistoia	*1*	*1*	*0*	*6*
North East	0	0	0	0
West	0	0	0	0
East	2	0	2	1
Siena	*1*	*0*	*2*	*1*
Arezzo	*1*	*0*	*0*	*0*
South	0	0	0	0
Total	17	6	12	23

programs. With regard to GY characterization, FD-C was most frequently found (Fig. 6 a). Few samples of FD-D were found in North West (Lucca district). Among BN, *tufB* type-b strains were significantly more frequent in all territories except South, were only *tufB* type-a was found (Fig. 6 b).

4. Conclusions

Even though a few FD infected plants were detected in the four years of monitoring, novel foci

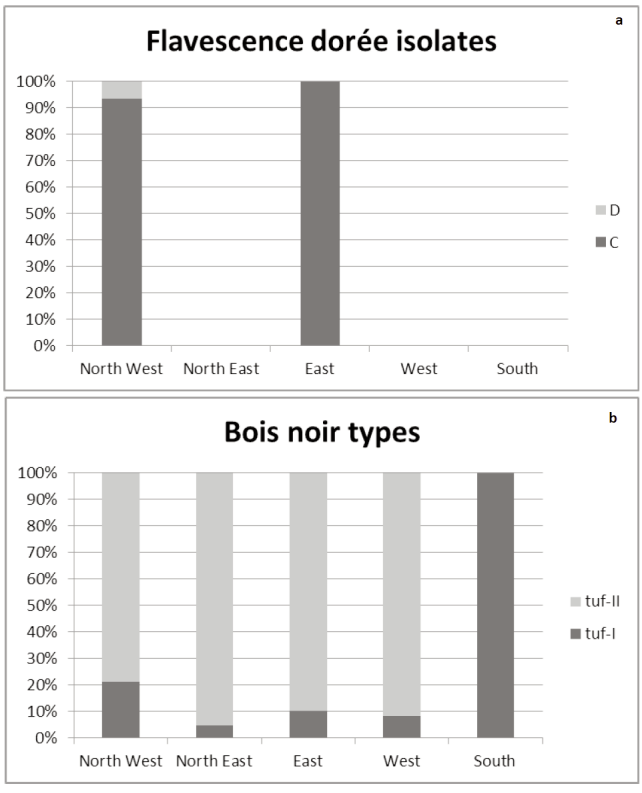

Fig. 6 - Distribution of (a) "flavescence dorée" and (b) "bois noir" subgroups and tufB types respectively in Tuscany.

continued to appear (17, 6, 12 and 23 in each year of survey), suggesting a more severe health condition of Tuscan vineyards and a dangerous distribution of FD in Tuscany.

Indeed, GY constitutes a serious concern for Tuscan viticulture, considering the repeated finding of novel FD foci in the northern part of Tuscany, the first detection of FD in the Eastern territories and the frequent presence of BN in all districts. Comparison between the 2012 and 2015 data in Lucca district indicate how the disease is spreading in the North West of Tuscany despite monitoring. In Lucca district, novel foci were observed each year, whereas the consistent findings of novel FD foci in the eastern district of North West in 2015 was also worrisome. Fortunately, no FD infected samples were found in North East, besides novel FD findings in the East.

With regard to GY characterization, the prevalence of FD-C confirms the results of surveys in North-Eastern territories of Italy (Veneto) (Borgo *et al*., 2001) and North-Western territories of Italy (Piedmont) (Marzachì *et al*., 2001). In Tuscany, *tufB* type-b was predominant over *tufB* type-a in most districts. This evidence is in accordance with data reported by Pierro *et al*. (2018), where the presence of the only *tufB* type-b was identified in a case vineyard in the Chianti Classico area (Tuscany). This strain has, as main host plant of the phytoplasma and of the vector, *Convolvulus arvensis* which high abundance was also reported in Tuscan vineyards (Marchi *et al*., 2015).

True positive rate of GY was overestimated in 2012, 2014 and 2015, probably due to simultaneous foliar symptoms caused by virus and fungal disease. Viruses, which are frequently found in Tuscany (Rizzo *et al*., 2012, 2015), as well as damage due to leafhopper, may mistake sampler. Nevertheless, the percentage of infection was comparable to those obtained in Northern Italy (Marzachì *et al*., 2001; Marzachì and Pacifico, 2006).

Eradication of FD from Tuscany seems a difficult task even in recently colonized territories, probably due to the jeopardized distribution of the pathogen. That may lead, in the subsequent years, to the discovery of many further foci characterized by only a few plants.

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short note

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Growth of potato genotypes under different silicon concentrations

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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 aim of this work was to verify the beneficial potential of Silicon on the growth of potato genotypes in order to select potato genotypes that best respond to Si application. Four potato genotypes were used: SMIJ319-7, Dakota Rose, SMIF212-3 and SMINIA793101, grown in hydroponic system. The plants were transferred to nutrient solutions with four Si concentrations: 0; 0.5; 2.5; and 5.0 mM as $NaSiO_3$. After seven days of exposure to treatments, leaf area, leaf number, shoot length, and fresh and dry weight of roots, stem and leaves were determined. The application of 0.5 mM Si promoted an increase in growth parameters of plants used in this work, mainly in leaf area, leaf number, and leaf and stem dry weight. However, the application of higher concentrations of Si (2.5 mM) promoted reduction in the growth parameters, mainly in leaf area. It was also possible to observe a genotypic variation with respect to Si, SMIJ319-7 and SMIF212-3 genotypes being the most responsive to Si. Therefore, the concentration of 0.5 mM Si is considered optimal for potentiating the growth of potato plants, and SMIJ319-7 and SMIF212-3 genotypes are the most responsive to Si.

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most produced food crops in the world, after rice, wheat and maize (Faostat, 2014), and because of its popularity it is known as "The king of vegetables" (Roy *et al.,* 2017). The current potato production in Brazil exceeds three million tons per year, with a planted area of more than 130 thousand hectares (Faostat, 2014).

Many variables affect the performance of potato plants. Among the ones manageable by man, nutritional management is one of the most important (Westermann and Davis, 1992). In southern Brazil, potato is cultivated predominantly in acid soils, poor in calcium, magnesium and with high aluminum (Al) and manganese contents. Accordingly, there is evidence that silicon (Si) interacts with aqueous Al, thereby reducing its bioavailability (and thus toxicity) and at the same time increasing the

bioavailability of the essential element phosphorus (Dietzel, 2002; Farooq and Dietz, 2015).

After oxygen, Si is the second most abundant element in lithosphere and soil, representing about 28% of Earth's crust (Gunes *et al.,* 2007). Silicon is considered an essential element only for some species belonging to Poaceae and Cyperaceae families (Liang *et al.,* 2005), but the beneficial effects of this element in growth, development, yield and resistance to disease have been observed in a wide variety of plant species (Ma, 2004). Yet it is not possible to demonstrate its essentiality to all higher plants due to the fact that there is no direct evidence that it participates of a molecule or of a constituent or metabolite essential for plants (Epstein, 1999).

The beneficial effects of Si to plants are most evident under conditions of stress (Ma and Yamaji, 2008). Published data suggest that Si increases tolerance to drought (Gong *et al.,* 2005), toxic metals (Gu *et al.,* 2011), UV-B radiation (Li *et al.,* 2004), salt stress (Liang *et al.,* 2003) and resistance of plants to pests and pathogens (Gao *et al.,* 2011). Silicon also alleviates mineral stress, such as manganese (Mn) and aluminum (Al) toxicity, and P deficiency (Iwasaki *et al.,* 2002).

A considerable amount of Si is found in various food products, like grains/cereals and their products (e.g. breakfast cereals, bread, beer), fruit and certain vegetables (bananas, currants, beans, lentils), and all natural waters (Bissé *et al.,* 2005). According to Nielsen (2009), a high consumption of Si may be beneficial, facilitating the absorption or the use of some minerals, including magnesium and copper, which are essential for growth and bone maintenance, cardiovascular health and wound healing.

There is no current information available on beneficial effects of Si on the growth of potato genotypes. Taking into account that about 40% of the world's arable soils have acid pH and toxic concentrations of Al (Ramanujan, 2014), including the soils in Rio Grande do Sul, and that potatoes are grown in a large area in this state, it is important to analyze the effect of Si on potato plant growth. The objective of this study was to analyze the effect of different Si concentrations on the growth parameters of potato genotypes, seeking to select potato genotypes more responsive to Si and the optimal Si concentration.

2. Materials and Methods

The tests were carried out at the Laboratory of Plant Biotechnology and in greenhouses belonging to the Department of Biology at the Federal University of Santa Maria. Four potato (*Solanum tuberosum* L.) genotypes were used: SMIJ319-7, Dakota Rose, SMIF212-3 and SMINIA793101.

Microtubers of four potato genotypes obtained from the Genetics Program and Improvement of Potato, UFSM, Santa Maria, RS, were propagated in plastic cups of 300 mL, using sand as substrate, and irrigated daily with nutrient solution. After about three weeks, 40 uniform plants of each genotype (shoot length of 5 cm) were chosen, the roots were washed in running water to remove the substrate and plants were transferred to hydroponic system consisting of a vessel with capacity of one liter. The plants were exposed to complete nutrient solution for three days for acclimatization. The nutrient solution was as follows (in µM): 6090.5 N; 974.3 Mg; 4986.76 Cl; 2679.2 K; 2436.2 Ca; 359.9 S; 243.592 P; 0.47 Cu; 2.00 Mn; Zn 1.99; Ni 0.17; B 24.97; 0.52 Mo; 47.99 Fe ($FeSO_4$/Na EDTA). After this acclimatization period, the plants were cultured for seven days in a new nutrient solution (pH 4.5±0.1) exposed to four different concentrations of silicon (Si): 0; 0.5; 2.5; and 5.0 mM, as $NaSiO_3$. This pH was used to ensure greater availability of Si, and to prevent its interaction with cations in the solution. The treatments were arranged in a randomized design, with 10 replicates per treatment and one plant for repetition. The nutrient solution with Si treatments was replaced every 48 hours and pH was adjusted daily.

Seven days after the start of exposure to treatments, the plants were collected to determine leaf area (with leaf area integrator AM 300, ADC BioScientific Ltd.), leaf number, shoot lenght (with scale graduated in millimeters) and dry weight of leaves, stem and roots.

For the statistical analysis, the data was checked for normal distribution of errors by the Anderson-Darling test and homogeneity of variances by the Bartlett test for all experiment variables. When these assumptions were met, it was proceeded to variance analysis and Scott-Knott test for treatments at 5% error probability, using Sisvar application (Ferreira, 2008).

3. Results and Discussion

For SMIJ319-7 and SMINIA793101 genotypes, the concentration of 0.5 mM silicon (Si) promoted an

increase in leaf area compared with control (without Si) (Fig. 1A). This result is consistent with studies of other species showing the Si ability, when applied at low levels (Barcelo *et al.,* 1993), to stimulate growth and development of plants, particularly under abiotic and biotic stress (Epstein, 1999; Ma, 2004; Liang et *al.,* 2007; Dorneles *et al.,* 2017).

The increase in leaf area promoted by Si can result in greater interception of solar radiation, and consequently greater accumulation of biomass in these plants. For SMIF212-3 and Dakota Rose genotypes, there were different responses in leaf area among treatments (Fig. 1A), indicating that there is a genotypic variation in relation to Si effects on potato plants. The concentrations of 2.5 and 5.0 mM Si promoted a reduction in leaf area in Dakota Rose genotype compared with control, suggesting that in this genotype, this parameter is more sensitive to high Si concentrations. Besides, the concentration of 2.5 mM of Si also promoted leaf area reduction for SMIJ319-7 and SMINIA793101 genotypes. Silicon is able to interact with essential ions to plants, such as Mg, Zn and Fe (Liang *et al.,* 2005; da Cunha *et al.,* 2008; Naeem *et al.,* 2014), thus the excess of Si may lead to the immobilization of these elements in the apoplast of these plants.

For the SMIJ319-7, SMIF212-3 and SMINIA793101 genotypes, 0.5 mM Si promoted an increase in the leaf number compared to the control (Fig. 1B), while for the Dakota Rose genotype there was no significant difference among treatments. The literature reports that Si plays a favorable role in growth by improving the acquisition of mineral nutrients (Lee *et al.,* 2010), possibly due to a kinetic relationship with some nutrient absorption, thereby causing an increase in biomass production. Thus, due to the pH level used in this work, the effect of Si should take place in the plant, which may lead to a better distribution of nutrients throughout the plant.

The presence of Si in the growth medium significantly influenced on shoot length of potato genotypes (Fig. 1C), where there was significant difference between Si concentrations and control (without Si). For the Dakota Rose genotype, in all Si concentrations, it was observed decreased shoot length compared to control (Fig. 1C). Besides, for the SMINIA793101 genotype, there was a reduction in plant height at 2.5 mM Si. On the other hand, Si concentration of 0.5 mM promoted increase in plant height for SMIJ319-7 and SMIF212-3 genotypes, thus this concentration can be optimal for the growth of potato plants. These data suggest that Si has significant and beneficial effect on this parameter for all genotypes, except for Dakota Rose, which did not obtain a positive response to Si application.

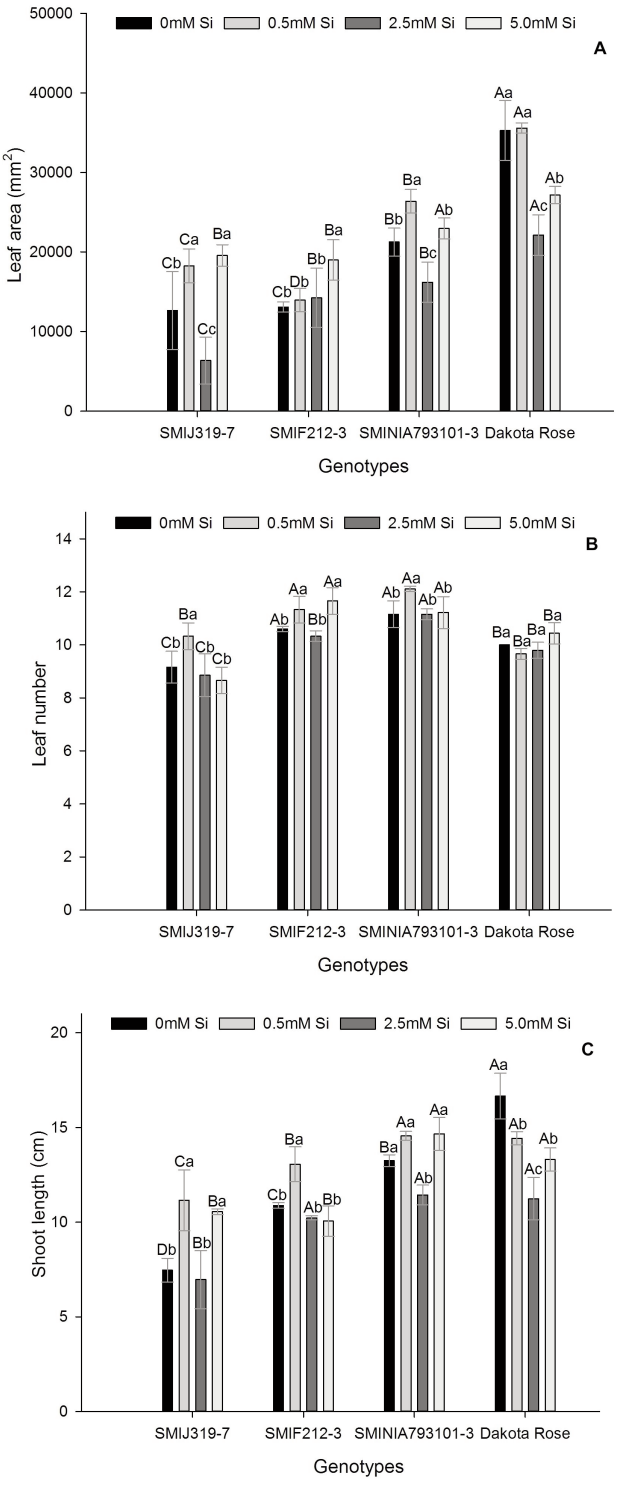

Fig. 1 - Leaf area (A), leaf number (B) and shoot length (C) of potato genotypes exposed to different silicon concentrations in growth medium. Different lowercase letters indicate significant differences between treatments in the same genotype. Different capital letters indicate significant differences between genotypes in the same treatment.

Silicon at 0.5 and 5.0 mM caused a significant increase in yield of leaf dry weight only for SMIJ319-7 and SMIF212-3 genotypes compared to the control (without Si) (Fig. 2A). In SMIJ319-7 plants, increased biomass accumulation induced by Si coincided with increase in leaf area, which may have promoted a greater area of interception of sunlight. For SMIF212-3 genotypes, the increase of leaf dry weight may be explained by Si accumulation in the leaves, because this genotype did not present increase in leaf area. The increase in biomass of potato plants observed in our study might be due to a higher rate of photosynthesis, chlorophyll content and increased activity of enzyme ribulose 1,5-bisphosphate carboxylase caused by Si (Lee *et al.,* 2010). On the other hand, 2.5 mM Si promoted a reduction on leaf dry weight for SMINIA793101 and Dakota Rose genotypes. This reduction in leaf dry weight accumulation caused by the application of 2.5 mM Si in the genotypes SMINIA793101 and Dakota Rose may be due to this concentration not being sufficient for the activation of possible Si transporters, which may be more active in higher concentrations. Some non-accumulating Si species like *Cucumbita moschata* Duch. and *Solanum tuberosum* present differences in the expression of Si transporters with increased application of this element (Mitani *et al.,* 2011 a, b; Vulavala *et al.,* 2016).

For the SMIJ319-7 genotype, Si concentrations (0.5 and 5.0 mM) promoted an increase in the production of stem dry weight compared to control (Fig. 2B). The concentration of 0.5 mM Si also promoted an increase in stem dry weight production for SMINIA793101 genotype. Silicon deposited in tissues can improve light trapping features for maintaining more upright the leaf blade (Epstein, 1999), resulting in increase of plant biomass. This deposition of Si in tissues may have contributed to the increase of biomass due to possible formation of a barrier to transpiracional flow that could lead to higher efficiency use of water (Lux *et al.,* 2002; Shi *et al.,* 2005). On other hand, for the SMIF212-3 and Dakota Rose genotypes, there was a negative effect of Si on the production of stem dry weight.

For the root dry weight, there was significant difference between the Si concentrations in all genotypes, where the presence of 0.5 mM Si promoted an increase in root dry weight compared to control (Fig. 2C). On the other hand, higher concentrations of Si (2.5 and 5.0 mM) generally promoted a reduction in root dry weight compared to control. This indicates that lower Si concentration (0.5 mM) is beneficial for this parameter, but higher concentrations may be detrimental to root dry weight. In addition, this accumulation of root dry weight is in agreement with low stem dry weight for the Dakota Rose genotype, indi-

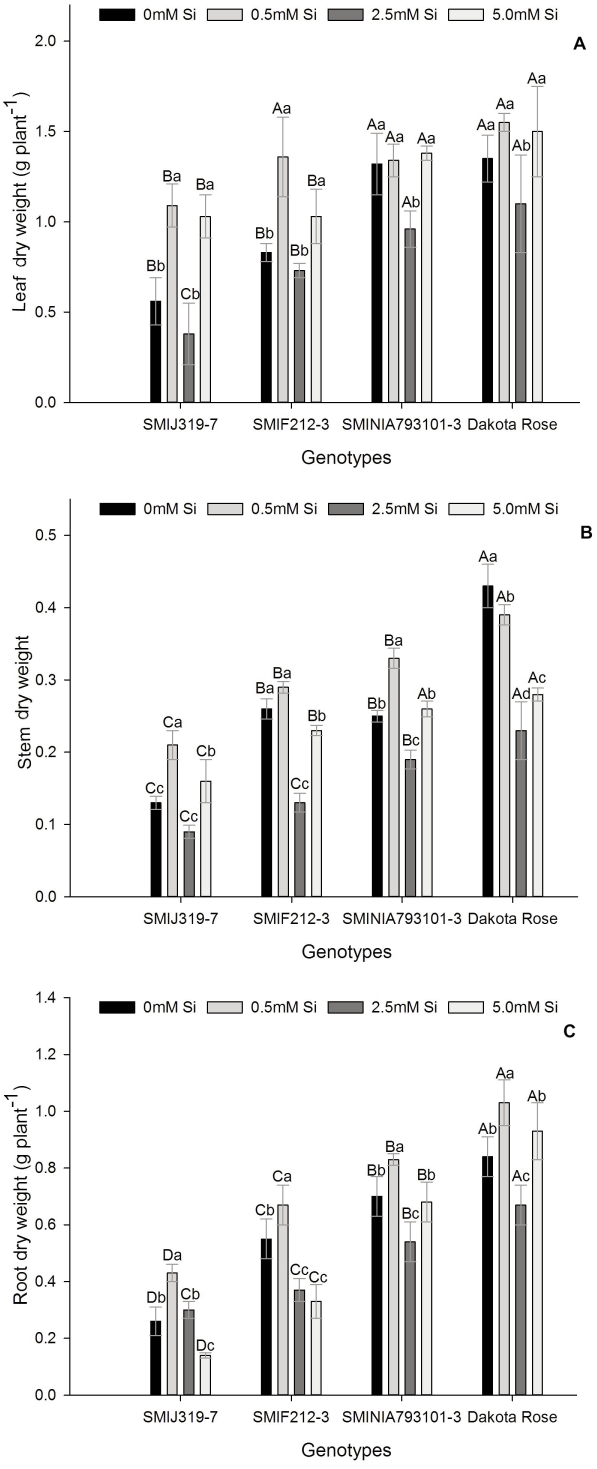

Fig. 2 - Leaf dry weight (A), stem dry weight (B) and root dry weight (C) of potato genotypes exposed to different silicon concentrations in growth medium. Different lowercase letters indicate significant differences between treatments in the same genotype. Different capital letters indicate significant differences between genotypes in the same treatment.

cating a Si effect on the mobilization of resources and their accumulation in roots.

The behavior pattern of the genotypes in relation to Si was similar. However, the Dakota Rose genotype has a higher root dry weight accumulation when compared to the other genotypes. Nevertheless, it is possible to observe that the effect of Si concentrations on root dry weight accumulation for the Dakota Rose and SMINIA793101 genotypes follow a similar behavior pattern. The same may be observed when comparing the behavior pattern of the SMIJ319-7 and SMIF212-3 genotypes, which are also similar. These data show that genotypes SMIJ319-7 and SMIF212-3 are more responsive to Si compared to genotypes Dakota Rose and SMINIA793101. These behavior patterns may be observed on all parameters of this work, and may be explained by differences on expression of putative Si-transporter, which may differ among genotypes and Si concentrations (Mitani *et al.,* 2011 a, b; Vulavala *et al.,* 2016).

At high concentrations (2.5 and 5.0 mM), silicon led to reduction of some growth parameters evaluated in the genotypes used in this study. This response may be due to Si effects on immobilizing and complex cations in plants apoplast (Shi *et al.,* 2005; Moussa, 2006; Farooq and Dietz, 2015). Some of these cations may be nutrients such as zinc, magnesium and manganese, thereby the reduced absorption by the plant may lead to nutritional stress in the long term.

In addition, the silicon deposited in leaves may reduce the transpiration avoiding water loss by plants (Hodson *et al.,* 2005; Farooq and Dietz, 2015). However, the excess of silicon on leaf surface may possibly reduce transpiration so that it reduces photosynthetic processes that depend on gas exchange and constant water flow.

Most research currently focus on Si potential in abiotic stresses mitigation (Dorneles *et al.,* 2016; Pavlovic *et al.,* 2016; Ashfaque *et al.,* 2017). However, studies demonstrating Si effects at high concentrations are scarce and show that high concentrations of this element may reduce the productive and qualitative potential of plants (Marodin *et al.,* 2016; De Souza Ferraz *et al.,* 2017). In addition, some plant species are specialized in absorbing, transporting and accumulating Si in their tissues due to the presence of specific transporters (Farooq *et al.,* 2015; Sanglard *et al.,* 2016). Such carriers may also be present in other species (Mitani *et al.,* 2011 b). Vulavala *et al.* (2016) demonstrated that the genotype 'Winston' of *Solanum tuberosum* has mechanisms of transport responsive to Si. It is possible that the differences in the responses of the genotypes of this study are due to differences in the expression of these transporters. This reinforces the importance of the present study, which provides data for future recommendations for Si application in potato plants.

4. Conclusions

The concentration of 0.5 mM of silicon may be considered optimal, since it induced an increase in growth parameters in potato plants, especially in leaf area, leaf number and biomass of leaves and stems. Furthermore, the most responsive genotypes to Si were SMIJ319-7 and SMIF212-3, possibly due to molecular characteristics such as presence of Si transporters. However, high Si concentrations may lead to unfavorable responses in some potato genotypes.

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