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Development of interspecific hybrids between *Habenaria radiata* and *Habenaria rhodocheila* complex

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Key words: apomixis, cross combination, PCR-RFLP, reciprocal crossing, seed germination.

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The authors declare no competing interests.

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Abstract: Reciprocal crosses between *Habenaria radiata* and *H. rhodocheila* complex were investigated to develop new hybrids. The fruit-setting frequency and seed germination in the cross combination of *H. radiata* × *H. rhodocheila* complex were higher than those of *H. rhodocheila* complex × *H. radiata.* The hybridity of the obtained progenies was confirmed through PCR-RFLP analysis of the rRNA gene. Cross combinations producing true hybrids, apomicts, or both were observed, indicating that both *H. radiata* and *H. rhodocheila* complex were facultative apomixis. The obtained hybrids, *H. radiata* × *H. rhodocheila* (orange flower), showed the intermediate plant form and flower shape of the parents, and both petals and lip were pale yellow.

1. Introduction

Habenaria is a large genus in the family Orchidaceae, consisting of more than 800 species distributed in tropical and subtropical areas such as Southern America, Southern and Central Africa, and East Asia (Pridgeon, 1992; Kurzweil, 2009; Pedron *et al*., 2012; Batista *et al*., 2013; Jin *et al*., 2014). *Habenaria* species show diverse plant forms, flower shapes, and petal colors. There are many *Habenaria* species having high ornamental value, but only a few species are commercialized.

In this study, we focused on two *Habenaria* species: *H. radiata* and *H. rhodocheila*. *Habenaria radiata* is a species native to Japan in the wetlands of Honshu, Shikoku, and Kyushu Islands. This species is low-temperature tolerant. The form of the flowers is unique and beautiful, and the white petals look like a white egret bird. This species has been used as ornamental pot plants (Kim *et al*., 2007, 2010; Mitoma and Kanno, 2018), but it can be used as cut flowers (Sinumporn *et al*., 2015). *Habenaria rhodocheila* is found in Southeast Asia, Laos, Myanmar, southeast China, Thailand, Malaysia, and the Philippines. The flowers of *H. rhodocheila* have a large lip and four lobes, with side lobes and oblique mid lobes. The lips show a wide range of color such as orange, pink, red, and yellow. Formerly, the pink-flowered genotype was accepted under the name *H.*

erichmichelii, and the yellow-flowered genotype was *H. xanthocheila*. The morphological characteristics are also different in each genotype, beside petal color (Kurzweil, 2009; Batista *et al*., 2013). *H. xanthocheila* is distinguished from *H. rhodocheila* in its tuber shape, i.e. *H. xanthocheila* has a crown-shaped tuber but *H. rhodocheila* has a round tuber (Cullen *et al*., 2011). Because these genotypes are very closely related, they are integrated into one species, called *H. rhodocheila* complex. In this report, we adopted the name *H. rhodocheila* complex and distinguished the genotypes only by the color of the petals. Producing hybrids between the two completely different *Habenaria* species, *H. radiata* and *H. rhodocheila* complex, could result in new hybrids having vigor, low-temperature tolerance, and beautiful flower shape with colorful petals.

Recently, many orchid species including *Habenaria* are at risk of extinction. The numbers of both *H. radiata* and *H. rhodocheila* complex are decreasing in their natural habitats, which are being destroyed through urbanization, agricultural use, ecological mismanagement of habitat, changes in climate conditions, and overcollection by people (Stewart and Kane, 2006; Mitsukuri *et al*., 2009; Tanaka *et al*., 2015). Supply of new interspecific hybrids with increased ornamental value is expected to reduce the illegal collection of the species in their habitats. There is little research on intraspecific cross breeding using these two *Habenaria* species. Only a successful of intraspecific cross between wild-type and petaloid-sepal genotypes in *H. radiata* was done (Kim *et al*., 2010; Mitoma *et al*., 2019). In this study, we carried out reciprocal crossing between *H. radiata* and *H. rhodocheila* complex, and evaluated the obtained progenies. This report is the first on successful interspecific crossing of *H. radiata* and *H. rhodocheila* complex.

2. Materials and Methods

Plant materials

Tubers of *H. radiata* 'Aoba' (HRA) (Fig. 1A) were planted in April, every year, in 12 cm plastic pots (5 tubers per pot) with sphagnum moss and tubers of *H. rhodocheila* complex (orange, pink, and yellow petal genotypes, RCO, RCP, and RCY, respectively) (Fig. 1B, 1C, 1D) were planted in 12 cm plastic pots filed with a medium consisting of Growing Mix (Metro Mix 350; Sun Gro Horticulture, MA USA): Kanuma (volcanic porous soil): Vermiculite, 1:2:1. Then, *H. radiata* were placed in a greenhouse in natural temperature with solar radiation. *H. rhodocheila* complex were placed in a growth chamber controlled at a constant temperature of 20°C with solar radiation.

Fig. 1 - Plant morphology of *Habenaria* species used in this study. (A) *H. radiata* 'Aoba', (B) *H. rhodocheila* complex (orange), (C) *H. rhodocheila* complex (pink), and (D) *H. rhodocheila* complex (yellow).

Interspecific cross and in vitro germination

A preliminary crossing experiment, HRA × RCO, was carried out in 2015. HRA and two *H. rhodocheila* complexes, RCO and RCY, were then cross-pollinated reciprocally in 2017. Five plants of each genotype were used in those cross combinations. Twenty plants of HRA and RCP were also cross-pollinated reciprocally in 2017. A total of six cross combinations were made (Table 1). The pollinia of mother plants were removed in advance to prevent self-pollination and the aimed pollinia of other plants were placed on the stigma. Reciprocal crosses were also conducted. Hand-pollinated flowers were labelled individually, and the capsules were harvested before dehiscence about 2 months after pollination. The capsules were surface sterilized with 70% (v/v) ethanol for 30 s and 0.1% (v/v) sodium hypochlorite solution for 15 min

Table 1 - Reciprocal crossing between *Habenaria radiata* and *Habenaria rhodocheila* complex

Cross combination	Number of flowers pollinated	Number of pod sets (%)	Seed germination	Number of plantlets tested PCR-RFLP	Number of true hybrids (%)
HRA × RCO	13	8 (61.5)	++++	36	36 (100)
RCO × HRA	2	2 (100)	-	-	-
HRA × RCP	28	16 (57.1)	+++	21	0 (0)
RCP × HRA	41	12 (29.3)	-	-	-
HRA × RCY	11	1 (9.1)	+	18	1 (5.5)
RCY × HRA	3	1 (33.3)	+	8	0 (0)

HRA = *H. radiate*; RCO = *H. rhodocheila* (orange petal); RCP = *H. rhodocheila* (pink petal); RCY = *H. rhodocheila* (yellow petal).
*++++ = Germination well, ++ = Germination fair, + = Germination poor, - = No germination.

and then rinsed three times with sterilized water. Seeds were removed from the capsules and mixed in a petri dish, and then a batch of seeds picked up with tweezers was placed on a seed germination medium in 5 cm petri dishes. The medium was MM (Malmgren, 1996) supplemented with 20 g/l sucrose and 0.7% agar, and adjusted to pH 5.75 prior to autoclaving at 0.103 MPa pressure and 121°C for 20 min. The cultures were kept at 20°C under dark conditions. Extract numbers of seeds placed on the medium was unknown, the germination was evaluated in four stages: well (+++), fair (++), poor (+) and no-germination (-). Six months after sowing, protocorms were transplanted to 6 × 6 cm plastic culture vessels with MM medium. The protocorm cultures were kept under light inflorescence lamps (FL40S. BRN; Toshiba Lighting & Technology Co. Ltd.) for 16 h with a light intensity of 31.5 µmol m^{-2} s^{-1}at 24°C. The protocorms were subcultured every month. After 12 months, the developed plantlets were acclimatized and planted in 12 cm plastic pots using the same growing medium as for the mother plants. The obtained progenies were grown in a growth chamber controlled at 20°C.

PCR-RFLP

The total DNA of both parents and progenies was extracted from 0.1 g of leaf tissue according to a modified ABBAS DNA extraction method (Abbas *et al*., 2013). PCR was performed in a 50 µl reaction mixture containing 70 ng of total DNA, 0.2mM each of rRNA gene specific primers (5'-ACA CAC CGC CCG TCGCTC CTA-3' and 5'-ACT CGA TGG TTC ACG GGA TTC TG-3'), 2.5 mM dNTPs, 20mM of 10× PCR *Ex Taq* buffer, and 5 U/µl of *Ex Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) according to Haruki *et al*. (1997). PCR was conducted under the following thermocycling conditions: 1 cycle of 96°C, 10 s; 25 cycles of 96°C, 10 s, 55°C, 30 s, 72°C, 60 s; and 1 cycle of 72°C, 10 min.

The amplified products of both the parents and the progenies were digested with selected restriction endonucleases (*Alu* I, *Hha* I, *Rsa* I, and *Sty* I; Nippon Gene Co. Ltd., Toyama, Japan) at 37°C for 1 h. The digested products were separated by electrophoresis in 1.8% agarose gels (Invitrogen, Carlsbad, California, USA) containing 0.1 µl/ml ethidium bromide solution and photographed.

3. Results

Pod set and seed germination in the reciprocal crossings

Six reciprocal cross combinations were made, and the pod set frequencies varied depending on both cross combinations and the ovule parents. The HRA × RCO cross combination resulted in 8 pod sets from 13 flowers (61.5%), and the opposite cross of RCO × HRA resulted in 2 pod sets from 2 flowers (100%). HRA × RCP had 16 pod sets from 28 flowers (57.1%), and the opposite cross RCP × HRA had 12 from 41 flowers (29.3%). HRA × RCY had only 1 pod set from 11 flowers (9.1%), and the opposite cross RCY × HRA had 1 from 3 flowers (33.3%) (Table 1).

All pods were harvested before dehiscence, and the seeds were cultured on MM medium without plant growth regulators. The seeds from the cross combination of HRA × RCO germinated well, but the seeds from RCO × HRA did not germinate. The seeds of HRA × RCP also had rather high germination, but the seeds from RCP × HRA did not germinate. Both cross combinations of HRA × RCY and RCY × HRA had poor seed germination (Table 1).

The sown seeds from HRA × RCO (Fig. 2A) swelled within 50 days of culture (Fig. 2B), developed protocorms around 90 days of culture (Fig. 2B), and the protocorms produced rhizoids (Fig. 2C). After being

subjected to light, the protocorms turned green (Fig. 2D), produced the first leaf within 120 days of culture (Fig. 2E), and then developed plantlets around 150 days of culture (Fig. 2F).

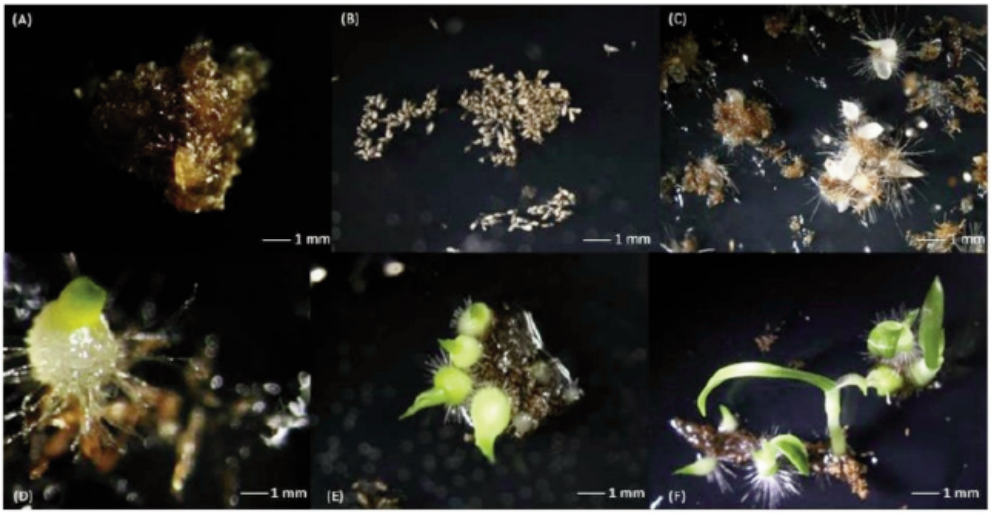

Fig. 2 - Seed development of *Habenaria radiate* × *Habenaria rhodocheila* on MM medium. (A) Sown seeds (day 0), (B) Protocorm development (50 days), (C) Enlarged embryo rupture testa and developed protocorm (3 months), (D) Green protocorm with protomeristem (3 months), (E) Emergence of first leaf (4 month), (F) Plantlets (5 months).

Confirmation of hybridity through PCR-RFLP analysis

To confirm the hybridity of the obtained progenies, PCR-RFLP analysis targeting the ribosomal RNA gene was used according to Haruki *et al*. (1997). The early growth stage of two progenies of HRA × RCO, named WXO1 and WXO2, were used. Expected single PCR product was amplified in all the tested plants. Three restriction enzymes (*Alu* I, *Hha* I and *Rsa* I) that showed polymorphism in the digested PCR products between both parents were applied. The band pattern of both WXO1 and WXO2 was intermediate between both parents (HRA and RCO), indicating that both WXO1 and WXO2 were true hybrids. Thirty-six HRA × RCO progenies in the *in vitro* stage were chosen randomly and their hybridity was tested in the same manner. The results showed that all the tested progenies were true hybrids (Table1) (Fig. 3A). In contrast, an early growth-stage progeny of HRA × RCP, named WXP1, showed a band pattern the same as the female parent (HRA) when the PCR products were digested with *Alu* I, *Hha* I, *Rsa* I, and *Sty* I, suggesting that the plants were apomicts. Twenty-one HRA × RCP progenies in the *in vitro* stage were tested in the same manner. The results showed all the tested progenies were apomicts (Fig.3B). One rapidly grown apomict was designed WXY1 and the one that was judged to be a true hybrid was designed WXY2. Moreover, the results of HRA × RCY (WXY) showed that the progenies included both apomixis and true hybrids (Fig. 3C). The progenies of the reciprocal cross RCY × HRA (YXW) showed the same band pattern as the female parent (RCY), suggesting that they were apomicts.

Morphological characteristics of the obtained progenies

The obtained progenies WXO1 and WXO2 (confirmed as hybrids through PCR-RFLP analysis), WXY1 (assumed to be an apomict), and the parent RCO were grown in a growth chamber controlled at 20°C. HRA grown in a greenhouse without heating was used for morphological comparison.

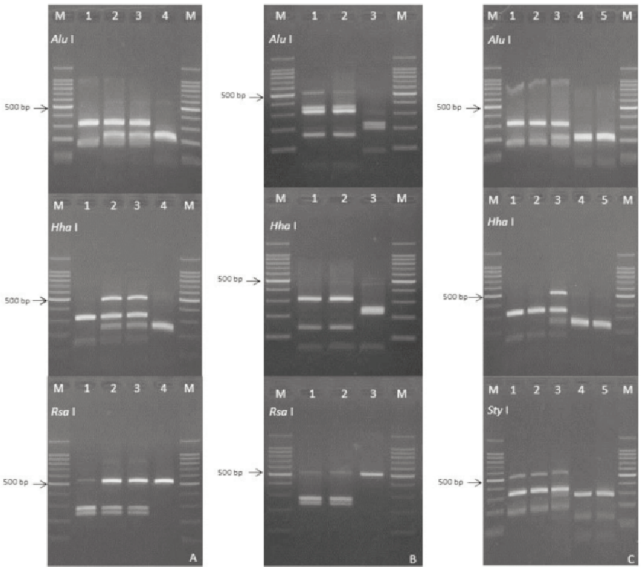

Fig. 3 - PCR-RFLP profile of parents and progenies. A: HRA × RCO. 1. HRA, 2. WXO1, 3.WXO2, 4. RCO. B: HRA × RCP. 1. HRA, 2. WXP, 3. RCP. C: HRA × RCY. 1. HRA, 2. WXY1, 3.WXY2, 4.YXW, 5. RCY.

Both WXO1 and WXO2 grew vigorously, and the first flowering was observed in WXO1 one year after transfer to *ex vitro* and in WXO2 two years after transfer to *ex vitro*. Both WXO1 and WXO2 showed an intermediate plant form (Fig. 4A, 4B, respectively) and leaf morphology of theirs parents. HRA had narrow light green leaves (Fig. 5A). RCO had wide lanceolate leaves with an undulate leaf margin, and the leaves were green or greyish green, sometimes with red-brown spots (Fig. 5D). WXO1 had lanceolate light green leaves, and WXO2 had lanceolate light green leaves with an undulate leaf margin (Fig. 5B, 5C, respectively). The inflorescence morphology of WXO1 and WXO2 was intermediate between the parents. WXO1 had an inflorescence with two flowers, and WXO2 had an inflorescence with eight flowers. *Habenaria radiata* produced two to four flowers (average 2.8 flowers) per inflorescence. RCO had eight to ten flowers (average 9.4 flowers) per inflorescence (Fig. 1B). The inflorescence morphology of WXY1 was similar to HRA, although it could not be accurately determined due to the poor growth of WXY1. HRA had a flower consisting of two pure white petals and a lip with three green ovate sepals. The lip had three main lobes; the two lateral lobes were highly fringed and the center lobe was simple (Fig. 6A). RCO had a flower consisting of two grey-brown orange petals and an orange lip and three sepals. The dorsal sepal was egg-shaped and the slanted lateral sepals sometimes rolled-in. The lip had three main lobes, with the two side lobes elliptical and the middle lip had two ovate-oblong lobes (Fig. 6B). The petals of both WXO1 and WXO2 were pale yellowish, which was an intermediate characteristic of the parents. The sepals of WXO1 and WXO2 were green, with the dorsal sepal being egg-shaped, the same as the female parent (HRA), and the lateral sepals were slanted and rolled-in, the same as the male parent (RCO). In addition, two petals were attached, forming an egg-shaped hood with the dorsal sepal. The lip had three main lobes, with the two lateral lobes being slightly fringed and the center lobe had two slightly ovate-oblong lobes, the same as the male parent (RCO) (Fig. 6D, 6E).The apomixtic progeny WXY1 produced flowers resembling *H. radiata* (Fig. 6F). One progeny derived from the cross combination of HRA × RCY (WXY2), confirmed as a true hybrid through PCR-RFLP analysis, showed an intermediate plant morphology between the parents (data not shown) but had not flowered because only one year had passed after acclimatization.

Fig. 4 - Plant morphology of hybrids. (A) WXO1, (B) WXO2, (C) WXY1.

Fig. 5 - Leaves of the parents and hybrids. (A) HRA, (B) WXO1, (C) WXO2, (D) RCO.

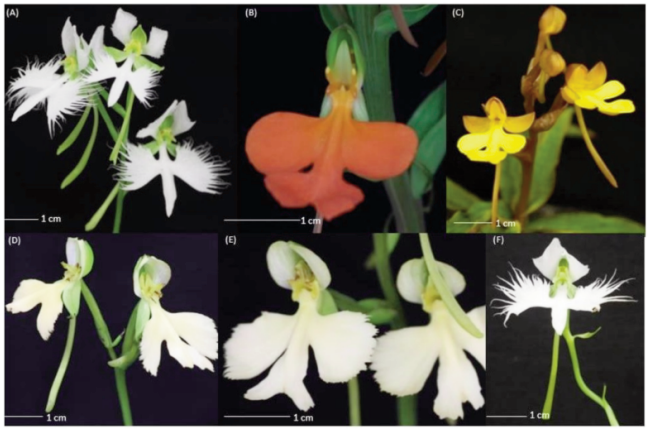

Fig. 6 - Floral morphology of the parents and hybrids. (A) HRA, (B) RCO, (C) RCY, (D) WXO1, (E) WXO2, (F) WXY1.

Plants in the genus *Habenaria* produce a storage organ having species-specific morphology. HRA produced stolons during the growing season, and new tubers formed at the top of the stolon. The HRA tuber was oval with a smooth surface (Fig. 7A). RCO produced a long oval tuber at the bottom end of the stem. The RCO tuber was bigger than that of HRA. The tuber had a rough surface and was densely covered with hair (Fig. 7C). Both WXO1 and WXO2 had

tubers with intermediate morphological characteristics of the parents (Fig. 7B) (Table 2).

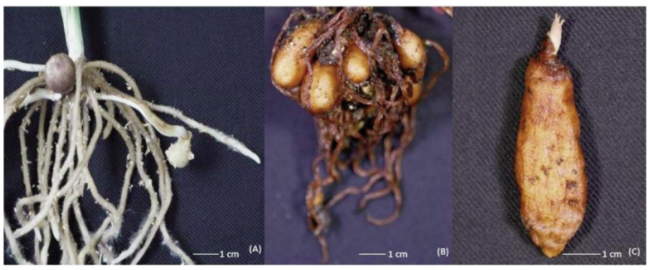

Fig. 7 - Tubers of the parents and hybrids. (A) HRA, (B) WXO2, (C) RC.

4. Discussion and Conclusions

Interspecific hybridization is a powerful breeding method that can produce new traits in ornamental plants including orchids. This study aimed to produce new *Habenaria* hybrids by using two different ecotype species (*H. radiata* and *H. rhodocheila* complex). The two species chosen in this study are different in not only plant morphology but also flowering physiology. Because the flowering time of *H. radiata* is during June to August under natural conditions, *H. rhodocheila* complexes were grown in a growth chamber controlled at 20°C to match the flowering time.

The fruit settings in *H. radiata* × *H. rhodocheila* complex were higher than in the opposite crosses. The germination of the obtained seeds was also different depending on the cross combination (Table 1). The seeds obtained from *H. radiata* × *H. rhodocheila* complex showed higher seed germination than those in the opposite crosses. Unilateral cross incompatibility is often observed in interspecific crossings including orchids (Johansen, 1990; Borba *et al*., 1999). The hybridity of the obtained progenies was investigated by using RFLP analysis. The results showed that all the tested progenies of HRA × RCO were true hybrids, whereas all the tested progenies of HRA × RCP and RCY × HRA were apomicts. Both true hybrids and apomicts were found in the progenies of HRA × RCY. The results of RFLP analysis were consistent with the observed plant morphological characteristics of the obtained progenies. The production of apomixis, including oblique and facultative, is known in some genera including *Habenaria* in the family Orchidaceae (Batygina *et al*., 2003). Zhang and Gao (2018) reported obligate apomixis in *H. malintana*. The present study showed that both true hybrids and apomicts appeared in *H. radiata* and *H. rhodocheila*. Successful crossing of different flower types of *H. radiata* has been reported (Kim *et al*., 2010). Adthalungrong *et al*. (2015) reported successful reciprocal crossing between RCP and RCY. These findings indicate that both *H. radiata* and *H. rhodocheila* do not have obligate apomixis. We consider that the facultative apomixis observed in this study is induced by interspecific crossing between distantly related species. In many plants, pollination is necessary for induction of apomixis (den Nijs and van Dijk, 1993). When HRA was used as a female parent, the frequency of apomict production varied depending on the pollen parents. The results suggest that the growth of the pollen tube in the ovary differed depending on the *H. rhodocheila* complex genotype. In HRA × RCY, only one pod was harvested, and both an apomict and true hybrid were obtained from the pod. This finding suggests that different embryogenesis, sexual and asexual, occurred at the same time. Further detailed morphological and genetic investigation of embryo development in interspecific hybridization is required to explain these phenomena. If the occurrence of apomixis is unpredictable in a practical breeding program, efficient selection of true hybrids is essential. The present study selected true hybrids at the early developmental stage of progenies through PCR-RFLP analysis. Selecting plants in the *in vitro* stage will be useful in a practical breeding program.

Table 2 - Morphology characteristics of two *Habenaria* species and interspecific hybrids

Name	Plant form (cm)		Leaves (cm)		Number of leaves	Flower (cm)		Stigma (cm)	Number of flowers	Inflorescences (cm)	Spur (cm)	Tuber (cm)		Storage organ characteristics
	Height	Spread	Length	Width		Length	Width					Length	width	
H. radiata (n=5)	13.8±2.4	15.1±0.4	6.9±0.7	0.5±0.1	5.0±3.0	3.2±0.1	2.2±0.1	0.3±0.1	2.8±0.4	18.8±2.0	3.3±0.8	1.5±0.1	1.1±0.2	Round-oval with many long stolons
H. rhodocheila (orange) (n=5)	22.3±3.1	23.4±0.6	9.5±1.9	2.2±0.4	6.4±0.2	3.1±0.0	2.3±0.1	0.4±0.1	9.4±1.2	25.8±2.4	3.5±0.5	3.6±1.5	1.5±0.2	Long oval
WXO1 (n=2)	10.8±2.8	9.3±1.0	3.2±0.7	0.8±0.1	4.0±1.0	2.4±0.2	1.6±0.2	0.4±0.1	1.5±0.5	12.9±3.4	3.1±0.1	1.4±0.1	1.0±0.1	Round-oval
WXO2 (n=2)	16.0±2.5	20.4±1.6	7.4±0.1	2.4±0.1	6.0±1.0	2.7±0.1	1.8±0.1	0.3±0.0	8.5±1.5	22.0±1.8	3.3±0.0	1.7±0.2	0.9±0.2	Round-long oval with many short stolons

We obtained new interspecific *Habenaria* hybrids, WXO1, WXO2, and WXY2. Both WXO1 and WXO2 showed intermediate morphological characteristics of the parents (Table 2). It is well-known in Orchidaceae that interspecific hybrids exhibit an intermediate morphological characteristic of parent. When the intermediate characteristics is observed in detail, the characteristics of either the female parent or the male parent often appear strongly in each organ as shown in this study. In case of *Ascocentrum ampullaceum* var. *aurantícum* × *Vanda coerulea*, the progenies showed flowers having pink petal color and spur which come from female parent and orange mottles on the petals come from male parent (Kishor *et al*., 2006). Interspecific hybrids between *Vanilla planifilia* and *V. aphylla* showed two types, light green plants without leaf resembling male parent and green plants with leaves resembling female parent (Divakaran *et al*., 2006). The flowers of WXO1 and WXO2 were pale yellow. The major flower pigment of *H. rhodocheila* complex is considered to be carotenoids (Sinumporn *et al*., 2015). In flowers that contain carotenoids as a main flower pigment, the flowers are orange when the amount of carotenoid is high and yellow when it is low (Kishimoto *et al*., 2007). It is assumed that the carotenoid content of the WXO1 and WXO2 petals is very low because one parent HRA has white petals, resulting in pale yellow petals in the hybrids. The interspecific hybrid of HRA × RCY named WXY2 showed intermediate morphological characteristics in leaves between parents (data not shown). Because that the hybrid was not large enough to give flowering, the floral characteristics was not determine yet. Interspecific hybrids often have flower color intermediate between the parents as reported in the reciprocal crossing progenies of RCP and RCY, which produced pale orange and pink flowers (Adthalungrong *et al*., 2015). The hybrids obtained in this study are new, but the ornamental value is not high enough. Further breeding steps such as backcrossing and self-pollination are required for flower pigment accumulation and flower form improvement. Furthermore, the temperature response of these hybrids is not yet clear because the resulting hybrids were grown under constant temperature conditions. It is necessary to clarify the characteristics of cultivation to evaluate the hybrids.

We obtained new interspecific hybrids between *H. radiata* and *H. rhodocheila* complex. The interspecific hybridization produced both true hybrids and apomicts. The apomicts were distinguished at the early developmental stage of the progenies through PCR-RFLP analysis. The obtained hybrids showed an interesting flower shape and color, which were intermediate between the parents. Further breeding processes, especially back crossing, is required to improve the ornamental value of the hybrids.

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AHS
Advances in Horticultural Science

Effect of foliar spray of calcium lactate on the growth, yield and biochemical attribute of lettuce (*Lactuca sativa* L.) under water deficit stress

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Key words: anthocyanin, antioxidant enzymes, leaf water status, nutrient uptake.

Abstract: The field experiment was conducted to evaluate the effect of foliar spray of calcium lactate (Ca) on fresh yield and biochemical attribute of lettuce (*Lactuca sativa* L.) under water deficit stress, in a split plot form based on a randomized complete block design with three Irrigation regimes (70, 85 and 100% ETc) and three calcium lactate treatment levels (0, 0.75 and 1.5 g L^{-1}) in three replicates. Results revealed that water deficit stress significantly reduced the growth and yield of plant, leaf relative water contents, excised leaf water retention and N, P and Mg absorption while led to increase anthocyanin, phenol and flavonoids contents, antioxidant activity, peroxidase and catalase activity and water use efficiency. The results of our research indicated that the application of CaL 1.5 g L^{-1} is capable of increasing lettuce yield, under field conditions with 30% less than optimal irrigation. CaL treatment showed a clearly protective effect in stressed plants, enhancing their leaf water status, antioxidant capacity and N and Ca contents in comparison to untreated plants. Therefore, feeding leaves by CaL with increasing antioxidant activity and nutrients content especially N led to increase growth and fresh yield of lettuce under normal irrigation and water deficit conditions.

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1. Introduction

Abiotic stresses such as high temperature, drought, salinity and chemical toxicity, are the most important limiting factors to crop productivity. Drought is undoubtedly one of the most important stresses that have huge impact on growth and productivity of the crops (Fahad *et al.*, 2017; Hussain *et al.*, 2018). Water stress is the most prominent abiotic stress limiting agricultural crop growth and productivity (Gholipoor *et al.*, 2013; Ihsan *et al.*, 2016). Deficit irrigation stress as a consequence of the progressive decrease in water availability has been a hot topic regarding food security during the last two decades (UNESCO, 2012). Growth and development of plants is influenced by reduction in turgor that result in decreased nutrient acquisi-

tion from dry soil (Luo *et al.*, 2011). Due to the threat of climate change, there is a need to limit the use of water resources in arid and semi-arid climates. It is therefore important to find new approaches to avoid crop productivity losses in 'limited fresh-water' areas.

Lettuce (*Lactuca sativa* L.), an annual plant of Asteraceae family, is considered as one of the most important salad vegetables as a cool season crop. Lettuce leaves contain vitamins C and E, carotenoids, phenolic acids with anti-free radical activity, and minerals with a lot of fiber, which are an important part of the human diet. Moreover, lettuce contains lactocin and lactucopicrin which improve the quality of sleep (Chakraborti *et al.*, 2002; FAOSTAT, 2016).

Since most of vegetable species are shallow-rooted, they are sensitive to mild water stress. In lettuce production, it is particularly important to preserve optimal growth through a well-scheduled irrigation program, where the harvested part of the plant is the photosynthetic leaf area, (Ahmed *et al.*, 2000; Casanova *et al.*, 2009). Its leaves have high water content and it is sensitive to mild water deficit stress due to its shallow root system (Kizil *et al.*, 2012). Therefore, in lettuce, new strategies will become critical to enhance productivity under deficit irrigation (Malcom *et al.*, 2012).

Foliar application of agro-chemicals has widely been used in agriculture as a rapid, low-cost and effective way for enhancing growth and productivity of many vegetable crops under water deficit stress especially green leafy vegetables like lettuce. Calcium lactate is considered as one of important agro-chemical which can be spray and play important roles in physiological and biochemical processes. Calcium (Ca) is the mineral nutrient most commonly decrease absorption under water deficit condition, so increasing the calcium content in the leafy vegetables could further improve Ca concentrations in plant tissues (Grusak, 2002).

Ca is an essential macronutrient for plant growth and development, and is considered as an important intracellular messenger, mediating responses to hormones, stress signals and a variety of developmental processes. Furthermore, Ca is an important component in the structure of cell walls and cell membranes (Hepler and Winship, 2010). Ca plays a role in the regulation of various mechanisms of plants under environmental conditions such as water stress, heat, cold and salinity. In addition, calcium signaling is required for acquisition of tolerance or resistance to the stress (Cousson, 2009). Positive effect of calcium in improving stress tolerance can be attributed to regulate of water status, antioxidant systems activity, osmolytes accumulation, improving photosynthetic pigment content, and nutritional balances (Kurtyka *et al.*, 2008). Ca plays an important role in oxidative stress signaling, linking H_2O_2 perception and induction of antioxidant genes in plants (Rentel and Knight, 2004). Ca participates in most cellular signaling processes (Sanders *et al.*, 2002) and interacts strongly with reactive oxygen species (Evans *et al.*, 2005).

Since the combined effects of Ca and water deficit stress have hardly been reported, the current study was, therefore, designed to evaluate the influence of foliar application of calcium lactate on the growth, yield and biochemical attribute of lettuce cv. New Red Fire under water deficit stress.

2. Materials and Methods

Experimental design

The field experiment was carried out at the Research farm of the Agriculture faculty, University of Zanjan, Iran, during 2017. The experiment was performed using a split plot based on a randomized complete block design with three Irrigation regimes (70, 85 and 100% ETc) as the main plot and three calcium lactate (CaL) treatment levels (0, 0.75 and 1.5 g L^{-1}) as the sub-plot in three replicates. The soil properties of experimental filed as well as average daily climatic data during the growing seasons was shown in Tables 1 and 2, respectively.

Plant material

Seeds of lettuce (*Lactuca sativa* L.) cv. New Red Fire was obtained from a "Takii seed" company. Lettuce seeds were sown in the nursery on the 2nd of August. Seedlings were transplanted at the 3-4 leaf stage when the seedlings were four weeks old with 25 cm spacing within row and 35 cm spacing between rows that there were about 11.5 plants per square meters (plants m^{-2}).

Table 1 - Soil physical and chemical properties on the site of experimental field

Soil texture	Organic matter (%)	pH	EC (dS m^{-1})	N (%)	Ca (g kg^{-1})	Na (g kg^{-1})	K (g kg^{-1})
Loam clay	0.94	7.4	1.49	0.07	0.12	0.13	0.20

Table 2 - Average daily climatic parameters of Zanjan Synoptic station during the growth seasons (2017) of lettuce

Meteorological parameter	May	June	July	August	September
Rainfall (mm)	0.01	1.11	5.00	0.00	0.02
Average temperature (˚C)	22.94	25.71	27.68	24.79	15.73
Minimum temperature (˚C)	11.29	16.8	17.61	14.68	7.89
Maximum temperature (˚C)	32.47	33.96	36.82	35.12	25.05

Irrigation treatments and calcium lactate applications

After plant establishment, lettuce plants were sprayed with different concentration calcium lactate at 6-7th leaf stage, 10 and 20 days after first spraying for 3 times, during the plant growth. Irrigation treatments were applied one week after the first spraying. All foliar sprayings time were the same and distilled water was used for control treatment. The three irrigation levels were calculated based on actual evapotranspiration (ETc): (1) control, irrigated 100% crop water requirement (I100), (2) deficit irrigation 85% ETc (I85), and (3) deficit irrigation 50% ETc (I50). The Water requirement of the plant for control treatment was estimated using long-term average daily data of meteorological parameters recorded at Zanjan Meteorological Station and following relation.

$$ET_C = ET_0 \times K_C$$

ET_C: Water requirement of lettuce (mm/day), ET_0: Evapotranspiration of grass reference plant (mm/day) and K_C: Vegetable coefficient of lettuce (no unit).

It is necessary to explain that ET_0 values were estimated based on the standard FAO-Penman-Monteith method. Table 2 shows the long-term average of meteorological parameters of Zanjan synoptic station during the period of plant growth which was used to calculate ET0 and ETC values. After calculating the ETC values, the net and gross irrigation water requirements of lettuce were estimated based on cropping intervals, type of irrigation system and irrigation interval and then give the plant at each irrigation time. Based on the calculations, the amount of irrigation water given to the control plants was estimated to be 895.7 $m^3.ha^{-1}$. Water requirement of other treatments was estimated and distributed based on the water requirement of control treatment and water stress (Allen *et al.*, 1998). All necessary management practices such as weeds control were done according to recommended practices during the crop growth.

Measurements

Anthocyanin content. Anthocyanin content in leaf tissue was determined according to the method of Mita *et al.* (2000). Fresh weight of leaves (0.1 g) was homogenized in methanol containing 1% (v/v) HCl and then was filtrated. The filtration was stored at 4°C for 24 hours in dark conditions. The absorbance of filtration was recorded at 550 nm using UV-vis spectrophotometer (Specorp 250 Jena-History) and the anthocyanin was expressed as µmol g^{-1} FW.

Total phenols and flavonoids contents

The fresh leaf tissue (2.0 g) was washed with deionized water, and homogenized in 80% cold methanol (20:80, V/V). The homogenate was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for the measurement of, total phenolic and flavonoid content. Total phenolics assay was carried out according to the procedure described in the literature (Meda *et al.*, 2005). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight based on a standard curve using gallic acid as standard. Total flavonoids were determined by the colorimetric method (Kim *et al.*, 2002). Quercetin was used as a reference standard, and the results were expressed as mg quercetin equivalents per 100 g fresh weight of leaf.

Antioxidant activity

As mentioned in the previous paragraph, 2.0 g of leaves were homogenated in methanol and then was centrifuged. The filtration was used to determine free radical scavenging activity using the 2, 2,-diphenyl-2-picryl-hydrazyl (DPPH) method at optical density 517 nm (Sun *et al.*, 2007). Antioxidant activity (%) was calculated using the following equation:

$$\text{Antioxidant activity} = A_{DPPH} - A_{sample\ (517\ nm)}/A_{DPPH} \times 100$$

Catalase (CAT) and peroxidase (POX) enzymes activity

Samples were taken from the fully expanded leaf and transferred to the laboratory in the ice. Leaf sample (0.5 g) was frozen in liquid nitrogen and ground using a porcelain mortar and pestle.

Catalase (CAT) activity was measured by following the decomposition of H_2O_2 at 240 nm with a UV spectrophotometer (Cakmak and Horst, 1991). Samples

without H_2O_2 were used as blank. The activity of CAT was calculated by the differences obtained at OD_{240} values at 30 second interval for 2 min after the initial biochemical reaction. Peroxidase (POX) activity was measured using modified method of the Tuna *et al.* (2008) with guaiacol at 470 nm. A change of 0.01 units per minute in absorbance was considered to be equal to one unit POX activity, which was expressed as unit g^{-1} FW min^{-1}.

Leaf water status (RWC, ELWR)

The fresh weight of young leaves (FW) was recorded and then was kept in Petri dishes for 24 hours immersed in distilled water. The turgid weight (TW) was measured after saturation of leaves with water. The leaves were dried at 70˚C to constant weight and then weighted (DW). Leaf relative water contents (RWC) were calculated according to the following formula reported by Hanson and Hitz (1982).

$$(\%)\ RWC = (FW-DW) / (TW-DW) \times 100$$

For the determination of excised leaf water retention (ELWR), The youngest leaves collected for each treatment were weighed to record fresh weight (FW), kept at room temperature (25˚C) for 6 hours and reweighed (WL). ELWR was calculated using the following formula suggested by Lonbani and Arzani (2011).

$$ELWR = [1-(FW-WL)/FW] \times 100$$

Nutrient contents

The lettuce leaf samples from each treatment were collected at the end of the experiment. For mineral analysis, leaf samples were taken and oven-dried at 70°C until constant weight. Then 0.3 g of the dry samples was taken and digested using a mixture of sulphuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) as described by Allen *et al.* (1974). All the studied elements were assayed in the digest of the concerned plant samples. Total nitrogen was determined using Kjeldahl method as described by Piper (1950). Phosphorus determination was done by complexing it with ammonium molybdate, which on reduction with ascorbic acid gives stable blue colour, the content of P was measured by spectrophotometer at 882 nm according to Watanabe and Olsen (1965). Potassium, calcium and magnesium content were analyzed by flame photometer (Chapman and Pratt, 1961).

Yield and water use efficiency (WUE)

Lettuce plants were weighed after harvest with a digital gravimetric scale. The average weight of single plant was calculated in grams and total yield was estimated in kg/m^2. Also water use efficiency (WUE) was obtained from the ratio of the amount of yield of each treatment to the amount of water consumed by the same treatment in $kg\ m^{-3}$.

Statistical analyses of the data

The analysis of variance (two-way ANOVA) and least significant difference (LSD) test ($P \leq 0.05$ and $P \leq 0.01$) used to compare means within each sampling date. The Statistical analysis and standard error calculation were carried out using SAS software (v. 9.1).

3. Results

Anthocyanin content

The data in Table 3 and figure 1, displays the anthocyanin contents of lettuce leaf applied with different concentrations of CaL under water deficit. Mean comparisons of data showed that deficit irrigation led to a significant increase in antioxidant activity compared to control. However, the effect of CaL on the anthocyanin contents was depended to the

Table 3 - Variance analysis (ANOVA) of effect of calcium lactate on physiological characteristics in lettuce under deficit irrigation

S.O.V	df	Mean of squares							
		Anthocyanin	Total phenols	Flavonoids	CAT activity	POX activity	Antioxidant activity	RWC	ELWR
Replication	2	1.003	0.002	0.308	0.057	0.003	16.725	8.614	23.677
Irrigation	2	2.162 **	6.187 **	11.932 **	1.953 **	0.851 **	132.47 **	42.799 *	238.260 **
Error (a)	4	5.648	0.135	0.330	0.009	0.001	23.524	9.036	14.106
Calcium lactate	2	4.292 *	1.057 **	24.265 **	0.702 **	0.052 *	318.416 **	186.691 **	420.124 **
Calcium lactate × irrigation	4	2.770 *	0.475 *	1.149 *	0.075 *	0.030 *	43.004 *	1.302 NS	29.661 *
Error (b)	12	6.388	0.121	0.338	0.023	0.008	9.686	9.323	6.176
Coefficient of Variation (%)	-	11. 18	2. 31	4.19	8.87	16.93	3.73	3.9	3.4

** and * represent significance at the 1 and 5% probability levels, respectively, and NS represents non-significance at $p<0.05$.

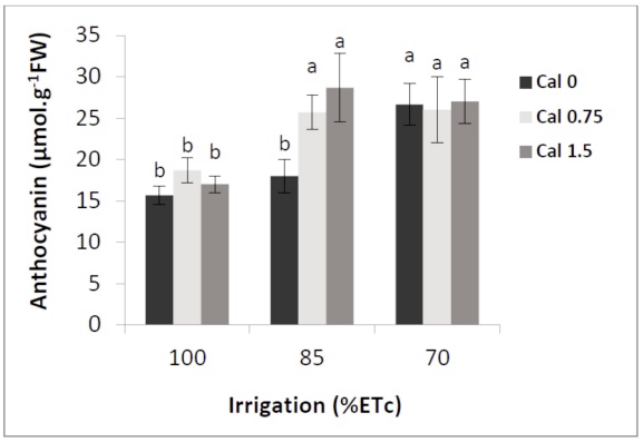

Fig. 1 - Effects of CaL treatments on anthocyanin content of lettuce under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

irrigation regime treatment. The highest value of anthocyanin content was obtained from plant treated with 1.5 g L^{-1} CaL under irrigation 85% ETc. According to the results, there was no significant difference between different levels of CaL under irrigation 100% ETc. However, anthocyanin content at 85% ETc was significantly increased by using CaL while under irrigation of 70% ETc with the increase of anthocyanin content did not show any significant difference between different levels of CaL.

Secondary metabolic products, which are intensively biosynthesized under drought, are antioxidants (Do Nascimento and Fett-Neto, 2010). Anthocyanin pigments as one of secondary metabolites and antioxidative systems play many important eco-physiological roles in plants, including roles in stress protection (Winkel-Shirley, 2002). Increased anthocyanin contents are thought to mask chlorophyll and/or act as a filter for preventing high light absorption by leaves and thus minimize photoinhibition (Farrant, 2000). Therefore anthocyanin accumulation in drought-stressed leaves confirms a possible protective role of anthocyanins as sun-screens and reactive oxygen species (ROS) scavengers in stressed plants (Merzlyak and Chivkunova, 2000), that similar results have also been reported by Jazizadeh and Mortezaei Nejad (2016) in chicory.

The obtained results indicated that CaL was effective in preserving and increasing anthocyanin content. These findings were in agreement with Abd-Elhady (2014) findings who also observed that CaL pretreatments proved to be effective for increasing the retention of anthocyanin in frozen strawberry.

Total phenols and flavonoids contents

The exposure to water deficit stress significantly (P<0.05) increased total phenols and flavonoids contents (Table 3, Figs. 2A, B). Besides, the results of the present study also showed that foliar application of CaL increased total phenols and flavonoids contents under normal and deficit irrigation, however, the effects of CaL was dependent to the irrigation levels. The maximum value of phenols and flavonoids contents was recorded in plant treated with 0.75 g L^{-1} CaL under irrigation 70% ETc. In all levels of irrigation, application of 0.75 g L^{-1} CaL had the greatest effect on total flavonoid content, although did not show significant difference with CaL 1.5 g L^{-1} under irrigation 70% ETc.

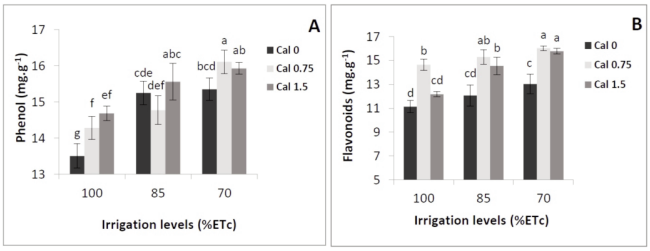

Fig. 2 - Effects of CaL treatments on total phenol and flavonoids contents of lettuce under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

Phenolic compounds include many secondary metabolites in plants that display antioxidant properties (Barbagallo *et al*., 2012). Some of the phenolic compounds, such as phenolic acids or flavonoids, are widely recognized in most of the plant species (Jwa *et al.*, 2006). Phenolic compounds are important because of their contribution to the nutritional quality attributes of fruits and vegetables such as color, astringency, bitterness and flavor (Vinson *et al.*, 2001). The role of phenols as antioxidant is supported by several researches and the recovery methods have a great importance for industrial use (Barbagallo *et al.*, 2012). Environmental stress can cause an increase in the content of phenolic compounds of cell (Weidner *et al.*, 2009).

Aghdam *et al.* (2013) reported that the total phenols and flavonoids contents increased in the cornelian cherry fruit with $CaCl_2$ treated. Their results suggested that $CaCl_2$ treatment may stimulate the accumulation of phenols and flavonoids fruits by activating their biosynthetic pathways. Biosynthesis of phenols such as flavonoids in plants carried out via the shikimate-phenylpropanoid pathways. Ca^{2+} plays

a direct role in the biosynthesis of phenols (Castañeda and Perez, 1996). CaL might be a potential molecule for activating phenyl propanoid-flavonoids pathways of fruits by increasing the PAL activity (Jacobo-Velazquez *et al.*, 2011; Aghdam *et al.*, 2013).

Catalase (CAT) and peroxidase (POX) enzymes activity

Significant differences among irrigation treatments were observed for CAT and POX enzyme activity (Table 3, Figs. 3A, 3B). The antioxidant enzyme activates increased with the decrease of irrigation water applied. As the results showed CAT and POX enzymes activity increased with increasing CaL concentration under deficit irrigation, although no significantly differences was observed in normal irrigation. The highest CAT and POX enzymes activity were recorded in plant treated with 1.5 g L^{-1} CaL under irrigation 70% ETc, which had no significant difference with 0.75 g L^{-1}.

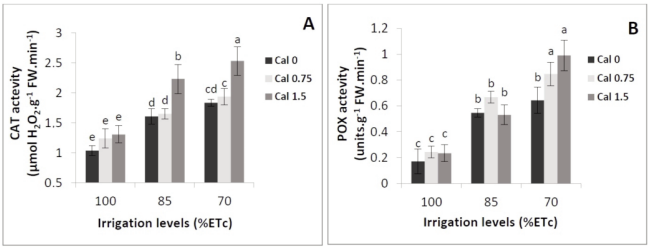

Fig. 3 - Effects of CaL treatments on catalase (CAT) and peroxidase (POX) enzymes activity of lettuce under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

The production of ROS (Vurukonda *et al.*, 2016) is another major factor that impairs plant growth under water deficit (Liting *et al.*, 2015). Plants employ a number of mechanisms, at molecular, cellular and physiological levels to persist stress condition (Shinozaki and Yamaguchi-Shinozaki, 2000). The activation of antioxidant enzymes is one of the major types of these mechanisms which enable plants to control ROS (Shahid *et al.*, 2014). CAT, ascorbate peroxidase (APX), superoxide dismutase (SOD) and POX are the key antioxidant enzymes involved in detoxification of superoxide and hydrogen peroxide (Kadkhodaie *et al.*, 2014).

A relationship between antioxidant enzymes activity and water stress or salinity tolerance was confirmed by comparison of a tolerant cultivar with a sensitive cultivar in several plant species, such as tomato (Mittova *et al.*, 2002).

Calcium is known to regulate different metabolisms in plants mediating signaling pathways, which modulate gene expression in response to stress and its adaptation (Upadhyaya *et al.*, 2011). Upadhyaya *et al.* (2011), observed POX activity was increased in the stressed plant as compared to controls, but recovering plants showed POX activity increasing after rehydration, which was enhanced by $CaCl_2$ and reported that $CaCl_2$ treatment resulted in increased non enzymatic antioxidant and enhanced activities of enzymatic antioxidant, including SOD, POX and CAT, and thus reduced ROS accumulation and lipid peroxidation ultimately leading to improved post-drought recovery potential in *Camellia sinensis*. Calcium applied alleviation of drought-induced damage has been clarified in numerous plants e.g. *Zoysia japonica* (Xu *et al.*, 2013), and *Phaseolus vulgaris* (Abou El-Yazied, 2011).

Antioxidant activity (AA)

AA was affected significantly by the irrigation treatments, and water deficit stress increased AA, which no significant difference was observed between irrigation 100 and 85%ETc (Table 3, Fig. 4). In present study, the exogenous application of CaL significantly (P<0.05) increased AA of lettuce under different irrigation regimes compared to control plant. The highest AA (93.03%) was observed in CaL 0.75 g L^{-1} under irrigation 85% ETc, however had no significant difference with CaL 1.5 g L^{-1} treatment under irrigation 70% ETc (Fig. 4).

The antioxidant activity in lettuce arises from phenolic compounds, secondary plant products, such as

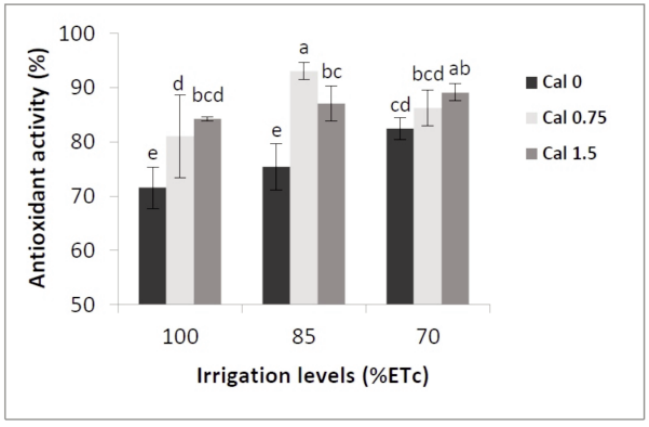

Fig. 4 - Effect of CaL treatments on antioxidant activity of lettuce under deficit irrigation. Values are means with standard errors (n = 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

flavonoids and phenols, and also anthocyanin. Also, the antioxidant activity strongly correlated to the presence of efficient oxygen radical scavengers, such as vitamin C and phenolic compounds (Tulipani *et al.*, 2008).

In current study a significant correlation was found between antioxidant activity and anthocyanins, phenols and flavonoids contents; which the anthocyanin, phenolic and flavonoids content and CAT and POX enzymes activity, as well as the total antioxidant activity also increased with increasing water deficit stress and Cal concentration. This finding described that phenolic compounds and anthocyanin, and antioxidant enzyme activity makes an important contribution to the antioxidant capacity in lettuce leaf. Velioglu *et al.* (1998) reported a strong relationship between total phenolic content and antioxidant activity in fresh fruits and vegetables.

Leaf water status (RWC, ELWR)

Based on the findings (Table 3, Figs. 5A, 6), deficit irrigation caused a significant reduction in RWC and ELWR contents. The application of CaL significantly ameliorated relative water content (RWC) and excised leaf water retention (ELWR) contents (Figs. 5B, 6). Mean comparisons of data, displayed that pretreatment with CaL markedly reduced the effects of water deficit stress and also improved ELWR under control irrigation and water deficit stress. The highest value of ELWR content was obtained in plant treated with CaL 1.5 g L^{-1} under irrigation of 85 and 100% ETc.

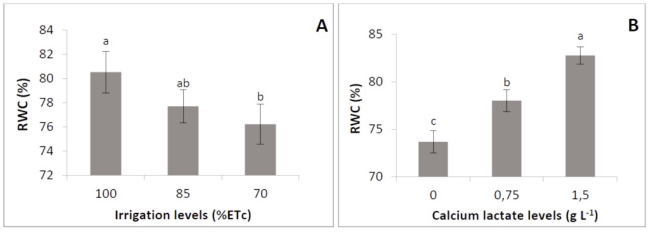

Fig. 5 - Effects of irrigation (A) and CaL (B) treatments on leaf relative water content (RWC) of lettuce. Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

RWC and ELWR are among the main physiological criteria that influence plant water relations and have been used for assessing drought tolerance (Xing *et al.*, 2004). Under drought stress, leaf RWC plays an important role in the tolerance of plants to stress by inducing osmotic adjustments due to the accumulation of osmoprotectants (Barnabás *et al.*, 2008; Zhang *et al.*, 2012). The maintenance of a high plant

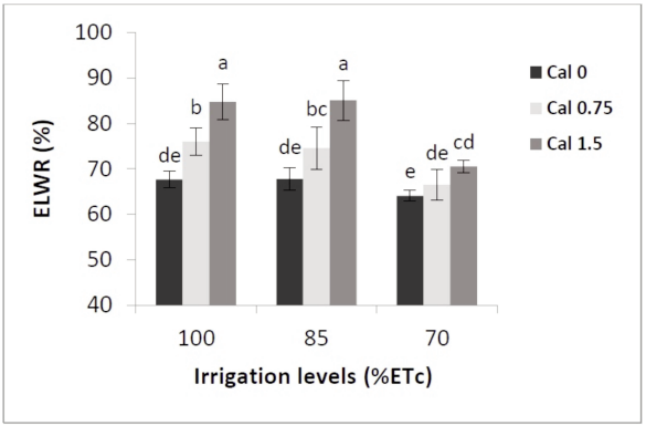

Fig. 6 - Effects of CaL treatment on excised leaf water retention (ELWR) content of lettuce under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that

water status during stress is a significant defensive mechanism to maintain enough water by minimizing water loss (e.g. caused by stomatal closure, trichomes, reduced leaf area, senescence of older leaves, etc.) and maximizing water uptake (e.g. by increased root growth) (Barnabás *et al.*, 2008). Because of the decrease in leaf area, the accumulation of chlorophyll has increased, but due to high transpiration, the plant loses more water and as a result, the RWC of leaf and consequently photosynthesis decreases (Farooq *et al.*, 2012). Farooqi *et al.* (2000) indicated that RWC of lemongrass leaves decreased in all the cultivars due to drought but after rehydration, RWC gradually increased to pre-stress level, which has also been reported in several crop species such as melon (Mani, 2014). As well as drought stress significantly decreased RWC and ELWR in spring safflower (Balian *et al.*, 2015).

Ruiz-Lozano and Azcon (1997) reported that calcium application significantly increased RWC in lettuce. The results of this research showed that the RWC of leaves increased with calcium application. Increasing relative water content means increasing water holding capacity, which can prevent water loss in leaves in a dry environment (Ma *et al.*, 2005).

Nutrient contents

According to the results (Table 4, Fig. 7), N content in lettuce leaves increased with increasing CaL concentration, indeed the highest value of N was obtained at CaL 1.5 g L^{-1} under irrigation 100 %ETc that had significant difference with deficit irrigation treatments (85 and 70% ETc), whereas in other treatments there were not any significant differences. Mean comparisons of data, showed that deficit irri-

Table 4 - Variance analysis (ANOVA) of effects of calcium lactate on nutrient contents and fresh yield in lettuce under deficit irrigation

Source of variations	df	Mean of Squares						
		N content	P content	K content	Ca content	Mg content	Fresh yield	WUE
Replication	2	0.030	0.008	0.008	0.002	0.004	206838.82	0.380
Irrigation	2	0.208 **	0.111 **	0.153 **	0.621 **	2.157 **	21349043.15 **	3.293 **
Error (a)	4	0.008	0.008	0.003	0.005	0.004	83934.71	0.183
Calcium lactate	2	1.158 **	0.024 NS	0.025 NS	0.291 **	0.095 **	6105376.18 **	10.144 **
Calcium lactate × Irrigation	4	0.116 **	0.006 NS	0.010 NS	0.047 **	0.018 *	413961.66 *	0.317 NS
Error (b)	12	0.012	0.006	0.008	0.004	0.005	101407.46	0.170
Coefficient of Variation (%)	-	1.47	3.48	1.43	2.09	3.4	2.82	2.78

** and * represent significance at the 1 and 5% probability levels, respectively, and NS represents non-significance at p<0.05.

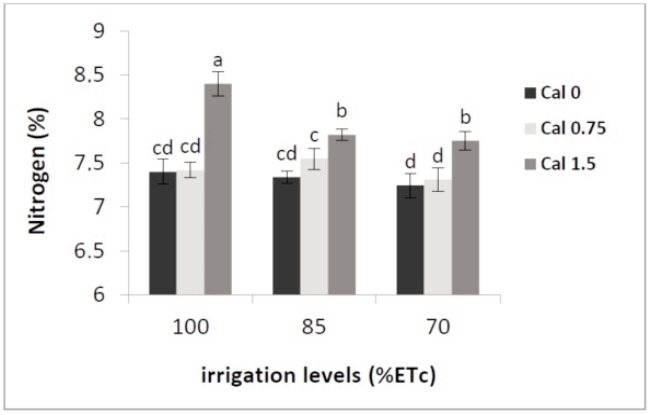

Fig. 7 - Effects of CaL treatments on nitrogen content of lettuce leaves under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

gation significantly increased P content in lettuce leaves and decreased K content compared to control irrigation (Table 4, Figs. 8A, 8B).

The Ca content increased with the deficit irrigation treatments, in particular with moderate deficit irrigation (85% ETc). Ca content in lettuce leaves increased in response to higher CaL concentration (Fig. 9A). In fact, the highest value of Ca was observed in treatments with application of 1.5 g L^{-1} CaL under irrigation 70 and 85% ETc. The overall effect of deficit irrigation on Mg content was negative, with a decrease of 0.79% (Fig. 9B) under deficit irrigation 70% ETc. Mg content in the leaves decreased when the concentration of CaL applied was increased. The lowest value of Mg content was obtained in plant applied with 1.5 g L^{-1} CaL under deficit irrigation 70% ETc.

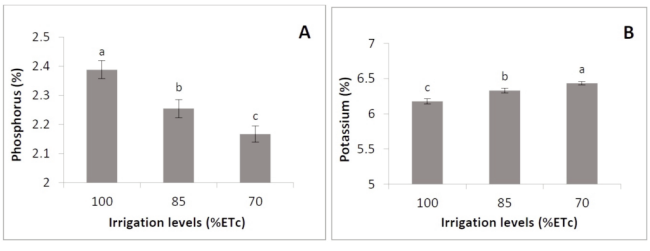

Fig. 8 - Effect of irrigation treatments on phosphorus (A) and potassium (B) contents of lettuce leaves. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

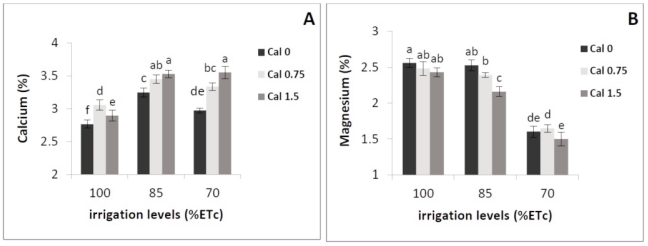

Fig. 9 - Effect of CaL treatments on calcium (A) and magnesium (B) contents of lettuce leaves under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

Drought stress and associated reduction in soil moisture can decrease plant nutrient uptake by reducing nutrient supply through mineralization (Sanaullah *et al.*, 2012), and nutrient diffusion in the soil (Chapin III, 1991; Lambers *et al.*, 2008). Drought can depress plant growth by reducing N and P uptake, transport and redistribution (Rouphael *et al.*, 2012). A majority of studies have indicated that plants decrease N and P uptake with a decline in soil moisture (Sardans and Peñuelas, 2012). N uptake was reduced in maize under stress conditions, which indicates that the absorption of nutrients is limited in conditions of water deficit stress, which may be reduced due to reduced transpiration rate, active transfer and membrane permeability (Naeem *et al.*, 2017). Owing to a reduction in stomatal conductance, photosynthesis and transpiration rates also

decrease, and CO_2 assimilation rates progressively decline in response to drought (Farooq *et al.*, 2012). Therefore, drought effects on plant may depend on the reduction in N and P uptake relative to the decrease in CO_2 assimilation (He and Dijkstra, 2014).

Based on the current findings, increasing K and Ca contents and decreasing Mg content of lettuce leaves under water deficit stress as compared to well-watered conditions that also reported by Tadayyon *et al.* (2018) in castor plants. Potassium has a positive correlation with the physiological effects of plants, such as water use efficiency, stomatal control, air and underground body biomass, and is likely to play an important role in photosynthesis (Sardans *et al.*, 2012). Increasing K content of leaves with decreasing irrigation rate maybe due to role of this cation in the regulation of osmotic pressure and stomatal control, (Zhao *et al.*, 2000). Nahar and Gretzmacher (2002) reported that with increasing deficit irrigation, Mg concentration in tomato tissues decreased, which is similar to results of the present study.

The same results were reported from other authors, that high concentrations of Ca often result in increased leaf Ca along with a marked reduction in leaf Mg (Nassery *et al.*, 1979; Borghesi *et al.*, 2011). As well as, Naeem *et al.* (2018) revealed that concentration of macronutrients (N, K, Ca) in maize grains was markedly improved by foliar supply of calcium which indicates its synergistic effect on uptake and translocation of these nutrients. Tuna *et al.* (2007) also observed leaf N, K and Ca content increased in tomato plants supplemented with calcium under stress conditions.

In safflower, by decreasing soil moisture K and Mg content decreased. Following this reduction, there was a significant increase in calcium concentration, which is justified by the antagonistic relationship between Ca and Mg (Vafaie *et al.*, 2013). Morard *et al.* (1996) reported an intense antagonistic relationship between Ca and Mg, that Mg transfer to leaves was affected by calcium.

Fresh yield

Lettuce plants grown under control and deficit irrigation conditions exhibited significant differences between CaL treatments in fresh yield (Table 4, Fig. 10). Water deficit stress caused significant reductions in yield. In fact, deficit irrigated plants showed a 6.8 and 15.8% decrease in fresh yield, respectively. As the results showed, with increasing CaL concentrations, lettuce yield significantly increased and reached to highest value (1.37 $kg.m^{-2}$) at CaL 1.5 g L^{-1} under irrigation 100% ETc (Fig. 10).

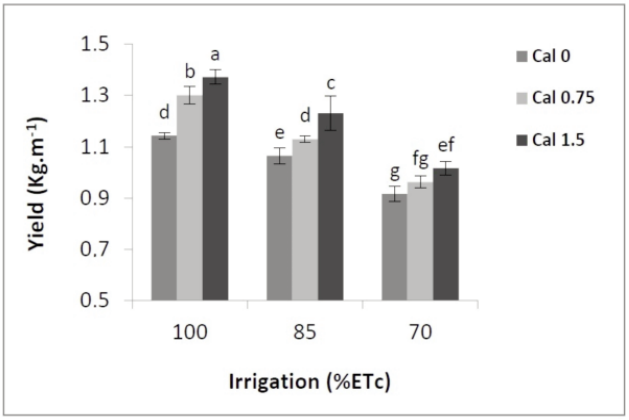

Fig. 10 - Effect of CaL treatments on fresh yield of lettuce under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different $P<0.05$.

Lettuce is one of the leaf-edible vegetables that it is extremely sensitive to water deficit stress due to shallow root system (Sabedze and Wahome, 2010). Our results are in agreement with many open-field studies on lettuce (Jiménez-Arias, 2019) and lettuce (Sayyari *et al.*, 2013). Deficit irrigation defined as a practice that applies water below full crop-water requirements, deliberately exposes plants to a certain level of moisture stress. It is well known that drought stress results in dehydration of the cell and osmotic imbalance that impairs numerous metabolic and physiological processes in plants (Mahajan and Tuteja, 2005). Reduction in fresh yield of lettuce with deficit irrigation might be attributed to the suppression of cell division and expansion, and growth due to the low turgor pressure and also closure of stomata leaf and more leaf senescence under drought stress (Sayyari *et al.*, 2013).

Foliar application of CaL enhanced fresh yield of lettuce. Naeem *et al.* (2013) reported that crop productivity and photosynthetic efficiency in *Senna occidentalis* was improved under Ca application. With low calcium availability, a reduction in bean plant height, leaf area and shoot and root growth has been reported (Leal and Prado, 2008). Foliar applied of chelated calcium enhanced the seed yield and related attributes in common bean under water-deficit conditions (Abou El-Yazied, 2011).

Water use efficiency (WUE)

According to the results (Table 4, Figs. 11A, 11B), irrigation and CaL treatments significantly affected WUE, but their interaction showed no significant differences. Water deficit stress significantly increased WUE and the highest WUE was recorded in 70% ETc deficit-irrigated plants that had no significant differ-

ence with deficit irrigation 85% ETc (Fig 8A). WUE increased with increasing CaL concentration and the highest WUE (15.8 kg m^{-3}) was obtained at 1.5 g L^{-1} CaL (Fig. 8B).

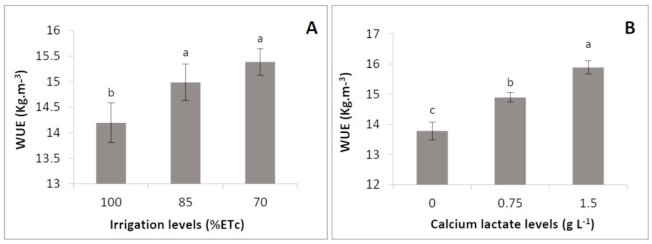

Fig. 10 - Effect of CaL treatments on fresh yield of lettuce under deficit irrigation. Values are means with standard errors (n= 3). Data were subjected to two-way ANOVA and different letters mean that values are statistically different P<0.05.

WUE, the physiological parameter of crop, describes the relationship between plant water use and dry matter production (Cai *et al.*, 2012). Regarding water resource constraints, it is essential to find ways to preserve water and increase water use efficiency in plants (Topcu *et al.*, 2007; Alenazi *et al.*, 2015). The highest WUE value was determined in 70% ETc irrigation. It was calculated that WUE values increased with the decrease in the amount of water. These results are similar to the previous finding of Şimşek *et al.* (2004), who reported that the maximum WUE for watermelon was obtained with low irrigation. With increased WUE, there is a greater biomass production per amount of water transpired, and less water is needed for growth and development (Nemali and van Iersel, 2008).

WUE is strongly related to photosynthetic activity and transpiration efficiency, and can be affected by irrigation (Monneveux *et al.*, 2006). Ca is directly involved in photosynthesis processes, and its deficit reduces the plant's biomass by reducing the efficiency of carboxylation and photosynthesis (Alarcon *et al.*, 1999). The results of the current experiment showed that N content was increased with Ca application, which increasing N content leads to increase dry matter production as well as the WUE. Therefore, Ca maybe increased the WUE by increasing the amount of N and Ca in lettuce plants.

4. Conclusions

The results obtained in this investigation proposed that lettuce is sensitive to water deficit stress during their entire growing period. Hence, it could be concluded that under water deficit, decrease in the relative water content in the leaves is related to the decrease markedly in the fresh yield. Application of CaL showed a clearly protective effect on yield of plants under water deficit stress. The result also revealed that treating the plants with calcium lactate led to increase N and Ca accumulation. CaL appears to promote water deficit tolerance by acting at different levels: leaf water status and antioxidant defenses, without evidence of toxic effects on the soil. Finally, calcium application was determined as an optimum strategy for most desirable traits that decrease stresses effect. However, further studies may be required to determine CaL application rates for optimal response of growth, yield and nutrients uptake.

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The effect of different colored netting on quantitative and qualitative traits of two foliage plant species (*Codiaeum variegatum* and *Aglaonema commutatum*)

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Key words: anthocyanin, brix°, croton, photoselective, yellow netting.

Abstract: Photoselective netting is a new group of colored netting developing in the past few decades. The effect of colored (red, green, and yellow) netting was studied on physiological traits of *Codiaeum* and *Aglaonema* in a trial in Flowers and Ornamental Plants Research Station of Lahijan, north of Iran. The trial was based on a split-plot experiment with two factors. The first factor was devoted to colored netting at four levels (no netting, green, yellow, and red) and the second factor was devoted to plant species at two levels of *Codiaeum* and *Aglaonema* based on a randomized complete block design with three replications. The results showed the positive effect of yellow netting on improving the vegetative capability of the plants, so that the highest plant height, shoot and root fresh and dry weight, and leaf area were observed in the plants grown under the yellow netting. Also, the highest anthocyanin, carotenoid contents, and catalase activity were obtained from the red netting and the highest Brix° and total chlorophyll from the red and yellow netting. According to the results, the highest vegetative growth rate was related to *Codiaeum*. The application of the colored nets provided the plants with more optimal growth conditions.

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1. Introduction

The average number of sunny days in Iran annually amounts to a significant value of 280 days, making it unavoidable to use shading to control incident radiation during sunlight exposure (Forghani and Kiani Abri, 2005). The concept of photo-selective shade netting was first devised in Israel and was tested on ornamental plants, vegetables, and fruit trees. Then, the idea gradually spread to the whole world to be applied to various plants, climatic regions, and agronomic practices (Shahak, 2008). So far, black netting has mostly been used for shading and transparent netting has been employed for protection against environmental or pest damages. A whole new group of protective nettings has been developed

to manipulate both the quality and quantity of radiation intercepted by plants and simultaneously protect them optimally (Shahak *et al.,* 2004 a, b).

The technology of colored nets that selectively filters sunlight and physically protects crops is based on specific nets in which color elements are used during fabrication. This technology seeks to absorb ultraviolet, blue, green, yellow, red, far-red, or near-infrared spectra. The direct light is intended to change into scattered light too. Colored nets can only alter the components of sunlight that penetrates through their plastic strings whereas solar rays that go across the holes are not manipulated (Shahak *et al.,* 2016). It has been documented that yellow and red, as well as grey, netting can significantly increase the productivity of *Capsicum annuum* versus black netting. These results were attributed to the increased number of fruits in a season in plants subjected to the netting (Shahak *et al.,* 2008). In another study by Shahak *et al.* (2016), it was revealed that yellow netting outperformed red netting in stimulating the vegetative growth of *Pittosporum* and among the studied nets, yellow netting exhibited much stronger strengthening effects than red netting. Other studies have also shown that blue netting reduced vegetative growth and induced dwarfness in leafy ornamental plants and cut flowers whereas red and yellow nets that reduced the intensity of blue light induced vegetative growth in plants (Shahak *et al.,* 2016). Photoselective nets can, according to studies, alter shade quality by scattering light and changing its spectral composition (Shahak *et al.,* 2008). Wang and Folta (2013) reported that the colored nets had a significant effect on increasing vegetative growth rate in foliage plants as compared to the black nets with a similar coefficient of shading (Oren-Shamir *et al.,* 2001). The results showed that the red and white nets increased the yield of Cordyline with respect to plant height, leaf number, biomass, leaf area, photosynthesis rate, and harvest index as compared to control. Also, plants under the colored nets exhibited a longer vase life than those in the open air, but there was no significant difference between nets with different colors. According to the results, white and red nets were better for the growth of Cordyline (Kumar Gaurav *et al.,* 2016). Since few studies have addressed the effect of colored netting on vegetative and physiological traits of foliage plants, the present paper explores the impact of color netting on vegetative traits of ornamental *Codiaeum variegatum* and *Aglaonema commutatum* plants in Flower and Ornamental Plant Research Station of Lahijan, Iran.

2. Materials and Methods

The effect of colored (red, green, and yellow) netting was studied on vegetative traits of *C. variegatum* and *Aglaonema commutatum* in Flowers and Ornamental Plants Research Station of Lahijan, north of Iran in the spring and of 2018. The trial was based on a split-plot experiment with two factors. The first factor was devoted to colored netting at four levels (no netting, green, yellow, and red) and the second factor was devoted to plant species at two levels of *C. variegatum* and *A. commutatum* based on a RCBD with three replications. Each experimental plot was composed of four plants. The light intensity in cloudy days was in the range of 4000-5000 lux, No shade net of 6000 lux and on sunny days in the range of 20,000-28,000 lux and No shade net of 30,000-35000 lux.

The recorded vegetative parameters included plant growth rate, plant height, shoot and root fresh and dry weight and leaf area. Dry weight was obtained oven-dried at 105°C for 24 h. To determine leaf area, the leaves length (L) and widest width (W) were measured, and the leaf area (A) was calculated by the following equation (Moll and Kamparth, 1997):

$$A = L \times W \times 0.75$$

The physiological traits included °Brix, chlorophyll *a*, *b* and total, carotenoid, anthocyanin, flavonoid, antioxidant capacity, catalase and peroxidase. The Brix° of the leaves was measured with an N-1α handheld refractometer (ATAGO Co., Japan). To measure chlorophyll contents of the treatments, 0.5 g of sample was weighed and ground in a mortar containing 50 ml 80% acetone. Then, the extract was infiltrated, adjusted to 50 ml, and poured into cuvettes. To determine chlorophyll content, it was read at 643 and 660 nm with a spectrophotometer. Chlorophyll *a* and *b* and total chlorophyll contents were estimated by the following equations (Mazumdar and Majumder, 2003):

$$\text{Total chlorophyll (mg/ml)} = 7.12(A_{660}) + 16.8\ (A_{643})$$
$$\text{Chlorophyll a (mg/ml)} = 993(A_{660}) - 0.777(A_{643})$$
$$\text{Chlorophyll b (mg/ml)} = 17.6(A_{643}) - 2.81(A_{660})$$

To measure the carotenoid level, the treatments were sampled. Then, 0.5 g was weighed from the sample and was ground in a mortar containing 50 ml 80% acetone. Then, it was infiltrated, adjusted to 50 ml , and poured into cuvettes. The extracts were read at 645, 663, and 660 nm and were placed in the following equation, denoted by *A*, to determine

carotenoid levels of the treatments (Mazumdar and Majumder, 2003):

$$\text{Carotenoid level} = 4.69(A_{660}) - 0.268(A_{643}) + 8.02(A_{663})$$

To measure anthocyanin content, 0.5 of the sample was taken and ground in a Chinese mortar containing 50 ml of hydrochloric-ethanol acid (85% ethanol 95% + 15% hydrochloric acid). Then, it was infiltrated, adjusted to 50 ml , and poured into cuvettes. They were placed in a refrigerator at 4°C for 24 hours followed by 2 hours in darkness. The extract was read at 535 nm with a spectrophotometer and was placed in the following equation to determine anthocyanin content (Mazumdar and Majumder, 2003):

$$\text{Total absorption} = \frac{e \times b \times c}{d \times a}$$

where a = sample weight (0.5 g), b = the volume taken for the measurement (5 ml), c = total volume (50 ml), d = the fraction taken for the sample 0.1, and e = absorption read at 535 nm.

$$\text{Total anthocyanin content of sample} = \frac{\text{Total absorption of sample}}{98.2}$$

To measure flavonoid content, 0.1 g of the sample was taken and ground in a mortar containing 85% methanol and was centrifuged at 8000 rpm for 5 minutes. Then, it was infiltrated and the supernatant was placed in a hot bath at 80°C for 10 minutes. Then, it was cooled down and its absor was read at 270, 300, and 330 nm with a spectrophotometer (Humadi and Istudor, 2008).

$$A = \varepsilon bc$$

where: A= absorption rate, ε= extinction coefficient 33000 $m.cm^{-1,}$ b= width of the cuvette (1 cm) and c= total flavonoid content.

In order to estimate antioxidant capacity, 1 g of the plant was wrapped in foil and was placed in liquid nitrogen for 2-3 minutes. Then, it was ground with 10 ml methanol 85% and the samples were placed in room temperature for one hour. Next, their extract was infiltrated and centrifuged for five minutes. Then, 150 ml was taken from it and was added with 850 µl DPPH. The solution was stirred fast and was kept in room temperature at the dark for 20 minutes. After placing the blank and resetting the instrument, first only DPPH was poured into the cuvette and it was read. Then, the sample was read at 517 nm by a spectrophotometer. The antioxidant capacity of the extracts was calculated by the following equation in terms of % inhibition in DPPH (Ramandeep and Savage, 2005):

$$\%DPPH = \frac{A_{cont} - A_{samp}}{A_{cont}} \times 100$$

where %DPPH = percent inhibition, A_{cont} = absorption rate of DPPH, and A_{samp} = absorption rate (sample + DPPH).

The activity of catalase (CAT) was measured through the following stages (Dazy *et al.*, 2008): 1 g of plant tissue that had been ground in 4 ml ethanol was added with (i) 0.01 mol phosphate buffer (pH = 7), (ii) 0.5 ml H_2O_2 0.2 mol, and (iii) 2 ml acid reagent (dichromate/acetic acid mixture). Then, its absorption was read at 610 nm with a spectrophotometer. To measure the enzymatic activity of peroxidase (POD), the extract was prepared as described above. Then, the variations of OD at 430 nm were read with a spectrophotometer once thirty seconds for two minutes (Chance and Maehly, 1955). Data were statistically analyzed with MSTATC Software Package, and the means were compared with the LSD test.

3. Results

According to the results of analysis of variance (ANOVA; Tables 1, 2), the simple effect of netting type and plant species and their interactions were significant ($p<0.01$) on plant height. The tallest plants were obtained from the yellow netting and the shortest from no net exposure. Also, means comparison for the interaction of 'netting type × plant species' revealed that the highest plant height was related to 'yellow net × *A. commutatum*' and the lowest to 'no net × *A. commutatum*'.

According to ANOVA (Table 1), plant growth rate was significantly ($p<0.01$) affected by shade netting, plant species, and 'shade netting × plant species'. Means comparison revealed that the highest growth rate was obtained from the yellow netting (Table 3) and the lowest from no-netting treatment. Means comparison for the interactive effect of 'shade netting × plant species' on plant growth rate showed that 'yellow netting × *C. variegatum*' had the utmost plant growth rate and 'no netting × *A. commutatum*' had the lowest one. According to ANOVA, leaf number was significantly influenced by netting type ($p<0.05$) and plant species ($p<0.01$), but the interaction of these two parameters was insignificant for this trait (Table 1). Means comparison revealed that the highest number of leaves was observed in the plants treated with the yellow net and the lowest in

the plants not treated with the nets (Table 3).

The effect of netting type and plant species was significant ($p<0.01$) on leaf size (leaf length and width), but the interaction of 'net type × plant species' was insignificant (Table 1). The yellow net was related to the highest leaf length and width and the control to the lowest leaf size (Table 3). As well, means comparison for the effect of plant species on leaf size indicated that *C. variegatum* produced the largest leaves (Table 4). ANOVA shows the significant ($p<0.01$) influence of netting type and plant species on leaf area, but the insignificant effect of 'netting type × plant species' on this trait (Table 1). The highest leaf area of 138.4 cm^2 was associated with the application of the yellow net, but control treatment (in which no net was applied) showed the lowest leaf area of 72.19 cm^2 (Table 3). Means comparison for the effect of plant species on leaf area revealed that *C. variegatum* had the highest leaf area (Table 4).

ANOVA revealed the significant ($p<0.01$) effect of shade netting type and plant species on shoot fresh and dry weight, but their interaction was significant

Table 1 - Analysis of variance for the effect of experimental factors on the studied morphological traits

Source of Variables	df	Means of squares											
		Final height	Growth	Initial leaf no.	Final leaf no.	Leaf no. Increase	Leaf length	Leaf width	Leaf area	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Replication	2	4.76 NS	6.74 *	0.58 NS	21.15 NS	14.86 NS	0.97 NS	0.51 NS	264.38 NS	25.99 NS	0.13 NS	1.26 NS	0.006 NS
Netting (A)	3	202.24 **	160.65 **	4.20 NS	189.84 *	153.79 **	41.01 **	7.09 **	4813.22 **	3492.35 **	17.12 **	35.24 **	0.173 **
Error	6	5.54	0.97	5.57	37.95	15.81	0.76	0.58	226.36	85.45	0.42	1.32	0.006
Plant (B)	1	1536.00 **	997.82 **	348.84 **	2095.34 **	734.27 **	173.35 **	19.26 **	16949.00 **	28295.47 **	138.67 **	0.39 NS	0.002 NS
AB	3	42.36 **	50.11 **	3.75 NS	22.95 NS	11.98 NS	13.57 NS	1.89 NS	1448.57 NS	529.42 *	2.59 *	6.80 *	0.032 *
Error	8	1.43	2.28	4.95	25.25	10.24	5.60	0.69	517.91	82.94	0.41	1.01	0.005
C.V. (%)		3.62	14.13	26.23	25.40	28.32	12.93	11.71	22.42	18.07	18.06	21.51	21.79

NS= insignificant difference; **= significant difference at the p<0.01 level; *= significant difference at the p<0.05 level.

Table 2 - Analysis of variance for the effect of experimental factors on the studied morpho-chemical traits

Source of Variables	df	Means of squares									
		Brix	Catalase	Peroxidase	Anthocyanin	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	Flavanoid	Antioxidant capacity
Replication	2	0.29 NS	0.001 NS	0.015 NS	1170.53 NS	1.81 NS	0.31 NS	1.62 NS	0.45 NS	0.001 NS	0.003 NS
Netting (A)	3	15.49 *	0.005 **	0.014 NS	16243.31 **	2.63 NS	0.95 NS	6.56 *	5.68 **	0.000 NS	0.008 **
Error	6	1.74	0.000	0.005	721.97	1.10	0.39	1.04	0.41	0.000	0.001
Plant (B)	1	532.04 **	0.000 NS	0.032 NS	6699.06 **	3.06 NS	0.06 NS	1.66 NS	0.36 NS	0.000 NS	0.003 *
AB	3	0.49 NS	0.000 NS	0.037 **	48512.77 **	2.80 NS	0.28 NS	2.13 NS	1.09 NS	0.001 **	0.007 **
Error	8	0.37	0.001	0.003	5179.48	1.19	0.27	1.92	0.47	0.000	0.000
C.V. (%)		6.97	48.63	13.11	28.83	75.90	56.85	57.60	31.23	10.53	36.02

NS= insignificant difference; **= significant difference at the p<0.01 level; *= significant difference at the p<0.05 level.

Table 3 - Means comparison for the effect of netting type on the studied traits

Netting	Final height (cm)	Growth (cm)	Leaf no.	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Leaf length (cm)	Leaf width (cm^2)	Leaf area	Sugar content (%)	Catalase (UNIT)[1]	Anthocyanin (mg/100 g)	Total chlorophyll (mg/g)	Carotenoid (mg/L)	Antioxidant capacity (DPPH%)
No netting	25.25 c	3.92 c	13.50 b	23.52 c	1.65 c	2.11 c	0.14 c	14.92 c	6.04 b	72.19 c	6.66 b	0.04 b	1.37 b	0.85 b	0.77 b	9 a
Yellow	39.33 a	16.50 a	27.17 a	82.05 a	5.75 a	7.89 a	0.55 a	21.17 a	8.50 a	138.4 a	9.68 a	0.04 b	2.62 b	2.90 a	2.60 a	2 b
Red	33.92 b	11.50 b	19.63 ab	50.25 b	3.52 b	4.82 b	0.34 b	19.17 b	7.33 ab	106.5 b	10.33 a	0.10 a	4.77 a	3.09 a	2.94 a	5 ab
Green	33.33 b	10.88 b	18.83 ab	45.74 b	3.20 b	3.84 bc	0.27 bc	18.00 b	6.46 b	88.92 bc	8.50 ab	0.03 b	1.20 b	2.77 ab	2.50 a	9 a

Similar letter(s) in each column show insignificant differences according to the LSD test.

[1] Peroxidase enzyme unit in µM /H_2O_2 consumed/min/mg.

Table 4 - Means comparison for the effect of plant species on the studied traits

Plant species	Final height (cm)	Growth (cm)	Leaf no.	Shoot fresh weight (g)	Shoot dry weight (g)	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Sugar content (%)	Antho-cyanin (mg/100 g)	Antioxidant capacity (DPPH%)
Codaeum variegatum	40.96 a	17.15 a	29.13 a	84.72 a	5.93 a	21 a	8.98 a	128.08 a	13.50 a	3.02 a	5 b
Aglaonema commutatum	24.96 b	4.25 b	10.44 b	16.05 b	1.12 b	15.63 b	6.19 b	74.93 b	4.08 b	1.97 b	7 a

Similar letter(s) in each column show insignificant differences according to the LSD test.

for this trait (p<0.05) (Table 1). The highest shoot fresh and dry weight was obtained from the yellow netting (Table 3) as means comparison for the effect of shade net type indicated. Likewise, means comparison for the interaction of netting type and plant species showed that 'yellow netting × *C. variegatum*' produced the highest shoot fresh and dry weight without any significant differences from that of 'red netting × *C. variegatum*' and 'green netting × C. variegatum'. The lowest shoot fresh and dry weight was obtained from 'no netting × *A. commutatum*' and 'green netting × *A. commutatum*' (Table 5). The effect of net type was significant on root fresh and dry weight at the p<0.01 level and the interaction of 'net type × plant species' was significant for these traits at the p<0.05 level (Table 1). Means comparison indicated that the highest root fresh and dry weights were obtained from the yellow netting and the lowest were obtained when the plants were barely exposed to radiation (Table 3). According to means comparison for the interactive effect of the treatments, 'yellow net × *A. commutatum*' produced the highest root fresh and dry weight whilst the lowest root fresh and dry weights were obtained from 'no net × *C. variegatum*', 'no net × *A. commutatum*', and 'green net × *A. commutatum*' (Table 5).

ANOVA indicated that degree Brix (°Bx) was significantly influenced by net type at the p<0.05 level and by plant species at the p<0.01 level, but the interaction of 'net type × plant species' was insignificant for this trait (Table 2). The highest degree Brix was related to the red net (10.33%) and yellow net (9.68%) and the lowest to control (6.66%) (Table 3). Means comparison for the effect of plant species indicated that *C. variegatum* had higher Brix of 13.50% (Table 4).

The effect of net type was significant (p<0.01) on catalase enzyme, but this enzyme content was not influenced by plant species and 'net type × plant species' (Table 2). The highest catalase enzyme content was obtained from the red netting showing insignificant differences from other treatments (Table 4). ANOVA showed that the interactive effect of 'net type × plant species' was significant (p<0.01) on peroxidase enzyme, but the effect of shading net and plant species was not significant (Table 1). The highest peroxidase enzyme content was obtained from 'no net × *A. commutatum*' and the lowest from 'red net × *C. variegatum*', 'green net × *A. commutatum*', 'green net × *C. variegatum*', 'yellow net × *A. commutatum*', and 'no net × *C. variegatum*' (Table 5).

Anthocyanin content was significantly (p<0.01) influenced by net type, plant species, and 'net type × plant species' (Table 2). Means comparison indicated

Table 5 - Means comparison for the interactive effect of 'netting type × plant species' on the studied traits

Plant species	Final height (cm)	Growth (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Peroxidase (UNIT)[1]
'No netting × *Codaeum variegatum*'	29.33 c	6.33 cd	44.32 bc	3.10 b	2.09 c	0.15 c	0.34 b
'No netting × *Aglaonema commutatum*'	21.17 d	1.50 d	2.72 d	0.19 c	2.14 c	0.15 c	0.61 a
'Yellow netting × *C. variegatum*'	49.33 a	25.33 a	118.2 a	8.27 a	6.68 ab	0.47 ab	0.43 ab
'Yellow netting × *A. commutatum*'	29.33 c	7.67 c	45.93 b	3.22 b	9.10 a	0.64 a	0.40 b
'Red netting × *C. variegatum*'	42.83 b	17.83 b	88.31 a	6.18 a	5.20 bc	0.36 bc	0.33 b
'Red netting × *A. commutatum*'	25.00 d	5.17 cd	12.19 cd	0.85 c	4.43 bc	0.31 bc	0.45 ab
'Green netting × *C. variegatum*'	42.33 b	19.08 b	88.10 a	6.17 a	5.21 bc	0.36 bc	0.39 b
'Green netting × *A. commutatum*'	24.33 d	2.67 cd	3.38 d	0.24 c	2.49 c	0.18 c	0.33 b

Similar letter(s) in each column show insignificant differences according to the LSD test.

[1] Peroxidase enzyme unit in µM /H_2O_2 consumed/min/mg.

that the highest anthocyanin content was obtained from the red net (Table 3), but the other treatments did not differ significantly to one another. According to means comparison for the effect of plant species on anthocyanin content, the highest content was observed in *C. variegatum* (Table 4). Also, means comparison for the interactive effect of 'net type × plant species' on anthocyanin content indicated that 'red net × *A. commutatum*' had the highest anthocyanin content and 'green net × *A. commutatum*' and 'no net × *A. commutatum*' exhibited the lowest one (Fig. 1).

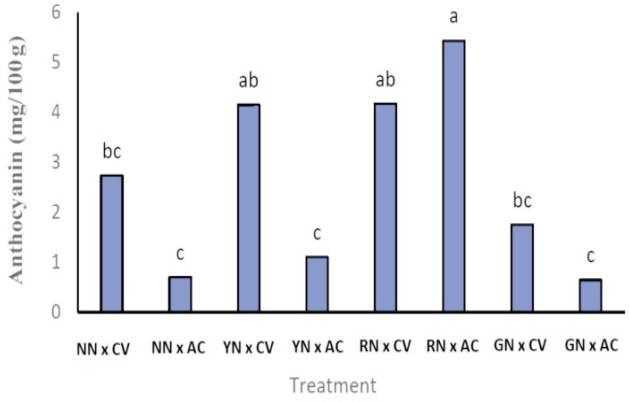

Fig. 1 - The effect of 'netting type × plant species' on anthocyanin content. NN= no netting, YN= yellow netting, RN= red netting, GN= green netting, CV= *Codaeum variegatum*, AC= *Aglaonema commutatum.*

The results of ANOVA showed that the effect of net type was significant ($p<0.05$) on total chlorophyll content, but the effect of plant species, net type, and 'net type × plant species' was insignificant on chlorophyll a and b and so was the effect of plant species and 'net type × plant species' on total chlorophyll (Table 2). Means comparison for the effect of net type on total chlorophyll (Table 3) indicated that the highest total chlorophyll was related to the red and yellow nets and the lowest to no-net treatment.

Shading net type influenced carotenoid content of plants significantly ($p<0.01$), but the trait was not significantly affected by plant species and 'net type × plant species' (Table 1). Means comparison showed that the plants grown under the red nets had a higher carotenoid content but without any significant differences from those grown under the yellow and green nets. The lowest carotenoid content was related to control (Table 3). The results of ANOVA showed that the interaction of 'net type × plant species' was significant ($p<0.01$) for flavonoid content, but the effect of net type and plant species was insignificant on this trait (Table 2). The highest flavonoid content was obtained from 'green net × *C. variegatum*' and the lowest from 'red net × *C. variegatum*' (Fig. 2).

Antioxidant capacity was significantly influenced by netting type and 'netting type × plant species' at the $p<0.01$ level and by plant species at the $p<0.05$ level (Table 2). *A. commutatum* plants grown under the yellow net and control plants had the highest antioxidant capacity, while the lowest capacity was related to the yellow net (Table 3). Also, it was found that *A. commutatum* had a higher antioxidant capacity than *C. variegatum* (Table 4). Means comparison for 'net type × plant species' revealed that the highest antioxidant capacity was related to 'no net × *A. commutatum*' and the lowest to 'yellow net × *C. variegatum*' and 'yellow net × *A. commutatum*' (Fig. 3).

4. Discussion and Conclusions

Based on our results the highest plant height was related to 'yellow net × *A. commutatum*'. In a study on the effect of colored nets on Cordyline, Kumar

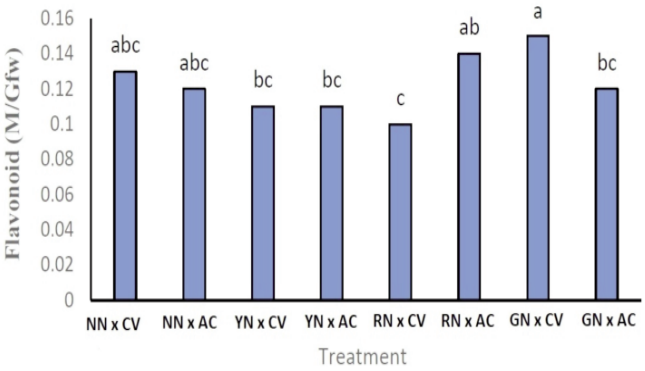

Fig. 2 - The effect of 'netting type × plant species' on flavonoid content. NN= no netting, YN= yellow netting, RN= red netting, GN= green netting, CV= *Codaeum variegatum*, AC= *Aglaonema commutatum.*

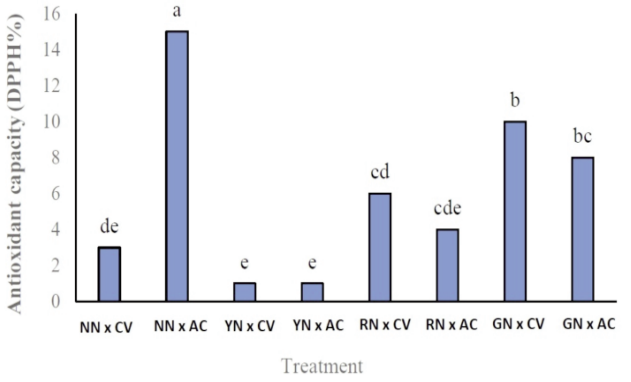

Fig. 3 - The effect of 'netting type × plant species' on antioxidant capacity. NN= no netting, YN= yellow netting, RN= red netting, GN= green netting, CV= *Codaeum variegatum*, AC= *Aglaonema commutatum.*

Gaurav *et al.* (2016) stated that plants grown under colored shade nets exhibited variable growth due to the spectral effect on plant growth. They reported that the Cordyline plants grown under white and red shade nets were taller than the control plants (not exposure to shade nets). It has been reported that both red and yellow shade nets enhanced the vegetative traits of Aralia including stem length and thickness, petals, and leaf dimensions and generally increased the yield of commercial cut flowers (Oren-Shamir *et al*., 2001; Shahak, 2008). Shahak *et al.* (2016), also, found that the yellow shading net induced vegetative growth of Pittosporum. The highest growth rate was observed in the plants exposed to the yellow nets. It has been argued that yellow nets outperform red nets in inducing vegetative growth, probably because of the inductive effect of supplementary artificial green light (Oren-Shamir *et al.*, 2003; Kim *et al.*, 2004). In a four-year research study on cut flowers in Besor Research Station (2000-2003), an increase was reported in stimulatory capacity and in growth under yellow and red nets (Shahak, 2008; Ovadia *et al*., 2009). There is a report that lettuce produced the highest number of leaves under colored nets (pearl and red nets) and the lowest number in control (no net application) (Ilić et al., 2017). It can be concluded that different plant species differ in their growth and development responses to the spectra generated by different colored nets. Leaf area is a crucial parameter for growth. It is defined as a plant's capacity to synthesize dry matter in terms of radiation use rate and photosynthesis rate. Kumar Gaurav *et al*. (2016) showed that leaf area of Cordyline was higher under colored shade netting than control (no shade net application). Colored nets influenced both the length of growth period and morphological traits of lettuce so that they enhanced leaf area index and shortened the length of growth period significantly (Ilić *et al.*, 2017).

In the present study, the yellow net resulted in higher fresh weight than the other nets and control. In addition, the highest plant growth was related to the yellow net, implying the impact of yellow nets on quantitative and qualitative traits of plants. This leads us to the conclusion that colored shade nets, especially yellow nets, can enhance plant biomass. In Kumar Gaurav *et al*.'s study (2016), the highest leaf fresh weight (85.88 percent higher in the red net than in the control) and leaf dry weight were obtained from the red net and overall, the colored nets had the strongest impact on this trait when compared to control (no net application).

Brix is a measure of sugar content of the solution and depends on radiation diffraction. It represents the percentage of solid material weight of a solution to the total weight of the solution. Our results revealed that flowers grown under the red and yellow shade nets had higher degree Brix and this may be related to the stimulatory effect of artificial green light under the yellow nets (Kim *et al*., 2004). Hydrogen peroxide is the most stable form of reactive oxygen species. It has been suggested that hydrogen peroxide is toxic to cells. Catalase and peroxidase play a crucial role in inhibiting the accumulation of hydrogen peroxide. These enzymes are abundant in aerobic microbes, but anaerobic microbes lack them (Singh, 2003). It has been documented that peroxidases are involved in many cell processes such as auxin metabolism, wood formation, traverse linkages in plant cell walls, response to environmental stresses, and so on (Yamasaki *et al.*, 1997). Thus, the higher catalase and peroxidase enzyme content in plant species plays a considerable role in inhibiting the accumulation of hydrogen peroxide in plants.

A laboratory trial has shown that three properties of light color, intensity and duration affect plant growth so that red/infra-red ratio is dictated by the duration and photo flux of radiation treatments and influences anthocyanin development and synthesis significantly (Mancinelli, 1990). Lefsrud *et al*. (2008) reported that anthocyanin content of lettuce was increased in plants exposed to red LED light. In the present study, plants grown under the red shade nets exhibited the highest anthocyanin content.

At intense radiation, chlorophyll degradation rate in plant leaf exceeds its synthesis rate, resulting in the loss of chlorophyll content due to the inhibition of chloroplast formation (Gonçalves *et al.*, 2001; Fu *et al.*, 2012). It has been documented that colored nets increase chlorophyll content in plants. For example, Alkalai-Tuvia *et al*. (2014) studied the effect of colored nets on peppers and reported that the chlorophyll content of the peppers grown under the pearl nets was significant higher than that of the peppers grown under the black nets.

The plants grown under the red nets had a higher carotenoid. It has been reported by Tinyane *et al.* (2013) and Selahle *et al.* (2014) that carotenoid content of tomatoes was increased under red and pearl shade nets. Kong *et al.* (2012) reported that the yellow net resulted in morphological changes and leaf carotenoid increase in peppers versus the red net, which may relate to the increase in green light con-

tent under the yellow net. Leaf carotenoid content was higher in lettuces grown under colored shade nets than control (no net) in Ilić *et al.*'s study (2017), which is consistent with our findings.

Phytochemical biosynthesis mostly depends on light quantity and quality as was observed in lettuce plants grown under black nets. In a study, plants grown under pearl nets had significantly higher total phenol content, flavonoids, and antioxidant properties than those grown under other nets (Ilić *et al*., 2017). In another study, an increase was observed in post-harvest flavonoid content in oregano, marjoram, and coriander under pearl nets (Buthelezi *et al*., 2016). Although phytochemical content decreases slightly after harvest, a high level of post-harvest phytochemical accumulation enables plants to maintain phytochemical quality in post-harvest period (Buthelezi *et al.*, 2016). The accumulation of antioxidant compounds in green plants depends on many parameters such as temperature, light quantity and quality, cultivar, growing season, and metabolic factors (Miller *et al*., 2010). The control of radiation quality by the red and pearl photo-selective netting resulted in keeping post-flowering antioxidant activity in vegetables (Kong *et al*., 2013). Kong *et al*. (2013) reported that peppers exhibited an elevated level of antioxidants under yellow nets implying that yellow and pearl nets were likely to enhance plant resistance to biotic stresses. It seems that plants respond differently to different light spectra and plant response to the elevated level of antioxidant capacity depends on the type of colored nets.

The results show that among the studied colored shade nets, the yellow net outperformed the other nets in improving the vegetative capacity of the studied plants. Also, the highest anthocyanin, carotenoid, and catalase contents were obtained from the red net and the highest degree Brix and total chlorophyll from the red and yellow nets. Overall, the application of the colored nets was more desirable for the plants than their non-application. It has been documented that the absorption rate of yellow-colored glass is higher in 360-200 nm (blue and violet) range that is lowly important radiation for photosynthesis than in 560-760 nm range that is photosynthetically important (Haghshenas and Ghiabaklou, 2009) because the latter range is severely intercepted by chlorophyll and increases photosynthesis. This can be a reason for the higher efficiency of the yellow netting. The absorption rate of violet to yellow range of visible light (360-600 nm) by red glass is very similar to yellow glass (Haghshenas and Ghiabaklou, 2009). This explains the change in plants' behavior to the radiation passing through the red net because it seems that the penetration of photosynthetically active radiation through red nets (i.e. red and orange portions of visible light) contributes to important plant functions such as photosynthesis and increases biomass.

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Different substrates for seedling production of *Euterpe oleracea* Mart.

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Key words: alternative substrates, morphological characteristics, seedling production.

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Abstract: The aim of this study was to evaluate the effect of substrates involving different combinations of soil, cattle manure, burnt rice husks, sand and commercial substrate on growth in the açai palm (*Euterpe oleracea* Mart.) under nursery conditions in the State of Roraima. The experiment was conducted in the Fruit-Farming Sector of Embrapa Roraima, located in the district of Boa Vista. The experimental design was completely randomised, with nine treatments and four replications of five plants per replication. The treatments were T1: sand, T2: commercial substrate (OrganoAmazon©), T3: 25% T1 + 75% manure, T4: 50% T1 + 50% manure, T5: 75% T1 + 25% manure, T6: 25% T1 + 75% burnt rice husks, T7: 50% T1 + 50% burnt rice husks, T8: 75% T1 + 25% burnt rice husks and T9: 25% soil + 25% sand + 25% manure + 25% burnt rice husks. The morphological characteristics plant height (H), stem diameter (SD) and number of leaves (NF) were evaluated every 30 days from transplanting to the end of the experiment (210 days). The root dry weight (RDW), shoot dry weight (SDW), total dry weight (TDW) and the Dickson quality index (DQI) were obtained at 210 days. The substrates composed of 50% sand + 50% manure and 25% soil + 25% sand + 25% manure + 25% burnt rice husks are indicated for the production of seedlings of *Euterpe oleracea* Mart., as they provide superior growth in plant height and stem diameter, and improve total dry-weight production in the plants.

1. Introduction

The species *Euterpe oleracea* Mart., known throughout the world as açaí, is a palm tree, native to the Brazilian Amazon, belonging to the Arecaceae family (Oliveira *et al.,* 2019). It has recently been the target of several studies, as it is a functional food rich in proteins, fibre, lipids, vitamins and minerals, and has antioxidant properties, making it an indispensable part of the most varied diets and recommended to those who practise physical exercise and enthusiasts of healthy living (Kang *et al.,* 2010; Oliveira *et al.,* 2019). According to Honorio *et al*. (2017), the açai palm is considered a species with multiple uses, however, its economic

potential is mainly related to marketing the fruit and stems, the origin of the palm heart. Since Brazil is the largest producer, consumer and exporter of these products, large-scale cultivation is attractive to both industry and rural producers, who can obtain a varied income from the products and by-products throughout the year (Monteiro *et al.,* 2019).

Considering the health benefits as well as the high added value of the products of this species, studies that contribute with information concerning cultivation are extremely relevant, especially as the açai palm occurs naturally in such areas as low-lying plains and flooded woodlands that have unique climate conditions which can compromise the initial planting stage of the crop if quality seedlings are not used (Neves *et al.,* 2019). Both the planting and maintenance of homogeneous plantations of the açai palm therefore require the acquisition of vigorous seedlings to guarantee a return on investment and success in establishing the plantation; for this, it is necessary to improve the techniques for producing açai seedlings of a high commercial standard (Silva *et al*., 2017).

The production of quality seedlings depends on various factors such as the composition of the substrate, which has the role of supporting the plants and providing suitable chemical and physicals conditions for the initial development of the roots and shoots (Bilderback *et al*., 2005). Normally, the large-scale production of seedlings makes use of commercial substrates; however, besides the high cost of acquiring the substrate, the great majority contain only small concentrations of nutrients and require the use of fertilisers, a factor that increases the costs of the activity (Olle *et al.,* 2012).

As an alternative, several authors propose the use of substrates consisting of agricultural by-products that can be used mixed with commercial substrate or other products, for example, rice husks, charcoal, coffee chaff and animal manure, as well as by-products from agroindustry, which vary according to the region (Rinaldi *et al.,* 2017).

According to Olle *et al*. (2012), the choice of substrate, in addition to considering the cost of acquisition and availability for seedling production, should be based on technical and scientific results that show the material is able to promote high rates of initial plant growth and survival after planting; this will reduce the costs of establishing the crop, making it possible to expand and/or set up new plantations. There are several materials that can be used as substrates for the production of seedlings of forest species, either alone or in combination, such as sand, soil, expanded clay, vermiculite, sawdust, rice husk, pine bark, bark fiber among others (Olle *et al.,* 2012). According to Silva *et al*. (2018) when compared to exclusive use, the combination of different materials may result in satisfactory results, especially regarding the maximization of seedling growth, a fact possibly related to the combination of factors that favorfavorable conditions for availability, absorption, translocation, and nutrient use by plants.

Based on the above, the aim of this study was to evaluate the effect of substrates involving different combinations of soil, cattle manure, burnt rice husks, sand and commercial substrate on growth in the açai palm (*Euterpe oleracea* Mart.) under nursery conditions in the State of Roraima.

2. Materials and Methods

The experiment was conducted in the Fruit-Farming Sector of Embrapa Roraima (at 2°23’45.31” N and 60°58’44.34” W), located in the district of Boa Vista in the State of Roraima. Mature fruit of the açai were harvested in the town of Anori, in Amazonas, and taken to the seed laboratory of Embrapa Roraima for the experiment.

The fruit was first pulped with the help of a pulp processor for mechanical extraction of the seeds, which were then washed in running water until the residue was completely eliminated, and kept at room temperature. The propagating material was later sown in a bed containing washed sand as a substrate for germination and initial development of the seedlings. Substrate moisture was maintained by manual irrigationwith distilled water, four times a day.

The process of seedling emergence began around 30 days after sowing. Once the seedlings had reached a height of approximately 2.0 cm, they were transplanted into polyethylene bags (17 x 22 cm) containing different combinations of commercial substrate, soil, cattle manure, burnt rice husks and sand according to the predetermined treatments. The plants were then housed in a nursery under 50% shading, and irrigated by sprinkler for five minutes, three times a day.

The experimental design was completely randomised (CRD), with nine treatments, four replications and five plants per replication, for a total of 180 plants. The treatments were T1: sand, T2: commercial substrate (OrganoAmazon©), T3: 25% T1 + 75%

manure, T4: 50% T1 + 50% manure, T5: 75% T1 + 25% manure, T6: 25% T1 + 75% burnt rice husks, T7: 50% T1 + 50% burnt rice husks, T8: 75% T1 + 25% burnt rice husks and T9: 25% soil + 25% sand + 25% manure + 25% burnt rice husks. According to Silva *et al*. (2009), a composite sample of substrate for each treatments was collected, air-dried and sieved through a 2.0 mm mesh, for chemical characterization, whose results are presented in Table 1.

The morphological characteristics of plant height (H), stem diameter (SD) and number of leaves (NL) were evaluated every 30 days from transplanting to the end of the experiment. Plant height was measured with a ruler graduated in centimetres (cm), and considered the height of the plant from the surface of the soil to the apex of the plant. The stem diameter was measured with the aid of a digital calliper in millimetres (mm), 1 cm above the surface of the substrate. At the end of the experiment (210 days after transplanting), the following plant characteristics were evaluated: shoot dry weight (SDW), root system dry weight (RDW) and total dry weight (TDW). For this, the plants were removed from the polyethylene bags, the roots were separated from the substrate by washing under running water and the shoots then separated from the root system. To obtain the dry weight, the two materials were placed in a forced air circulation oven at ±70°C to constant weight. The Dickson Quality Index (DQI) was then determined (Dickson *et al*., 1960). The data were submitted to analysis of variance (ANOVA) and the mean values of the treatments compared by Tukey's test at 5% probability with the aid of the SISVAR software.

3. Results and Discussion

The analysis of variance revealed significant differences between the plants submitted to the different combinations of substrates for all the morphological characteristics under evaluation (Table 2), demonstrating that the different combinations can have a direct influence on the growth characteristics of plants of the açai palm (*Euterpe oleracea* Mart.) under nursery conditions.

It can be seen from the result of the mean-value comparison test that the plants grown in T4, comprising 50% sand + 50% cattle manure, obtained on average greater or statistically equal values for all the morphological characteristics under evaluation. A similar result, except for the variable SD that was slightly lower, was obtained for the plants submitted to T9, a substrate consisting of a mixture of 25% soil + 25% sand + 25% manure + 25% burnt rice husks.

Among the components in the treatments (T4 and T9) that resulted in better results for all the morphological characteristics under evaluation, cattle manure and sand should be highlighted. These results reinforce those obtained in an experiment by Menezes and Oliveira (2009) with the organic production of açai seedlings; the authors determined that the greatest growth in seedling height was obtained in the substrate containing cattle manure.

Honorio *et al*. (2017), carrying out the test for germination and emergence in seeds of the açaí in alternative substrates, suggested a combination of cattle manure + sand (1:1) as the most efficient in promoting the variables of physiological seed quality. Araujo

Table 1 - Chemical composition of the different combinations of soil, cattle manure, rice husks, and commercial substrate using açaí seedlings (*Euterpe oleracea* Mart.) growing seedlings, under nursery conditions

Treatments	H potential	OM dag/kg	K mg/dm³	P mg/dm³	Ca cmol/dm³	Mg cmol/dm³	Al cmol/dm³	H+Al cmol/dm³	Zn mg/dm³	Fe mg/dm³	Mn mg/dm³	Cu mg/dm³	B mg/dm³	S mg/dm³
T1 *	6.7	2.6	108.0	145.0	10.4	0.5	0.0	1.0	26.7	40.4	139.0	1.1	0.6	18.8
T2	5.7	10.0	312.0	263.9	13.8	7.4	0.0	1.9	26.9	62.3	160.2	0.6	0.7	49.1
T3	5.8	6.2	112.0	314.9	10.2	5.0	0.0	1.7	24.4	13.5	90.9	0.6	0.8	50.7
T4	6.5	4.0	92.0	218.2	10.0	2.9	0.0	1.3	23.5	20.3	107.0	0.8	0.8	34.9
T5	6.2	3.8	92.0	151.2	9.9	1.6	0.0	1.2	24.3	27.9	111.2	1.0	0.7	25.9
T6	6.1	4.9	106.0	71.7	12.2	1.4	0.0	1.9	16.5	13.5	88.6	0.3	0.5	17.2
T7	6.6	4.3	120.0	93.0	11.0	0.9	0.0	1.1	20.8	18.0	127.3	0.6	0.6	17.7
T8	6.7	3.7	122.0	132.9	11.0	0.7	0.0	1.1	23.3	33.5	132.3	0.8	0.4	19.4
T9	6.4	4.1	120.0	170.2	9.9	1.8	0.0	1.2	23.1	15.1	100.5	0.7	0.6	28.1

* T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25% burnt rice husks.

Table 2 - Mean values for plant height, stem diameter, number of leaves, root dry weight, shoot dry weight, total dry weight and Dickson Quality Index, in açaí seedlings (*Euterpe oleracea* Mart.) grown in different combinations of substrates under nursery

Substrate	Plant height (cm)	Stem diameter (mm)	Number of leaves	Root dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Total dry weight (g plant^{-1})	Dickson quality index
T1	8.50 e *	3.77 f	3 e	2.14 c	2.39 c	4.53 c	1.34 c
T2	16.47 bc	6.80 c	5 b	6.67 bc	2.09 c	8.76 bc	1.56 c
T3	17.43 b	6.18 d	4 c	25.99 a	20.90 a	46.89 a	12.94 a
T4	24.19 a	8.21 a	5 a	28.62 a	13.76 ab	42.38 a	12.37 a
T5	15.35 c	5.77 d	4 c	10.24 b	9.39 bc	19.63 b	5.49 b
T6	11.47 d	5.00 e	4 d	5.05 bc	4.46 c	9.50 bc	2.99 c
T7	9.71 de	3.88 f	3 e	3.50 c	2.82 c	6.32 bc	1.91 c
T8	11.02 d	3.93 f	3 e	4.37 bc	3.17 c	7.54 bc	2.14 c
T9	24.16 a	7.57 b	5 a	29.00 a	21.26 a	50.26 a	12.81 a
Mean	15.37	5.68	4.00	12.84	8.91	21.76	5.95
CV %	22.01	16.06	14.76	24.16	29.91	31.38	27.10

* Mean values followed by the same lowercase letter in a column do not differ at 5% probability by Tukey's test. T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25% burnt rice husks.

et al. (2019) describes how cattle manure is a basic substrate for obtaining vigorous seedlings, since its main function is to improve water retention, in addition to supplying macro and micronutrients and increasing their availability to the plants, resulting in improvements in seedling performance.

According to Olle *et al*. (2012), another basic component to be used in small proportions when combining substrates for plants is sand, as it provides the ideal conditions for aerating the substrate and gives good drainage. Furthermore, promoting the use of this material is based on its low cost and easy availability in some regions.

The superior results obtained for RDW, SDW, TDW and DQI in the plants submitted to T3, composed of 25% sand + 75% cattle manure, should also be pointed out. According to Menegatti *et al.* (2019), the DQI is a good indicator of plant quality, since robustness and the balance of biomass distribution in the plants are considered in its calculation and it includes several parameters which are regarded as important; the higher its value, the better the quality standard of the seedling.

However, since the performance of the plants in T3, despite an expressive DQI, was poor for the other characteristics under analysis (H and SD), and considering their importance in evaluating seedling quality, this shows the need for combining characteristics to obtain a better evaluation.

Conversely, plants submitted to treatment T1, comprising 100% sand, displayed inferior values for all the morphological characteristics under evaluation. As mentioned above, sand is a conditioner to be used in small proportions in the composition of a substrate, as it is practically an inert material and not associated with any nutrients that can be made available to the plant. In addition, sand shows high drainage potential, which may reduce water availability to the plant, making it impossible to maintain all the physiological processes essential to growth.

Furthermore, according to Smiderle *et al*. (2015), high-density materials such as sand, when used alone or in large proportions within a mixture, are unsuitable due to their excessive weight, which makes it difficult to handle the plants in their containers and to trade or transport them to their final planting site.

Similar to the results obtained for the plants submitted to the substrate of 100% sand, the T7 substrate, comprising a mixture of 50% sand + 50% burnt rice husks also resulted in poor plant performance for the morphological characteristics under evaluation. According to Smiderle *et al*. (2015), caution is needed when using combinations of burnt rice husks and sand in the composition of plant substrates, since both materials allow high water drainage and may result in water deficiency in the plants, which would compromise the processes of photosynthesis and respiration and consequently the maintenance of cellular elongation, resulting in smaller plant growth (Souza *et al*., 2020).

As a result of the water deficit and the reduction in photosynthetic rates, the plants accumulate a smaller amount of biomass, thereby showing a reduction in total dry mass - the behaviour shown by the plants in T1 and T7 (4.53 and 6.32 g plant^{-1} respectively). The plants grown in these two substrates had a total dry mass of less than around 6.5 times the dry mass found in the plants from the treatments considered superior, T9 and T4 (50.26 and 42.38 g plant^{-1} respectively).

The total dry weight is the sum of SDW and RDW; the higher this value, the better the quality of the produced seedlings (Adamipour *et al*., 2019). According to Chiomento *et al*. (2019), the total dry weight indicates the hardiness of a seedling, with the highest values representing more lignified and hardier seedlings, which may take hold faster under field conditions soon after planting.

It should be emphasized that the satisfactory morphological characteristics showed by plants grown in the commercial substrate (OrganoAmazon©) (T2) can be related to the chemical composition of the substrate, which showed higher levels than the other treatments for the macro and micronutrients, including organic matter (OM), which when decomposed tends to release nutrients, especially nitrogen, and other mineral elements such as phosphorus, magnesium, calcium, sulphur (Aalipour *et al*., 2019). However, it is also assumed that this substrate did not result in plants with superior morphological characteristics to the other plants due to the pH of the substrate, which was less than 6.

According to Aalipour *et al*. (2019), soils with a pH below 6 tend to reduce the availability of some nutrients, compromising the absorption and supply of the required amount of each element to enhance some of the physiological processes that culminate in growth.

The growth in plant height and stem diameter of the plants cultivated in different combinations of substrates throughout the period of the experiment can be seen in figure 1 and 2, respectively. All the curves, irrespective of the morphological characteristic under evaluation, show a linear trend, but the plants in T4 and T9 showed faster growth when compared to the plants of the other treatments being tested, achieving greater values for these variables by the end of the experiment (Table 3).

Chiomento *et al.* (2019), among the morphological characteristics under evaluation, the stem diameter is the most indicated for evaluating the survival capacity of the seedling in the field, due to a greater capacity for the formation and growth of new roots. According to Nascimento *et al*. (2019), the substrate that provides the seedlings with a balance between stem diameter growth and height, also provides greater robustness and more resistance to the adverse conditions found in the field, resulting in a higher survival rate and consequently reducing the costs of replanting.

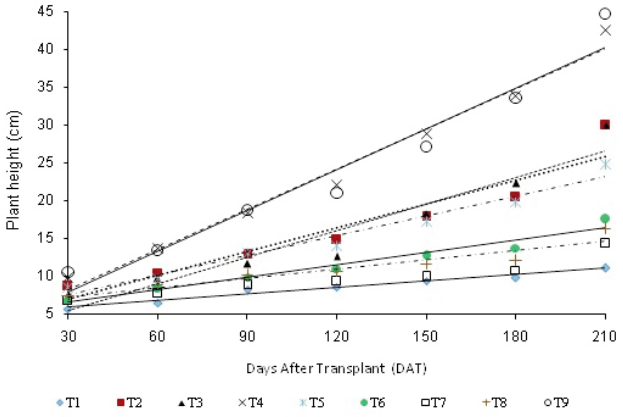

Fig. 1 - Effect of different combinations of substrates on plant height in açai seedlings (*Euterpe oleracea* Mart.) under nursery conditions. T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25% burnt rice husks (n=4).

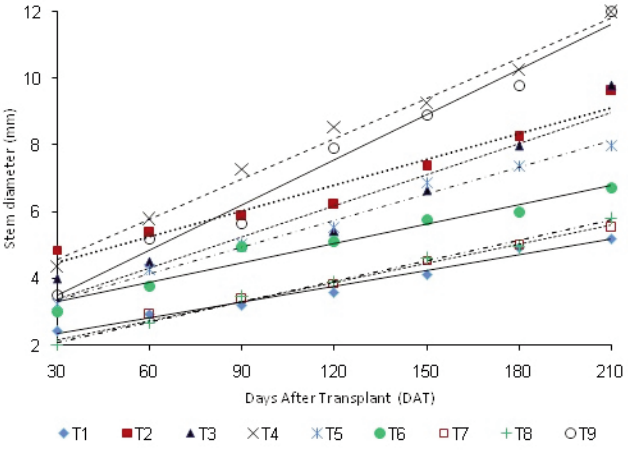

Fig. 2 - Effect of different combinations of substrates on stem diameter in açai seedlings (*Euterpe oleracea* Mart.) under nursery conditions. T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25% burnt rice husks (n=4).

Table 3 - Mean values for plant height (cm), stem diameter (mm), number of leaves and nivel significative for treatments obtained in açaí seedlings (*Euterpe oleracea* Mart.) grown in different combinations of substrates under nursery conditions

Treatments	Plant heigh (average)	Stem diameter (average)	Number of leaves (average)
T1	8.50 e *	3.77 f	3.24 e
T2	16.47 bc	6.80 c	4.56 b
T3	17.43 b	6.18 d	4.03 c
T4	24.19 a	8.21 a	5.01 a
T5	15.35 c	5.77 d	4.30 bc
T6	11.47 d	5.00 e	3.71 d
T7	9.71 de	3.88 f	3.13 e
T8	11.03 d	3.93 f	3.23 e
T9	24.16 a	7.57 a	4.97 a
DMS	1.78	0.48	0.31
Error	0.40	0.11	0.07
CV %	22.01	16.06	14.76

* Means followed by the same small letter in the column do not differ from one another by the Tukey test (p≤0.05%). T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25% burnt rice husks.

According to Silva *et al*. (2017), the presence of manure may have improved both the structural characteristics of the soil and the water retention, and may also have acted as a nutrient reservoir, including larger quantities of nitrogen, nutrient which may be indirectly discriminated for variable O.M. (Table 1). For Figueiredo *et al*. (2019), N is an essential element for components of the photosynthetic system, such as the chlorophylls, proteins and enzymes that allow satisfactory rates of carbon assimilation to be maintained (Taiz *et al.*, 2017; Figueiredo e*t al.*, 2019), and thereby guarantee the production of photoassimilates that drive plant growth.

In turn, Cattle manure mixed with soil has been widely used as a substrate for the production of seedlings of various species, as it provides ideal nutritional conditions for plant development, demonstrating that the presence of organic material in the substrate may be decisive in the growth of seedlings in the nursery and better initial start-up in the field (Steffen *et al*., 2010; Silva *et al*., 2017).

Considering further the relationship between the chemical characteristics of the substrate in each treatment and growth over time, it can be seen that treatments T1 and T7, which had lower values for these two morphological characteristics (H and SD), had low levels of organic matter and Mg.

This result can be understood in view of the materials used in the substrate of each treatment. T1, consisting entirely of sand, can be described as a substrate devoid of mineral nutrients; this may have compromised the production of several enzymes, proteins and other compounds which are indispensable to the metabolism of the plant.

Whereas T7, a substrate composed of a mixture of sand and burnt rice husks (1:1), induced higher values for the variables H and SD, these are not statistically superior to obtained for T1 (Table 3 and 4), suggesting that burnt rice husks, being an organic material, may have provided the plants with a certain level of nutrients.

However, according to Smiderle *et al*. (2015), sand and burnt rice husks degrades relatively slowly, which together with the high drainage it exhibits,

Table 4 - Linear equations, R^2 values and significance for the plant heigh, stem diameter and number of leaves of açai seedlings *(Euterpe oleracea* Mart.) grown in different combinations of substrates under nursery conditions

Treatment	Plant heigh		Stem diameter		Number of leaves	
	Equation	R^2 (sign)	Equation	R^2 (sign)	Equation	R^2 (sign)
T1	0.0286x + 5.0629	0.96 **	0.0157x + 1.88	0.97 **	0.0137x + 1.60	0.98 **
T2	0.1055x + 3.805	0.90 **	0.0257x + 3.7129	0.95 **	0.0139x + 2.8857	0.81 **
T3	0.1173x + 1.9929	0.90 **	0.0311x + 2.4486	0.92 **	0.0161x + 2.10	0.93 **
T4	0.177x + 2.95	0.98 **	0.0403x + 3.3686	0.98 **	0.0098x +3.828	0.47 *
T5	0.0869x + 4.921	0.96 **	0.0266x + 2.5514	0.98 **	0.0130x + 2.728	0.51 *
T6	0.0545x + 4.927	0.96 **	0.0195x + 2.7043	0.98 **	0.0121x + 2.257	0.83 **
T7	0.0358x + 5.4	0.89 **	0.0191x + 1.5857	0.98 **	0.0133x + 1.5286	0.99 **
T8	0.0411x + 6.1	0.89 **	0.0205x + 1.4543	0.99 **	0.0121x + 1.7714	0.98 **
T9	0.1799x + 2.571	0.95 **	0.0452x + .1329	0.98 **	0.0100x + 2.771	0.51 *

** significative for P<0.01; * significative for P<0.05. T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25% burnt rice husks.

may lead to leaching of the available nutrient content, explaining the smaller growth.

It should be noted that the low levels of Mg in treatments T1 and T7 may also have compromised plant growth in these substrates, since Mg is the central atom in the chlorophyll molecule. Substrates with less available Mg therefore tend to a reduced synthesis of this molecule. The reduction in leaf chlorophyll decreases the capacity for light absorption, promoting the fixation of a smaller amount of carbon, which reduces the production of carbohydrates and consequently reduces the energy for maintaining the rate of plant growth (Cartelat *et al*., 2005).

The number of remaining leaves per plant over the experimental period for each substrate under test can be seen in figure 3. The treatments showed different behaviour according to the materials used in the substrate. Treatments T9 and T4 displayed a quadratic trend when adjusting the regression equations, showing that throughout the growing period the number of leaves per plant tend to decrease due to the fall of older leaves.

These results suggest that plants submitted to these treatments (T4, consisting of 50% sand + 50% cattle manure, and T9, a substrate consisting of 25% soil + 25% sand + 25% manure + 25% burnt rice husks) throughout the development period, employ a greater amount of energy for growth in H and RD, instead of increasing the number of leaves.

A reduction in the number of leaves during plant growth can be beneficial, since after transplanting the seedlings in the field, the smaller number of leaves per plant will result in lower rates of transpiration enabling the plant to direct the energy produced during the process of photosynthesis towards root growth and development, initially helping the plants to take hold under climate conditions which differ from those of the greenhouse.

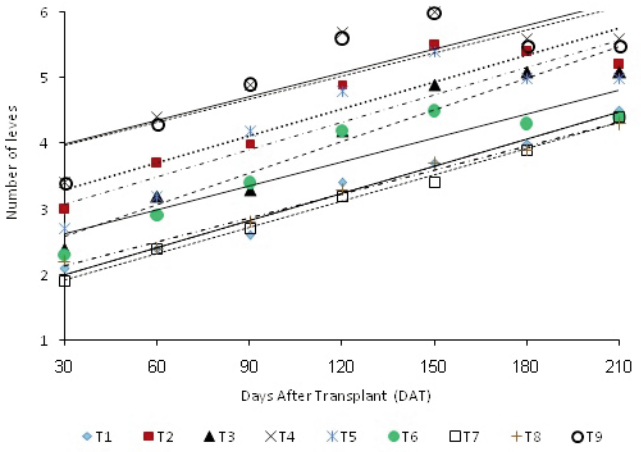

Fig. 3 - Effect of different combinations of substrates on the number of leaves per plant in açaí seedlings (*Euterpe oleracea* Mart.) under nursery conditions. T1= sand, T2= commercial substrate (OrganoAmazon©), T3= 25% T1 + 75% manure, T4= 50% T1 + 50% manure, T5= 75% T1 + 25% manure, T6= 25% T1 + 75% burnt rice husks, T7= 50% T1 + 50% burnt rice husks, T8= 75% T1 + 25% burnt rice husks, T9= 25% soil + 25% sand + 25% manure + 25%

4. Conclusions

The use of different materials in the substrate composition influenced the growth of *Euterpe oleraceae* Mart., seedlings, highlighting for treatment 50% sand + 50% manure and the most diverse composition (25% soil + 25% sand + 25% manure + 25% burnt rice husks).

The substrates which consisted of the combinations 50% sand + 50% manure and 25% soil + 25% sand + 25% manure + 25% burnt rice husks are indicated for the production of seedlings of *Euterpe oleracea* Mart., as they provide superior growth in plant heightand stem diameter, and improve total dry-weight production in the plants.

These treatments suggest the plants employ a greater amount of energy for growth in H and SD, instead of increasing the number of leaves, and this can be a strategy will result in lower rates of transpiration enabling the plant to direct the energy produced during the process of photosynthesis towards growth faster and ensuring high field survival rates.

These results could have of great interest to producers of seedling *Euterpe oleracea* Mart., as they show an increase in the quality of the seedlings produced, which is an advantage when planting, as better-quality seedlings tend to take hold faster and display better growth in the field, besides helping to reduce production costs.

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AHS
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Cefixime manages internal bacterial contamination during tissue culture operation

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Abstract: Large-scale propagation of *Pyrus communis*, which is a difficult-to-root species, is dependent on tissue culture technique. However, internal bacterial contaminations are an obstacle in tissue culture of fruit tree species. For this purpose, this investigation was conducted with several experiments to manage bacterial contamination. First, gram test for bacterial contamination related to *Pyrus* shoots proliferating was conducted. Gram test revealed that bacteria contaminating pear shoots were negative gram. Then, we investigated the application of cefixime (0, 100, 300 and 500 mg L^{-1}) or ampicillin (0, 100, 300 and 500 mg L^{-1}) for managing bacterial contaminations. It was found that the contaminated area on medium containing 500 mg L^{-1} cefixime (63.585 mm^2) was lower than other treatments (803.84 mm^2). Therefore, cefixime at 500 mg L^{-1} was selected to control the bacterial contamination. Next, different procedures were used included shaking with (1: sterile distilled water, 2: 500 mg L^{-1} cefixime and culturing in media with 500 mg L^{-1} cefixime, 3: 500 mg L^{-1} cefixime, culturing and subculturing in media with 500 mg L^{-1} cefixime 4: Disinfection). The third procedure was known the best due to the low bacterial contamination percentage and rate also the healthy growth of plants. Finally, the effect of gibberellic acid at 0 and 1 mg L^{-1} was investigated to compensate for shoot growth reducing in the presence of cefixime. 1 mg L^{-1} gibberellic acid improved the growth indices in the presence of cefixime.

1. Introduction

Fire blight, the most devastating disease of pear, leads to the death of the whole pear tree through the systematical infection in all underground and aerial parts of the tree (Vanneste, 2000; Evrenosoğlu *et al.,* 2019). From the horticultural science perspective, the revival of the pear orchards is dependent on large-scale propagation. The majority of cultivated pear is *Pyrus communis* (Morgan *et al.,* 1994) and *P. communis* cv. Williams is sensitive to fire blight (Abdollahi *et al.,* 2010). However, *Pyrus communis* is difficult-to-root (Zhu *et al.,* 2003; Sun *et al.,* 2011); therefore, the tissue-culture technique can support large-scale propagation of pear.

One of the most important factors in tissue culture is the control of microbial contamination. Plant immune system acts against pathogens. There are three steps of plant defense responses included the response in the step of entry, establishment, and spread of pathogens. Overall, plant immune systems can be classified into cell wall reinforcement and programmed cell death. In pathogen entry condition, cell wall reinforcement is efficient and in pathogen establishment and spread conditions programmed cell death can be restrictive (Abramovitch and Martin, 2004). When pathogens suppress the plant defenses responses, the plant was invaded by pathogens and contaminated. One of the phytopathogens is bacteria; they cause serious troubles *in vitro* conditions. Some of the modes of bacteria actions to suppress the host defenses refer to the use of type III effector proteins and toxins (Abramovitch and Martin, 2004) as well as, type IV secretion systems to inject effector proteins into cells (Angot *et al.,* 2007). Tissue culture technique is very sensitive to special pathogenic factors and all of the microbes in air condition and equipment. In other words, all of the microbes infect cultures with the aim of nutrition (Nadha *et al.,* 2012). Internal infections in plant cultures had often harmful effects for shoot proliferation, shoot rooting and quality of plant growth (Nadha *et al.*, 2012). In tissue culture, the selection of the appropriate antibiotic is important. There are several reports about the use of antibiotics to manage the bacterial contamination (Phillips *et al.,* 1981; Falkiner, 1990; Kneifel and Leonhardt, 1992; Barrett and Cassells, 1994; Falkiner, 1997; Nadha *et al.,* 2012). Cefixime is an antibiotic belonging to cephalosporin class. The mode of action of cephalosporins is related to inhibiting the cell wall biosynthesis so that this class arrests the formation of peptide bonds (Kohanski *et al.,* 2010).

Affect sites of various antibiotics are different; therefore the efficiency of different antibiotics is different in the removal of bacteria. There are some observations for different antibiotics affect sites in the previous studies, such as, inhibiting of cell wall synthesis that is related to benzylpenicillin and phosphomycin, inhibiting protein synthesis related to chloramphenicol and streptomycin, inhibiting of RNA and DNA synthesis related to rifampicin and nalidixic acid (Phillips *et al.,* 1981). One of the side effects of antibiotic application in plant tissue culture is the reduction of growth. However, in control of bacterial infection in *Guadua angustifolia*, streptomycin sulfate decreased growth shoot, but kanamycin caused intensive growth with high-quality; therefore, the effects of antibiotics are different on plant growth (Nadha *et al.,* 2012). The management of contamination in tissue culture leads to the prevention of waste of time and energy. In this investigation, we examine the use of antibiotics to manage the bacterial contaminations coupled with the use of GA_3 to compensate for the poor growth of the plant in the presence of antibiotic in the media.

2. Materials and Methods

Plant materials

Three months old proliferating micro-shoots of *Pyrus communis* cv. Williams, as the most common cultivar in the world, exhibited bacterial contamination. Contaminated micro-shoots were picked to investigate the experiments of bacterial control for the large-scale production of *P. communis*. These micro-shoots were maintained in MS medium (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 3% sucrose.

Gram test of bacteria

Two drops, approximately 50 µL, of a 3% (W/V) solution of potassium hydroxide were placed on a clean glass slide as outlined by Ryu (1940). Bacterial cells were transferred from culture media aseptically with a flat wooden toothpick and placed into the drop of KOH with rapid, circular agitation. After 5-8 sec, the toothpick was alternately raised and lowered just off the slid surface to detect a stringing effect. It was considered gram-negative bacteria if drop viscosity increased within 15 sec (Suslow *et al.,* 1982; Schaad *et al.,* 2001).

Antibiotic selection test

Contaminated shoots were cultured on MS medium (Murashige and Skoog, 1962) supplemented with either ampicillin 0, 100, 300 and 500 mg L^{-1} or cefixime at 0, 100, 300 and 500 mg L^{-1}. Vessel cultures with 32 mm inner diameter were maintained at a constant temperature of 25±1°C and in 16/8 h light/dark photoperiod (45 µmol m^{-2} s^{-1}) using cool white fluorescent lamps (Sylvania, Germany). After a week, the contaminated area was measured in each treatment.

Bacterial contamination removing

In the before step, we selected the proper antibiotic (cefixime at 500 mg L^{-1}). Then, we used four different procedures using cefixime at 500 mg L^{-1} to control any eventual bacterial contamination. For each procedure, 3 micro-shoots were cultured in a

candle jar as a replicate.

1) Shaking of contaminated shoots with sterile distilled water (control);

2) The first, shaking of contaminated shoots with cefixime at 500 mg L^{-1} then, cultured in media with cefixime at 500 mg L^{-1}. Finally, sub-culturing in free antibiotic media;

3) The first, shaking of contaminated shoots with cefixime at 500 mg L^{-1}, then, cultured in media with cefixime at 500 mg L^{-1}. Finally, sub-culturing in media with cefixime at 500 mg L^{-1};

4) Disinfection of contaminated shoots (immersing in 1% hypochlorite sodium for 5 min then rinsed with sterile water three times).

MS medium (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 3% sucrose were used for each procedure. Cultures were maintained at a constant temperature of 25±1°C and in 16/8 h light/dark photoperiod (45 µmol m^{-2} s^{-1}) using cool white fluorescent lamps (Sylvania, Germany). After 30 days, several traits were evaluated: percentage of fungal contamination and bacterial contamination (BC), bacterial contamination rate (BCR) and general health.

Based on the following equation (E1) bacterial contamination rate was counted per each micro-shoot in each candle jar:

$$BCR = \sum \frac{Ni}{Di}$$

where BCR= Bacterial contamination rate, N_i = Number of the contaminated shoot in each day, D_i = Day number.

Rescued shoot improvement

Micro-shoots related to the best procedure were transferred to MS medium (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 3% sucrose, 500 mg L^{-1} cefixime and gibberellic acid (GA_3) treatments. The concentrations of GA_3 were 0 and 1 mg L^{-1}. pH was adjusted at 5.8 with NaOH prior to autoclaving at 98 kPa and 121°C, and the media were solidified using 0.8% agar. Cefixime antibiotic and GA_3 added to the media after autoclaving by filtering. Related traits of this experiment were included: the percentage of new growth, the percentage of proliferation, the average number of bud and leaf, as well as, the average shoot length.

The evaluation of declined antibiotic dose

After the six months using cefixime at 500 mg L^{-1}, proliferated shoots were divided into two groups. Each group of plants was cultured in media with cefixime at either 500 mg L^{-1} or 250 mg L^{-1}. pH was adjusted at 5.8 with NaOH prior to autoclaving at 98 kPa and 121°C, and the media were solidified using 0.8% agar. Cefixime antibiotic and GA_3 were added to the media after autoclaving with the syringe filter (pore size: 0.22 µm). After 10 days, the percentage of bacterial contaminations and bacterial contamination rate were measured per each micro-shoot in each candle jar based on (E1).

Rooting micro-shoots

After six months, micro-shoots were transferred to ½-strength QL (Quoirin and Lepoivre, 1977) medium supplemented with 1.5 mg L^{-1} Naphthaleneacetic acid (NAA) and 500 mg L^{-1} cefixime. Cultures were maintained a week in dark conditions, then, transferred to 16/8 h (light/dark) photoperiod and light intensity of approximately 45 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) emitted by cool-white fluorescent tubes in 35% relative humidity.

Statistical analysis

These experiments were arranged as a completely randomized design with three replications. The data were statistically analyzed using a one way ANOVA test and means were compared with the Duncan test at the 5% level of confidence. All of the statistical tests were performed using SAS (Statistical Analysis System) software v9.1. All of the percentage data were transformed to Arcsin √x.

3. Results and Discussion

The result of the gram type detection showed contamination of this investigation is related to gram-negative bacteria. In the antibiotic selection step, we compared ampicillin and cefixime. We used 0, 100, 300 and 500 mg L^{-1}concentrations of each antibiotic. Ampicillin is a common antibiotic in tissue culture, and its activity spectrum is related to gram-positive and gram-negative bacteria, whereas the cefixime is antibiotic acting against gram-negative bacteria. In antibiotic selection test, results revealed 500 mg L^{-1}cefixime could overcome contamination better than other treatments. The contaminated area on medium containing 500 mg L^{-1}cefixime (63.585 mm^2) was lower than other treatments (803.84 mm^2). Therefore, we selected 500 mg L^{-1} cefixime to control the contamination for later experiments.

ANOVA revealed that the difference between the four procedures was significant ($P \leq 0.01$) for bacterial contamination percentage and the rate of bacterial

contamination (Table 1). Four procedures were used for the survival and rescuing of shoot from bacterial contamination. The third procedure (the first, shaking of contaminated shoots with 500 mg L^{-1} cefixime; then, cultured in media with 500 mg L^{-1} cefixime. Finally, sub-culturing in media with 500 mg L^{-1} cefixime) was known the best due to the low bacterial contamination percentage, rate and finally the healthy and fresh growth of plants. These indices in other procedures were not desirable; as the highest percentage of bacterial contamination was observed in the first procedure (shaking with sterile distilled water). All of the procedures led to necrotic plants except for procedure 3 (Table 2). The presence of antibiotic in media effected on plant growth and weakened their growth; therefore, we used GA_3 and its effect evaluated on plants growth. GA_3 application in media containing 500 mg L^{-1} cefixime had a significant effect on the percentage of new growth, the average of bud number, the average shoot length and the average of leaf number ($P<0.05$) (Table 3). Without the application of GA_3 were not observed any proliferation and new bud formation; while in media containing GA_3, 22.53% proliferation and 1.46 the average number of bud were observed (Table 4). As well as, the results showed the decrease in the antibiotic dose to 250 mg L^{-1} cefixime increased BCP

Table 1 - Analysis of variance traits under study

Source of variance	DF	Means square	
		Bacterial contamination	Rate of bacterial contamination
Treatment	3	2963.807 **	45.48649 **
Error	8	7.350.308	0.5436

NS= no significant; **= significant at the 0.01 level of probability according to Duncan Test.

Table 2 - Evaluation of different procedure to come over bacterial contamination during the culture

Procedure	Fungal contamination (%)	Bacterial contamination (%)	Bacterial contamination rate	General health after 30 days
1	0	90 ± 0	9.47 ± 0.77	Necrotic leaves
2	0	74.55 ± 2.42	4.93 ± 0.119	Necrotic leaves
3	0	17.01 ± 1.22	0.45 ± 0.053	Green leaves and healthy
4	0	57.85 ± 1.56	7.47 ± 0.323	Necrotic leaves

1= shaking with sterile water.
2= the first, shaking of contaminated shoots with cefixime (500 mg/l) then, culturing in media with antibiotic. Finally, sub-culturing in free antibiotic media.
3= the first, shaking of contaminated shoots with cefixime (500 mg/l), then, culturing in media with antibiotic. Finally, sub-culturing in with antibiotic media.
4= disinfection (immersing in 1% hypochlorite sodium for 5 min then shaking with sterile water for three times.
Values are mean ± standard error.

Table 3 - Analysis of variance of traits under study

Source of variance	DF	Means square				
		Percentage of new growth	Percentage of proliferation	Average of bud number	Average shoot height	Average of leaf number
Treatment	1	514.20 **	761.53 **	3.22 **	60.16 *	54.0 *
Error	4	6.022	4.53	0.0066	7.33	0.33

**= significant at the 0.01 level of probability according to Duncan Test.
*= significant at the 0.05 level of probability according to Duncan Test.

Table 4 - Evaluation of Gibberellic acid in MS medium along with PGRs and 500 mg/L cefixime on secondary growth traits

Concentration (mg/L)	New growth (%)	Proliferation (%)	Average bud number	Average shoot height (mm)	Average leaf number
1	43.93 ± 1.23	22.53 ± 1.74	1.46 ± 0.07	9.66 ± 2.03	10.33 ± 0.33
0	25.41 ± 1.58	0 ± 0	0 ± 0	3.33 ± 0.88	4.20 ± 0.33

Values are mean ± standard error.

to 41% and BCR to 1.2 (Table 5). Therefore, using the antibiotics at 500 mg L^{-1} should continue because the plants grow without bacterial contamination only in the presence of 500 mg L^{-1} cefixime (Fig. 1). Nadha *et al.* (2012) stated the removal of kanamycin from the medium did not result in resumption contamination after 10 days (Nadha *et al.,* 2012); while other literature mentioned that the usage of antibiotics for inhibiting the bacteria growth has impermanent impact and removal of antibiotics has accompanied by resumption contamination (Falkiner, 1990; Barrett and Cassells, 1994; Falkiner, 1997; Leifert and Cassells, 2001) confirming the results of this experiment. In the consumption of antibiotic, resistant-bacteria theory is undeniable. Despite long-term using of cefixime, about six months, it could not only remove bacterial contamination, but also act without any resistant-bacteria. Finally, rescued shoots were able to produce healthy roots.

Table 5 - Evaluation of decreasing of cefixime on BCR and BCP after ten days

Traits	Cefixime concentration (mg/L)	
	250	500
Bacterial contamination rate	41 a	0 b
Bacterial contamination percentage	1.2 a	0 b

Different letters in columns indicate significant difference between treatments at 5% level.

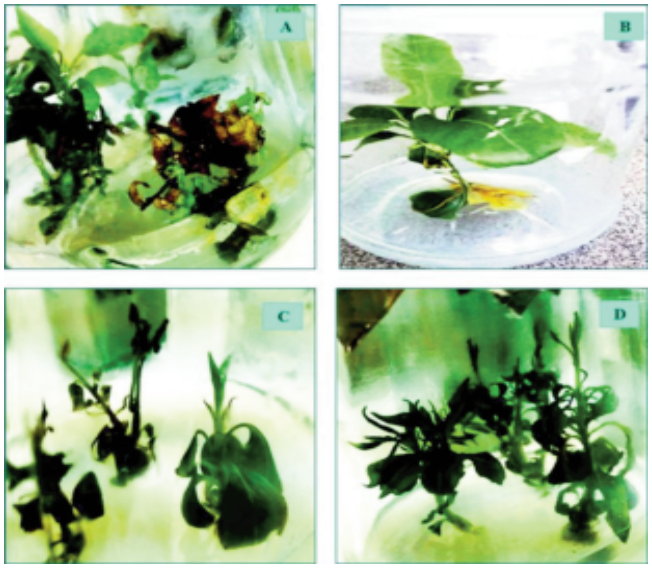

Fig. 1 - Contaminated shoots *in vitro* culture (A). Contaminated shoot after sub-culturing on media without cefixime (B). New growth after using GA3 treatment (C, D)

Leifert and Cassells (2001) mentioned alternatives for the antibiotic in their review. These alternatives included medium acidification and autotrophic culture (e.g. culture without carbohydrate) (Leifert and Cassells, 2001). In other literature were noted to activating of endogenous bacteria as a result of sub-culturing in media with cytokinins (Kneifel and Leonhardt, 1992). However, plant tissue culture without cytokinins, carbohydrates and with the modification in media acidity is impossible. In this regards, this investigation showed with the presence of cefixime in media containing cytokinins, carbohydrates could manage the bacterial contamination. Based on the results of this investigation, cefixime at 500 mg L^{-1} had not any toxicity effect on growth and proliferation. Cefixime is an antibiotic belonging to cephalosporins class. The cephalosporin antibiotics have been introduced as the appropriate antibiotic plant tissue culture since they have low eukaryote toxicity (Mathias and Boyd, 1986) which our results emphasize this point.

4. Conclusions

Bacterial contamination incidence is common and unavoidable during the in vitro propagation of fruit tree species. This investigation presented a procedure to manage the bacterial contamination of *P. communis* cv. Williams during the *in vitro* culture. Based on the results of this investigation, cefixime at 500 mg L^{-1} could control the bacterial contamination. The use of antibiotic in a medium is associated with a decrease in the growth of plants. This side effect of antibiotic was managed with the application of GA_3 at 1 mg L^{-1}. Therefore, we suggest cefixime at 500 mg L^{-1} for *in vitro* propagation of fruit trees.

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Improving water productivity and yield of onion crop by combining early planting and straw mulch under different irrigation levels in dry Mediterranean region

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Abstract: In response to the Sustainable Development Goals (SDGs) adopted by United Nations, combining using straw mulching, the proper crop planting date, and regulated deficit irrigation (RDI) is fundamental to adapt to climate change and to promote sustainable agriculture in the dry Mediterranean region. Two-year pot experiment under field conditions (2017 and 2018) was conducted in Damascus Countryside, Syria (altitude 600 m), to evaluate the onion crop response to early planting, irrigation level, and straw mulching. Treatments composed of three different planting dates with 28-day intervals (two early dates in February and March, and the traditional date in April), three irrigation levels (100, 80, and 60% of crop evapotranspiration, ETc), and two types of soil cover (with and without wheat straw mulch), with three replicates. Findings revealed that the seasonal ETc decreased from about 900 mm under current practice (planting in April without mulch) to only about 550 mm under both straw mulch and earliness in planting. Large bulb yield increases (more than double) were also obtained. Moreover, early planting using straw mulching significantly enhanced the onion crop response to RDI, even at 60% of ETc. Combining early planting in February, straw mulching, and full irrigation represents the best agricultural management.

1. Introduction

Onion (*Allium cepa* L.) is an important crop worldwide. The environmental conditions such as photoperiod and temperature mainly affected its growth, development, and bulb yield. The agronomic practices such as planting date and irrigation water availability have also an effect on onion crop production (Brewster, 2008; Khokhar, 2014; Mubarak and Hamdan, 2018 a). Onion crop thrives best when temperatures are cool during early development period and then warmer and sunny during maturity. Hence, planting date has a profound impact on onion crop growth and development. Early planting date tends to have a longer onion growing season before bulb initiation ensuing larger plants. However, large plants are more likely to become sensitive to the cold stimulus resulting in bolting

(formation of seed stalk followed by flowering), which represents a highly unfavourable feature for onion bulb production. Large plants are also related with split bulbs. However, late-date-planted onions start forming bulbs before reaching satisfactory plant growth to support the final size of bulbs. This would produce small bulbs, and therefore, decreasing the bulb yield (Brewster, 2008; Rohini and Paramaguru, 2016). Thus, determining the proper onion crop planting date is central to adapt to the regional climate changes.

Onion crop has a shallow rooting system, and therefore, it is considered as a sensitive crop to soil water deficit than other deep-rooted crops. Water productivity (WP, also known as water use efficiency, WUE) is usually used to recognize the environments or management practices by which the yield per unit water can be optimized. In the dry regions where water resources are limited as in the dry Mediterranean region, improving water productivity and crop production represents also a main challenge for agricultural water management.

Mulch has been widely adopted because of its agro-pedo-ecological benefits. It constitutes of synthetic (plastic films) or natural (plant residues as wheat or rice straw) materials. Both materials reduce the loss of soil water through evaporation. However, unlike plastic films, straw mulch allows rain and irrigation water to penetrate and to reach the soil. This conserves soil water content, and thereby reducing irrigation water requirements, promoting rooting system development, and increasing crop growth, development and yield (Vavrina and Roka, 2000; Gimenez *et al.*, 2002; Mubarak and Hamdan, 2018 b). From eco-environmental point of view, the use of plastic mulch would not be justified at low crop prices and/or very high plastic films costs, especially it requires to be removed after use annually. The reuse of plastic films is not practical agronomically and technologically. This could increase the harmful environmental impacts from plastic components. For these reasons, straw mulching present an eco-environmentally sustainable choice. Unlike plastic films, straw mulch could incorporate into the soil ecosystem, where it is expected to biodegrade. Thus, straw mulching could be considered as a slow-acting organic fertilizer, improving soil fertility and soil physical properties, and consequently, crop yield (Khaledian *et al.*, 2010, 2011).

Deficit irrigation (DI) in combination with mulching could be considered as a key water-saving technique that would help in meeting both water scarcity and sustainable crop production (Fereres and Soriano, 2007; Chai *et al.*, 2016). The effects of deficit irrigation under mulching on onion yield have been documented (Vavrina and Roka, 2000; Igbadun *et al.*, 2012; Patel and Rajput, 2013; Tsegaye *et al.*, 2016; Mubarak and Hamdan, 2018 b). Several studies showed that it is better to fractionate the water stress during the cropping season (Regulated deficit irrigation, RDI) rather than applying a water stress during the critical stages of crop growth period (Kadayifci *et al.*, 2005; Patel and Rajput, 2013). For example, deficit irrigation given at 75% of crop evapotranspiration (ETc) was recommended for onion crop production (Tsegaye *et al.*, 2016).

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

As the onion crop production is already limited by the water availability and local climate, moving towards feasible tools (such as using regulated deficit irrigation under mulching) and agronomic practices (such as determining the proper crop planting date) adapted to the regional climate change is urgently needed for better water saving and cultivation period of the crop (FAO, 2011; Khokhar, 2014; Zinkernagel *et al.*, 2015).

In this context, and in response to the ambitious Sustainable Development Goals (SDGs) proposed by United Nations to adapt to climate change and to promote sustainable agriculture, the present study aimed to assess the interactive effects of various planting dates, different irrigation levels, and straw mulching on onion crop production. The outcomes may introduce appropriate agronomic alternatives to meet the ever increasing demand for onions and to

save irrigation water in the dry Mediterranean area. Moreover, results may contribute to make regulated deficit irrigation with straw mulching familiar for most farmers, and to stimulate them to adopt these techniques in their fields.

2. Materials and Methods

Pot experiments were conducted under open field conditions at the Deir Al-Hajar Agricultural Experiment Station, Damascus Countryside, Syria (33°20′ N, 36°26′ E, altitude 600 m), for different planting dates during February to May in two consecutive years 2017 and 2018. The site is located within a dry Mediterranean area, in which the total annual rainfall is about 120 mm, and the annual reference evapotranspiration is about 2000 mm. Some climatic data for the studied site collected during the growing seasons were fairly close to those averaged over the last 16 years (from 2000 to 2016) as can be shown in Table 1. For this reason, testing different planting dates during two years seemed somewhat adequate.

The soil is classified as a clay loam (29.5% clay, 42.7% silt, and 27.8% sand). Both volumetric soil water contents at permanent wilting point (*PWP*) and field capacity (*FC*) are 0.18 and 0.36 m^3 m^{-3}, respectively. Some chemical and physical soil properties are: pH of 8.0; ECe of 0.34 ds m^{-1}; organic matter of 1.00%; available P of 5.7 ppm; NO_3^- of 28.3 ppm; NH_4^+ of 12.6 ppm.

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

Four different planting dates separated with 28 days were tested: PS1 (early February), PS2 (early March), PS3 (early April), and PS4 (early May). Unfortunately, onion bulb sets which were planted in May (PS4) in both studied years did not properly germinate, and therefore, they were ignored. Within a year and at each planting date, the experiment was laid out following a 2×3 factorial experiment arranged in a randomized complete block design (RCB design) with two modes of soil cover and three irrigation levels, replicated three times. The soil covering comprised of two distinct types. The first one was with mulching using 40 g of wheat straw per pot (about 8 t ha^{-1}); and the second one was with no mulching. The irrigation levels composed of IL100 (full irrigation, 100% ETc), in which plants received 100% of the crop evapotranspiration; and the root zone was replenished to field capacity. IL80 and IL60 treatments (regulated deficit irrigation) were irrigated at the same frequency as in IL100 but with water amounts equal to 80 and 60% of the ETc as calculated in IL100, respectively. In other words, the three watering treatments received at each irrigation event 1.0, 0.8 and 0.6 times the soil water depletion

Table 1 - Some climatic data for the experimental site during both studied years (2017 and 2018) and the 16 years average (from 2000 to 2016)

Variable	Year	Feb.	Mar.	Apr.	May	Jun.	Jul.
Minimum temperature (°C)	2017	4.0	6.2	9.7	14.4	17.2	20.6
	2018	5.7	7.9	10.0	15.6	18.2	19.8
	2000-2016 average	4.0	6.8	10.1	14.1	17.6	19.3
Maximum temperature (°C)	2017	14.7	18.7	26.2	31.6	35.7	40.6
	2018	18.8	24.3	27.2	31.5	34.6	36.9
	2000-2016 average	15.7	20.6	25.3	30.4	35.0	37.4
Mean temperature (°C)	2017	9.1	14.0	19.2	24.9	28.4	31.1
	2018	12.7	17.3	19.9	25.7	27.7	28.8
	2000-2016 average	10.6	15.0	18.1	23.6	27.7	29.4
Relative air humidity (%)	2017	69.3	74.4	63.1	57.9	56.3	56.0
	2018	68.8	65.0	54.8	51.5	59.6	55.6
	2000-2016 average	75.0	64.1	60.9	56.5	56.3	60.7
Precipitation (mm)	2017	11.6	42.6	0.0	0.0	0.0	0.0
	2018	30.3	1.0	14	9.9	0.0	0.0
	2000-2016 average	31.0	31.6	5.9	4.2	0.0	0.0

occurred in the full irrigation treatment (IL100), respectively. Irrigation water was added 3 times per week. Each experiment was started on the planting day with a wet soil at field capacity as measured by pot's weight. The pots were weighed before and after each irrigation event. The water amount depleted (mm) between two successive irrigation events (ETc) was regulated by weight and estimated using (Eq. 1) as:

$$ETc = (W_1 - W_2)/(P_w \times S) \quad [1]$$

where *ETc* = the crop evapotranspiration between two irrigation events (mm); W_1 = the weight of the pot (kg) after irrigation (the soil water content in the pot was at the field capacity); W_2 = the weight of the pot (kg) just before the next irrigation event; ρ_w = the water density (g cm^{-3}); and *S*= the pot soil surface area (m^2). The daily crop evapotranspiration (mm day^{-1}) was estimated by dividing the ETc calculated using Eq. (1) by the number of days between two successive irrigations. The seasonal crop evapotranspiration was the summation of the daily ETc, which represented the total crop water requirements during a growing season. Irrigation water amounts, which were applied to the non mulching treatments, were based on the IL100 treatment under no-mulching conditions; whereas those applied to the straw mulching treatments were based on the IL100 treatment under mulching conditions.

For each planting date, phosphorous and potassium were applied as basal application at planting day; wherease, nitrogen fertilizer was splited into two equal applications, and added during early vegetative stage. Irrigation was stopped when almost 70% of leave-head dropped as signs of maturity. The onions were lifted to field cure. Then the leaves were cut leaving about 2.0 cm tops above the bulb. The length, diameter, and weight of both matured onion bulbs from each pot were measured. The sum of weights of both bulbs represent the bulb yield per pot (Y-pot), and was expressed as g pot^{-1}. Water productivity (WP, kg m^{-3}) and irrigation water use efficiency (IWUE, kg m^{-3}) were calculated using equations [2] and [3] (Mubarak *et al*., 2018). WP is the relationship between yield and seasonal evapotranspiration (ETc). Whereas, IWUE is the relationship between yield and the total amount of irrigation water applied (I, Liter pot^{-1}).

$$WP = Yield/ETc \quad [2]$$

$$IWUE = Yield/I \quad [3]$$

Within a year, a combined analysis of data over planting seasons was carried out to examine the interaction between planting season and the studied treatments (Gomez and Gomez, 1984). The analysis of variance (ANOVA) was conducted using the DSAASTAT add-in version 2011 (Onofri, 2007). Mean comparison was made using the LSD test at the 1% level. Trend comparison (regression analysis) was also performed. Data was presented and illustrated according to the rules described by Gomez and Gomez (1984).

3. Results

As mentioned above, onion bulb sets planted in May did not properly germinate. This could be explained by the fact that onion is a vegetative overwintering stage in its life cycle, i.e., it grows best when temperatures are cool during early development period (Brewster, 2008). Therefore, it is not recommended to delay planting onion sets after April in the dry Mediterranean area.

Bulb shape indicators

The shape of onion bulbs was represented herein by two indicators: bulb length (BL) and diameter (BD). The ANOVA revealed that the main effects of all studied factors (planting date, soil cover system, and irrigation levels) highly significantly affected bulb shape indicators in both years (Table 2). Within a year, the data under each factor were pooled over the other factors as can be seen in Table 3. Results indicated that both indicators (BL and BD) found in 2017 were comparable with their homologues in 2018.

Early planted onion sets (PS1) produced the tallest onion bulbs (7.9 and 8.5 cm in 2017 and 2018, respectively) than the other planting dates, while the later planted sets (PS3) produced bulbs significantly shorter by 10-22% than those in PS1 and PS2, in both years. Moreover, onion sets grown under straw mulching produced bulbs considerably taller by about 14% in average than those grown under no-mulching conditions. With regard to irrigation levels, the higher value of BL was observed under full irrigation (IL100); then, bulb length decreased as the water application rate decreased. The mean value of BL reduced by about 8 and 25% in 2017, and by about 3 and 14% in 2018, when onion sets were planted in March (PS2) and April (PS3), respectively (Table 3).

Table 2 - Analysis of variance of the data of crop responses as affected by planting date, soil cover system, and irrigation level (significance of *Fisher* test)

Source of variance	df	BL	BD	Y-pot	WP	IWUE
			2017			
Planting date (PS)	2	***	***	***	***	***
Rep. within PS	6					
Soil cover system (SC)	1	***	***	***	***	***
PS × SC	2	***	NS	NS	***	***
Irrigation level (IL)	2	***	***	***	***	***
PS × IL	4	NS	NS	NS	NS	NS
SC × IL	2	NS	NS	NS	NS	NS
PS × SC × IL	4	NS	NS	NS	NS	NS
Pooled error	30					
Total	53					
CV (%)		5.88	6.12	9.14	9.07	9.26
			2018			
Planting date (PS)	2	***	***	***	***	***
Rep. within PS	6					
Soil cover system (SC)	1	***	***	***	***	***
PS × SC	2	NS	NS	NS	***	***
Irrigation level (IL)	2	***	***	***	***	***
PS × IL	4	NS	***	NS	NS	NS
SC × IL	2	NS	**	NS	NS	NS
PS × SC × IL	4	NS	NS	NS	NS	NS
Pooled error	30					
Total	53					
CV (%)		6.90	3.83	5.89	5.49	5.45

*** = significant at 1‰ level, ** = significant at 1% level, NS = non-significant at 1% level. df = degree of freedom, BL = Bulb length, BD = Bulb diameter, Y-pot = bulb yield per pot, WP = water productivity, IWUE = irrigation water use efficiency.

On the other hand, the planting date×soil cover system (PS×SC) interaction effect on bulb length was found to be also significant at the 1‰ level only in 2017 (Table 2). To examine the nature of this interaction, the BL data of planting dates were compared under both soil cover systems. The values of BL under straw mulching were 8.23, 7.78, and 7.03 cm, and under no-mulching condition were 7.62, 7.05, and 5.38 cm, for PS1, PS2, and PS3, respectively. The mean values of BL in PS1 and PS2 under no-mulching were smaller by 8-10% than those under straw mulching. While in the traditional planting date in April (PS3), BL under no-mulching was 31% smaller than that with straw mulching. This difference could explain the nature of PS×SC interaction.

The largest BD was produced from onion sets planted early in February (PS1) in both year: 4.7 and 4.0 cm in 2017 and 2018, respectively. It then significantly reduced as the planting date delayed. The mean values of BD from PS3 were shorter by 35% in 2017 and 23% in 2018 than those from PS1. Furthermore, using straw mulching led to a significant increase in BD by 17% compared with no-mulching conditions. Also, significant differences in BD were observed in relation to irrigation levels. The maximum values of BD of 4.5 cm in 2017 and 4.6 cm in 2018 were found under full irrigation condition (IL100). Then, they significantly decreased as the irrigation level decreased. The effect of decreasing water application rate by 40% of ETc (as in IL60 treatment) resulted in a decreased BD by about 48% relative to those in IL100, in both years (Table 3).

Only in 2018, both PS×IL and SC×IL interactions were highly significant (Table 2). To examine the nature of PS×IL interaction, the BD data of planting dates were compared under irrigation levels (data not shown). The decline in BD as planting date delayed was found to be more severe under full irrigation compared with water stress conditions. Also, to examine the nature of SC×IL interaction, the BD data of both soil cover systems were compared under irrigation levels (data not shown). The increase in BD as irrigation level increased was found to be more severe under straw mulching compared with no-mulching conditions. These differences could explain the nature of both interactions.

Table 3 - Mean comparisons of crop responses as influenced by planting date, soil cover system, and irrigation level for both studied years

Tested factor	BL (cm)	BD (cm)	Y-pot (g pot^{-1})	WP (kg m^{-3})	IWUE (kg m^{-3})
			2017		
Planting date					
PS1 (February)	7.9 a	4.7 a	119.74 a	4.60 a	5.71 a
PS2 (March)	7.4 b	3.7 b	110.52 b	4.09 b	4.26 b
PS3 (April)	6.2 c	3.0 c	83.79 c	2.82 c	2.87 c
LSD 0.01	0.4	0.2	8.77	0.32	0.36
Soil cover system					
with mulch	7.7 a	4.1 a	123.07 a	5.14 a	5.80 a
without mulch	6.7 b	3.5 b	86.30 b	2.53 b	2.76 b
LSD 0.01	0.3	0.2	7.16	0.26	0.30
Irrigation level					
IL100 (100% ETc)	7.9 a	4.5 a	141.77 a	4.39 a	4.76 a
IL80 (80% ETc)	7.3 b	3.9 b	105.11 b	3.95 b	4.39 b
IL60 (60% ETc)	6.3 c	3.1 c	67.11 c	3.18 c	3.70 c
LSD 0.01	0.4	0.2	8.77	0.32	0.36
			2018		
Planting date					
PS1 (February)	8.5 a	4.0 a	122.32 a	4.96 a	5.44 a
PS2 (March)	7.8 b	3.9 a	115.42 b	4.21 b	4.46 b
PS3 (April)	7.0 c	3.5 c	92.83 c	3.13 c	3.30 c
LSD 0.01	0.5	0.1	5.95	0.21	0. 22
Soil cover system					
with mulch	8.2 a	4.1 a	126.54 a	5.37 a	5.81 a
without mulch	7.3 b	3.5 b	93.83 b	2.83 b	2.99 b
LSD 0.01	0.4	0.1	4.86	0.17	0.18
Irrigation level					
IL100 (100% ETc)	8.2 a	4.6 a	148.65 a	4.52 a	4.86 a
IL80 (80% ETc)	8.0 a	3.8 b	110.53 b	4.17 b	4.48 b
IL60 (60% ETc)	7.2 c	3.1 c	71.39 c	3.60 c	3.87 c
LSD 0.01	0.5	0.1	5.96	0.21	0.22

* Means followed by the same letter within a year and column for each tested factor are not significantly different according to LSD at 1% level. BL = Bulb length, BD = Bulb diameter, Y-pot = bulb yield per pot, WP = water productivity, IWUE = irrigation water use efficiency).

Bulb yield per pot (Y-pot)

Analysis of variance shown in Table 2 indicated that the main effects of the three studied factors on Y-pot were significant at the 1‰ level. However, none of the three-factor or two-factor interactions were significant at the 1% level in both studied years. The data under each factor were averaged over all levels of the other factors for mean comparison purposes (Table 3). In both years, earliness in planting date resulted in an increase in the bulb yield. The mean yield of bulbs which were planted in February (PS1), were the highest with 119.7 and 122.3 g pot^{-1} in 2017 and 2018, respectively. Y-pot significantly decreased with delayed planting. It reduced considerably by 8 and 30% in 2017 and by 6 and 24% in 2018 when onion sets were planted in PS2 and PS3, respectively, compared with sets planted in PS1.

In addition, Y-pot was found to be increased significantly by 30% in 2017 and 26% in 2018 when straw mulching was used relative to no-mulching conditions (Table 3).

On the other hand, decreasing water application rate resulted in a significant decline in the bulb yield. The lowest mean values of Y-pot (67.1 g pot^{-1} in 2017 and 71.4 g pot^{-1} in 2018) were observed under sharp deficit irrigation when only 60% of ETc was applied to irrigate onion plants. Then, Y-pot was highly improved with increasing irrigation level. Y-pot increased by about 110 and 56% when onion plants were irrigated by 100 and 80% of ETc, respectively, compared with those irrigated by only 60% of ETc, in both years.

However, for presentation and discussion purposes, all experimental data of Y-pot under all studied treatments were demonstrated in figure 1a for 2017 and 1b for 2018. Trend analysis indicated that the relationships between Y-pot and irrigation level (as a percentage of ETc) for each planting date and under both soil cover systems were linear (equations not presented). As can be seen in figure 1, the rate of increasing yield with increasing irrigation water level was similar regardless of the planting date adopted under both soil cover systems. This confirmed the lack of interaction as found by ANOVA (Table 2). As shown also in figure 1, the maximum value of bulb yield was recorded in the treatment combining between planting in February under straw mulching and 100% of ETc (IL100) conditions. The yield of bulbs produced under such conditions could be attained about 185g pot^{-1} in both years (Fig. 1). Moreover, irrigating onion plants with only 60% of seasonal ETc (IL60) could produce bulb yield comparable with that under 80% of ETc (IL80), provided using straw mulching and planting onion sets as early as possible.

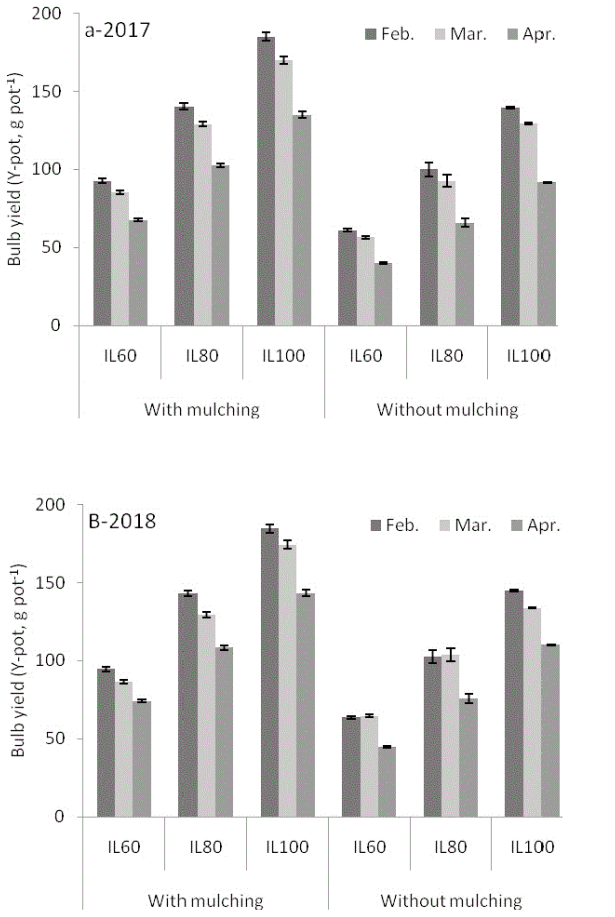

Fig. 1 - Response of onion bulb yield (Y-pot) in 2017 (a) and in 2018 (b) to planting date (February, March, and April), soil cover system (with and without mulching), and irrigation level (IL100, IL80, and IL60). Error bar represents one standard deviation.

Water use parameters

As mentioned above, irrigation treatments (IL100, IL80, and IL60) received at each irrigation event 100, 80 and 60% of the amount of soil water depleted under IL100 conditions, respectively. Irrigation water amounts and seasonal crop evapotranspiration (ETc) for the studied treatments are shown in Table 4 for both years. The seasonal ETc were close to the applied water amounts, because water amounts added to the pots were equal to the depleted water amounts, as regulated by weight. As can be seen, both irrigation water amounts and crop water consumption were greatly decreased when straw mulch was applied. For the three planting dates (PS1, PS2, and PS3), an average of 30% of water was saved when straw mulching was used compared with non-mulching conditions, regardless of the tested irrigation level. Moreover, early planting resulted in a noticeable decrease in both irrigation water amount and crop water use compared with the other planting dates (Table 4). For instance, onion crop planted in February (PS1) required irrigation amount 20-30% lesser than that planted in the traditional planting date (April, PS3). These results highlight the need for changing cultural practices and adopting early planting in order to conserve water resources.

The ANOVA detected that both crop water productivity (WP) and irrigation water use efficiency (IWUE) were significantly influenced by the main effects of planting date, soil cover system, and irrigation level at the 1‰ level, within both years. The interaction between planting date and soil cover system (PS×SC interaction) was also significant at the 1‰ level (Table 2). It is worth to examine the nature of this interaction. Figure 2 illustrates the data of both WP and IWUE under different planting dates (as averaged over all irrigation levels)under each system of soil covering, for both years. Regression analysis indicated that the relationship between both traits (WP and IWUE) and planting date were linear with significant values of R^2 at the 1% level, under both straw mulching and no-mulching conditions. For each trait, both representative straights were not parallel, but did not intersect over the studied period. The slope of straight is about two times greater under straw mulching than that without mulching. This indicates that the enhancements in both WP and IWUE due to the earliness in planting date, could be dou-

Table 4 - Irrigation water amount (without rainfall) and crop evapotranspiration (ETc) as influenced by planting date (PS1, PS2, and PS3), soil cover system, and irrigation level, for both studied years

Parameters	Soil cover	Irrigation level	PS1 (Feb.)	PS2 (Mar.)	PS3 (Apr.)
		2017			
Irrigation water amount (mm)	Without mulch	IL100	705.3	798.8	899.6
		IL80	561.0	649.9	734.2
		IL60	416.7	502.6	570.8
	With mulch	IL100	458.4	561.4	636.0
		IL80	363.9	461.3	524.9
		IL60	269.4	361.2	413.8
Crop evapotranspirationETc	Without mulch	IL100	800.2	819.1	909.2
		IL80	664.5	671.8	745.7
		IL60	525.4	528.1	586.2
	With mulch	IL100	552.5	578.0	641.5
		IL80	459.9	479.8	532.5
		IL60	367.5	387.3	429.9
		2018			
Irrigation water amount (mm)	Without mulch	IL100	706.5	809.6	886.8
		IL80	572.3	655.8	718.3
		IL60	431.0	493.9	540.9
	With mulch	IL100	494.6	566.7	620.8
		IL80	400.6	459.0	502.8
		IL60	301.7	345.7	378.7
Crop evapotranspirationETc	Without mulch	IL100	760.3	848.1	924.3
		IL80	615.8	687.0	748.7
		IL60	463.8	517.3	563.8
	With mulch	IL100	548.4	605.2	658.3
		IL80	444.2	490.2	533.2
		IL60	334.5	369.2	401.5

bled if straw mulching is used. This could explain the existence of the interaction between planting date and soil cover system.

The obtained data of both WP and IWUE under all tested treatments were shown in figure 3a and 3b for WP, and in figure 4a and 4b for IWUE, in 2017 and 2018, respectively. Also, the mean values under each factor were presented in Table 3 for mean comparison purposes. Both WP and IWUE as derived from the traditional planting date (PS3, in April) were the lowest: 2.82 and 3.13 kg m^{-3} for WP, and 2.87 and 3.30 kg m^{-3} for IWUE, in 2017 and 2018 respectively. Earliness in planting date resulted in a noticeable increase in both efficiencies. For instance, when onion sets were planted early in February (PS1), WP and IWUE were significantly augmented by 63 and 99% in 2017, and by 59 and 65% in 2018, respective-

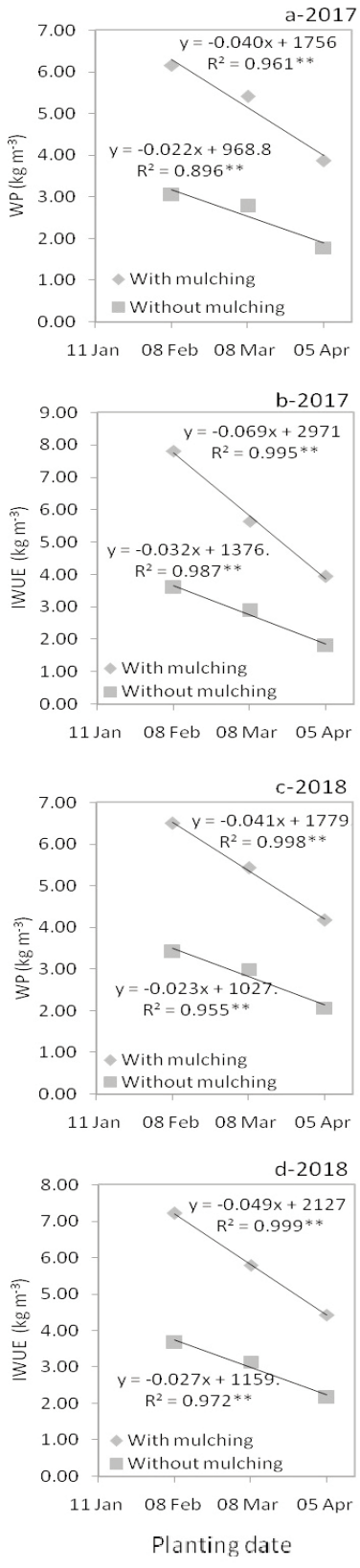

Fig. 2 - For both years, responses of both (a and c) crop water productivity, WP, and (b and d) irrigation water use efficiency, IWUE, to planting dates under both soil cover systems. Regression equations are fitted and coefficient of determination (R2) is given under each system of soil cover. ** = significant at 1% level.

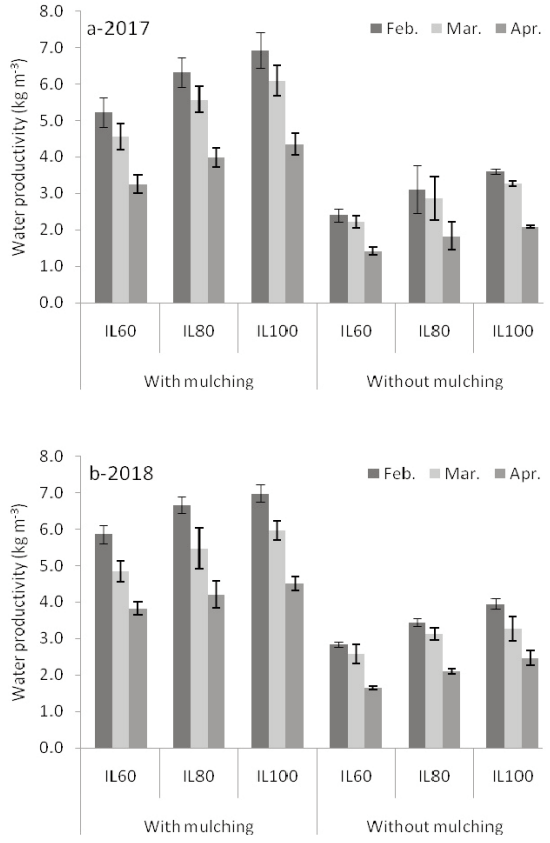

Fig. 3 - Response of water productivity (WP) in 2017 (a) and in 2018 (b) to planting date (February, March, and April), soil cover system (with and without mulching), and irrigation level (IL100, IL80, and IL60). Error bar represents one standard deviation.

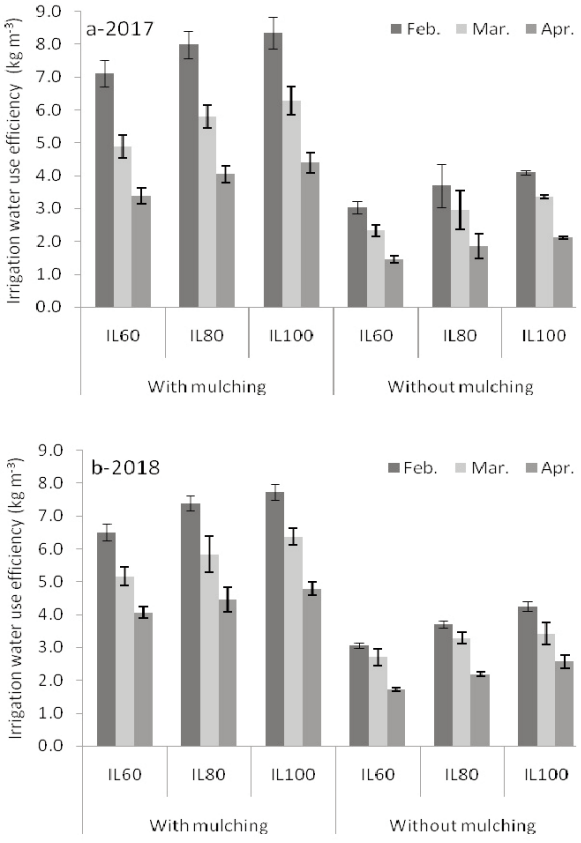

Fig. 4 - Response of irrigation water use efficiency (IWUE) in 2017 (a) and in 2018 (b) to planting date (February, March, and April), soil cover system (with and without mulching), and irrigation level (IL100, IL80, and IL60). Error bar represents one standard deviation.

ly. Furthermore, both WP and IWUE were found to be enhanced considerably when straw mulching was used: they were two times more than those under no-mulching conditions regardless of the planting dates or irrigation levels chosen. In addition, increasing water application rate resulted in an efficient use of water. The maximum values of both WP and IWUE (4.39 and 4.76 kg m^{-3} in 2017, and 4.52 and 4.86 kg m^{-3} in 2018, respectively) were recorded under full irrigation treatment. They then declined dramatically with decreasing irrigation level.

Trend analysis revealed that both WP and IWUE were linearly related to the irrigation level (as % of ETc) under both soil cover systems, regardless of the planting date considered, as shown in Figures 3 and 4 (mathematical equations not presented). Such developed linear functions could be invested for predicting the targeted values of WP and IWUE under similar climatic conditions in the dry Mediterranean area. For instance, the best agricultural management suggested to have maximum values of both traits (WP and IWUE) is to plant onion sets in mulched soil in February under full irrigation conditions. The values of WP and IWUE produced under such conditions could reach in average 6.95 and 8.05 kg m^{-3} (Figs. 3 and 4).

4. Discussion and Conclusions

As the tested onion variety is an oval- to elongated-shape onion, the larger the bulb size (both length and diameter), the better the bulb shape for appearance and marketing purposes. The onion bulb size was found to be increased when onion sets were planted early under straw mulching and 100% of ETc conditions. This could be related to the soil water availability. Under water stress, the soil is drier and relatively more compacted. Its mechanical resistance may limit the growth of the bulb and cause it to grow longitudinally. Moreover, straw mulching can reduce

the soil evaporation and conserves soil humidity (Kirda, 2000; Fereres and Soriano, 2007; Igbadun *et al.*, 2012), and consequently, may encourage developing onion bulbs to grow in both length and diameter directions. Thus, the recommended agricultural management to produce better shape of onion bulbs for consumers, is to plant onion sets in February under straw mulching and full irrigation level conditions. Similar results about the role of straw mulching in enhancing the bulb shape indicator were reported by Mubarak and Hamdan (2018 b).

The bulb yield, and both WP and IWUE were also found to be maximized when onion sets were planted early, under straw mulching and full irrigation. These results could be explained by the fact that the early planting partially covers the late winter time in which onion plants grow well and there would be relatively plenty of water available, compared with the rest of the year from spring to the end of summer. Onion sets planted early also had enough time to benefit from cool period during the vegetative stage, which improved photosynthesis, and therefore production, compared with the actual practice followed by farmers (planting in April). This finding is in agreement with similar results obtained by Hamma (2013) and Rohini and Paramaguru (2016). Also, both irrigation water amount and crop water consumption were found to be greatly decreased, saving about of 30% of water when straw mulch was used compared with no-mulch conditions, irrespective of irrigation level. Under current practices of planting in April without mulching, farmers are not in favor of fully irrigating their crops even if the yield is reduced, due to the huge irrigation water needs (about 900 mm). Thus, using straw mulch could regulate such case. Our research results indicated that onion plants planted in February and grown under straw mulch could be fully irrigated with only 550 mm. The favorable impact of mulching was preveiously reported (Vavrina and Roka, 2000; Igbadun *et al.*, 2012; Hamma, 2013; Tsegaye *et al.*, 2016; Mubarak and Hamdan, 2018 b). In fact, mulching decreases evaporation from soil surface, remaining more water available for plants (Kirda, 2000; Fereres and Soriano, 2007; Igbadun *et al.*, 2012). This could also moderate the severity of wetting-drying cycle between irrigations, and therefore, yield could be improved (Vavrina and Roka, 2000; Gimenez *et al.*, 2002; Mubarak and Hamdan, 2018 b). Moreover, Khaledian *et al.* (2010 and 2011) showed that increasing in crop yield could be also attained under straw mulching due to the enhancements in both soil fertility and soil physical properties.

Results indicated that the tested onion variety was very sensitive to regulated deficit irrigation. Many reports cited similar results that onion yield was optimized under full irrigation rather than under regulated deficit irrigation (Bekele and Tilahun, 2007; Kumar *et al.*, 2007; Nagaz *et al.*, 2012; Igbadun *et al.*, 2012). For example, Nagaz *et al.* (2012) observed that irrigating onion crop with 60% of ETc resulted in considerable reduction in bulb yield, dry matter, and bulbs per hectare, compared with those irrigated by 100% or 80% of ETc. However, an important finding of our experiments is that the onion crop response to regulated deficit irrigation was found to be significantly enhanced when straw mulching is used. For example, irrigating with only 60% of ETc using straw mulching resulted in WP and IWUE much higher even than those irrigated by 100% of ETc without mulching (Figs. 3 and 4). The irrigation water saving in such treatment (deficit irrigation using straw mulching) could be used to irrigate additional cropped area. Patel and Rajput (2013) reported similar outcome that with 40% deficit irrigation throughout the growing period, a water saving obtained could be utilized to irrigate additional ½ ha.

To conclude, onion crop was found to be responsive to early planting and straw mulching, so that both onion bulb size and yield were significantly enhanced, compared with those obtained under the traditional agricultural practices (planting in April without mulching). Both crop water productivity and irrigation water use efficiency were also considerably increased; and the seasonal crop water requirements obviously decreased. Our research results suggest that the best agricultural management is to plant onion sets in mulched soil in February under full irrigation conditions. Moreover, early planting date and straw mulching improved the response of onion crop to the regulated deficit irrigation. This could be an appropriate agronomic alternative to meet the ever increasing demand for onions and to save irrigation water. Onion bulb responses were predicted to be increased linearly with the increment in water application rate and with the earliness of planting date, with an obvious better preference under straw mulching. Both experimental data and the developed equations could be used for predicting onion crop responses under similar agro-pedo-climatic context without carrying out any additional experiments. Moreover, they could be used as a tool for rational management of limited irrigation water.

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Production of seed-propagated compact potted *Corylopsis* plant in one year

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Abstract: The feasibility to produce compact *Corylopsis sinensis* var. *calvescens* and *C. coreana* plant in a 10 cm pot in one year from transplanting seedlings with maximized number of short shoots and inflorescences was investigated. *Corylopsis sinensis* var. *calvescens* was selected as a suitable species to produce compact plant with inflorescences. Slow release fertilizer (SRF) at a rate of 0, 0.125, 0.25, and 0.5 g per pot was applied to the surface of the growing medium (Expt. 1). Shoots were pinched 2 (Feb. 28), 4, 6, and 8 weeks (May 16) (Expt. 2) after transplanting, and ancymidol, paclobutrazol, chlormequat, and daminozide plant growth retardants were treated (Expt. 3). Application of a SRF at 0.5 g per pot and pinching four times at 2-week intervals before May 16 effectively increased the flowering percentages and the number of stems with inflorescences, to accelerate flowering, and also produced a compact plants. Paclobutrazol at 10-20 mg/L applied as soil drench was effective in inhibiting stem elongation in the first year; however, higher concentrations should be avoided to prevent excessive reduction in the growth of shoots and production of malformed inflorescences.

1. Introduction

The genus *Corylopsis* Siebold & Zucc., commonly known as Winter Hazel that flowers early in the spring in China and Korea, is a shrub or small tree. Most of the *Corylopsis* species grows tall reaching a height of 2-4 meters (Bean and Anisko, 2014). Flowers are bisexual and seeds are produced. Among 29 species, 19 species are endemic in China (Zhang *et al*., 2003). *Chinensis sinensis* Hems. var. *calvescens* Rehder & E.H. Wilson is growing in the mountains in Guangxi, Sichuan, and Jiangxi, among other provinces in China (Zhang *et al*., 2003) and *C. coreana* Uyeki (Son *et al*., 2016) in a rather restricted area in Korea. All species are deciduous shrubs producing light yellow pendant racemes (inflorescence) measuring

about 5 cm in length, followed by appearance of leaves (Fig. 1).

Corylopsis, one of many germplasms native and indigenous to China that includes an endangered *Acer pentaphyllum* Diels (Roh *et al*., 2008 b), are not well known to horticulturist, growers, and landscape industry, but has a great potential to develop as a new ornamental plant. Although *Corylopsis* may be available from rooted cuttings or tissue-cultured propagules for a mass propagation (Moon *et al*., 2002; Koh and Lim, 2006), the success of the rooting of cuttings depends on the season when cuttings were collected and may not provide a large number of plants (Kwon *et al*., 2011) and acclimatization of tissue culture propagules is not easy (Moon *et al*., 2002; Koh and Lim, 2006). Therefore, seeds are a viable alternative source for mass propagation and for forcing seedlings to flower. Suitable species should first be identified and then excessive stem elongation must be controlled using plant growth retardants (Currey and Lopez, 2016), and pinching combined with plant growth retardant treatment (Jeong, 2000).

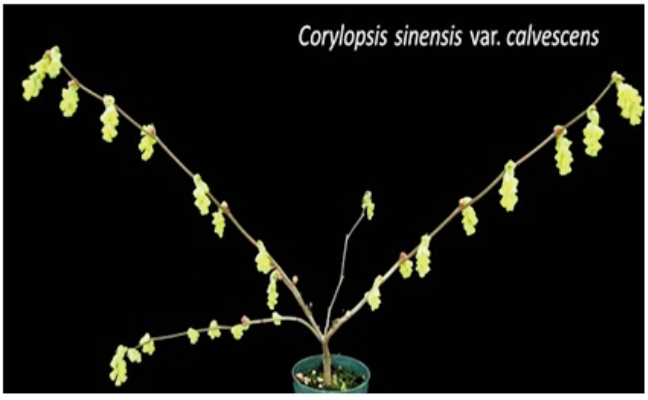

Fig. 1 - Appearance of *C. sinensis* var. *calvescens* at anthesis produced in a 10 cm pot from seeds. Clusters of flower buds (inflorescences) are well developed on long shoots.

Stem elongation can be inhibited by practices such as pinching shoots in many floral and ornamental plants, resulting in short plant height (Lee *et al*., 2006; Latimer and Whipker, 2013). Growth and flowering is also affected by treatment with plant growth regulators. Among the many growth retardants, ancymidol (α-Cyclopropyl-α-(4-methoxyphenyl)-5-pyrimidinemethanol), daminozide (Butanedioic acid mono(2,2-dimethylhydrazide), chlormequat (2-Chloro-*N,N,N*-trimethylethanaminium chloride), and paclobutrazol [(2RS, 3RS)-1-(4-Chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) penta-3-ol] have been used in many floral and ornamental plants to inhibit stem elongation (Currey and Lopez, 2016). Generally, ancymidol and paclobutrazol is effective when applied as a soil drench, and daminozide and chlormequat when applied as a foliar spray.

Germination of *Corylopsis* seeds as affected by warm and cold stratification and the X-ray imaging to separate full seeds from empty seeds is well documented (Kim *et al*., 2015, 2017, 2018). To produce compact and flowering *Corylopsis* plants in small pots in one year after transplanting seedlings, selection of proper species and the most suitable cultural practices should be identified. However, there is no report on the growth and flowering of *Corylopsis* starting from small propagules regardless of propagation methods: seed propagation, rooting of cuttings, and *in vitro* propagation. Shortening the total production time from 2-3 years to one year while ensuring the qualities of plants at flowering from seedlings in *Lilium longiflorum* Thunb. bulbils in *L.* ×*elegans* Thunb. and tissue-cultured propagules in interspecific hybrids between *L. longiflorum* and *L.* ×*elegans* was reviewed (Roh, 1992, 1996).

Production of *Corylopsis* in a small pot with inflorescence will attract and enable consumers to purchase at the nursery or the garden center in the early spring, and then plant them in the garden to enjoy the beauty of flowers for many years. The objectives of this research were (1) to select the species to grow starting from seeds in small pots, and to study the effect of (2) slow release fertilizer (SRF) treatments, (3) the pinching frequencies, and (4) plant growth retardant treatments to produce compact seed-propagated *Corylopsis* plants in 10 cm pots in one year from transplanting seedlings.

2. Materials and Methods

Preliminary field evaluation to select a suitable species for final evaluation

Seeds of 45 accessions (data not presented) including *C. glabrescens* (NA50804, Longwood 1997-0068B, Longwood Chimes), *C. spicata* (NA37208, NA40102, Arnold 7950A), *C. pauciflora* (NA37205, Longwood 1944-0213*H), and *C. vietchiana* (NA37208, NA65619) were sown between Oct. 2 and Nov. 2 and planted into 10 cm pot filled with ProMix BM (Premier Horticulture Inc., Quakertown, PA, USA) between Mar. 1 and Apr. 2, and grown in the field. The final evaluation based on the number of plants that flowered and the growth habits, *C. coreana* and *C. sinensis* var. *calvescens* (NA 57391) (Roh *et al*.,

2008 a) were selected (Table 1) for evaluation in the next three experiments.

Effect of slow release fertilizer treatment on growth and flowering (Expt. 1)

About 200 seeds each of *C. sinensis* var. *calvescens* and *C. coreana* were sown on Oct. 20, 2009 in a 15 cm pot and received temperature treatments [20°C (Oct. 21 - Dec. 1) and 5°C (Dec. 2, 2009 - Feb. 16, 2010)]. One seedling was transplanted per 10 cm pot filled with ProMix BM on Mar. 4, 2010. On Mar. 18, 2010 when seedlings formed 4 nodes, the main shoot was pinched leaving two pair of leaves.

Slow release fertilizer (SRF; Osmocote, 14 N - 6.2 P - 11.6K; Scotts Co., Marysville, OH, USA) was applied to the surface of the growing medium at transplanting seedlings at a rate of 0, 0.125, 0.25, and 0.5 g per 10 cm pot (Table 2). During the culture, plants were fertilized with 1.33 g/L of 15N - 7P - 12.8K water soluble fertilizer once a month.

Greenhouse day temperature was maintained at 21-22°C on Oct. 1, 15-17°C on Nov. 1, 13-14°C on Nov. 16, 10-12°C on Dec. 1, 7-8°C on Dec. 16, 2010 and at 4-5°C on Jan. 1, 21-24°C on Apr. 16, 2011, and was raised by 2.5°C every 2 weeks until Sept. 1, 2011. Night temperature was maintained 2°C lower than the day temperature. The number of weeks to flower counted from the date of transplanting seedlings to pots, and the number and length of nodes with inflorescences from the three longest shoots, and the number of nodes with 2 inflorescences was recorded from 15 plants per treatment. Flowering date was recorded when two florets each from two inflorescences reached anthesis, and data were subjected to the regression analysis for each species using Statistical Analysis System program (SAS, 2002).

Effect of pinching frequencies on growth and flowering (Expt. 2)

After sowing about 200 seeds as described in Expt. 1, seedlings were transplanted. On Mar. 18, 2010, 0.25 g of slow release fertilizer was applied to the surface of growing medium and the effect of pinching frequencies on *C. sinensis* var. *calvescens* was evaluated. Shoots were either not-pinched or pinched 2 (Feb. 28), 4 (Apr. 18), 6 (May 2), and 8 weeks (May 16) after transplanting as outlined (Table 3). To the surface of the growing medium at transplanting seedlings 0.8 g of slow release fertilizer per pot was applied, and plants were fertilized with 1.33 g/L of 15N - 7P - 12.8K water soluble fertilizer once a month.

The number of weeks to flower, and the total number of shoots with flowers and flower buds, and the length and number of flowers from the first and second longest shoots was recorded from 15 plants per treatment. The number of days to flower was counted from the date of transplanting. Data were subjected to the analysis of variance (ANOVA) and means were compared with Tukey's honestly significant difference (HSD) test.

Effect of growth retardants treatment on growth and flowering (Expt. 3)

Corylopsis sinensis var. *calvescens* seeds were sown and transplanted, and pinched as described in Expt. 1, and pinched again on May 26, 2010. Plants were grown in greenhouse maintained at 18-21°C/16-19°C (day/night) and then in greenhouse maintained at 22-25°C/20-23°C until July 6. To the surface of the growing medium at transplanting seedlings 0.8 g of slow release fertilizer per pot was applied, and plants were fertilized with 1.33 g/L of 15N - 7P - 12.8K water soluble fertilizer once a month.

Growth retardants were applied on Jul. 7, when new shoots were about 5-8 cm long. Each pot was treated with 25 mL of ancymidol [0.026% active ingredient (a.i.)] and paclobutrazol (0.4% a.i.) at 0, 10, 20, 40, and 80 mg/L was applied as a soil drench. Daminozide (85% a.i.) and chlormequat (11.8% a.i.) at 0, 2,500,

Table 1 - Evaluation of flowering and growth habit in the field *C. coreana* and *C. sinensis* var. *calvescens*

Species	Seed harvest	2008		2009		
		Germination	Transplanting	Flowering [z]	No. of plants [y]	Growth characteristics [x]
C. coreana	2007	Mar. 6	Mar. 12	Mar. 19-25 (Mar. 21)	9 (21)	Upright (12), prostrate/upright (1)
C. sinensis var. *calvescens*	2007	Mar. 1	Apr. 2	Mar. 27-Apr. 7 (Apr. 2)	14 (16)	Upright (13), prostrate/upright (1)
	2008	Feb. 26	Apr. 8	Mar. 22-Apr. 3 (Mar. 28)	16 (16)	Upright (16)

[z] Range and mean of flowering.
[y] Plants that produced inflorescence and the total number of plants evaluated (parenthesis).
[x] Number of plants (parenthesis) showing upright and prostrate growth characteristics.

5,000, 7,500, and 10,000 mg/L was applied as a foliar spray, and 200 mL of solution was applied to 15 plants. On Nov. 20, 2010, plants were grown in a greenhouse maintained at 4-5°C for cold treatment until Mar. 1, 2011.

Dates of flowering, when two florets from an inflorescence reached anthesis were recorded and the lengths of two longest shoots (shoot length A) per plant and shoots longer than 3 cm were counted on Jan. 16. Plants were moved outdoors on Mar. 27, and the new growth of two longest shoots (shoot length B) was also recorded on May 10, 2011. Data collected from 15 plants per treatment were analyzed by two-way ANOVA with plant growth retardants and concentration as variables.

3. Results

Selection of a suitable species for final evaluation

Following evaluation of 45 accessions including *C. glabrescens,C. spicata*, *C. pauciflora*, and *C. vietchiana* (data not presented), *C. sinensis* var. *calvescens* and *C. coreana* showing upright growth characteristics of shoots and flowering response were selected-for the final evaluation. All accessions grew taller than 1.3 m and spread over 65 cm wide in case of *C. spicata*, but with a few inflorescence (data not presented). The selection criteria were based on the number of plants that had flowered exhibiting upright growth characteristics. In less than one year counting from the time of transplanting, 14 from 16 *C. sinensis* var. *calvescens* plants flowered showing up-right growth (Table 1). *Corylopsis coreana* was also selected for its large foliage for its good fall foliage color, even though only nine out of 21 plants had flowered.

Effect of slow release fertilizer treatment on growth and flowering (Expt. 1)

The number of weeks to flower in 52 to 53 weeks in *C. sinensis* var. *calvescens* and *C. coreana* was not affected by the rate of SRF treatments (Table 2). The number of total shoots and of shoots with inflorescences increased linearly with SRF treatment from 2.5 to 4.1 in *C. coreana* and from 2.1 to 4.1 in *C. sinensis* var. *calvescens*. The lengths of the three longest shoots also increased in both species, from 11.9 to 25.6 cm for the longest shoot, from 4.8 to 15.8 cm for the third shoots in *C. coreana,* and from 12.8 cm to 29.3 cm for the longest shoot in *C. sinensis* var. *calvescens*.

The number of nodes with inflorescences in all the three shoots of *C. coreana* received 0.5 g SRF treatment was 0.3 or less than 0.3 and there was only one node with more than 2 inflorescences. However, the number of nodes with inflorescence produced and the number of nodes with 2 inflorescences was higher in *C. sinensis* var. *calvescens* than in *C. coreana.* The number was increased to 4.8 nodes in the first

Table 2 - The effect of slow release fertilizer treatment on the growth and flowering of *Corylopsis coreana* and *C. sinensis* var. *calvescens* [z]

Species	Slow release fertilizer (g/10 pot)	No. of weeks to flower [y]	No. of total shoots	Length (cm) of three longest shoots (SH)			No. of nodes with inflorescences [x]		
				SH 1	SH2	SH 3	SH1	SH2	SH 3
C. coreana	0	52	2.5	11.9	6.7	4.8	0.1	0.1	0.0
	0.125	52	3.3	14.4	9.8	7.4	0.3	0.1	0.0
	0.25	53	4.6	20.3	13.6	10.1	0.1	0.0	0.0
	0.5	52	4.1	25.6	22.0	15.8	0.1	0.3	0.3
Regression analysis - linear effect [w]		NS	*	**	**	**	NS	NS	NS
C. sinensis var. *calvescens*	0	53	2.1	12.8	6.7	4.5	0.1	0.0	0.0
	0.125	53	2.9	14.3	9.9	7.5	0.8	0.7	0.0
	0.25	52	3.4	21.3	13.0	10.4	3.1	2.8	1.8
	0.5	52	4.1	29.3	21.8	19.8	4.8	4.4	2.7
Regression analysis - linear effect [w]		NS	**	**	**	**	*	*	*

[z] There was a significant difference between two species; data for each species were subjected to the linear regression analysis.
[y] The number of weeks to flower was counted from the date of transplanting seedlings.
[x] Nodes with inflorescence that were formed on new growth by pinching.
[w] Non-significant (NS), significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

shoot and to 4.4 nodes in the second shoot following treatment with 0.5 g of SRF and the number of nodes with more than 2 inflorescences was also increased. *C. sinensis* var. *calvescens* treated with 0.125, 0.25, and 0.5 g SRF has the potential to produce a small potted plant (Fig. 2).

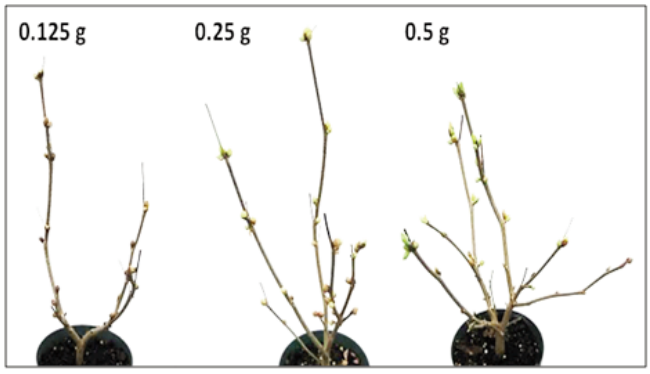

Fig. 2 - Appearance of the *Corylopsis sinensis* var. *calvescens* plants in a 10 cm pot treated with 0.125, 0.25, and 0.5 g of slow release fertilizer per pot prior to leaf emergence and anthesis.

Effect of pinching frequencies on growth and flowering (Expt. 2)

Regardless of frequencies and timing of pinching, the flowering of *C.sinensis* var. *calvescens* occurred in 53 weeks (Table 3). Flowering ranged between 73 and 93%, and the highest flowering rate was recorded when pinched for 4 times at 2, 4, 6, and 8 weeks, yielding a significantly higher number of shoots with inflorescences (5.3) and consequently the highest number of inflorescences (22.1) compared with the control.

The length of the first and the second non-pinched shoot was 49.3 and 33 cm, respectively, with a difference of 16.3 cm (Table 3). However, when pinched 4 times, the lengths were 38.0 and 31.8 cm with a difference of 6.2 cm. The number of inflorescences in non-pinched and pinched shoots, which was 9.2 and 7.1 in the first shoot and 4.0 and 6.9 in the second shoot, respectively, did not vary significantly. However, the difference in the number of inflorescences (0.2) between the first and the second shoot was significantly lower in the pinched shoot compared with the non-pinched shoot (5.2). In general, when pinched, the difference in the inflorescences between the first and the second shoot was less than 1.9, which was significantly less than that of the control.

Effect of growth retardant treatments on growth and flowering (Expt. 3)

When plants were treated with ancymidol, chlormequat, and daminozide, flowering took 22 to 28 days regardless of treatment concentrations, which was not significantly different from that of control (Table 2). However, soil drench treatments with paclobutrazol (20 mg/L or higher concentrations) took longer than 36 days. Flowering percentage was higher than 60% when plants were treated with ancymidol, chlormequat, and daminozide, regardless of treatment concentrations.Treatment with 20 mg/L of paclobutrazol severely inhibited the extension of peduncle bearing inflorescence triggering the death of inflorescence immediately after

Table 3 - The effect of pinching frequencies on growth and flowering of *Corylopsis sinensis* var. *calvescens*

Pinching [z]				No. of weeks to flower [y]	Flowering %	Total shoots no. with inflores-cences	Flowers (Total No.)	Shoot length (cm)			No. of nodes withinflorescences [x]		
2 Weeks	4 Weeks	6 Weeks	8 Weeks					First shoot	Second shoot	Difference	First	Second shoot	Difference
x	x	x	x	53	87	2.9	17.2	49.3	33.0	16.3	9.2	4.0	5.2
o	x	x	x	53	87	2.1	12.8	49.0	38.8	10.2	6.9	6.8	0.1
o	o	x	x	54	73	2.4	13.1	47.1	35.5	11.6	6.4	4.8	1.6
o	o	o	x	53	87	3.5	18.5	41.6	35.2	6.4	6.8	5.7	1.1
o	o	o	o	53	93	5.3	22.1	38.0	31.8	6.2	7.1	6.9	0.2
o	x	o	o	53	87	4.3	17.3	32.4	29.2	3.2	5.7	5.2	0.5
o	o	x	o	54	80	3.1	10.4	37.3	26.0	11.3	4.7	3.9	0.8
o	x	o	x	53	73	3.3	14.8	39.5	29.8	9.7	6.5	6.3	0.2
o	x	x	o	53	87	3.3	16.3	39.0	22.2	16.8	7.6	5.7	1.9
Level of significance [w]													
HSD at P<0.05				NS	-	0.94	3.58	8.25	5.61	5.82	4.27	3.84	1.59

[z] Not pinched (x) or pinched (o) 2 (Feb. 28), 4 (Apr. 18), 6 (May 2), and 8 weeks (May 16) after transplanting.
[y] The number of weeks to flower was counted from the date of transplanting seedlings.
[x] Nodes with inflorescence induced by pinching.
[w] Non-significant (NS) or significance at $P \leq 0.05$, F-test.

emergence following leaf emergence (Fig. 3). Therefore, days to flower were estimated on the date of leaf emergence. Flowering percentage was significantly reduced to less than 30% when plants were treated at 20 and 40 mg/L paclobutrazol.

When shoot lengths following 80 mg/L ancymidol were recorded on Jan. 16 (26 weeks after growth retardant treatment), the length of the first and the second longest shoots was significantly reduced from 26.3 cm to 15.6 cm and from 16.4 cm to 12.2 cm, respectively (Table 4).

The length of the two longest shoots treated with daminozide and chlormequat showed similar trends as observed in plants treated with ancymidol. The length of the two longest shoots was significantly reduced to 12.9 cm and 9.8 cm following treatment with 10 mg/L paclobutrazol (Fig. 3), responding to the quadratic effect of concentrations. The length of shoot B showing new growth on May 10 was significantly inhibited to less than 5.0 cm when treated with paclobutrazol. The number of shoots longer than 3 cm varied from 4.8 to 5.7, from 3.8 to 4.8, 4.3 to 5.5, and 5.0 to 3.9 upon treatment with ancymidol, chlormequat, daminozide, and paclobutrazol, respectively. The numbers were not affected by concentrations of these three retardants (data not presented).

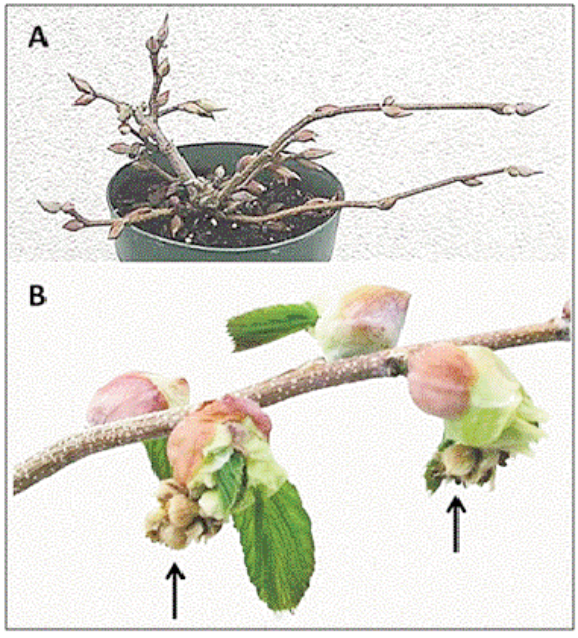

Fig. 3 - *Corylopsis sinensis* var. *calvescens* in a 10 cm pot treated with 25 mL of 40 mg/L paclobutrazol (A). Blasted inflorescences (arrow) and emerging of dark green leaves of reduced size indicate excessive doses of paclobutrazol (B). Photographed on Mar. 27, 2011.

4. Discussion and Conclusions

Successful acclimatization rate of *in vitro* propagated *C. coreana* was low (Moon *et al*., 2002) and limited time of the season to propagate by rooting of cuttings (Kwon *et al*., 2011) are the limiting factors for mass propagation to secure sufficient and uniform propagules for experiments, and further, reports are not available on flowering of *in vitro* propagules and rooted cuttings. This clearly indicates that seeds can be used as a propagule to produce sufficient number of seedlings to produceflowering plants in a year from transplanting seedlingsby various cultural practices reported in this study. The morphological characteristics of *C. sinensis* var. *calvescens* are suitable to produce in 10 cm pots compared with *C. coreana*, if stem elongation can be controlled and many shoots with well-developed inflorescences can be formed (Fig. 1).

Growth and flowering as influenced by slow release fertilizer (SRF)

There is a clear difference between *C. coreana* and *C. sinensis* var. *calvescens* responding to SRF application per 10 cm pot. Responding to the increased rates of slow release fertilizer, especially at 0.5 g SRF application, and *C. sinensis* var. *sinensis* is recommended to produce as a 10 cm potted plant in one year as the number of inflorescences and of nodes with more than two inflorescences are increased. Production of *C. coreana* may not be recommended due to fewer numbers of nodes with inflorescences and only 0.1 node produced more than 2 inflorescences.

Growth and flowering as influenced by pinching and growth retardant treatments

Stem length is one of the limitations to produce compact *C. sinensis* var. *calvescens* in small pots, which can be reduced either by pinching or growth retardant treatments. Manual or mechanical pinching is associated with increased labor costs. Treatment with growth retardant may not induce branching when compared with pinching.

Pinching shoots four times in 2, 4, 6, and 8 weeks prior to May 2 is an effective cultural practice to produce compact plants for small pots without affecting days to flowering and flowering percentage. The increase in the number of shoots with inflorescences, the total number of inflorescences, and the number of nodes with inflorescences may result from an increased number of shoots that are formed prior to the development of inflorescence, which may occur

Table 4 - Growth and flowering of *Corylopsis sinensis* var. *calvescens* as influenced by growth retardant treatments

Plant growth regulator	No. of days to flower (Mar. 1) (flowering percent)	Shoot lenght A (cm) (Jan. 16, 2011 [z])		Shoot length B (cm) of new growth (May 10, 2011)	
		FIR [y]	SEC	FIR	SEC
Ancymidol (soil drench) (mg/L)					
0	22 (60)	26.3	16.4	21.0	20.0
10	23 (80)	28.4	17.5	20.2	19.4
20	24 (70)	27.9	21.7	20.2	20.0
40	25 (70)	23.9	19.1	21.9	17.9
80	25 (60)	15.6	12.2	16.5	16.3
Regression analysis	NS	L*	L*	NS	NS
Paclobutrazol (soil drench) (mg/L)					
0	22 (60)	24.7	20.0	24.0	20.0
10	28 [w] (50)	12.9	9.8	5.0	3.5
20	38 [w] (30)	11.2	8.1	1.7	1.2
40	36 [w] (10)	10.3	7.9	1.3	0.8
80	36 [w] (30)	11.0	8.5	1.1	0.6
Regression analysis [x]	-	Q**	Q**	Q**	Q**
Chlormequat (foliar spray) (mg/L)					
0	22 (70)	28.9	20.3	23.1	20.9
2.5	22 (80)	35.1	24.9	22.9	20.1
5	25 (70)	26.5	20.0	18.3	18.1
7.5	27 (60)	23.7	14.5	17.9	17.0
10	25 (70)	24.3	18.5	20.2	19.9
Regression analysis	NS	L*	NS	NS	NS
Daminozide (foliar spray) (mg/L)					
0	23 (60)	29.2	23.0	21.0	19.9
2.5	27 (80)	33.3	23.5	23.5	18.5
5	25 (70)	28.5	22.0	22.5	18.9
7.5	25 (60)	29.3	22.7	22.7	19.1
10	27 (70)	25.5	19.5	21.2	18.5
Regression analysis	NS	L*	NS	NS	NS
Level of significance [v]					
Growth retardant (PGR)	NS	***	***	***	***
Concentratio	NS	**	*	*	*
PGR × Concentration	NS	**	**	**	**

[z] Data collected from over-wintered plants.

[y] Length of the first (FIR) and second (SEC) longest shoots.

[x] Analysis was not carried out due to the estimated days of flowering and low flowering percentage, and regression analysis was performed for each growth retardant. Linear (L) and quadratic (Q) effect.

[w] Days to flower following paclobutrazol treatment were recorded upon leaf emergence following the death of inflorescence. Generally, about 15 days elapsed between flowering and the appearance of leaf emergence.

[v] Non-significant (NS) or significance at $P<0.05$ (*), 0.01 (**) or 0.001 (***), F-test.

after May 2. Since the time of floral bud initiation has not been examined anatomically, it requires further studies. Increase in the number of lateral shoots and flowers were increased as the pinching frequencies in *Sedum rotundifolium* D.B. was increased (Jeong, 2000). The length of shoots exceeding 30 cm following pinching is considered excessive for producing *Corylopsis* in a 10 cm pot.

Flowering of *C. sinensis* var. *calvescens* plants treated with ancymidol, chlormequat, and daminozide that produced higher than 60% plants with inflorescences regardless of treatment concentrations did not differ significantly from that of control. The longest shoot length (A) on Jan. 16 responding linear effect to ancymidol, chlormequat and daminozide was the shortest, especially when treated with 80 ppm ancymidol. Ancymidol is,therefore, recommended for *C. sinensis* var. *calvescens* in 10 cm pot. Since the length of new shoot (B) on May 16 was not affected, the effect of these three plant growth

retardants may not last long when compared with paclobutrazol. A single application of ancymidol is not effective and may require two treatments to produce quality *Mussaenda* 'Queen Sirikit' as a short-stemmed potted plant without reducing the number of flowers per plant and delaying the flowering (Cramer and Bridgen, 1998). Application of ancymidol requires further testing since growth retarding effects of ancymidol do not persist as reported in *L. lancifolium* Thunb. (Roh, 1979) and shoot length is increased under a long day photoperiod during June or July as observed in *L. longiflorum* (Roh and Wilkins, 1977).

Soil drench treatment with paclobutrazol 20 mg/L or higher concentrations which took longer than 36 days to flower (Table 4) compared with 22 days with the control. When treated with 20 mg/L of paclobutrazol, the extension of peduncle-bearing inflorescence was severely inhibited resulting in the death of inflorescence immediately following leaf emergence, thus lowering the flowering rate from 60% to 10% (Fig. 3). Shoot length was arrested under any paclobutrazol treatment which is undesirable.

Although 10-20 mg/L paclobutrazol as a soil drench is considered effective to reduce shoot length, the growth of new shoots and inflorescence development is significantly arrested even a year later, which may require double treatments at low concentrations, i. e., 5 mg/L to avoid severe growth retardation and malformation of inflorescence. A single foliar spray of 500 mg/L paclobutrazol may be used to test produce compact flowering plants as reported in *Rhododendron* hybrids, which is a woody ornamental (Wilkinson and Richards, 1991).

Generally paclobutrazol was not effective in *Mussaenda* at 0.125-0.25 mg a.i. per pot as a soil drench compared with ancymidol and daminozide (Cramer and Bridgen, 1998), which is considered effective in producing compact plants with accelerated flowering although 0.4 g a.i. per pot increased the number of flowers, producing malformed and unacceptable of *Rhododendron* 'Sir Robert Peel' (Wilkinson and Richards, 1991). Shoot length of poinsettia (*Euphorbis pucherrima* Wild. ex Klotzch) was reduced by daminozide and chlormequat treatments without affecting the flowering (Lewis *et al*., 2004). Combined treatment with chlormequat and daminozide can also be considered as reported to be effective to retard stem elongation of zonal (cutting) geraniums [*Pelargonium ×hortorum* (L.H. Bailey)] (Tayama and Carver, 1990).

Due to the long-lasting inhibitory effect of paclobutrazol on shoot elongation and reduction in leaf size when applied as a soil drench, a malformation of inflorescence and formation of inflorescences after leaf emergence result in a lower percentage of plants with inflorescences in both species in this study and in other woody ornamentals such as *Dissotis rotundifolia* (Sm.) Triana and *Tibouchina forthergillae ×pilosa* (Hawkins *et al*., 2015). Therefore, paclobutrazol is not recommended to use as a soil drench in *Corylopsis.* The optimum dosages require further study comparing the effect of soil drench and foliar spray. A spray treatment of paclobutrazol may be considered as the quality of *Dianthus caryophyllus* L., cv. Mondriaan was improved (Bañón *et al*., 2002).

This is the first report providing practical and horticultural strategies to produce flowering *Corylopsis* plants in small pots with a great potential to utilize under-utilized native plants as ornamental and nursery plant starting from seeds. *Corylopsis sinensis* var. *calvescens* indigenous to China is a suitable species as compared to *C. coreana* native to Korea to produce from seeds with application of slow release fertilizer at 0.5 g per pot and pinching for four times at 2-week interval before May 16 to reduce shoot elongation, to increase flowering percentage, and to accelerate flowering with increased number of inflorescences.

Treatments with ancymidol as a soil drench and daminozide and chlormequat as a foliar spray at all concentrations evaluated in this study were not effective to produce compact plants as compared to paclobutrazol treatment. Soil drench treatment with paclobutrazol at 10-20 mg/L is considered effective in reducing shoot elongation the first year. However, the inhibitory effects of paclobutrazol last longer than a year, and appear resulting in malformation of inflorescences in the second year. Therefore, investigation on selecting appropriate treatment methods andconcentrations of paclobutrazol to reduce shoot elongation without triggering malformation of inflorescence is needed. The time of floral initiation and development in relation to pinching treatment needs to be determined as well.

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Varietal differences in sweetness value and flesh juiciness among persimmon cultivars

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Key words: breeding stock, *Diospyros kaki* Thunb., flesh juiciness, sweetness value.

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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: To promote persimmon breeding project, we analyzed the sugar composition (a ratio of sucrose to hexose sugars, SH ratio) and flesh juiciness of 43 persimmon cultivars (*Diospyros kaki* Thunb.) consisting of 24 pollination-constant non-astringent (PCNA)-types and 19 non-PCNA-types, together with other fruit quality traits. The cultivar collection includes newly-released cultivars after 1990 and commercially-produced local cultivars in Japan. These cultivars were broadly classified into three types: sucrose accumulators, intermediate accumulators, hexose accumulators. Analysis of variance showed that the genotypic effect on the SH ratio and flesh juiciness is high with negligibly small environmental variance, indicating that SH ratio and flesh juiciness can be determined by a one-year trial without tree replication. Highly varietal diversity in the SH ratio and flesh juiciness was observed within and between persimmon cultivar types. Sweetness value (SSC × SH ratio) of the cultivars/selections seems to be a useful predictor of fruit sweetness. In terms of palatability, however, persimmon cultivar's improvement should be performed on the sweetness value in association with flesh juiciness.

1. Introduction

Persimmon (*Diospyros kaki* Thunb.) is believed to have originated in Eastern Asia, is produced worldwide including in Azerbaijan, Brazil, China, Iran, Israel, Italy, Japan, Korea, New Zealand, and Spain (FAOSTAT, 2017). A number of local varieties has been developed in China, Korea, and Japan during a long history of domestication (Parfitt *et al.*, 2015; Sato and Yamada, 2016; Yesiloglu *et al.*, 2018). Persimmon cultivars can be classified into four types: pollination-constant astringent (PCA); pollination-variant astringent (PVA); pollination-variant non-astringent (PVNA); pollination-constant non-astringent (PCNA), based on seed formation, change in flesh color, and nature of astringency loss (Hume, 1914; Ikeda *et al.*, 1985; Yonemori *et al.*, 2000). Among these types, fruits of PCA- and PVA-type cultivars are always astringent without postharvest treatment such as application of carbon dioxide gas or ethanol vapor. PVNA-type cultivars require pollination and seed formation to lose astringency as well as flesh

browning. Fruits of PCNA-type cultivars naturally lose their astringency because they terminate accumulating tannins at early fruit development stage. In particular, PCNA-type persimmon cultivars are highly desired for fresh consumption on a worldwide level.

Regarding eating quality of PCNA-type cultivar, the great stress is laid upon the evaluation of soluble solids content (SSC, °Brix) using refractometer because sugars generally contribute a very large proportion to the SSC in the ripening stage (reviewed by Giordani *et al*., 2011). However, Ban *et al*. (2010) and Mitani *et al.* (2015) postulated that flesh juiciness is a decisieve parameter to determine the texture of persimmon fruit, where fruits with higher firmness had a tendency to be less juicy as observed in apple (Harker *et al*., 2003). On the other hand, the percentage sucrose in flesh significantly affects the sweetness of fruit, such as East Asian pear (Kajiura *et al*., 1979), oriental melon (Zhang and Li, 2005), peach (Suzuki *et al.*, 1990; Cirilli *et al.*, 2016) and strawberry (Sone *et al*., 2000). Thus, understanding how varietal differences influence sugar composition will provide valuable information for genetic improvement of the palatability of persimmon fruit in future breeding programs. Giordani *et al*. (2011) classified worldwide persimmon cultivars such as 'Atago', 'Fuyu', 'Rojo Brillante' and 'Triumph' into three groups based on the cluster analysis of sugar composition. To date, however, there has been no comprehensive research on the sugar composition of the recent Japanese PCNA- and non-PCNA-type cultivars. Since 1990, new PCNA- and non-PCNA-type persimmon cultivar have been released in Japan by the National Institute of Fruit Tree Science (Yakushiji and Nakatsuka, 2007; Yamada *et al*., 2012 a, b). These new cultivars have large sized fruit, brilliant skin color, and are highly palatable, but their sugar composition remains largely unknown.

Asakuma and Shiraishi (2017) showed that genotypic effect of sugar composition (SH ratio) and flesh juiciness is significantly high, whereas the year, genotype × year interaction, among trees within genotype, and tree × year interaction is small or negligible. In experimental field of the current study, we have preserved a Japanese persimmon cultivar collection of both PCNA- and non-PCNA-type cultivars as breeding stocks since 1980. Although the total number of preserved cultivars is less than 50, the cultivar collection includes newly-released cultivars after 1990 and commercially-produced local cultivars in Japan. In the present study, we (1) re-confirmed small year variability in the sugar composition and flesh juiciness by the analysis of variance, and (2) discussed varietal differences in the sugar composition and flesh juiciness among persimmon cultivars in association with palatability.

2. Materials and Methods

Plant materials for yearly variation of sugar composition

Four PCNA-type cultivars of Japanese persimmon ('Akiou', 'Fuyu', 'Matsumotowase-Fuyu', 'Taishu') were used. These cultivars are grown for commercially marketable fruit production by normal cultural practices, including pruning, flower and fruit thinning, irrigation, soil and pest management (Yamada, 2006) in an open-field of the Fukuoka Agricultural and Forestry Research Center, Fukuoka, Japan (33°50′ N and 130°57′ E). According to the reference value derived from our annual survey from 2008 to 2015 (Table S1), mature representative eight fruits per tree were sampled from three trees per genotype for late-October to late-November in 2014 to 2016 seasons depending on each cultivar's optimal ripening time and skin color.

The statistical fixed-effect model (Table S2) that we adopted to express the phenotypic value (Asakuma and Shiraishi, 2017) is:

$$P_{ijkc} = m + G_i + Y_k + (GY_{ik}) + T_{ij} + (TY_{ijk}) + E_{ijkc}$$

where P_{ijkc} is the phenotypic value of the *c*th fruit of the *j*th tree of the *i*th genotype in the *k*th year; *m* is the overall mean; G_i is an effect contributed by the *i*th genotype; Y_k is an effect of the *k*th year; GY_{ik} is the interaction between the *i*th genotype and the *k*th year; T_{ij} is an effect of the *j*th tree of the *i*th genotype; TY_{ijk} is the interaction between the *j*th tree of the *i*th genotype and the *k*th year; and E_{ijkc} is an effect of the *c*th fruit of the *j*th tree of the *i*th genotype in the *k*th year. ANOVA provided the variance associated with genotype (σ_g^2), among years (σ_y^2), genotype × year interaction (σ_{gy}^2), among trees within genotypes (σ_t^2), tree × year interaction (σ_{ty}^2) and among fruits within tree (σ^2).

Plant materials for varietal difference in sugar composition

A total of 43 of Japanese persimmon cultivars consisting of 23 PCNA-types (Fig. S1), 9 PVNA-types (Fig. S2), 6 PVA-types (Fig. S3), and 5 PCA-types (Fig. S4) was analyzed in 2016. Cultural practices were con-

formed to *Plant materials for yearly variation of sugar composition*. Depending on the optimal ripening time of the cultivar, five to eight mature fruits per one-tree of each cultivar were harvested from late-September to early-December in 2016 season according to the reference values based on above-described cultivation records (Table S1). Classification of fruit ripening time was performed in accordance with the definitions by Yamada *et al.* (1995). Fruit shape index, FSI (longitudinal diameter/transverse diameter) was defined according to Maeda *et al.* (2018) with slight modifications (Fig. S5). Cracking of apex and calyx-end of fruit was examined by UPOV guideline (UPOV, 2004). Astringency removal of PCA- and PVA-type cultivars was performed by treatment with either ethanol vapor or carbon dioxide gas depending on the cultivar (Yamada, 2006). The sampled fruit was weighed, and each fruit was horizontally cut to measure fruit skin color, flesh firmness, flesh juiciness, soluble solids content (SSC, °Brix), and sugar composition.

Analysis of fruit quality traits

Fruit skin color, flesh firmness, flesh juiciness, soluble solids content, and sugar composition was measured according to Asakuma and Shiraishi (2017). Fruit skin color around fruit apex was measured using a chromameter (CR-300, Minoruta, Tokyo, Japan), and expressed as the value of color chart following formula:

$$\text{Color chart (CC)} = -9.485 \text{ Ln (hue angle)} + 44.503, R^2 = 0.9278.$$

Flesh firmness (kg) was determined by a handheld universal pressure tester with a 5.0-mm-diameter ×10.0-mm-height columnar plunger (KM-5, FUJIWARA SCIENTIFIC, Tokyo, Japan). Fifteen to 20 g of peeled flesh was weighed and wrapped in one layer of medical gauze. After hand-pressing (only one press) for 15 s, the squeezed juice (flesh juiciness) was measured using a 25-mL mess cylinder and expressed as mL g^{-1} FW. Soluble solids content (SSC) of the resulting juice samples was determined as the °Brix value using a portable calibrated electronic refractometer (PAL-1, Atago, Tokyo, Japan).

For the analysis of sugar composition, 8 to 10 g of peeled flesh was weighed and transferred to a 50-mL heat-tolerant tube and partially screw-capped. The flesh sample was immediately microwave-irradiated at 730 W for 60 s before extracting the sugars. The irradiated sample was ground in a laboratory blender with ~40 mL deionized water. The puree was centrifuged at 5000 × *g* at 25°C for 10 min. The resulting supernatant was brought to 50 mL with deionized water and filtered through a 0.45-µm filter. Sugar composition was analyzed using a HPLC (LC-10A, Shimadzu, Kyoto, Japan) consisting of a SCL-10A system controller, LC-10AD pumps, a CTO-10A column oven, and a RID-10A refractive index detector. The column (SCR-101N, 7.9 × 300 mm, Shimadzu, Kyoto, Japan) was operated at 60°C with 0.8 mL min^{-1} of water. The injection volume was 10 to 20 µL.

3. Results and Discussion

Variation in sugar composition

HPLC profiles of sugar composition were obtained from persimmon cultivars examined with three major peaks assigned as sucrose, glucose, and fructose, respectively, thereby expressing as the SH ratio, a ratio of sucrose to hexose sugars (Hirano *et al.*, 1995). As shown in figure 1, we classified persimmon cultivars into three types according to Zheng and Sugiura (1990) with slight modification; sucrose accumulators (percentage sucrose ≥55.1%, SH ratio≥ 1.23), intermediate accumulators (percentage sucrose in 45.0 to 55.0%, SH ratio in 0.82 to 1.22), hexose accumulators (percentage sucrose ≤44.9%, SH ratio ≤0.81). Previous studies have shown that the

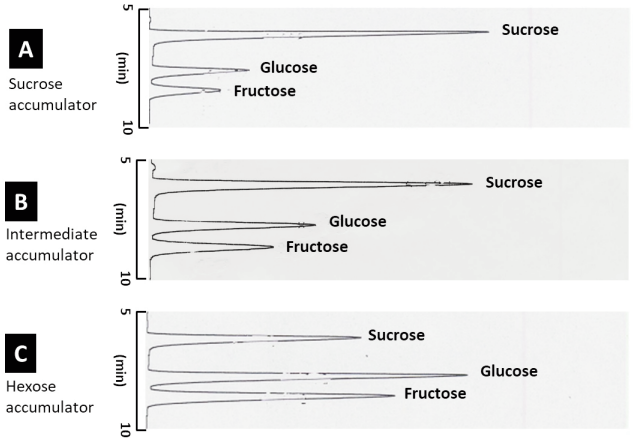

Fig. 1 - Sugar composition of persimmon cultivars from the HPLC analysis. A: Sucrose accumulators ['Fuyu (PCNA-type)', 'Akagaki (PVNA-type)', 'Atago (PCA-type)']; B: Intermediate accumulator ['Maekawajiro (PCNA-type)', 'Saefuji (PVNA-type)', 'Aizumishirazu (PVA-type)']; C: Hexose accumulators ['Soshu (PCNA-type)', 'Nishimurawase (PVNA-type)', 'Hiratanenashi (PVA-type)'] PCA: pollination-constant astringent PVA: pollination-variant astringent PVNA: pollination-variant non-astringent PCNA: pollination-constant non-astringent.

sucrose percentage in persimmon cultivar ranged between 12 and 70%, resulting in varietal difference in the sugar composition or SH ratio (Tsuji and Komiyama, 1987; Zheng and Sugiura, 1990; Hirano *et al.*, 1995; Hirai *et al.*, 2004; Suzuki *et al*., 2010; Asakuma and Shiraishi, 2017). Sugar profiles of PCNA- and non-PCNA-types from well-known cultivars such as 'Atago' (sucrose accumulator), 'Hiratanenashi' (hexose accumulator), 'Soshu' (hexose accumulator) and 'Fuyu' (sucrose accumulator) in previous reports are in agreement with those in the present study.

Table 1 shows highly varietal difference in the SH ratio among the PCNA-type cultivars, especially for Mid- to Late-Oct. ripening ones. In cultivars as sucrose accumulators, large amounts of sucrose recorded in 'Sodawase (SH ratio = 3.29)' followed by 'Okugosho (SH ratio = 2.35)', 'Hanagosho (SH ratio = 2.17)', 'Suruga (SH ratio = 2.02)', 'Fuyu (SH ratio = 1.93)', and 'Shinshu (SH ratio = 1.89)'. In the hexose accumulator, large amounts of hexose were present in 'Kishu (SH ratio = 0.21)', 'Soshu (SH ratio = 0.23)', 'Izu (SH ratio = 0.35)', followed by 'Tenjingosho (SH ratio = 0.61)', 'Taishu (SH ratio = 0.64)' and 'Taiga (SH ratio = 0.67)'. Among the remainders, sucrose and hexose seemed to be accumulated approximately equal amounts with 1.10 in SH ratio ('Maekawajiro'), 1.03 in SH ratio ('Misatogosho'), and 0.96 in SH ratio ('Reigyoku').

Table 1 - Sugar composition of pollination-constant non-astringent (PCNA) type of Japanese persimmon cultivars in 2016

Fruit ripening time/ cultivar or selection	Sugar composition (g 100 g^{-1} FW)				Sugar composition (%)			Type of sugar accumulation	SH ratio	Sweetness value	Flesh juiciness (mL g^{-1} FW)
	Sucrose	Glucose	Fructose	Total	Sucrose	Glucose	Fructose				
Early-Oct.											
Soushu	1.98	4.96	4.60	11.5	17.2	43.0	39.9	Hexose	0.21	3.1	0.27
Mid-Oct.											
Izu	3.03	4.53	4.22	11,8	25.7	38.5	35.8	Hexose	0.35	5.3	0.29
Shinshuu	9.32	2.62	2.32	14.3	65.4	18.4	16.3	Sucrose	1.89	32.3	0.22
Late-Oct.											
Sodawase	11.66	1.90	1.64	15.2	76.7	12.5	10.8	Sucrose	3.29	58.9	0.27
Reigyoku	6.44	3.59	3.12	13.2	49.0	27.3	23.7	Intermediate	0.96	16.1	0.36
Taiga	5.25	4.21	3.68	13.1	40.0	32.0	28.0	Hexose	0.67	11.1	0.32
Kanshu	5.93	3.87	3.48	13.3	44.7	29.1	26.2	Hexose	0.81	13.2	0.17
Kishu	2.66	5.90	5.56	14.1	18.8	41.8	39.4	Hexose	0.23	3.4	0.24
Taishu	5.30	4.52	3.76	13.6	39.0	33.3	27.7	Hexose	0.64	10.1	0.35
Maekawajiro	6.83	3.48	2.71	13.0	52.5	26.7	20.8	Intermediate	1.10	18.2	0.22
Tenjingosho	6.00	5.33	4.46	15.8	38.0	33.8	28.2	Hexose	0.61	10,5	0.17
Early-Nov.											
Akiou	9.14	3.40	2.68	15.2	60.1	22.3	17.6	Sucrose	1.57	27.6	0.38
Misatogosho	7.37	4.18	2.96	14.5	50.8	28.8	20.4	Intermediate	1.03	18.6	0.23
Uenishiwase	7.63	3.35	2.48	13.5	56.7	24.9	18.4	Sucrose	1.31	20.8	0.13
Matsumotowase-Fuyu	9.16	2.85	2.41	14.4	63.5	19.8	16.7	Sucrose	1.74	26.8	0.27
Mid-Nov.											
Mushirodagosho	5.53	3.77	3.57	12.9	43.0	29.3	27.7	Hexose	0.75	10.8	0.33
Youhou	5.99	4.17	4.11	14.3	42.0	29.2	28.8	Hexose	0.72	12.2	0.18
Late-Nov.											
Fuyu	9.79	2.75	2.32	14.9	65.9	18.5	15.6	Sucrose	1.93	31.7	0.27
Okitsu-20 (Ro-19)	8.64	3.61	3.06	15.3	56.4	23.6	20.0	Sucrose	1.30	25.5	0.31
Taiho	7.92	3.50	2.88	14.3	55.4	24.5	20.1	Sucrose	1.24	21.2	0.36
Early-Dec.											
Okugosho	9.43	2.14	1.88	13.5	70.1	15.9	14.0	Sucrose	2.35	43.0	0.24
Suruga	8.86	2.26	2.13	13.3	66.9	17.1	16.1	Sucrose	2.02	35.4	0.26
Hanagosho	9.03	2.20	1.96	13.2	68.5	16.7	14.9	Sucrose	2.17	37.1	0.25

Fruit ripening time (*Late-Sep., Early- to Late-Oct., Early- to Late-Nov., Early-Dec.*) was classified according to Yamada *et al.* (1995).
SH ratio= sucrose [g 100 g^{-1} FW] / hexoses (glucose + fructose) [g 100 g^{-1} FW].
Sweetness value= soluble solids content (reference value in Table S1) x SH ratio.

In the PVNA-type cultivars (Table 2), 'Nishimurawase' (SH = 0.47) was hexose accumulator, whereas the sucrose accumulators had SH ratios, varing from 1.83 ('Akagaki') to 2.77 ('Rendaiji'). The SH ratio of intermediate accumulators ranged from 0.86 ('Fudegaki') to 1.22 ('Zenjimaru'). PCA-type cultivars (Table 3) were all sucrose accumulators, except for 'Kawazokogaki' in SH ratio of 1.12 (intermediate accumulator). Conversely, most PVA-type cultivars (Table 3) could be classified as hexose accumulators, with SH ratios ranging from 0.39 ('Tonewase') to 0.67 ('Hiratanenashi') with the exception of 'Koshu-

Table 2 - Sugar composition and other fruit traits of pollination-variant non-astringent (PVNA) type of Japanese persimmon cultivars in 2016

Fruit ripening time/ cultivar or selection	Sugar composition (g 100 g^{-1} FW)				Sugar composition (%)			Type of sugar accumulation	SH ratio	Sweetness value	Flesh juiciness (mL g^{-1} FW)
	Sucrose	Glucose	Fructose	Total	Sucrose	Glucose	Fructose				
Late-Sept.											
Nishimurawase	3.47	3.22	4.16	10.9	32.0	29.7	38.3	Hexose	0.47	7.1	0.16
Early-Oct.											
Akagaki	8.13	2.24	2.2	12.6	64.7	17.8	17.5	Sucrose	1.83	29.3	0.20
Mid-Oct.											
Fudegaki	5.45	3.29	3.06	11.8	46.2	27.9	25.9	Intermediate	0.86	14.0	0.21
Ganzan	8.56	2.38	2.11	13.1	65.6	18.2	16.2	Sucrose	1.91	31.1	0.18
Oomiyawase	10.17	1.90	1.83	13.9	73.2	13.7	13.2	Sucrose	2.73	46.1	0.20
Saefuji	7.12	3.93	3.55	14.6	48.8	26.9	24.3	Intermediate	0.95	16.4	0.28
Late-Oct.											
Rendaiji	8.45	1.58	1.47	11.5	73.5	13.7	12.8	Sucrose	2.77	41.0	0.20
Early-Nov.											
Zenjimaru	8.05	3.41	3.20	14.7	54.9	23.3	21.8	Intermediate	1.22	21.5	0.25
Early-Dec.											
Shogatsu	9.84	1.90	1.80	13.5	72.7	14.0	13.3	Sucrose	2.66	46.3	0.19

Fruit ripening time (*Late-Sep., Early- to Late-Oct., Early- to Late-Nov., Early-Dec.*) was classified according to Yamada *et al.* (1995).
SH ratio= sucrose [g 100 g^{-1} FW] / hexoses (glucose + fructose) [g 100 g^{-1} FW].
Sweetness value= soluble solids content (reference value in Table 1S) x SH ratio.

Table 3 - Sugar composition of pollination-constant astringent (PCA) and pollination-variant astringent (PVA) type of Japanese

Fruit ripening time /cultivar or selection	Type of astringency	Sugar composition (g 100 g^{-1} FW)				Sugar composition (%)			Type of sugar accumulation	SH ratio	Sweetness value	Flesh juiciness (mL g^{-1} FW)
		Sucrose	Glucose	Fructose	Total	Sucrose	Glucose	Fructose				
Mid-Oct.												
Ichidagaki	PCA	10.43	3.59	3.13	17.1	60.8	20.9	18.2	Sucrose	1.55	30.5	0.35
Tonewase	PVA	3.77	5.13	4.48	13.4	28.2	38.3	33.5	Hexose	0.39	5.9	0.25
Late-Oct.												
Saijo	PCA	8.69	2.12	1.97	12.8	68.0	16.6	15.4	Sucrose	2.12	34.8	0.28
Kawazokogaki	PCA	7.80	3.80	3.15	14.8	52.9	25.8	21.4	Intermediate	1.12	18.1	0.31
Early-Nov.												
Hiratanenashi	PVA	5.54	4.50	3.81	13.8	40.0	32.5	27.5	Hexose	0.67	11.3	0.22
Koshuhyakume	PVA	9.62	2.18	1.88	13.7	70.3	15.9	13.7	Sucrose	2.37	41.0	0.31
Hagakushi	PCA	8.56	2.32	2.34	13.2	64.8	17.5	17.7	Sucrose	1.84	32.4	0.26
Taigetsu	PVA	4.34	4.74	4.25	13.3	32.6	35.6	31.9	Hexose	0.48	7.5	0.43
Taiten	PVA	5.93	3.80	2.61	12.3	48.1	30.8	21.2	Intermediate	0.93	15.7	0.46
Mid-Nov.												
Aizumishirazu	PVA	5.85	3.52	3.40	12.8	45.8	27.6	26.6	Intermediate	0.85	12.7	0.21
Atago	PCA	8.15	2.02	1.78	12.0	68.2	16.9	14.9	Sucrose	2.14	33.8	0.23

Fruit ripening time (*Late-Sep., Early- to Late-Oct., Early- to Late-Nov., Early-Dec.*) was classified according to Yamada *et al.* (1995).
SH ratio= sucrose [g 100 g^{-1} FW] / hexoses (glucose + fructose) [g 100 g^{-1} FW].
Sweetness value= soluble solids content (reference value in Table 1S) x SH ratio.

hyakume' (SH = 2.37), which was classified as a sucrose accumulator. 'Taiten' was an intermediate accumulator in SH = 0.93.

Despite the small number of cultivars examined in the present study, there are no general relationships between astringency type (PCNA, PVNA, PVA, and PCA) and sugar accumulation type. In terms of biochemical consideration, varietal difference in the SH ratio can be explained by the degree of sucrose cleavage due to the activity of vacuolar acid invertase (Hirai *et al.*, 1986; Zheng and Sugiura, 1990). Furthermore, recent transcriptional studies on the sugar accumulation-related key genes postulated that varietal differences in the SH ratio may be resulted from the balance between sucrose synthase and vacuolar acid invertase activities in persimmon fruit (Suzuki *et al.*, 2010; Shiraishi and Asakuma, 2019). To date, the genetic mechanisms controlling sucrose accumulation in persimmon fruit remain unclear. However, we hypothesize that dominance of sucrose accumulation over hexose accumulation in persimmon fruit on the basis of our ongoing breeding program (unpublished data).

Environmental variance in sugar composition, flesh juiciness and SSC

Table 4 shows the contribution of variance from each trait to the total variance. The variance of genotype (σ_g^2) was high for SH ratio in 66.9% and flesh juiciness in 61.7%. As a whole, the variance of year (σ_y^2), among trees within genotype (σ_t^2), genotype × year (σ_{gy}^2) and tree × year (σ_{ty}^2) interactions were small or negligible, varying from 0.0 to 6.6% of the total variance. Similar to previous report (Asakuma and Shiraishi, 2017), the present results indicated that adding year or tree replications will not be efficient in reducing the environmental variance for SH ratio and flesh juiciness. Mitani *et al.* (2015) also showed that genotypic effect of flesh juiciness is significantly high. It is thus considered that the genotypic effect on the SH ratio and flesh juiciness is high with negligibly small environmental variance, and that these traits can be determined by a one-year trial without tree replication. In contrast, σ_g^2 of SSC was small in 14.6%, followed by σ_y^2 in 12.5% and σ_{gy}^2 in 8.8%. Other variance components, σ_t^2 and σ_{ty}^2 of SSC were small or negligible with 1.5 and 4.2%, respectively. Furthermore, the ratio of $\sigma_t^2/(\sigma_y^2 + \sigma_{gy}^2)$ is calculated as 0.07. If the ratio exceeds 1.0, tree replications should be required. However, our results indicate that repeated yearly measurements are more efficient than replicated trees to estimate the genetic variance of SSC as observed in grape (Sato *et al.*, 2000) and persimmon (Yamada *et al.*, 1993)

Using variance components in Table 4, the error variance (σ_E^2) of each trait can be obtained by the following equation (cf. Yamada *et al.*, 1993):

$$(\sigma_y^2/3) + (\sigma_{gy}^2/3) + (\sigma_t^2/3) + \{\sigma_{ty}^2/(3\times3)\} + \{\sigma^2/(3\times3\times8)\}.$$

The σ_E^2 of SH ratio, flesh juiciness, and SSC is 0.01366, 0.00014, and 0.16003, respectively. Broad-sense heritability ($\sigma_g^2/\{\sigma_g^2+\sigma_E^2\}$) results in high for SH ratio in 0.95 and flesh juiciness in 0.97, whereas low for SSC in 0.63. In general, a high broad-sense heritability means that most of the variation among genotypes is caused by genetic variation and not environmental variation. Knowing the heritability can be of value when the breeder will make an effective selection. In this study, the high heritability of SH ratio and flesh juiciness is useful to discriminate genetic sweetness and juiciness of persimmon fruit, respectively. Yamada *et al.* (1993) elucidated that an increase in yearly repetition instead of tree replications substantially reduced σ_E^2 in the measurements for SSC and fruit weight to clarify the genetic properties of genotypes. In the present study, SSC of each

Table 4 - Estimates of variance component and their percentage to total variance obtained from the analysis of variance

Variance components	SH ratio	Flesh juiciness	Solube solids content (SSC)
σg^2 (genotype)	0.2636 (66.9%)	0.0052 (61.7%)	0.2725 (14.6%)
σy^2 (year)	0.0260 (6.6%)	0.0000 (0.0%)	0.2326 (12.5%)
σgy^2(genotype× year)	0.0000 (0.0%)	0.0003 (3.5%)	0.1642 (8.8%)
σt^2(among trees within genotype)	0.0062 (1.6%)	0.0000 (0.0%)	0.0285 (1.5%)
σty^2(tree × year)	0.0161 (4.1%)	0.0000 (0.0%)	0.0791 (4.2%)
σ^2(among fruit within tree)	0.0819 (20.8%)	0.0029 (34.8%)	1.0869 (58.3%)

Negative value was assumed to be zero
SH ratio= sucrose/hexoses (glucose + fructose)

cultivar/selection was evaluated as the reference value based on one tree with more than five years field trials (Table S1).

Varietal difference in sweetness value and flesh juiciness

Sugars represent a crucial component of fruit edible quality, principally conferring sweetness, one of the main attributes influencing the degree of consumer acceptance. The ratio of constitutive sugars determines the sweetness of fruits; the higher the sucrose percentage, the stronger the organoleptic perception of sweetness in Asian pear (Kajiura *et al.*, 1979), oriental melon (Zhang and Li, 2005), peach (Cirilli *et al.*, 2016) and strawberry (Sone *et al.*, 2000). In this study, we proposed a new index entitled "sweetness value" evaluating fruit sweetness by the equation: SSC × SH ratio. In place of Table S3, SSC value in Table S1 was used for calculation of sweetness value because of the above-described environmental error. As shown in Tables 1-3, the sweetness value varied due to the propotional level of sucrose content, ranging from 3.1 to 58.9 in PCNA-, 7.1 to 46.3 in PVNA-, and 5.9 to 41.0 in PVA- and PCA-type cultivars. Corresponding to sugar accumulation type, hexose accumulators exhibited lower sweetness value in 3.1 ('Soushu') to 11.1 ('Taiga'), whereas sweetness values of sucrose accumulators were higher in 20.8 ('Uenishiwase') to 58.9 ('Sodawase'). These results indicate that sweetness value seems to be a useful predictor of fruit sweetness in persimmon genotype. In our previous sensory tests (Asakuma and Shiraishi, 2017), the less-sweet genotypes exhibited SH ratios below 0.3, while highly-sweet genotypes had SH ratios exceeding 1.0. Given that the SSC of genotype is around 16 (average value in 16.6 of 43 persimmon cultivars/selections in Table S1), sweetness value of highly- and less-sweet genotype is expected as exceeding 16 and below 4.8, respectively. For instance, its sensory sweetness of 'Kishu' has been evaluated to be lower than that of 'Fuyu' by several persimmon breeders and growers, although 'Kishu' fruit has normally around 16 in SSC, which is comparable to 'Fuyu' (Yamada *et al.*, 2009). In fact, sweetness value of 'Kishu' was 3.4 in contrast to that of 'Fuyu' in 31.7 (Table 2), which is in agreement with above-mentioned sensory sweetness.

However, there can be inconsistencies when evaluating the eating quality between sweetness scores and cultivars having different flesh juiciness, particularly less juice content (data not shown). In the present study, highly varietal difference in flesh juiciness was observed, ranging from 0.13 ('Uenishiwase') to 0.38 ('Akiou') mL g^{-1} FW of PCNA type (Table 2), 0.16 ('Nishimurawase') to 0.28 ('Saefuji') mL g^{-1} FW of PVNA type (Table 3), and 0.21 ('Aizumishirazu') to 0.46 ('Taiten') mL g^{-1} FW of PVA and PCA type (Table 4). In general, the harder the flesh of fruits, the more chewing is required to breakdown the tissue and the longer it takes to release the juice (Harker *et al.*, 2003). In peach (Suzuki *et al.*, 1990), sweet cherry (Dever *et al.*, 1996), and carrot (Horie and Hiramoto, 2009), flesh sweetness is significantly promoted by high juiciness. Similarly, Ban *et al.* (2010) and Mitani *et al.* (2015) revealed that flesh juiciness is considered to be crucial mouth-feel attribute in the overall taste of persimmon fruit. Asakuma and Shiraishi (2017) proposed that the new descriptor of flesh juiciness of persimmon fruit as "very juicy" (≥0.30 mL g^{-1} FW), "juicy" (0.21-0.29 mL g^{-1} FW) and "slightly juicy" (≤0.20 mL g^{-1} FW) based on the sensory juiciness. From this perspective, three sucrose accumulating PCNA-type cultivars/selections ('Akiou', 'Okitsu 20', and 'Taiho') are considered to be promising breeding stocks because of the high sweetness value (21.2 to 27.6) and high juiciness (0.31 to 0.38 mL g^{-1} FW) together with large fruit size and brilliant fruit color (Tables S1 and S3). Thus, in terms of palatability, persimmon cultivar's improvement will be effectively performed using a combination of sweetness value and flesh juiciness.

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Salicylic acid effects on some physio-chemical properties and secondary metabolite accumulation in *Mentha piperita* L. under water deficit stress

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Abstract: Salicylic acid (SA) play important roles in different physiological processes of plants such as plant growth, stress response, plant adaptation and secondary metabolite accumulation. This study was conducted to evaluate the effect of exogenous SA applications on the growth measurements such as fresh and dry weight of aerial part and lead dry weight, biochemical properties (membrane permeability, lipid peroxidation, Proline content and ROS scavenger enzymes) and secondary metabolite accumulation (total phenolic and the flavonoid and essential oil content) in peppermint (*Mentha piperita* L.) plants grown at different levels of water deficit stress (Field capacity: FC). For this aim, three different water deficit stress [no stress (100% FC), mild stress (75% FC) and moderate stress (50% FC)] and four different SA concentrations (0, 1, 2 and 2.5 mM) were applied to peppermint plants. Results showed that all of the measured parameters were affected by the water deficit stress and SA application. By elevating the level of water deficit stress, fresh and dry weights of aerial parts and dry leaf weight decreased. Increasing in the water deficit stress level from mild to moderate stress resulted to reduce the essential oil content while proline, lipid peroxidation, total phenolic contents, flavonoid content, and antioxidant enzyme activities increased depending on water deficit stress. Exogenous application of SA obstructed the negative effects of water deficit stress by decreasing the lipid peroxidation and membrane permeability and improving the antioxidant enzymes activities. Essential oil content increased significantly in plant treated with SA grown under water deficit stress conditions. Application of 2 or 2.5 mM of SA enhanced the plant growth and development without any toxic effects and increased significantly the total phenolic content, dry leaf weight, and the essential oil content in stressed and even in control (100% FC) peppermint plants.

1. Introduction

Peppermint (*Mentha piperita* L.) is considered as one of the most important medicinal and aromatic herb in the world cultivated in many

countries such as India, Italy, France, China, Hungary and United States among others (Lawrence, 2007). During the last two decades peppermint cultivation has experienced a remarkable increase, mainly due to the rediscovery of medicinal properties of peppermint essential oil, which has a lower risk of side effects compared to synthetic drugs (Kumar and Kumar-Gupta, 2008; Herro and Jacob, 2010). Furthermore, *M. piperita* is used as flavoring agent for beverages (phenolic and flavonoids compounds group, including eriocitrin, hesperidin and luteolin 7-O-rutinoside) (McKay and Blumberg, 2006; Hossain *et al*., 2010) and food industry (Essential oil and different type of plant exteract), as a fragrance (higher amounts of high-volatile monoterpenes such as menthone and isomenthone (Rohloff, 1999), as insecticide (Karamaouna *et al*., 2013) and fungicide in many industrial products. Essential oil of peppermint contains very important monoterpenes (Essential oil of peppermint contain menthofuran, menthol, menthone, isomenthone, pulegonecaryophyllene, betacaryophyllene, neomenthol, 1,8-cineole, sabinene and limonene) with antibacterial, antifungal (Edris and Farrag, 2003), antioxidant and cytotoxic activities (Mimica-Dukic *et al.*, 2003; Hussain *et al*., 2010). Due to markedly importance of peppermint, maintaining a high and constant essential oil production for industries requirements and supplying market demands (Silva, 2002) under unfavorable conditions such as water deficit stress is very important.

Water deficit stress is the major yield limiting factor for many agriculture crop plants affecting many biochemical, morphological and physiological parameters. Many research studies indicated that drought stress influenced the growth, yield, secondary metabolite production and composition in different aromatic and medicinal plants. Furthermore, between the abiotic stresses, drought stress can exacerbates the effect of the other stresses in plants and increase the effects of other stresses such as salinity, cold or hot stress (Khalid, 2006; Azhar *et al*., 2011; Verma and Shukla, 2015). In order to reduce the harmful effects and to increase the resistance of plants to the drought stress, some exogenous plant growth regulator applications such as salicylic acid (SA) can be used (Lee *et al*., 2019). Salicylic acid, as a plant messenger molecule, plays a non-enzymatic antioxidant role, regulates many physiological and biochemical mechanisms as cell expansion, vascular differentiation, vegetative and reproductive development, seed germination, flowering, fruit set and secondary metabolite accumulations during stress occurrence (Arfan *et al*., 2007; Hayat and Ahmad, 2007). This compound belongs to the group of the phytochemicals with beneficial effects on human health (Hayat and Ahmad, 2007). In addition, SA is a phenolic compound that has a crucial role in plant defense against pathogenic agents (Dempsey and Klessing, 1994; Hayat *et al*., 2010). Apart from growth-promoting and health related effects of SA in different plants for instance in *Artemisia annua* L. (Aftab *et al*., 2010), *Cuminumcy minum* (Rahimi *et al*., 2013) sweet basil (*Ocimum basilicum* L.) and marjoram (*Majorana hortensis*) (Gharib, 2007) and *Salvia macrosiphon* (Rowshan *et al*., 2010), SA is also reported to confer resistance to plants against various stresses e.g. in *Matricaria chamomilla* (Kovácik *et al*., 2009), and *Vigna radiate* (Khan *et al*., 2014 a).

This experiment was conducted to determine the effect of different concentrations of exogenous SA applications on some physiochemical properties and secondary metabolite accumulation in *M. piperita* plants grown under different level of water deficit stress.

2. Materials and Methods

Plant materials, SA and water deficit stress treatments

Plants were initiated from rhizome segments of *M. piperita* obtained from a commercial nursery located in Alborz province, Karaj. The plants were grown in a greenhouse at the University of Persian Gulf in pots with a diameter of 10 cm and irrigated every 2 days during the first 30 days. The daily temperature inside the greenhouse was within optimal ranges for peppermint growth (20-25°C). Fertilization was carried out at 15 and 25 days after planting. Each pot was fertilized with a solution containing $Ca(NO_3)_2$ (1.12 g/L), $MgSO_4$ (0.45 g/L), KNO_3 (0.35 g/L), KH_2PO_4 (0.30 g/L), Ferric EDTA (0.06 g/L), and M_nSO_4 (0.01 g/L). Thirty-day old seedlings were transferred to plastic pots with a diameter of 30 cm filled with soil, cocopeat and perlite (1:1:1; v/v) and kept in an incubation room where the temperature was 25°C ± 1; the relative humidity was 60-80% and photoperiod of 16 h/d. The first SA (Synonym: 2-Hydroxybenzoic acid; Linear Formula: $C_7H_6O_3$ (CAS 69-72-7), Sigma-Aldrich) treatments (0, 1, 2 and 2.5 mM) was applied to 35 days old plants by spraying aerial parts of the plants. The stock solutions were prepared by dissolving SA in ETOH and final volume was maintained by

distilled water containing 0.02% of Tween 20 as surfactant. Control plants were sprayed with distilled water including 0.02% of Tween 20 and EtOH (100%) as in SA solutions. The 2th, 3th and 4th SA application were repeated at days 50 and 65 and 80 days after transplanting on peppermint plants exposed in three different irrigation (100, 75 and 50 FC) treatments as FC (100% field capacity - FC), mild stress (75% FC), and moderate stress (50% FC). Throughout cultivation period, moisture levels in the growth media were controlled by daily weighting following the procedure of Yadav *et al*. (2014). Briefly, to calculate the amount of water necessary to bring each soil to determined FC, a 50 g soil sample from randomly chosen pots were collected and the water content was determined by drying at 100°C at 24 h after the pots were watered. The percentage of soil water content was calculated according to Yadav *et al*. (2014) method. Nutrient and water leaching from pots was captured in dish placed under each pot and the leachate was returned to the soil before the addition of any water. At the end of the experiment, all plants within each pot were harvested and then analyzed.

Determination of biochemical and physical properties

The growth response of the plants to elicitor treatments and water deficit stress were determined by measuring the fresh and dry weights of aerial parts per plant.

Lutts *et al.* (1996) method was used for determining the membrane permeability of the excised leaves at the end of the experiment by using a conductivity meter. After harvesting (from three plants per treatment), 5 leaves (randomly chosen full developed leaves from upper part of the plant) were cut into 1 cm segments. Leaf segments were washed with three changes of deionized water to remove surface adhered electrolytes. Samples were placed in 20 ml vials contain deionized water and incubated at 25°C on a rotary shaker (100 rpm). After 24 h incubated at 25°C, the electrical conductivity of the bathing solution (L_1) was determined by using a conductivity meter. Then samples were autoclaved at 120°C for 20 min and the electrical conductivity (L_2) was obtained. The membrane permeability was obtained using L_1/L_2. All measurements were made in triplicate.

Lipid peroxidation in leaves was determined by estimating the malondialdehyde (MDA). For malondialdehyde (MDA) determination, 1 g leaf sample (full developed leaves were collected from the upper part of each plant) was homogenized in 5 ml 1% trichloroacetic acid and centrifuged at 10000 *g* for 10 min. The amount of MDA in the supernatant was estimated by the thiobarbituric reaction as described by Madhava *et al.* (2000). MDA concentration was calculated from the absorbance at 532 nm by using the extinction coefficient of 155 Mm cm^{-1}. All measurements were made in triplicate.

Proline content of treated plant was determined as described by Bates *et al*. (1973). A 1:1:1 solution of proline, ninhydrin acid and glacial acetic acid was incubated at 100°C for 1 hour. For proline colorimetric determinations, the reaction was arrested in an iced bath and the cromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined in a BioMate spectrophotometer (Thermo-Spectronic). After the analyses, following equation: (g proline in extract/115.5) g^{-1} sample = mol g^{-1} FW) was used for calculation the proline concentration from a standard curve.

Enzyme assays

At the end of experiment, leaves were collected and frozen in liquid nitrogen and stored at -80°C before enzyme extraction. ROS scavenger enzymes were extracted as described by Zhang and Kirkham (1996) with some modifications. All operations were carried out at 4°C. Intact leaves were ground using mortar and pestle under liquid nitrogen in cold 50 mM sodium phosphate solution (pH 7.5) containing 250 mM Sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpolypyrrolidone in a 6:1 proportion (w/v). The homogenate was then filtered and centrifuged at 25,000 *g* for 20 min at 4°C. Then solid ammonium sulfate $(NH_4)_2SO_4$ added to the supernatant to make up 80% saturated solution and allowed to stir gently for several hours at 4°C. After centrifugation (28,000 *g* for 45 min at 4°C), pellets, were resuspended in a small volume of 50 mM of sodium phosphate (pH 7.5) and used for enzyme assays.

The superoxide dismutase (SOD) activity was assayed by observing the inhibition of photochemical reduction of nitro blue tetrazolium according to Krishnan *et al*. (2002) protocol. One mL of assay mixture consisted of 75 µM nitro blue tetrazolium, 2 µM riboflavin, 50 mM Na-P buffer (pH 7.8) 0.1 mM EDTA, 13 mM Methionine, and enzyme extract. The samples were kept 30 cm under a light source (4000 lux) for 15 min and the reaction started during this time. The reaction was stopped by switching off the light. A non-irradiated reaction mixture, served as a control which was run in parallel. The absorbance was read at 560 nm.

The Ascorbate peroxidase (APX) activity (ε= 2.8 mM^{-1} cm^{-1}) was determined from the decrease in A290, due to the H_2O_2-dependent oxidation of ascorbate using procedure of Zhang and Kirkham (1996). One mL reaction mixture contained 50 mM Na-P (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, and enzyme.

The catalase (CAT) activity (ε H_2O_2 = 39.4 mM^{-1} cm^{-1}) were assayed spectrophotometrically according to Zhang and Kirkham (1996) method by monitoring the change in A240 due to the decreased absorption of H_2O_2. The reaction mixture contained enzyme extract, 50 mM Na-P, pH 7.0, and 15 mM H_2O_2 (in 1 mL final volume). The reaction was initiated by addition of H_2O_2.

Secondary metabolites determination

At the end of the experiment, in all plants within each pot leaves (1 g full developed leaves were collected from the upper part of the each plant) were collected and frozen in liquid N2 immediately and stored at -80°C before analysis. Folin-Ciocalteu colourimetric method was used for determining the total phenolic content of the peppermint extract (Singleton and Rossi, 1965). Samples were transferred into the different test tube and mixed thoroughly with Folin-Ciocalteu reagent (5 ml). After 5 mins, 4 ml of 7.5% sodium carbonate (Na_2CO_3) was added and allowed at room temperature to react for 2 hrs. The absorbance was measured using spectrophotometer at 765 nm. Samples were measured in three replicates. The flavonoid content was determined according to Liu *et al.* (2002) by aluminium trichloride method using Catechin. A volume of 125 µL of extract is added to 75 µL of a 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min, and then 150 µL of aluminium trichloride (10%) was added and incubated for 5 min, followed by the addition of 750 µL of NaOH (1M). The final volume of the solution was adjusted to 2500 µL with distilled water. After 15 min of incubation the mixture turned to pink and the absorbance was measured at 510 nm.

Total essential oil content

Hydro distillation method was used.In this method, at least 10 g of dried *M. Piperita* shoot related to the treatment immersed in 150 cc water were submitted to hydro-distillation with a Clevenger-type apparatus for 3 h (until no more essential oil was obtained). The essential oil was collected, dried with anhydrous sodium sulfate, and stored at 4°C until used.

Statistical analysis

This experiment was performed in factorial arrangement based a completely randomized design with 3 replicates per treatment and one plant per replicates in which pots were placed on the benches in the incubation room. The factors included different SA concentration (0, 1, 2 and 2.5 mM) and water deficit stress in 3 levels. Data were analyzed by SPSS 19 software, and differences among treatments were determined using Tukey's test ($p<0.05$).

3. Results and Discussion

Growth measurement

Foliar application of salicylic acid (SA) and water deficit stress affected peppermint plants in different ways, including physiological and biochemical properties. Some physiological disorders such as leaf yellowing and abscission were observed in plants at sever water deficit irrigation. Also, the high concentration of SA caused burning and drying the edge of some mature leaves in plants (data not shown). The Limited water supply is one of the major abiotic factors that adversely affect agricultural crop production worldwide. Water deficit stress significantly reduced the growth measurements (fresh and dry weights of aerial parts of plants and leaf dry weight) in peppermint plants at 50% FC (Table 1). However, SA treatments were not caused any growth reduction but in plants under water deficit stress, prevented the growth reduction rate. Highest growth values were

Table 1 - Effect of foliar spray of SA on some growth parameters

Irrigation treatment	SA (mM)	Fresh weight of aerial parts (g)	Dry weight of aerial parts (g)	Dry weight of leaf (g)
Control	0	30.74 b	5.47 b	2.61 bc
	1	31.31 b	7.15 a	3.45 b
	2	42.28 a	8.40 a	4.33 a
	2.5	41.24 a	8.34 a	4.08 a
FC (75%)	0	20.92 cd	4.56 c	1.93 d
	1	22.25 cd	4.75 bc	2.13 cd
	2	26.66 cde	4.84 bc	2.83 d
	2.5	27.71 c	4.65 c	1.91 d
FC (50%)	0	19.25 cde	4.44 c	1.58 d
	1	19.92 cde	4.26 c	1.62 d
	2	18.32 e	3.95 c	1.92 d
	2.5	19.21 de	3.91 c	1.64 d

* Differences between means indicated by the same letters are not statistically significant ($p\leq0.05$).

obtained from the plants treated only with 2 mM of SA. Khorasaninejad *et al*. (2011) also observed significant reduction in growth parameters of peppermint (*M. piperita* L.) under water stress. SA treatment in non-stress and even in stress conditions showed positive effect on the growth measurements in comparison to the control (Table 1). Different concentrations of SA (from 10^{-5} to 10^{-3} M) improved growth parameters (plant height, number of branches, nodes, and leaves per plant, as well as leaf area) in basil (*Ocimum basilicum*) and marjoram (*Origanum majorana*) plants (Gharib, 2007) and these authors suggested that such effects could be related to increased photosynthetic activity. The beneficial effects of SA on the growth measurements especially in term of dry weight of mint in this study paralleled with the results of Khodary (2004) and Hayat and Ahmad (2007). This growth enhancement suggests that SA affects various physiological and biochemical processes, including photosynthesis, water processes, ion homeostasis, antioxidant capacity generates and a wide array of metabolic responses in plants (Hayat and Ahmad, 2007). Also, SA modulates several physiological responses such as maintain Indole-3-acetic acid (IAA, 3-IAA) and gibberellin levels, inhibit ethylene biosynthesis and prevent auxin oxidation in plants (Pierpoint, 1994), which could be one reason for the SA-enhanced vegetative growth of peppermint in our study.

Essential oils

Peppermint essential oil (EO) is a rich source of monoterpenes such as menthol and menthone used in pharmaceutical, food, cosmetic and cleaning industries (Kamatou *et al*., 2013) and its commercial value depends on the essential oil contents. In this study, essential oil content was affected with both water stress and SA application (Table 2). Essential oil content increased from 1.146% (control plants) to 1.192% (mild stress treated plant) and decreased to 0.782% in moderate stress treated plant (Table 3). Exogenous application of SA alleviated this adverse effect especially in moderate stress condition and also SA application enhanced the EO yield in mild stress condition. In control irrigation conditions, 2 mM SA treatment increased total essential oil content to 1.256% while application of SA at higher concentrations caused a reduction in total essential oil content. Under water deficit stress conditions, SA significantly increased the total essential oil content when compared to the untreated plants. Particularly, 2 and 2.5 mM of SA increased essential oil content more than 2-fold compared to the untreated plants in the presence of moderate water deficit stress.

Responses of plant to environmental stress such as drought stress in terms of volatile emissions are species specific and depend on the duration and intensity of the stress period (Jord´anm *et al*., 2003). EO yield increased under mild water stress in this study. In medicinal and aromatic plants, the effect of

Table 2 - Effect of foliar spray of SA on biochemical properties of peppermint plants grown at different level of FC

Irrigation treatment	SA (mM)	Membrane permeability (s)	Lipid peroxidation (nM g^{-1})	Proline (mol g^{-1})
Control	0	37.11 def	5.81 d	0.58 ab
	1	27.43 efg	6.35 d	0.42 bc
	2	28.32 efg	5.38 d	0.28 c
	2.5	21.43 fg	6.05 d	0.66 ab
FC (75%)	0	41.02 cd	14.48 a	0.67 ab
	1	53.74 c	12.01 a	0.59 ab
	2	50.26 cd	6.09 d	0.4 bc
	2.5	25.49 fg	7.11 cd	0.62 ab
FC (50%)	0	87.04 a	11.71 ab	0.83 a
	1	41.52 cde	11.29 ab	0.79 a
	2	74.28 b	8.66 c	0.59 ab
	2.5	49.91 cd	7.03 cd	0.76 a

* Differences between means indicated by the same letters are not statistically significant (p≤0.05).

Table 3 - Effect of foliar spray of SA on phenolic and essential oil contents of peppermint plants grown at different level of FC

Irrigation treatment	SA (mM)	Total phenolic content (mg g^{-1})	Flavonoid content (mg GAE/g)	Total essential oil content (%)
Control	0	8.37 e	17.7g	1.146 d
	1	16.51 d	22.2e	1.153 d
	2	14.28 de	38.7d	1.256 c
	2.5	14.32 de	34.81e	1.092 e
FC (75%)	0	28.08 ab	37.2d	1.191 d
	1	22.71 bc	42.3c	1.201 d
	2	18.08 cd	47.4b	1.418 b
	2.5	17. 94 cd	48.2b	1.482 b
FC (50%)	0	29.39 ab	40.7c	0.782 f
	1	31.99 a	55.3a	1.010 d
	2	18.28 cd	53.2a	1.597 a
	2.5	33.65 a	55.7a	1.575 a

* Differences between means indicated by the same letters are not statistically significant (p≤0.05).

water deficit stress on essential oil yield is variable and depends on plant species. For example, drought stress had a negative effect on the essential oil yield of *M. piperit*a (Khorasaninejad *et al*., 2011). By contrast, water stress had a positive effect on *Salvia officinalis* and showed a higher concentration of monoterpenes such as cineole, camphor and α/ß-thujone (Nowak *et al*., 2010). Increasing in essential oil yield under stress condition may be related to induction changes in morphological or physiological traits such as high trichome density and smaller leaf size that prevent excessive water loss and allow them to survive in arid or semi-arid environmental conditions (Nobel, 1999). Foliar spray with Triazole (A triazole refers to any of the heterocyclic compounds with molecular formula $C_2H_3N_3$, having a five-membered ring of two carbon atoms and three nitrogen atoms) decreased the leaf area in *M. pulegium* (Hassanpour *et al*., 2012) and increased trichome density in *Bougainvillea spectabilis* (Mansouri and Kurup, 2009). The results suggest that the stimulation of essential oil production under drought stress could be due to low allocation of carbon to the growth and high terpene concentrations under stress conditions. Plant growth regulators (PGRs) favorably affect the yield and quantity of essential oil in lemon grass, rose grass, peppermint, spearmint, and sage (Shukla and Farooqi, 1990; Khan *et al*., 2014 b, 2015). Among different concentration of SA tested on *M. piperita* in none stress condition, 2 mM of SA showed the most effective in improving the essential oil content. The positive effect of SA on essential oil content and essential oil yield may be ascribed to the improvement in overall plant growth and metabolism by SA. It seemed that SA, considered to be a signaling molecule, might be involved in the signal transduction system, leading to the balance and improved quantity of the secondary metabolites, i.e., essential oil (Hayat and Ahmad, 2007). Thus, SA-mediated enhanced plant growth, photosynthesis, and the overall plant metabolism might account for oil accumulation in the present study. These results corroborate with the findings on *Artemisia annua*, *Salvia macrosiphon*, *Cumium cyminum*, *Ocimum basilicum*, and *Majorana hortensis* (Gharib, 2007; Aftab *et al*., 2010; Rowshan *et al*., 2010; Rahimi *et al*., 2013). Moderate stress significantly decreased essential oil content in current study (Table 3). Similarly, Khorasaninejad *et al*. (2011) reported significant decreases in total essential oil content in water stressed peppermint plants. These results suggest that a high level of water stress can suppresses the essential oil biosynthesis pathway in peppermint.

Reduction in photosynthesis rate and/or any changes in metabolic pathway could be caused some disorders of oil biosynthesis resulted in reduction in oil content (Srivastava *et al*., 1998). In control irrigation condition, foliar application of SA at 2 mM concentration resulted to increase in essential oil content of plants compared to the control plants while higher concentrations of SA decreased the oil content. Another interesting result is that SA foliar applications increased the essential oil content in water stressed plants. Under water deficit stressed conditions, essential oil contents of peppermint plants treated with SA were higher in comparison with the group exposed to water stress alone. The highest oil content was found in plants treated with SA at 2.5 mM concentration grown under moderate water stress. In this condition, oil content was more than 2 fold greater than that of plants under combination of moderate water stress and 0 mM of SA. According to the commercial importance of peppermint, our results represented that peppermint plants can be grown in mild or in moderate stress in the presence of SA applications.

Membrane permeability, lipid peroxidation and proline content

Both water deficit stresses resulted to a significant increase in the levels of membrane permeability, a sign of injury to the cells (Table 2). The maximum increase in membrane permeability was obtained in plants subjected to 50% FC and SA treatments reduced significantly this increase was in under water stress. The maximum reduction in membrane permeability was observed in plants treated with 2.5 mM SA. Our results indicated that water stress increased the membrane permeability levels of peppermint plants and they were alleviated significantly by foliar applications of SA.

Water stress significantly increased the lipid peroxidation compared to the control irrigation plants (Table 2). The maximum lipid peroxidation content was recorded in plants grown under high level of water deficit stress. However, SA treatments, especially at 2 mM concentration, significantly overcame the toxicity generated by water stress alone and almost leveled the values with those of control. However, SA did not affect the lipid peroxidation level in control irrigation plants.

The proline content increased by increasing the water stress but foliar spraying of SA at 2 mM concentration upon water stressed plants reduced the

proline content.

Ion leakage is known as a index for evaluating the cell wall damage caused from stress. Membrane permeability increased in line with the elevating levels of water deficit stress in this study. Also, there is a close relationship between producing oxidative damage under water stress condition of cell membrane permeability. However SA significantly decreased the membrane permeability in plants under water deficit stress (Table 4). This result can approve the capability of SA spraying treatment in negative effect of drought stress. Water deficit stress resulted to the displacement of membrane proteins, cellular compartmentalization disruption, loss of membrane selectivity, integrity and a loss of activity of enzymes (Mahajan and Tuteja, 2005).

Malondialdehyde is one of the main known indicator or reliable biomarker for lipid peroxidation, which is part of the oxidative damage at cell level. Malondialdehyde increase the cell permeability by deterioration of membrane integrity. Our results showed that there is a significant relationship between lipid peroxidation and water deficit stress (Table 4). Under water deficit stress conditions the amount of lipid peroxidation increased in peppermint plants, moreover, it was determined that MDA level increased as a result of deterioration of the cell structure. Karray-Bouraouia *et al*. (2009) and Karlıdag *et al*. (2011) reported a positive correlation between stress condition and lipid peroxidation. Water deficit stress caused considerable membrane injuries leading to membrane lipid peroxidation. Foliar spray of SA did not show any significant changes in lipid peroxidation content in control irrigation condition while it significantly decreased lipid peroxidation content in water stress-treated plants. The results showed that SA had an important role in membrane integrity and maintenance of membrane structure via preventing damages caused by water deficit stress. Low membrane lipid peroxidation in drought-exposed and SA (0.5 mM)-supplemented *T. aestivum* reported by Kang *et al*., (2012).

Table 4 - Effect of foliar spray of SA on antioxidant enzyme activities of peppermint plants grown at different level of FC

Irrigation treatment	SA (mM)	SOD (unit mg protein^{-1})	CAT (mol min^{-1} mg protein^{-1})	APX (mol min^{-1} mg protein^{-1})
Control	0	25.17 cd	6.44 cde	311.99 c
	1	17.47 ef	3.11 e	153.71 d
	2	12.31 f	6.63 cde	189.48 d
	2.5	21.19 de	4.15 de	279.11 c
FC (75%)	0	30.88 bc	15.92b	392.95 b
	1	21.17 de	8.52 c	158.21 d
	2	23.46 d	7.43 cd	264.01 c
	2.5	21.13 de	5.41 cde	314.64 c
FC (50%)	0	41.79 a	21.33 a	501.28 a
	1	19.94 de	16.21 b	183.23 d
	2	31.95 b	9.15 c	331. 53 c
	2.5	47.61 a	9.58 c	483.12 a

* Differences between means indicated by the same letters are not statistically significant (p≤0.05).

Amino acids play an important role in plant development and metabolism. Proline, an amino acid, plays main roles in plants exposed to stressful conditions (like water deficit stress, low temperature, salinity, heavy metal exposure and UV radiations, etc) such as osmoprotective, protein compatible hydrotrope, scavenging free radicals and buffering cellular redox potential, energy supply, stabilize the function and structure of protein and DNA, alleviating cytoplasmic acidosis and maintaining appropriate NADP+/NADPH ratios compatible with metabolism, metal chelator, antioxidant properties and a signaling molecule (Naidu *et al*., 1991; Bassi and Sharma, 1993; Schat *et al*., 1997; Hare *et al*., 1998; Rhodes *et al.*, 2002; Kavi Kishor *et al.,* 2005; Munns, 2005; Sharma and Dietz 2006; Ashraf and Foolad, 2007; Chookhampaeng, 2011). So, overproduction of proline is a common physiological response of plants against stressful environment. In this study, proline contents were increased with increasing water deficit stress (Table 4). So, more proline accumulation has been associated with increasing the stress tolerance of plant. In general, results showed that drought stress affected the proline biosynthesis in the leaves. The foliar spray of SA on stressed plants cause in reduction of proline content but these declines were not statistically significant in some concentration. It could be due to positive effects of SA in maintaining the membrane maintaining cell turgor or osmotic balance as an osmotic regulator (Wang and Zeng, 1993) or in stabilizing cell membranes, preventing electrolyte leakage and bringing concentrations of ROS within normal ranges.

Phenolic and flavonoids contents

Total phenolic and total flavonoid contents were increased under water deficit stress significantly as compared to control irrigation (100% FC) (Table 3). Minimum phenolic content was measured in control plants while its level increased with increasing the

water deficit stress intensity and SA application. Foliar application of SA (2 mM) under control irrigation increased total phenolic content less than two times compared to controls. Moreover, not only water stress but also SA applications enhanced total phenolic contents compared to controls. The plants under moderate stress were sprayed with a solution of 2.5 mM SA showed the highest phenolic contents. Phenolic compounds as stress markers accumulate under stressful environment. It is believed that soluble phenolics compounds act as scavengers of ROS and membrane stabilizer during abiotic stress (Moyer *et al.,* 2002; Taiz and Zeiger, 2006). The increase in total phenolic contents in our study is similar to that observed by El-Danasoury*et al.* (2010) in a study with *Mentha spicata* and Queslati *et al.* (2010) with *Mentha pulegium* under salinity stress. Also, foliar SA application resulted to increase in total phenolic contents in control plants. Foliar SA application at 2 mM was the most effective dose under stress free conditions for peppermint plants. In water stressed plants, foliar spry of SA generally have positive effects on phenolic accumulations. Especially at moderate stress and 2 and 2.5 mM concentrations of SA showed the highest amount of phenolics. Pepper-mint treated with SA or drought stress showed an increase in total phenolic and flavonoid content in our study. This effect was previously reported in ginger (*Zingiber officinale*) plants, where the total phenolic content increased by approximately 20% after treatment with 10^{-3} M SA (Ghasemzadeh and Jaafar, 2012). Also, Figueroa-Pérez *et al*. (2014) previously reported in peppermint plants, where the flavonoids, increased by approximately 93%, 100% and 56% treated with 0.5, 1.0 and 2.0 mM SA, respectively, when compared with the control. Flavonoids were increased in water deficit stress condition and SA treated plants in our study. Flavonoids are the main bioactive secondary metabolites in plants and they can serve as scavengers of ROS by locating and neutralizing radicals before they damage the cells and are thus important for plants under adverse environmental conditions (such as wounding, drought, metal toxicity, and nutrient deprivation) (Løvdal *et al.,* 2010; Agati *et al.,* 2012). Plants often respond to environmental stresses with an increase in the endogenous SA level (Janda *et al*., 2014). SA is a promising compound for the reduction of stress sensitivity in the practical agriculture (Horváth *et al.,* 2007).

ROS enzyme activity

The activities of SOD, CAT and APX enzymes were investigated in the leaves of peppermint plants. SOD, CAT and APX enzymes activities significantly increased in parallel to the water stress in the present study. Activity of SOD was found at 25.17 unit/mg protein, 30.88 unit mg protein^{-1} and 41.79 unit mg^{-1} protein in plants under control, mild and moderate stress, respectively (Table 4). In general, foliar application SA on stressed and non-stressed plants alleviated the activity of SOD. However, 2.5 mM of SA increased the SOD activity in plants under control and moderate stress condition. Also, CAT activity increased in the plants under water deficit stress (Table 4). In control irrigation plants, foliar spray of SA treatments did not show a significant change in CAT activity whereas they substantially decreased the CAT activity in mild and moderate stress. APX activity almost showed the similar trend with SOD activity and its activity increased with water deficit stress, gradually (Table 4). SA application at 2.5 mM concentration had no significant effect in terms of the reducing the APX activity in control and moderate stress treatments. The maximum APX activities were found in under moderate stress without SA application or with 2 mM SA while the highest reductions in APX activity content were recorded in plants treated with 1 mM SA in both mild and moderate water stress treatments. Developing enzymatic and non-enzymatic antioxidant defense mechanisms against reactive oxygen species is the main strategy of plants in stressful condition (Noctor *et al.,* 2002; Gong *et al.,* 2005; Cheeseman, 2007). In this study, ROS enzyme activities enhanced by increasing the intensity of water stress and APX enzyme activity increased with increasing stress conditions and the highest increases occurred in plants under moderate stress condition (Table 4).

4. Conclusions

According to the obtained results, it may be concluded that prolong water stress especially under moderate water deficit stress had negative effect on peppermint. Under water deficit stress, peppermint plants showed a significant decline in growth measurement in term of fresh and dry weights of aerial parts and leaf dry weight while increased antioxidant enzyme activities, the membrane permeability and lipid peroxidation. In addition, water stress enhanced total phenolic and flavonoids contents while significantly decreased the total essential oil content in moderate stress condition (mild stress increased the total essential oil content but, it was not significant).

However, the findings of the present study showed that foliar spray of SA increased grown parameters in non-stress plants while there were no significant effects of SA on water stressed plant. Severity of water stress has a great impact on the physiological and biochemical process of plants. SA applications in this study reduced the severity of both water stress condition and decreased the level of grown parameters reduction rate. SA significantly reduced the water stress-induced negative effects by improving the antioxidant enzyme activities and decreasing the lipid peroxidation and membrane permeability. In terms of the secondary metabolites, SA treatment increased the total phenolic contents in control plants while, especially in plants exposed to water stress, total essential oil content increased. SA contributed to the plant growth and development without any toxic effects and 2 mM of SA significantly increased the essential oil content in non-stressed plants. Also, it was determined that 2 mM of SA for control irrigation and 2 and 2.5 mM of SA for both water stress condition were the optimum concentrations in terms of dry leaf weight, total phenolic contents and essential oil content in peppermint plants.

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The toxicity potential of Ag nanoparticles synthesized from *Cordia myxa* L.

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Abstract: Plant-mediated nanoparticles synthesis is considered as one of the appealing options in bio-nanoparticles synthesis, however, there is contradictory information about the positive or negative impacts of nanoparticles on plants. Investigating the toxic effects of Ag NPs on model plants, such as onion, can reveal the probability of damage. Thus, the present study was conducted to compare the germination indices and biochemical parameters of edible onion seeds treated with different concentrations of two types of silver nanoparticles (green synthesized and chemical synthesized) to examine the oxidative stress. Based on our results, the interaction between leaf extract and silver salt resulted in a color change from pale yellow to dark brown, the first sign of the AgNPs formation. The green AgNPs treatment improved the onion germination indices. The green AgNPs-exposed seeds displayed a no-significant reduction in protein content and same protease activity as the control treatment, but chemical AgNPs-treated displayed a significant rise of protease and reduction in protein content in concentration more than 0.06 gL^{-1}. In chemical AgNPs-treated seeds both peroxidase and catalase displayed an ascending linear trend and the most activities belonged to chemical AgNPs at 0.05 g L^{-1} (315.62 and 51.45 µmol $min^{-1}g^{-1}$ FW, respectively). Both nanoparticle types made an increase in MDA, but green AgNPs did not significantly differ with control treatment. It can be concluded that the green synthesis of nanoparticles is a safe and suitable alternative for chemical-synthesized metal nanoparticles.

1. Introduction

The production, manipulation, and use of nanoparticles (NPs), due to their distinctive and definite capabilities, has become one of the most attractive research topic in different area of science, including biology, chemistry, engineering, medicine, physics, agriculture and food. Nano-dimension structures involved in many aspects of plant biology such as ionic transport and molecular transmissions. The diameter of plant cell wall pores are in the range of 5 to 20 nm. Furthermore, plasmodesmata, the intracellular channels to facilitate molecular transitions, are also nano-scale (50 to 60 nm in diameter) (Zambryski, 2004).

The nanotechnologies based-products are expected to upturn (Maynard *et al*., 2006; Rejeski and Lekas, 2008). Nanotechnology applications in agricultural sector involve in precision farming, smart feeding, enrichment of food quality, products packaging, products labeling, nano-pesticide, nano-fertilizer, and nano-herbicide (Thiruvengadam *et al*., 2018). The different chemical and physical methods are applied to the synthesis of nanoparticles such as sol-gel, electrochemical, hydrothermal, sono-chemical, and microwaves techniques. Chemical methods had low efficiency, need high temperature, high pressure and high energies during the reaction process. Besides they need the expensive reagent and complex equipment. Furthermore, chemical methods require some non-degradable chemical components, as reducing or stabilizing agents, which finally caused environmental pollution (Senapati *et al*., 2012).

The potential of biological materials to synthesize metal nanoparticles has provided a low-cost and eco-friendly route. Biological synthesis or green synthesis of nanoparticles, uses biological material as the reducing and capping agents (Veerasamy *et al*., 2011). The green synthesis uses microorganisms such as fungus, bacteria (Ahmad *et al*., 2005), yeast and actinomycetes (Kowshik *et al*., 2003) or macro-organisms e.g., plants and algae, as intermediate agents (Niemeyer and Mirkin, 2004; Dubey *et al*., 2010; Prasad *et al*., 2012).

Plant-mediated nanoparticles synthesis is considered as one of the appealing options in bio-nanoparticles synthesis, due to high variety and abundance, no need for a complicated growth conditions, bulk-biomass production, no need for special nutrient media, cost-effectiveness, richness of various effective metabolites, simple single-step synthesis process and suitable for the large-scale synthesis of nanoparticles. Polyphenolic contents of the plant act as reducing agents and eventually act as coating and stabilizing agent of nanoparticles (Dubey *et al*., 2010).

Cordia myxa (L.) or sapistan-tree, a member of Boraginaceae family, is native to tropical areas of Asia. It well documented that *C. myxa* contained flavonoids, glycosides, sterols, terpenoids, saponins, phenolic acids, alkaloids, tannins, coumarins, resins ; and mucilage (Inas *et al*., 2011; Rashed *et al*., 2014): a suitable candidate for green NPs synthesis.

However, there is contradictory information about the positive or negative impacts of nanoparticles on plants. Titanium dioxide nanoparticles increased the dry and fresh weight of spinach by increasing the light absorption, boosting rubisco enzyme activity (Lin and Xing, 2007) andrising the nitrogen metabolism (Yang *et al*., 2007). Zinc and copper nanoparticles showed significant differences in growth, and toxicity symptoms in treated plants (Lee *at al*., 2008; Monica and Cremonini, 2009; Musante and White, 2012; Prasad *et al*., 2012). Titanium nanodioxide (40 µg ml^{-1}) improved seed germination, dry and fresh weight of onion seedling; however, the higher concentrations (50 µg ml^{-1} and more) had a reverse result (Raskar and Laware, 2013). Conversely, this nanoparticle did not affect the length and quantity of the onion root (Klancnik *et al*., 2011). The symptoms have depended on the form and coating type of nanoparticles (Barrena *et al*., 2009).

Due to the growing rate of production and release of nanoparticles in nature (as Nano-fertilizers, Nano-toxins, and Nano-carriers), there are increasing concerns about the possibility of toxicity and oxidative damage (whether chemical or green synthesis) of these particles on the ecosystem. The WHO (World Health Organization) and FAO (The Food and Agriculture Organization) at the meeting on the application of nanotechnologies in the food and agriculture sectors, in Rome in 2010, recognized the potential concern of nanotechnology in agriculture and food sectors. The main concern was about the exponential developing global knowledge on nanotechnology and its applications, which may affect the balance of advantage to risk (FAO/WHO expert meeting report, 2010).

The Ag NPs is known to have antioxidant, antibacterial and antifungal properties and may be used in food, medicine and cosmetics products (El-Nour *et al*., 2010; Abdel-Aziz *et al*., 2014). So, investigating the toxic effects of these particles on model plants, such as onion, can reveal the probability of damage. Thus, the present study was conducted to compare the germination indices and biochemical parameters of edible onion seeds who treated with different concentrations of two types of silver nanoparticles (green synthesized and chemical synthesized) to examine the oxidative stress.

2. Materials and Methods

Research site and plant material collection

The current study was conducted at the Horticultural Laboratory of Agriculture and Natural Resources Faculty, Chemical Laboratory of Science

Faculty, Central Laboratory (University of Hormozgan, Bandar Abbas, Iran) and Molecular Research Center (Hormozgan University of Medical Sciences, Iran), during 2018.

Preparation of Cordia myxa leaf extract

Leaves of *Cordia myxa* L. (a member of Boraginaceae family), were collected from Rooydar city, Hormozgan Province, Iran (57° 6′ E, 60° 3′ N, Elevation: 50 m, RH: 70%, Mean temperature: 28±1°C) during winter, 2018. The healthy leaves were choosing, then washing in tap water followed by rinsing in distilled water and air drying (6 days under the shade conditions at 24±1°C). The dried leaves were powdered using an electric grinder. The leaf extract was prepared by mixing 10 g of dried powder along with 150 mL of deionized water and then heating the mixture at 80°C for 30 min. The solution was pre-filtered through Whatman No. 42 filter paper and re-filtered through Whatman No. 1 filter paper. The obtained extract was collected and stored in the refrigerator (Samari *et al*., 2018).

Synthesis and purification of silver nanoparticles (AgNPs) using C. myxa leaf extract

For AgNPs synthesis, 2.0 ml of *C. mixa* leaf extract was added to 25 ml of 7.0 mM aqueous solution of $AgNO_3$ (Merck, Germany) and the reaction pH was adjusted to 11.0 using NaOH. The mixture was continuously stirred at room temperature (24±1°C) for 3 h. The yellow-mixture turned to dark-brown (Samari *et al.*, 2018). The green synthesized AgNPs were centrifuged at 10000 rpm for 30 min and rinsed with deionized water. Then, the obtained sediments were re-dispersed in deionized water to get rid of any free phyto-molecules. This process (centrifugation and re-dispersion) was repeated three times to ensure the purification of nanoparticles and separation of unbonded compounds.

Characterization of silver nanoparticles

Preliminary characterization of the green synthesized AgNPs was carried out using a S-3100 UV-Vis spectrophotometer (Scinco, Korea, which is operated at a resolution of 2 nm and is equipped with a 10 mm quartz cuvette).

An aliquot of the dried purified pellets, obtained from centrifugation, was used for X-ray diffraction (XRD). The XRD pattern was obtained using a powder X-ray diffractometer (Bruker D8 Advance powder diffractometer) with Cu-Kα radiations (λ=1.5406 nm, in a 2θ range from 20° to 80°). Fine configuration of the green synthesized AgNPs was measured using a transmission electron microscopic examination (TEM). For this, the colloidal AgNPs were allowed for sonication and then a thin film was prepared on the carbon coated grid (Cu Mesh 300). TEM observations were made with (Zeiss-EM10C) operated at an accelerating voltage of 80 kV.

Preparation of colloidal solution of AgNPs (green and chemical synthesized)

The chemical AgNPs wasprovided from Iranian Nano-biotechnology company. Chemical and green nanoparticles were dispersed separately in distilled water and placed in an ultrasonic apparatus (SONICA® Ultrasonic Cleaners) for 30 min for homogenization. Then, colloidal solutions containing each nanoparticle were prepared at the concentrations of 0, 0.03, 0.06, 0.012, 0.025 and 0.05 g L^{-1}.

For germination assay, red onion seeds (*Allium cepa* L.), disinfected (0.04% of sodium hypochlorite solution, 3 min), rinsed in distilled water (3 times, each for 1 min), dipped in Nano-solutions for 30 min and finally rinsed in distilled water. Seeds were then cultured in sterilized glass Petri dishes (80 mm in diameter, covered with filter papers). Each Petri dish contained 100 seeds.

Germination indices

Germination percentage and germination value. Germination test was conducted under a laboratory condition (24±1°C). The seeds were placed on 80 mm petri dishes and were covered with filter papers. The seeds were irrigated daily with double distilled water. Counting was done by the emerge of the roots (the seed with root length equal or more than seed diameter were assumed as germinated). The counting continued until 3 days fixed germination. The germination indices including germination percentage and germination value were calculated using the equations (1) and (2) (Okoro, 1976).

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

$$GV = MDG \times PV \quad (2)$$

where GV is germination value and MDG is mean daily germination and calculated by the dividing of germination percentage by the number of days to the end of the test. PV or peak value, derived from all the cumulative germination percentages on any day divided by the number of days to reach these percentages.

The length of radicle and plumule. Seedling root and stem length was measured using a ruler (express in mm).

Biochemical analysis

Preparation of enzyme and protein extract. About 0.5 g of the root was homogenized in liquid nitrogen. Then, 1.0 ml of extraction buffer (in 100 ml: containing of 50 mM potassium phosphate buffer with pH: 7.0, 0.0372 g EDTA and 1.0 g PVP) was added and centrifuged (15 min, 12000 rpm, 4°C). At last, the supernatant was used to assay protein content, and the activity of catalase, peroxidase, and protease (Dhindsa *et al*., 1981).

Protein content assay. The Bradford method (1976) was used to determine protein content. Briefly, 1.0 ml of Bradford reaction solution (containing 0.01 of Coomassie Brilliant Blue G250, 5.0 ml of 96% ethanol and 10.0 ml of 85% phosphoric acid) was added to 50 µl of protein extract. The optical absorption of the extracts was determined by a spectrophotometer (Cecil CE2501 model) at 595 nm with a plastic cuvette (Bradford, 1976).

Peroxidase activity assay. After adding 33 µl of the enzyme extract to 1.0 ml of the peroxidase reaction solution (containing 13 mM guaiacol, 5 mM hydrogen peroxide and 50 mM phosphate potassium buffer at pH=7.0), the absorption of the extracts was recorded at 470 nm (Chance and Maehly, 1995).

Catalase activity assay. For catalase assay 50 µl of the enzyme extract was mixed with 1.0 ml catalase reaction solution (containing 50 mM phosphate potassium buffer at pH=7.0 and 15 mM hydrogen peroxide). The absorption was recorded at 240 nm (Dhindsa *et al*., 1981).

Protease activity assay. After adding 350 µl of 50 mM sodium phosphate buffer (pH=7.5) and 800 µl of 1% casein (W/V) to 50 µl of the enzyme extract, the mixture was incubated under laboratory condition (24±1°C) for 10 min. Then, 400 µl of 10% trichloroacetic acid (w/v) was added and the mixture re-incubated for 20 more min. Finally, after centrifugation (10000 rpm, 5 min), the optical absorption of extracts was determined at 280 nm with a quartz cuvette (Kwmbhavi *et al*., 1993).

Malondialdehyde assay (MDA). About 0.1 g of the root was homogenized with 5.0 ml of 1% trichloroacetic acid. The extract was then centrifuged (10000 rpm, 5 min) and the supernatant (250 µl) was placed in a water bath (95°C for 30 min), after adding one ml of MDA (containing 20% trichloroacetic acid and 5% thiobarbituric acid). The samples were placed on ice and then re-centrifuged (1000 rpm, 5 min). Finally, the optical absorption of the extracts was recorded at 532 and 600 nm (Alexieva *et al*., 2001).

Total phenol assay. The total phenol was assayed using the Spanos and Wrolstad (1990) technique; about 0.1 g of root sample was homogenized with 15.0 ml of 80% methanol. The extract was then centrifuged (10000 rpm, 10 min). Then, 490 µl of distilled water and 500 µl of Folin reagent were added to 10 µl of the supernatant and were placed under dark condition (24±1°C, for 3 min). Subsequently, 500 µl of 1% sodium carbonate (1.0 g sodium carbonate - 100 ml distilled water) was added to each sample and re-placed under dark condition (24±1°C) for 30 more min. Finally, the absorbance of extracts was determined at 765 nm.

Antioxidant capacity (DPPH assay). The DPPH assay was followed by Brand-Williams *et al.* (1995) procedure. Briefly, about 0.1 g of root sample was homogenized with 15.0 ml of 80% methanol and incubated under dark laboratory condition (24±1°C, for 24 h). Then, the extracts were centrifuged (10000 rpm, 10 min). After adding 40 µl of methanol, 350 µl of DPPH and 1550 µl of 80% methanol to 600 µl of the supernatant, the samples were kept under the dark condition at 4°C, for 20 min. Finally, the optical absorption was recorded at 517 nm and the antioxidant capacity was calculated using the equation (3):

$$\text{Antioxidant capacity\%} = [(A_{cont} - A_{samp})/A_{cont})] \times 100 \quad (3)$$

where A_{cont} and A_{samp} are the standard and sample optical absorption.

Data analysis

The research was conducted as a factorial experiment based on complete random design, with six replications (each replicon contains 100 seed). The statistical analysis was done using SAS Version 9.1.3 (SAS Institute Inc. Cary, NC, USA, 1990). The factors were AgNPs type (green AgNPs and chemical AgNPs) and AgNPs levels (0, 0.03, 0.06, 0.012, 0.025 and 0.05 g L^{-1}). The Shapiro-Wilks test confirmed the data normality (procedure: Proc Univariate, SAS). The Multivariate Analysis of variance, considering the AgNPs type and AgNPs levels as independent variables were done (procedure: Proc Glm, SAS). Pillai's trace test confirmed the variance homogeneity (procedure: Proc Glm, SAS). Tukey's test was performed for mean comparison (procedure: Files, Sedit, Factor, Range, P<0.01, MSTATC). Excel 2013 was used to draw the figures.

3. Results

Synthesis and characterization of C. myxa synthesized AgNPs

The interaction between leaf extract and silver salt resulted in a color change from pale yellow to dark brown, the first sign of the AgNPs formation. This process occurred under room temperature (24±1°C) and lasted 3 hours (Fig. 1).

UV-Vis Spectroscopy is one of the most extensive techniques to confirm the production of nanoparticles. The UV-Vis spectrum of synthesized AgNPs using *C. myxa* leaf extract (Fig. 2A) showed a significant peak with λ_{max}around 410 nm due to SPR (Surface Plasmon Resonance) of AgNPs.

TEM images were used to detect the surface morphology and size distribution of synthesized AgNPs by *C. myxa* leaf extract. TEM micrographs of the AgNPs confirmed the spherical shape of particles, 3-10 nmsize, well-distributed and little aggregation in solution (Fig. 2B); however, the average size of particles was found to be 5.8 nm.

The XRD patterns of synthesized AgNPs showed prominent Bragg reflections at 2θ values of 38.1, 44.25, 64.55 and 77.2 (Fig. 3A), which is related to the (111), (200), (220) and (311) Bragg reflections of face-centered cubic (FCC) AgNPs. SEM analysis confirmed the nano dimension of chemical AgNPs (Fig. 3B).

Germination indices

Germination percentage and germination value. The germination percentage was influenced by the type and concentration of nanoparticles (Table 1).

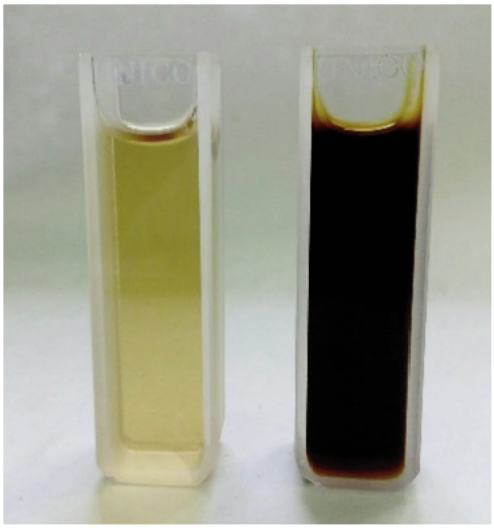

Fig. 1 - The mixture of *C. myx*a leaf extract and $AgNO_3$ (Left), green-synthetized AgNPs colloidal solution (Right).

The green AgNPs treatment improved the onion germination. Most germination percentage belonged to green AgNPs (100% in 0.05 g L^{-1}), and the least value

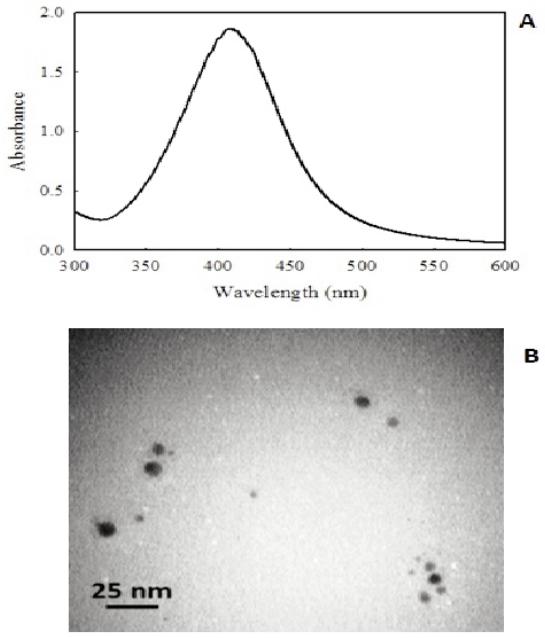

Fig. 2 - The SPR spectrum of *C. myxa* synthesized with the AgNPs (A), and TEM image of AgNPs synthetized by *C. myxa* leaf extract (B).

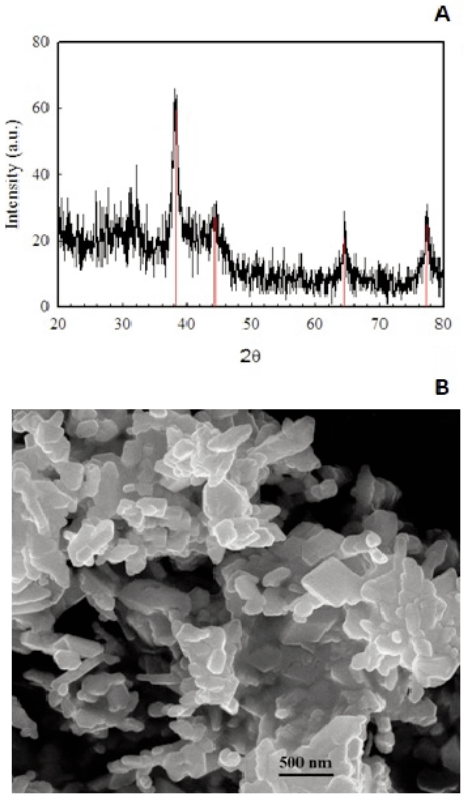

Fig. 3 - The XRD patterns of the *C. myxa* synthesized AgNPs (A) and electron microscopic scanning image of chemical AgNPs (B).

(70%) belonged to chemical AgNPs treatment at concentrations more than 0.06 g L^{-1} (Fig. 4A).

The germination value also influenced by the interaction of treatments (Table 1). Green AgNPs treatment did not differ with control, while treatment with the chemical AgNPs caused a significant reduction in the germination value (from 1.88 in controlreached to 0.12 in the 0.05 g L^{-1}). Most and least germination values (1.90 and 0.12) were observed in onion seeds treated with the high concentration of green AgNPs and chemical AgNPs, respectively (Fig. 4B).

The length of radicle and plumule. The effect of type and concentration of AgNPs was significant on the length of plumule (Fig. 5). There was no significant difference between the green AgNPs and the control. Even, the chemical AgNPs was not different from the control up to the concentration of 0.06 g L^{-1}. The higher amount of chemical AgNPs, reduced the

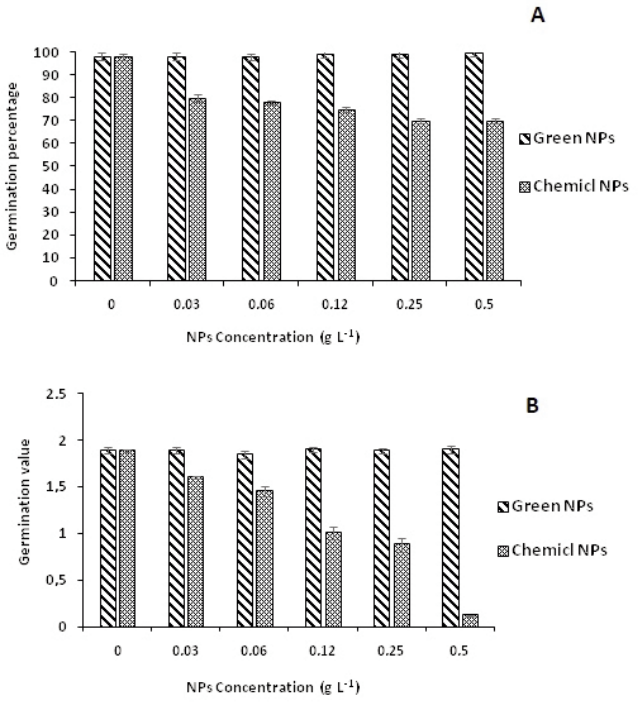

Fig. 4 - The influence of AgNPs type and concentration on germination percentage (A) and germination value (B) in *Allium cepa*. Means ± SD of six replicates. The same letter denotes lack of a statistically significant difference (Tukey, p<0.01).

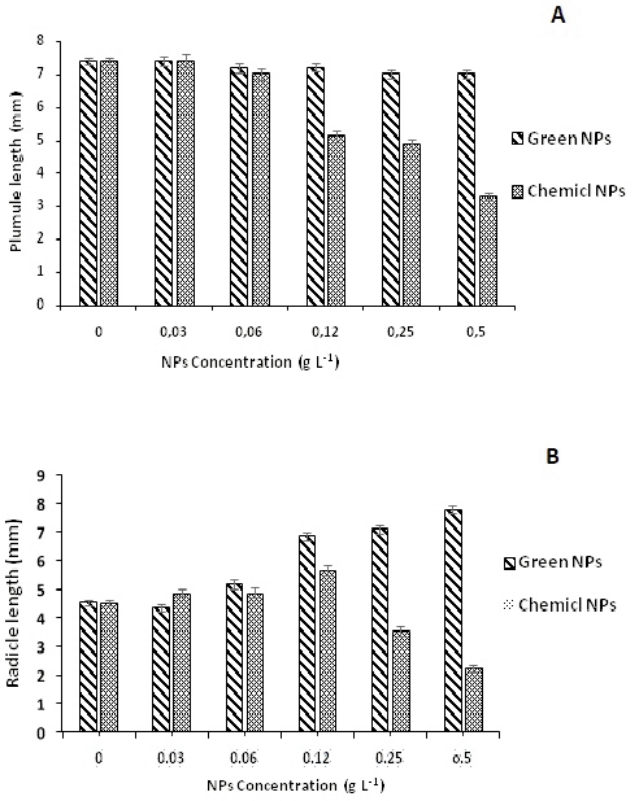

Fig. 5 - The influence of AgNPs type and concentration on plumule length (A) and radicle length (B) in *Allium cepa*. Means ± SD of six replicates. The same letter denotes lack of a statistically significant difference (Tukey, p<0.01).

Table 1 - The interaction of AgNPs type and concentration on *Allium cepa* biochemical parameters

SOV	Germination percentage	Germination value	Radicle length	Plumule length	Protein	Peroxidase	Catalase	Protease	MDA	Phenol	IC_{50}
NPs type	4408.33 ** (z)	89.89 **	284.21 **	926.64 **	0.11	540853.35 **	3600.65 **	9151.33 **	0.112 *	886.31 **	14.57
NPs concentration	3.80	0.07	9.98 **	5.11 **	0.27 *	40965.25 **	13476.80 **	1.40 *	0.030 **	17.84	34.52 *
NPs type × NPs concentration	15.53*	0.31 **	4.90 **	1.70	0.15*	38719.77 **	7566.10 **	1.87 *	0.020 **	17.89 **	21.90 **
Error	13.10	0.26	1.17	1.92	0.16	5852.71	683.03	1.14	0.020	55.16	24.61

(z) The mean square values are given.
* and **: state significant at 5 and 1% respectively
AgNPs type ×AgNPs concentration: states the interaction of AgNPs type and AgNPs concentration

plumule length (from 7.40 mm in the control treatment reached to 3.28 mm in the 0.05 g L^{-1} of chemical AgNPs) (P≤0.01) (Fig. 5A). The radicle length had a different pattern in response to the type and concentration of the nanoparticles. Green AgNPs improved radicle length (from 4.53 mm in control treatment reached to 7.76 mm in high concentration) (P<0.01). The chemical AgNPs made a decreasing trend in this parameter and reached it to 2.22 mm in the concentration of 0.05 g L^{-1} (Fig. 5B).

Biochemical indices

Protein content and protease activity. Despite the significant effect of two AgNPs types on protein content and protease activity (Table 1, P≤0.01), the interaction between AgNPs type and concentration had a diverse pattern (Fig. 6A and B). The green AgNPs-exposed seeds displayed a no-significant reduction in protein content (from 1.1 to 1 mg g^{-1}). The reduction of protein content in chemical AgNPs-treated seeds was dose-dependent (Fig. 6A). Green AgNPs-treated seeds had the same protease activity as the control treatment. Chemical AgNPs displayed a significant rise in concentration more than 0.06 gL^{-1} (from 1.77 µmol min^{-1} g^{-1} FW in control treatment to 6.21 µmol min^{-1} g^{-1} FW at the high concentration) (Fig. 6B).

Antioxidant enzymes (peroxidase and catalase activities). By the results, the activity of both antioxidant enzymes was influenced by the type and concentration of the AgNPs (Table 1). Green AgNPs-treated seeds indicated no significant difference with control. In chemical AgNPs-treated seeds both enzymes displayed the linear trend, so most peroxidase and catalase activities belonged to chemical AgNPs at 0.05 g L^{-1} (315.62 and 51.45 µmol $min^{-1}g^{-1}$ FW, respectively) (Fig. 7A and B).

Malondialdehyde (MDA), phenol and DPPH (antioxidant capacity). The MDA influenced by AgNPs treatment (Table 1) and the less value was observed in control treatment (0.03 mg g^{-1} Fw). Both nanoparticle types made an increase in MDA, but green AgNPs did not significantly differ with control treatment. Also, the content of MDA had a linear relation

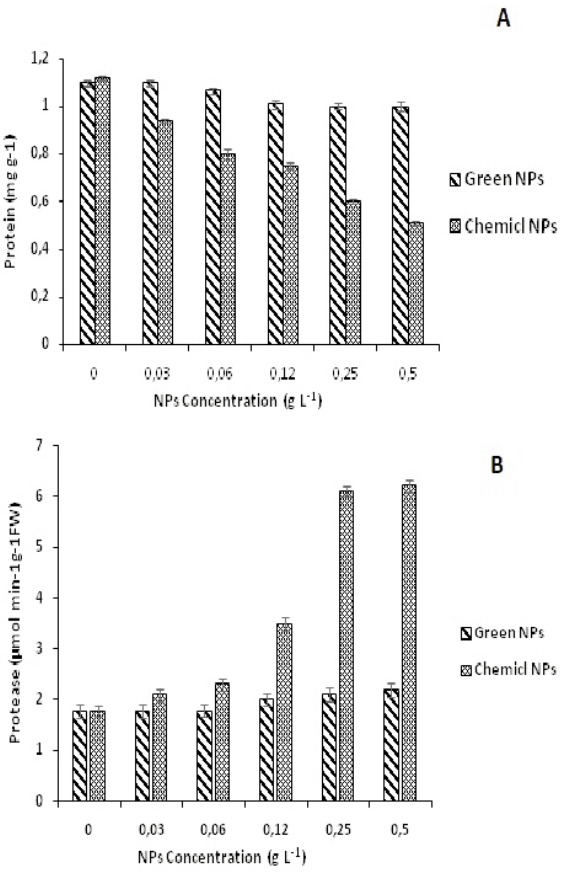

Fig. 6 - The influence of AgNPs type and concentration on protein content (A) and activity of protease (B) in *Allium cepa*. Means ± SD of six replicates. The same letter denotes lack of a statistically significant difference (Tukey, p<0.01).

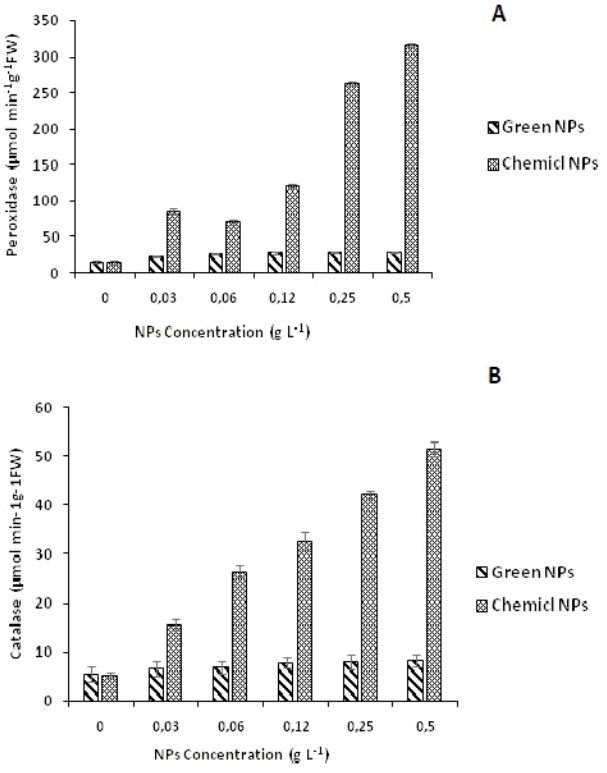

Fig. 7 - The influence of AgNPs type and concentration on activities of peroxidase (A) and catalase (B) in *Allium cepa*. Means ± SD of six replicates. The same letter denotes lack of a statistically significant difference (Tukey, p<0.01).

with chemical AgNPs concentration (from 0.03 mg g^{-1} Fw in the control reached to 0.25 mg g^{-1} FW at high concentration of chemical AgNPs) (Fig. 8A).

The type and concentration of AgNPs influenced phenol content. The most value was assigned to chemical AgNPs (15.16 mg Gallic g^{-1} FW at 0.05 g L^{-1}), and the least value to control treatment (4.10 mg Gallic g^{-1} FW) (Fig. 8B).

The amount of antioxidant capacity was also affected by NPs treatments (Table 1). This parameter was expressed in mg g^{-1} fresh weight based on the-half-maximal inhibitory concentration (IC_{50}). The control value (10.67 mg g^{-1} FW) followed a descending trend in both AgNPs types. It reached to 8.97 mg g^{-1} FW in green AgNPs while to 1.01 in chemical AgNPs (Fig. 9).

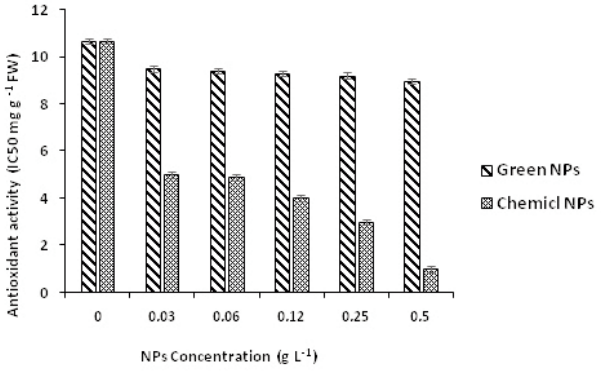

Fig. 9 - The influence of AgNPs type and concentration on antioxidant capacity in *Allium cepa*. Means ± SD of six replicates. The same letter denotes lack of a statistically significant difference (Tukey, p<0.01).

4. Discussion and Conclusions

Plants contain flavonoids, phenols, aroma, latex and alcohols, and some of these compounds are responsible for the reduction of metal ions and production of metal NPs from the metal salts. It has been stated that the leaf extract of *Cordia* species possesses phenolic and flavonoids derived compounds such as robinin, rutin, datiscoside, hesperidin, dihydrorobinetin, chlorogenic and caffeic acid (Al-Ati, 2011). The hydroxyl and ketonic groups of such compounds, construct chelate structures by binding to metal ions (Issaabadi *et al*., 2016). In present work, the leaf extract of *C. myxa* was used as a reducing and stabilizing agent for phyto-synthesis of AgNPs. This eco-friendly procedure was free-hazard and non-toxic. The successful synthesis of green AgNPs from plant extracts has already reported in *Chenopodium album*, *Camellia sinensis* and *Rhus coriaria* (Song and Kim, 2009; Dwivedi and Gopol, 2010).

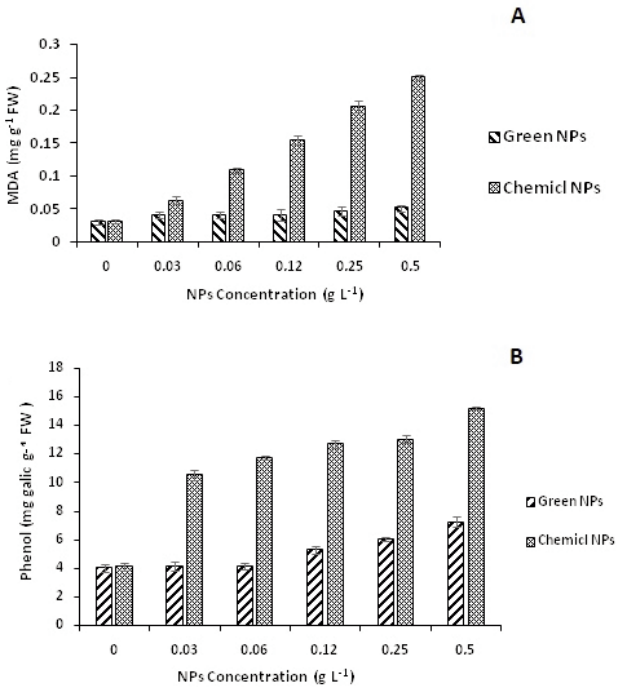

Fig. 8 - The influence of AgNPs type and concentration on MDA (A) and total phenol (B) in *Allium cepa*. Means ± SD of six replicates. The same letter denotes lack of a statistically significant difference (Tukey, p<0.01).

The NPs synthesis basis is oxidation of hydroxyl, carbonyl, and aldehyde and subsequently, reduction of metal ions during the neutralization of the electric charge (Sivaraman *et al*., 2009). In present research the *C. myxa* extract turned to dark brown, due to the surface plasmon resonance of AgNPs and confirmed the successful synthesis of the AgNPs (Kasthuri *et al*., 2009; Roopan *et al*., 2013). In this study, the synthesis process lasted 3 hours at room temperature condition. Other reports have also mentioned that this rapid NPs synthesis dose not require high temperatures (Sivaraman *et al*., 2009). The FCC crystal structure of green synthesized AgNPs was found by comparing the obtained XRD data with JCPDS File No. 04-0783 (Sutradhar and Saha, 2016), clearly indicate the highly crystalline structure of green AgNPs.

Nanoparticles play an important role in many areas of biology, chemistry and agriculture (Navarro *et al*., 2008), but their adverse potential on the environment is being subjected to extreme discussions. NPs penetrate to various parts of the plants, some stored within the cell and some in extracellular space (Lee *et al*., 2008). High AgNPs concentrations pass through the cell by diffusion, cause mitochondrial

mal-function, generation of ROS (He *et al*., 2012; Roh *et al*., 2012). On the other hand, easy penetration of nanoparticles into the seed shell develops water absorption and increases the activity of rubisco enzyme, which finally simplifies germination (Gao *et al*., 2006). In the present study, *Cordia myxa* AgNPs increased onion germination percentage and value. The improvement of seed germination has reported in nano-metal treated spinach (Gao *et al*., 2006), maize (Lin and Xing, 2007) and peanut (Prasad *et al*., 2012).

The AgNPs have to penetrate plant cell walls, the natural sieves, and roots plasma membranes to enter the xylem tissues and then dislocated to stems and finally leaves (Dietz and Herth, 2011). In fact, the roots are the first target for lethal materials (Sresty and Rao, 1999). There are disagreeing reports regarding the effects of nanoparticles on the plant root and system. The NPs treatment made a decrease in radish root length (Wang *et al*., 2015), but an increase in the shoot and root length of rice (Hao *et al*., 2016). Also, ZnO NPs caused root elongation in radish, lettuce, corn, and cucumber (Lin and Xing, 2007). ZnO NPs (500 mg L^{-1}) increased soybean's root length, while higher concentrations resulted in a significant reduction (Lopez-Moreno *et al*., 2010). The root cap cells of AgNPs-exposed *Lolium multiflorum* were damaged and malformed, which lastly reduced root growth and dry matter (Yin *et al*., 2011).

Plant root inter-connect with physical and chemical factors of the root zone. The elongation zone of onion root may act as a sensitive receiver for external signals. The length and morphology of onion roots is an important parameter that reflects the toxicity of the chemical compounds (Odeigah *et al*., 1997). It has reported that AgNPs lessened root length of onion whichrelatedto a reduction in water absorption and cell division (Kumari *et al*., 2009). In this study, an inhibition in radicle and plumule development in chemical AgNPs-treated seeds indicate its toxicity potential. Moreover, the root length reduced more than the shoot length in AgNPs-treated samples.

The toxicity of AgNPs in biological systems is closely related to its surface oxidation, releasing the Ag ions, and interaction with macromolecules (Reidy *et al*., 2013), especially with sulfur-containing molecules e.g. proteins, due to silver- sulfur strong tendency (Liu *et al*., 2011). The nano-dimension plant pores, the surface charge of NPs (more negative AgNPs limit the cell-particle interactions and made lower toxicity) and coating type (chemical or biological) influence the intensity of AgNPs' toxicity (Choi and Hu, 2008; El Badawy *et al*., 2011).

In the present study, protein content and protease activity of green AgNPs treated seeds did not significantly differ from the control. However, the high concentration of chemical AgNPs obviously decreased protein content and boosted protease activity. This alignment related to the role of protease in protein catabolism. High concentrations of AgNPs denature the membrane and release LPS (lipopolysaccharide) and purines. Then limit the proton mobility, react with thiol groups of some enzymes, deactivate enzymes, bind to protein groups, denature proteins, produce hydrogen peroxide, which finally causes oxidative stress (Hwang *et al*., 2008; Zhu *et al*., 2008).

The regulated production of free radicals in organisms maintains the oxidation and reduction homeostasis cycle; however, there is a group of antioxidant enzymes who prevent and deactivate ROS. Also, extracellular antioxidant molecules, such as ascorbate, scavenge free radical molecules (Shams *et al*., 2011). Increasing the activity of antioxidant enzymes, catalase and peroxidase, inplant's exposed to the chemical metal NPs treated plants (Krishnaraj *et al*., 2012; Singh *et al*., 2013; Wang *et al*., 2015; Cvjetko *et al*., 2017) confirms our results.

Nanoparticles interact with the cell membrane (Khan *et al*., 2011), then depending on their nature and concentration, reduce the destructive effects of oxidative stress, cause programmed cell death (Lei *et al*., 2008). Silver nanoparticles could damage cell division, cause chromatin bridge, disturbed metaphase, make multiple chromosomal breaks and final cell disintegration (Kumari *et al*., 2009). According to Burman *et al.* (2013), zinc oxide NPs linearly increased ROS and MDA content. The MDA content of tomato and tobacco plants who exposed to AgNPs was higher than control plants (Cvjetko *et al*., 2017). Also, an increase in phenol content has already been reported in metal NPs treated plants (Singh *et al*., 2013). IC_{50} is a measure of the potency of a substance in inhibiting the biological function and indicates how much of this substance is required to inhibit the biological process. In our research, the chemical AgNPshad IC_{50}around 8 times more than green AgNPs, which confirms the more toxicity risk of chemical AgNPs. The same trend was observed in green AgNPs synthesized from Aegle marmelos extract (Patil *et al*., 2015). The results of the present work displayed that green AgNPs significantly enhanced antioxidant abilities by stimulating polyphenols and ascorbic acid in onion.

Globally, incredible changes in agricultural production patterns have taken place, through the application of modern labor-saving technologies, mechanization, and improved crop varieties. In sustainable agriculture, the application of nano-fertilizers is a talented option to provide the food needed for the growing population worldwide. Green synthesis of nanoparticle as an environmental-friendly technique, by minimizing and reducing hazardous material, gradually has introduced itself in the commercial production of nanoparticles. The present research used an eco-friendly and low-cost methodology, without the use of any danger or lethal chemicals for AgNPs synthesis from leaf extract of *C. myxa* under room temperature conditions. The phenolic and flavonoid contents of leaf extract acted as reducing and stabilizing agent in nanoparticles synthesis and AgNPs with a good quantity and stability were synthesized.

Various bioassays are available to assess the relative toxicity of chemicals in different organisms. However, there is no single test that can detect the damage of classes of chemical compounds. A germination test is a sensitive tool used in physiological and cytogenetic studies. According to our findings, the green nanoparticle synthesis, not only had no oxidative effect on onion germination parameters, but also some stimulant effect was observed. The chemical nanoparticle motivated the plant's defense responses, by inducing oxidative stress and limited germination in a dose-dependent manner. As a final conclusion, it can be noted that the low-cost and easy synthesis of nanoparticles from plant sources, is a safe and suitable alternative for chemical-synthesized metal nanoparticles.

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Indirect shoot organogenesis and *in vitro* root formation of *Antirrhinum majus* L. by using of sodium nitroprusside

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Key words: Callus, nitric oxide, plant growth regulator, Snapdragon.

Abstract: The aim of this study was to determine the effect of different concentrations of sodium nitroprusside (SNP) on *in vitro* shoot organogenesis from hypocotyl explant derived from *in vitro* grown seedling as well as root formation of *Antirrhinum majus* L. (Snapdragon). In the first experiment, different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 2.26, 4.52, and 6.79 µM) were used for callus formation. The highest callus fresh weight (1.86 g) as well as callogenesis frequency (93.34%) were observed in Murashige and Skoog (MS) medium containing 4.52 µM 2,4-D. In the later experiments, various concentrations (0, 10, 20, 30, 40, and 50 µM) of sodium nitroprusside (SNP) were applied for shoot regeneration from callus that derived from hypocotyl segments. Based on our results, MS medium supplemented with 4.44 µM 6-benzylaminopurine (BAP) plus 0.49 µM 3-indolebutyric acid (IBA) along with 30 µM SNP had the highest shoot organogenesis frequency (93.34%) and shoot number (6.33) from callus. In root induction experiment, different concentrations (0, 20, 40, 60, 80, and 100 µM) of SNP were applied and MS medium containing 60 µM SNP was the best treatment for root induction. The survival rate of plantlets was more than 95% in acclimatization stage. The present study describes an efficient regeneration system for Snapdragon.

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1. Introduction

Snapdragon (*Antirrhinum majus* L.) is known as one of the most significant ornamental plants which has worldwide values as cut flowers, herbaceous landscape plants, and flowering potted plants (El-Nashar, 2017). Also, snapdragon has high commercial values with its wide range of color, shape, structure, and size (Weiss *et al.*, 2016). The commercial propagation of snapdragon is via seeds. The seed propagation cannot ensure the whole genetic uniformity so seed-propagated plants may indicate undesired phenotypes, quality, and regeneration potential. Therefore, plants

might be selected randomly without taking necessary care. These features of seeds exert a negative impact on sexual production of this plant (Jaworski *et al*., 2016). Therefore, the development, as well as improvement of *in vitro* culture techniques in this ornamental plant, is of high paramount (Hesami and Daneshvar, 2016). A rapid regeneration pathway for *A. majus* could be useful for commercial propagation of nursery and cut-flower industries as well as breeding programs (Sheyab *et al*., 2010). Also, *in vitro* culture of this plant is necessary for producing high quality/price ratio flower. Moreover, genetic engineering by using biolistic or *Agrobacterium* methods could be known as a viable alternative in traditional breeding methods for developing distinguished snapdragon cultivars in order to satisfy market demands (Davies *et al.,* 2013). On the other hand, the efficiency of gene transformation in snapdragon, obtained via these methods, remains low in this ornamental plant because of the lack of efficiency *in vitro* propagation protocols (Azadi *et al.*, 2016). According to the previous study, Sheyab *et al.* (2010) indicated that high applicability of transformation in *A. majus*is completely depended on the propagation procedures. Therefore, the use of *in vitro* culture for producing Snapdragon could reduce these problems that occur in the commercial production of this plant (Atkinson *et al.*, 1989; Sheyab *et al*., 2010).

Adjusting the culture medium with suitable plant growth regulators (PGRs) in various combinations and concentrations could enhance the propagation potential of various genotypes and explants (Hesami *et al*., 2017 a, b; Jafari *et al*., 2017; Hesami *et al*., 2018 a, b, c; Hesami and Daneshvar, 2018 a, b; Hesami *et al*., 2019 a, b, c). Thus, it is significant to improve the propagation protocols by using suitable PGRs in order to overcome difficulties associated with clonal regeneration and gene transformation strategies to satisfy the increasing demand for *A. majus* (Newbury, 1986; Sheyab *et al*., 2010; Hesami and Daneshvar, 2016).

Nitric oxide is known as a messenger molecule for regulating plant development (Neill *et al*., 2003; Hesami *et al*., 2019 d). This molecule has recently been characterized as one of the phytohormones (Leterrier *et al.*, 2012). Nitric oxide is known as a ubiquitous bioactive molecule that mainly contributed to various plant developmental processes such as fruit ripening, flowering, organ senescence, and germination (Jimenez-Quesada *et al*., 2017). The exterior usage of nitric oxide might improve the tolerance of plants under various stresses such as temperature, heavy metals, ultraviolet radiation, drought, and salinity (Laspina *et al*., 2005; Qiao and Fan, 2008). The activation rate of nitric oxide has been evaluated by the exogenous usage of sodium nitroprusside instead of using NO gas directly because of some technical difficulties (Sarropoulou and Maloupa, 2017). In recent years, nitric oxide is used for developing *in vitro* plant propagation (Rico-Lemus and Rodríguez-Garay, 2014). Kalra and Babbar (2010) indicated that nitric oxide could enhance the regeneration response via increasing the number of meristems and recommended that nitric oxide regulates the gene expression related to differentiation of meristems. Also, Sarropoulou and Maloupa (2017) recommended that nitric oxide exert a powerful impact on cell division and also it could be involved in shoot organogenesis and proliferation. Han *et al*. (2009) and Sarropoulou *et al*. (2014) showed that *in vitro* shoot proliferation as well as root formation of plantlets were promoted significantly by applying SNP to the MS medium in *Malus hupehensis* and cherry rootstocks, respectively. Although there are few studies about the effect of nitric oxide on improving *in vitro* shoot organogenesis (Han *et al*., 2009; Xu *et al*., 2009; Kalra and Babbar, 2010; Tan *et al*., 2013; Sarropoulou *et al*., 2014; Arun *et al*., 2017; Ghadakchiasl *et al*., 2017; Sarropoulou and Maloupa, 2017), there is no research evidence on the effect of this molecule on shoot organogenesis of snapdragon. Thus, the aim of this study was to evaluate the effect of sodium nitroprusside (SNP) on indirect shoot organogenesis as well as root formation that derived from hypocotyl explants of snapdragon in order to reduce the time of *in vitro* shoot propagation.

2. Materials and Methods

The seeds of snapdragon were washed under tap water for 30 min. Further surface sterilization treatments were conducted in a laminar airflow chamber. The seeds were surface sterilized with 70% ethanol for 10 seconds and soaked for 10 min in 10% (v/v) NaOCl. Afterward, the seeds were washed three times in sterilized distilled water. Subsequently, the sterilized seeds were inoculated on one-tenth strength MS medium. After 8-10 days, seeds were germinated, and the hypocotyl segment from *in vitro* seedling was used as a source of explant for the latter experiment.

The MS medium containing 3% (w/v) sucrose, 0.6% (w/v) agar was used as basal medium. The basal

medium was fortified with different PGRs, and pH 5.8 adjusted with 1 N NaOH before autoclaving at 121°C for 20 min. All growth regulators except sodium nitroprusside (SNP) were added before autoclaving. SNP was added after autoclaving by filtering. All cultures were maintained at 25±2°C with 55-60% relative humidity, and 16 h photoperiod (65 µmol m^{-2} s^{-1}) that provided by cool white fluorescent light.

Hypocotyl explants (0.5-1.0 cm) from 1-week-old *in vitro* seedlings (Fig. 1 a) were inoculated on MS medium supplemented with various concentrations (0, 2.26, 4.52, and 6.79 µM) of 2,4-D for callus formation. All of the culture vessels were kept at 25±2°C in the absence of light. Data of callus formation frequency (%) and callus fresh weight (g) were measured after four weeks of culture.

Calli were cultured in the regeneration medium containing 4.44 µM BAP plus 0.49 µM IBA supplemented with different SNP concentrations (0, 10, 20, 30, 40, and 50 µM). The shoots regeneration frequency and the number of shoots per callus were determined after 5 weeks of treatment.

Shoots with 0.5-1.5 cm in length were transferred to MS medium supplemented with 1 mg/l GA_3 (elongation medium) for 4 weeks. Then, the elongated shoots (2-3 cm elongation) were chosen and transferred to the half strength MS medium containing 3% (w/v) sucrose, 0.6% (w/v) agar and different concentrations (0, 20, 40, 60, 80, and 100 µM) of SNP. Rooting percentage (%) and root number including main and secondary roots were evaluated after 30 days.

Plantlets with well-developed root system were removed from the media, washed thoroughly with sterile water and transplanted into potting mixture containing autoclaved perlite and cocopeat mixture (1:1) and covered with transparent plastic to maintain high humidity. The plastic sheets were removed after 4 weeks in order to acclimatize plantlets to greenhouse condition, and the plants were shifted to pots comprising garden soil.

All experiments were performed with a total of 10 replicates per treatment and were repeated 3 sets. The data were analyzed by ANOVA using SAS version 9.3 followed by Duncan's multiple range test (DMRT, $P<0.05$).

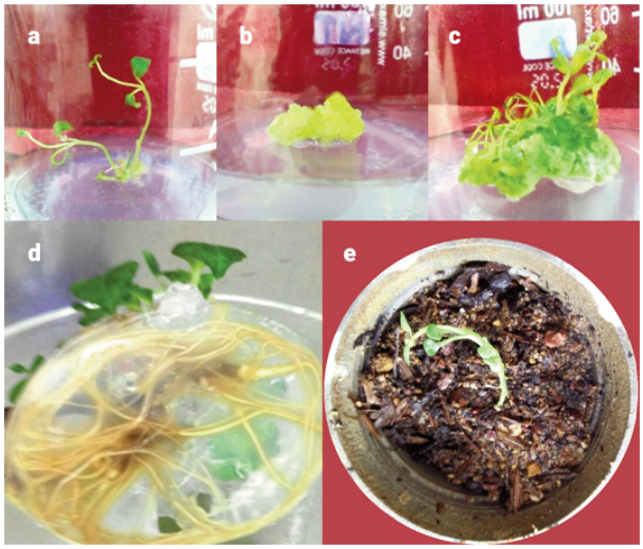

Fig. 1 - *In vitro* shoot regeneration through indirect organogenesis from seedling derived hypocotyl segments of *Antirrhinum majus* L. (a) Seedling from *in vitro* seed germination; (b) Yellow-greenish and friable callus induction on MS + 4.52 µM 2,4-D; (c) Shoot regeneration from callus on MS medium containing 4.44 µM BAP plus 0.49 µM IBA along with 30 µM SNP; (d) *In vitro* root formation on MS + 60 µM SNP; (e) Acclimatized regenerated plants after four weeks.

3. Results and Discussion

The callogenesis experiment was conducted in order to figure out the most suitable and efficient concentration of 2,4-D for callus formation. The result of this study indicated that the maximum percent of callus induction (93.34%) and callus weight (1.86 g) (Fig. 2) were achieved on MS medium containing 4.52 µM 2,4-D (Fig. 1b). In the agreement with our result, Sangwan and Harada (1975) showed that acceptable callus formation of Snapdragon through stem explant was achieved in MS medium containing 4.52 µM 2,4-D. The earlier study proved the positive effect of 2,4-D on the callus formation,

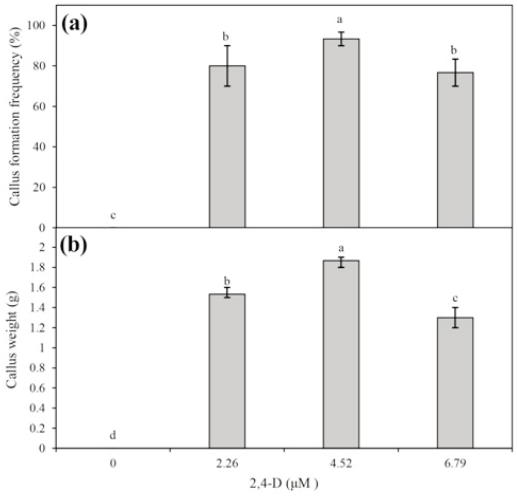

Fig. 2 - Effect of different concentrations of 2,4-Din MS medium on (a) callus formation frequency and (b) callus fresh weight of *A. majus*. Means followed by the same letter are not significantly different at $P<0.05$ as determined by Duncan's multiple range test; Vertical bars: standard error.

and also this study reported that 2,4-D may be involved in endogenous IAA metabolism regulation by inducing some specific proteins and controlling DNA methylation (Pan *et al*., 2010). However, in another study, the callus formation of *A. majus* via hypocotyl explant was obtained on MS medium with different concentrations of NAA plus 10% coconut milk (Atkinson *et al.,* 1989).

By increasing sodium nitroprusside from 10 µM to 30 µM, shoot regeneration was improved (Fig. 3). Also, the maximum frequency of shoot organogenesis (93.34%) and shoots number (6.33) were observed in MS medium supplemented with 30 µM SNP (Fig. 1c, Fig. 3). However, the higher level (more than 30 µM) of sodium nitroprusside might limit the shoots number and shoot organogenesis frequency. Calli can grow in MS medium supplemented with 50 µM sodium nitroprusside. These obtained results recommended that sodium nitroprusside can promote shoot organogenesis in proper doses. Our results indicated that sodium nitroprusside completely promoted shoot organogenesis from hypocotyl segments in MS medium along with 4.44 µM BAP plus 0.49 µM IBA. Thus, BAP and SNP appear to have a synergistic effect on shoot regeneration. The effect of NO on *in vitro* organogenesis is completely associated with cytokinins (Arun *et al*., 2017). It has previously been shown that NO might interact with auxin and cytokinin, linking the regulation of cell division to differentiation during the de-differentiation and re-differentiation of plant cells (Ghadakchiasl *et al.*, 2017; Karalija *et al*., 2017). Tun *et al*. (2001) observed that NO plays a potential role in mediating plant hormone (auxin and cytokinin) signal transduction during growth and development. Carimi *et al*. (2005) found that BA stimulates the release and accumulation of NO in plant suspension cell cultures. Therefore, in the present study, SNP may have functioned as an intermediary for adventitious shoot differentiation and regeneration, as suggested by Han *et al*. (2009) in *Malus hupehensis*.

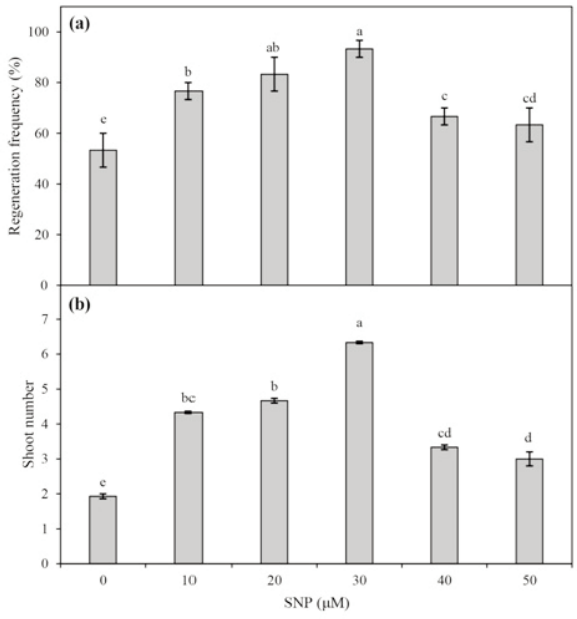

Fig. 3 - Effect of different concentrations of SNP in MS medium containing 4.44 µM BAP plus 0.49 µM IBA on (a) regeneration frequency and (b) shoot number of *A. majus*. Means followed by the same letter are not significantly different at P<0.05 as determined by Duncan's multiple range test; Vertical bars: standard error.

Our results showed that MS medium supplemented with 1 mg/l GA_3 caused shoot elongation. NO (precursor of SNP) has been reported to influence several plant developmental events in which gibberellins (GAs) play crucial roles such as seed germination, hypocotyl elongation, acquisition of photomorphogenic traits, and primary root growth (Beligni and Lamattina, 2000). However, the actual interaction between NO and GAs has been described for only a limited number of these physiological events. In fact, most of our current knowledge of the mechanisms underlying the interplay between GAs and NO is restricted to the regulation of seed germination (Neill *et al*., 2003) and the inhibition of hypocotyl elongation during seedling de-etiolation (Lozano-Juste and León, 2011). NO has been described as acting upstream of GAs (Bethke *et al*., 2007), regulating both biosynthesis and perception/transduction of GAs (Lozano-Juste and León, 2011).

There was no root formation in the MS medium without sodium nitroprusside while adding SNP promoted root formation significantly. By increasing the concentration of SNP from 0 to 60 µM, the root formation frequency (100%) and roots number (8.33) (Fig. 4) were increased significantly (Fig. 1d). However, the roots number was decreased when the SNP level was over 60 µM. Root formation is known as the meristematic development of tissues after removing the primary root system (Dash *et al*., 2017; Jafari *et al*., 2017). It was indicated that nitric oxide was involved in the response of auxins during root induction in cucumber (Pagnussat *et al*., 2003) and another report demonstrated that a NO-mediated cGMP dependent pathway was involved in this process (Pagnussat *et al*., 2003). In order to form the root meristem, auxins promoted parenchyma cells dedifferentiation and entrance to cell division (Klerk *et al*., 1995; Fujita and Syono, 1996). Also, Gouvea *et*

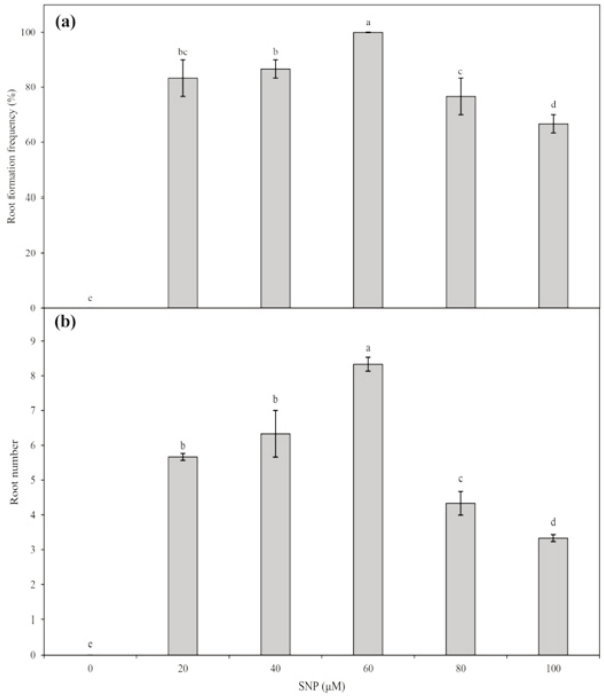

Fig. 4 - Effect of different concentrations of SNP in MS medium on (a) root formation frequency and (b) root number of *A. majus*. Means followed by the same letter are not significantly different at P<0.05 as determined by Duncan's multiple range test; Vertical bars: standard error.

al. (1997) suggested that the role of nitric oxide in signal transduction pathways for root elongation is similar to the role of auxins in this step. Therefore, it became clear that nitric oxide might have an interaction with auxins in regulating cell division to differentiation in ''de-differentiation'' and ''re-differentiation'' steps of plant cells (Ötvös *et al*., 2005). The positive effect of nitric oxide on improving root induction is reported in various species (Huang and She, 2003; Correa-Aragunde *et al.,* 2004; Han *et al*., 2009). Sarropoulou *et al.* (2014) recommended that nitric oxide could (a) produce an antioxidant condition that protects auxins from deteriorations as well as oxidation, (b) speed up cell expansion in order to improve rooting in plants, (c) serve as a downstream messenger in the IAA signaling pathway, (d) regulate enzyme activities or cell-cycle genes that are associated with auxin signal transduction, and (e) reduce the lignification of cell wall. By using exogenous sodium nitroprusside, the root induction in mung bean was promoted significantly (Huang and She, 2003). Furthermore, sodium nitroprusside can induce root hair induction in lettuce (Lombardo *et al*., 2006), and development of lateral roots in tomato (Correa-Aragunde *et al*., 2004).

Plantlets that had well-developed roots were transferred successfully into small pots consisting of perlite and cocopeat mixture (1:1). Our results showed that the rooted plants had 95% survival rate in the acclimatization stage. Afterwards, within 20 days after transferring plantlets to the greenhouse, the normal growth of plantlets was resumed (Fig. 1e). Similar to our results, Hesami and Daneshvar (2016) indicated that by acclimatization of the snapdragon plantlets in the perlite and cocopeat mixture (1:1), 90% survival rate was obtained.

In conclusion, we have developed a method for indirect shoot organogenesis from hypocotyl explants of *A. majus*. It is of note that SNP, a donor of NO, has a direct effect on *in vitro* shoot differentiation and rooting of the snapdragon explants. SNP may interact with auxin and cytokinin, linking the regulation of cell division to cell differentiation during the dedifferentiation and redifferentiation of plant cells. The improvement of ornamental plant by conventional methods (hybridization, inbreeding and mass selection) is time and labor consuming, depends on the existing gene pool(s) and violently influenced by environmental conditions. On the other hand, callus culture can be utilized as a powerful tool for genetic cell transformation via somaclonal variation and promoting mutagenesis and genetic engineering that can be either more rapid than traditional breeding and leading to new genes and genotypes. The indirect plant regeneration system developed for *A. majus* provided a step towards the application of such methodology, for this ornamental plant. Moreover, this protocol is rapid with induction of callus to acclimatizing of plantlets to greenhouse completed within 21 weeks.

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Short note

Determining the main agronomic traits of snake melon (*Cucumis melo* var. *flexuosus* L.) fruits as affected by genotypic differences

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Key words: breeding programs, fruit colour, powdery mildew, resistance, snake cucumber, yield.

Abstract: The variability affecting the main agronomic traits of 10 snake melon genotypes (*Cucumis melo* var. *flexuosus* L.) (three F1 hybrids, six breeding lines and the widely grown and consumed traditional cultivar Mornagui), grown under greenhouse conditions during 2014 and 2015 seasons, was examined and compared. Their resistance to powdery mildew was also checked. The main production-driving agronomic traits varied significantly (P<0.05) depending on the genotype. The major skin colour determined visually was different among the considered genotypes. The hybrids (H5 and H12) and the breeding lines (L1, L2, L5, L6 and L9) had dark green skin. The fruits of the hybrid H7 were dark and light green and the breeding line L4 has intense and dark green fruits whereas cv. Mornagui was characterized by medium light green fruits. Regarding fruit shape, the hybrids H7 and H5 produced straight fruits whereas the hybrid H12 and the breeding lines; L2, L4, L5, and L9 and the cv. Mornagui had elongate fruits. However both breeding lines L1 and L6 were characterized by straight elongate fruits. The F1 hybrid H5 was the most productive (3.6 Kg/plant and ≈ 21 fruits/plant for total yield and number of fruit/plant respectively). These findings are useful for further breeding programs aiming to develop new powdery mildew resistant snake melon cultivars with satisfying agronomic traits.

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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

Snake melons (*Cucumis melo* var. *flexuosus* L.), belong to the Cucurbitaceae family largely distributed and consumed since the antiquity across large geographical area (Pandey *et al.*, 2010; Paris, 2012). The *flexuosus* distinguishes from other *C. melo* varieties by the twisted long to very long fruits, typically exceeding the 4:1 length-to-width ratio, characterized by a slightly hairy white or light green exocarp (rind) often furrowed by more or less deep grooves running lining the fruit surface. The rind covers a creamy white or pale green flesh (mesocarp and endocarp) containing numerous whitish edible seeds, deliquescent at maturity. Because the stem is rather thin, the plant is usually supported on trellises, where the snake-like fruits grow on vines attaining up to 120 cm lengths (Burger *et al.*, 2010).

Snake melon fruits are known all around the world with different trivial names: "alficoz" in Spain, "tortarello" or "cetrangolo" in Italy, "fakous" or "fegous" in Arabic Maghreb countries, "agoor" in Soudan, "acur", "hıtta" or "hıtı" in Turquie, "kakri" in India, "uri" in Japan and Armenian cucumber, "yard-long melon", "serpent-melon" or "Gutah" elsewhere (Solmaz *et al.*, 2016). Snake melon fruits are generally consumed raw at the immature stage of ripening with a preference for straight long and thick green fruits in the Mediterranean region. Here, this crop has been appreciated since antiquity for their crisp texture and refreshing, slightly acidic and non-sweet flavour, as revealed by their recurrent depiction in wall painting, sculptures and mosaics dating back to ancient Egypt and Roman Empire, as well as by the presence of references in the Islamic and Jewish literature (Paris *et al.*, 2011; Paris, 2012). The fruits are stomachic and seeds are also traditionally utilized as antitussive, digestive, febrifuge and vermifuge (Duke and Ayensu, 1985).

Besides the differences in skin colour and in the longitudinal furrowing of the fruits, snake melons genotypes differ also in various productivity-driving and quality traits including early marketable and total yield, numbers of fruits per vine and resistance to main pest diseases (eg. fusarium and powdery mildew) (Pandey *et al.*, 2010). Powdery mildew is the main fungal disease affecting similarly greenhouse and open-field grown cucurbits and particularly snake melon. This disease is easily, recognizable by the whitish powdery fungal growth developing on many parts of the plant and fruits (Sitterly 1978; Zitter *et al.*, 1996) . The disease is primarily caused by two fungal species around the world: *Golovinomyces orontii* and *Podosphaera xanthii* and are considered as an important limiting factor for snake melon production (Fernández-Ortuño *et al.*, 2006; Bellón-Gómez *et al.*, 2015). Therefore, growers and breeders are increasingly looking for resistant genotypes in order to overcome this problem although these fungal species are developing also new strains more virulent.

Previous research on snake melons mainly focused on the effect of salinity, sowing period and harvesting intervals on fruit yield, the phenological phases and morphological traits of the plants, the influence of different rootstocks on production in soilless cultivation, the *in vitro* plant regeneration, seed priming, the variability among genetic, morphological, vegetative, fruit and yield parameters of germplasm, the impact of breeding hermaphrodite lines on yield and, least but not last, the identification of fruit morphological and quality traits by QTL mapping (Reviewed in Ilahy *et al.*, 2019).

Genotypic differences strongly influence the microbial community composition in the rhizosphere (Aydi-Ben-Abdallah *et al.,* 2019). Pre- and post-harvest manipulations also significantly impact quality traits of horticultural crops (Siddiqui *et al.*, 2015, Siddiqui and Singh, 2015; Ilahy *et al.*, 2018). Furthermore, in snake melon genotypes carrying resistance to powdery mildew, the quality traits might be affected with respect to ordinary and susceptible genotypes. Thus, this study investigates the genotypic variability affecting the main agricultural traits of different snake melon genotypes (three F1 hybrids, six breeding lines and a traditional reference cv. Mornagui) grown under greenhouse condition. The overall aim is to select the most promising genotypes (in terms of agronomic traits and mildew resistance), to be used in further snake melon breeding programs

2. Materials and Methods

Plant material

Ten powdery mildew resistant (PMR) snake melon genotypes were used in our study (Three hybrids; H7, H5 and H12), six breeding lines; L1, L2, L4, L5, L6 and L9) and the largely grown and consumed snake melon cultivar Mornagui. The breeding lines were heirlooms landraces previously selected for their higher productivity and powdery mildew resistance levels as well as desirable agricultural traits generally

preferred by the consumer (length and skin colour).

The (F1) hybrids were developed in a line x line mating design, in which parents characterized by high levels of powdery mildew resistance (L2 and Mornagui), were crossed with the selected breeding lines (L1, L5 and L6) characterized by good powdery mildew resistance coupled with high productivity and desirable phenotypic traits. The breeding lines and the obtained hybrids were exchanged with different laboratory working on the same topics in Italy, Hungary and India.

Plug trays were used to grow snake melon seedlings at the beginning of December 2013 and 2014. Four weeks later seedlings were transferred into a sandy soil (Fig. 1) under unheated water-impermeable plastic screens greenhouse, recommended for growing vegetable crops particularly cucurbits during winter cycles, using 125 and 150 cm as in- and between-row separations respectively at the region of Teboulba (35°63'N, 10°95'E) in the coastal zone of Tunisia. The experimental design was a randomized complete block design (RCBD) with three blocks (replication). Each replication consisted of 10 plants per genotype. They were subjected to agricultural practices commonly adopted by high-yielding farmers in this region. The production methods were in accordance with those reported recently in Ilahy *et al.* (2019).

Fig. 1 - Overview of the considered snake melon trial.

Determination of the agronomic characteristics

Powdery mildew resistance was determined on the basis of visual evaluation of the developed symptoms. Fruits were harvested weekly at the immature stage of ripening. Yield was determined following counting and considering all fruits on each plant. Early yield (agricultural output discriminating cultivars with precocious production with respect to others) and total yield [Kg of fresh weight (FW)/plant], number of fruit per plant (N. fruits/plant), average fruit weight (g) were determined on all the trials plants. In addition, fruit length and diameter were also recorded for all fruits. Fruit length and diameter (cm) were determined using a Vernier caliper.

Experimental design and statistical analysis

The effect of genotypic differences on the agronomic traits of snake melon was assessed by analysis of variance (ANOVA). When a significant difference was detected, means were compared using the least significant difference (LSD) test ($p<0.05$). All statistical analysis was performed using SAS Version 6.1 software (SAS Institute, Cary, NC, USA). Agglomerative Hierarchical Cluster analysis was used to determine differences and similarities among the genotypes, and the distance measure used was Euclidean distance computed between each population by the Ward method.

3. Results and Discussion

The main agronomic traits of the snake melon hybrids and breeding lines grown under greenhouse during the productive seasons 2014 and 2015 are reported in Table 1. Pooled data of the two years were analyzed. Plants of the different genotypes exhibited visually an important vigour with exceptional foliage cover (Figs. 1, 2).

The major skin colour was visually different among the evaluated genotypes. The hybrids H5, and H12 and the breeding lines L1, L2, L5, L6 and L9 had dark green skin (Fig. 2). The hybrid H7 was dark and light green and the breeding line L4 has intense and dark green fruits whereas cv. Mornagui was characterized by medium light green fruits. Regarding fruit form, the hybrids H7 and H5 produced straight fruits and H12, L2, L4, L5, 'Mornagui' and L9 had elongate fruits. However both breeding lines L1 and L6 were characterized by straight elongate fruits.

The early and total yields (expressed as Kg /plant), the number of fruits per vine, as well as the average

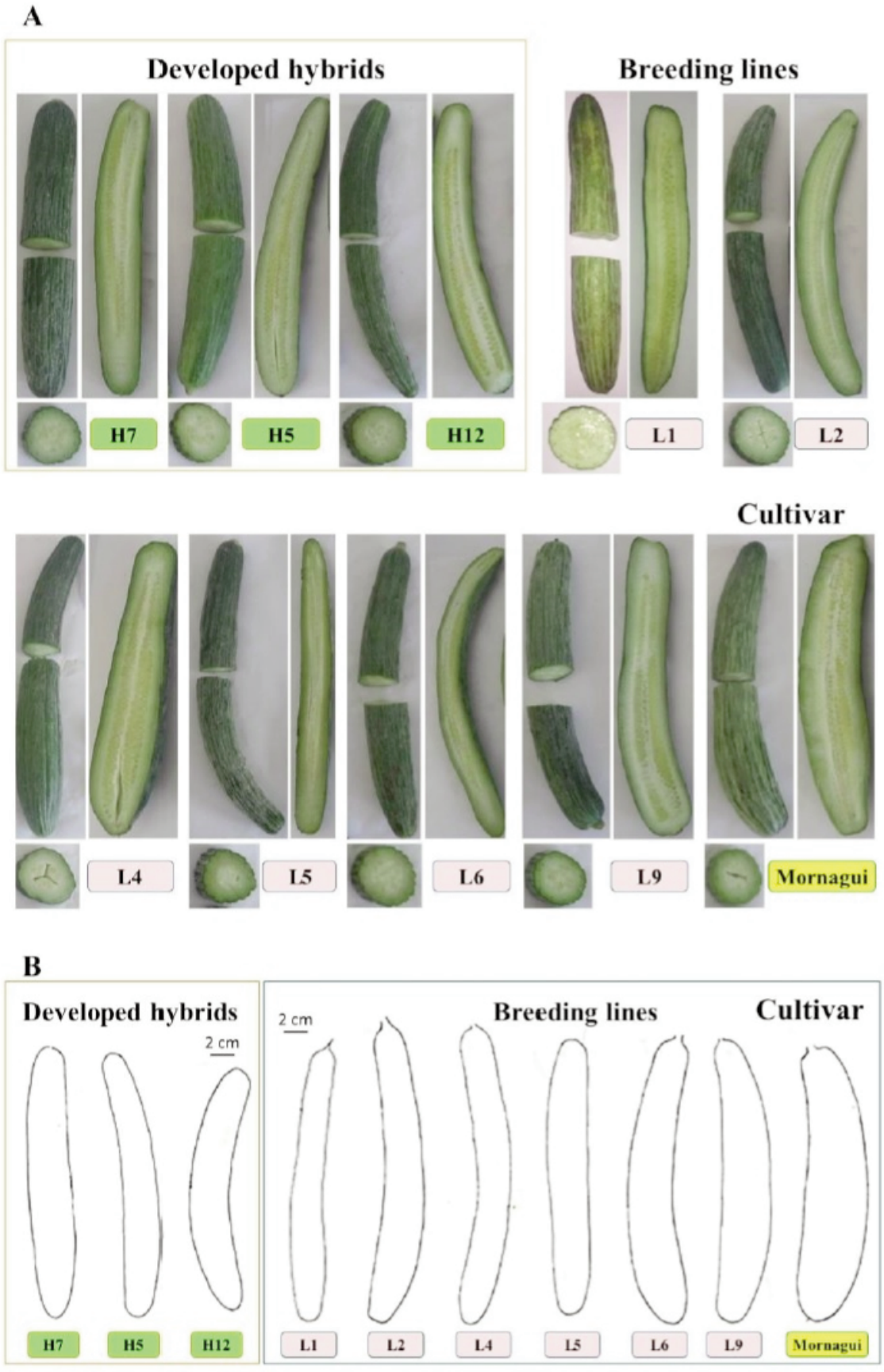

Fig. 2 - External appearance, longitudinal and cross sections (A), and schematic representation of the morphology (B) of the different snake melon genotypes peponides under analysis (hybrids, breeding lines and the traditional cultivar Mornagui).

fruit weight, length, diameter and length/width ratio varied significantly (P<0.05) between genotypes (Table 1). Early yield ranged from 0.9 Kg per plant for the breeding line L5 and the cultivar Mornagui to 1.5 and 1.7 Kg per plant for the hybrids H5 and H7, respectively. The breeding line L9 had the lowest total yield (1.9 Kg per plant) and 10.7 fruits per plant, while the highest values were recorded for the hybrid H5 (3.6 Kg per plant and 20.7 fruits per plant). The average fruit weight ranged between 151.0 g to 184.3 g for the breeding line L6 and Mornagui cultivar, respectively. In agreement to the low weight, the breeding line L6 produced short fruits (19 cm length as average) with the lowest length/width ratio (5.14), while the fruits of the hybrid H5 and of the breeding line L1 were the longest (32.0 and 32.6 cm, respectively) and showed the highest length to width ratio (8.36 and 8.65, respectively). Fruit diameter is an important agricultural trait for snake melon as consumers generally prefer long fruits with low diameter (width). The lowest fruit diameter (3.3 cm) was measured for the breeding line L5 and the highest value (4.2 cm) was obtained for the hybrid H7 and cv. Mornagui. To our knowledge, these presented data are among the first reports on snake melon horticultural traits. Nevertheless, with respect to total yield, our data exceed the range of values (0.5-3.5 kg/plant) reported by Aydi-Ben-Abdallah *et al.* (2019) and (0.3-2.0 Kg per plant) reported by Abdelmohsin *et al.* (2015) for open field and greenhouse grown monoecious cultivars of the *flexuosus* group (Alimin, PI222187 and Snake melon) and for the hermaphroditic breeding lines generated by crossing the *flexuosus* parents with the melon accession Paul, while they fell within the range reported by the same authors for number of fruits per vine (2-25). No correspondence was found, instead, between our findings and the number of fruits per plant (5.8-7.2) and fruit length (33-90) measured in five stable inbred lines of cultivated landraces of snake melons grown in open field at Wad Medani (Sudan) by Yousif *et al.* (2010); while they were in accordance with those of Ali-Shtayeh *et al.* (2017) for fruit weight (53-201 g) and length/width ratio (3-9) in a study comprising 50 accessions of snake melons grown in different open fields across the West Bank, and were slightly lower than the length (48.3 cm) and width (10.7 cm) values reported by Ramamurthy and Waters (2015) for a pale-green fleshed, non-sweet accession (USDA PI435288) grown it the fields of the Lincoln Agronomy Research Farm of the University of Nebraska.

Powdery mildew resistance visual evaluation showed that the snake melon line L9 exhibited similar resistance level to the ordinary cultivar Mornagui and also to the hybrid H5 (L2 x L1).

Table 1 - Agronomic characteristics evaluated in snake melon hybrids, breeding lines and cultivar Mornagui grown under greenhouse conditions during the seasons 2014 and 2015 (pooled data of the two years were analyzed)

Genotypes	Major skin colour	Fruit form	Early yield (Kg/plant)	Total yield (Kg/plant).	Number of fruits/plant	Fruit weight (g)	Fruit length (cm)	Fruit diameter (cm)	PMR*
Developed hybrids (Line x Line)									
H7 (Mornagui X L5)	Dark/light green	Straight	1.7±0.1a	3.4±0.5 ab	20.3±2.9 ab	164.0±2.3	29.8±1.2 b	4.2±0.2 a	+
H5 (L2 X L1)	Dark green	Straight	1.5±0.1 a	3.6±0.3 a	20.7±2.0 a	173.6±1.2	32.0±1.0 a	3.7±0.1 bcd	+ +
H12 (Mornagui X L6)	Dark green	Elongate	1.4±0.2 b	3.0±0.2 abc	18.4±0.3 abc	169.0±16.7	22.0±0.6 e	4.1±0.2 ab	+
Breeding lines									
L1	Dark green	Straight elongate	1.4±0.3 b	3.3±0.6 ab	19.4±3.8 abc	172.7±6.1	32.6±0.1 a	3.9±0.3 abc	+
L2	Dark green	Elongate	1.1±0.1 b	2.7±0.1 abcd	16.0±0.6	166.3±3.7	31.2±0.1 ab	3.8±0.1 abcd	+
L4	Intense dark green	Elongate	1.3±0.1 ab	2.7±0.2 bcd	17.0±1.5 abc	153.6±2.7	27.5±0.3 c	3.8±0.4 abc	+
L5	Dark green	Elongate	0.9±0.2 b	2.1±0.2 cd	13.7±1.8 cd	154.6±5.2	19.2±0.1 f	3.3±0.1 d	+
L6	Dark green	Straight elongate	1.0±0.1 b	2.2±0.1 cd	14.7±0.7 bcd	151.0±2.6	19.0±0.6 f	3.7±0.1 bcd	+
L9	Dark green	Elongate	1.0±0.2 b	1.9±0.5 d	10.7±1.8 d	174.0±12.1	22.5±0.1 e	3.6±0.3 cd	+ +
Cultivar Mornagui	Medium/light	Elongate	0.94±0.1 b	2.5±0.2 bcd	13.6±1.2 cd	184.3±12.9	25.2±0.6 d	4.2±0.3 a	+ + +

The data refer to visually assessed snake melon fruits and are the average of at least 10 independent replicates.
Mean ± SD followed by the same letters do not differ significantly (LSD test, P<0.05).
* PMR is the evaluation of the Powdery mildew resistance, based on visual evaluation of the symptoms developed.

The dendogram from the hierarchical ascending classification (Fig. 3) has discriminated snake melon genotypes into 3 main clusters (level of trencature 5). The first cluster comprised 4 genotypes (the hybrid H5 and the breeding line L1 in a sub-cluster and the hybrid H7 and the breeding line L2 in the other sub-cluster) characterized by the most producing yield and the most elongated fruits. The second main clusters comprised 3 genotypes (the hybrid H12, the breeding line L9 and the traditional cv. Mornagui) mainly characterized by intermediate producing yields and intermediate fresh weights. The third cluster comprised the breeding lines L5, L6 and L4 characterized by the small fruit weight and the small fruit length.

4. Conclusions

The variability detected, for the main agronomic traits, in different snake melon genotypes (F1 hybrids and breeding lines) can be useful for further conventional snake melon breeding programs aiming to improve their agronomic traits and could contribute to the breeding of powdery mildew-resistant elite cultivars suitable for greenhouse and open field conditions.

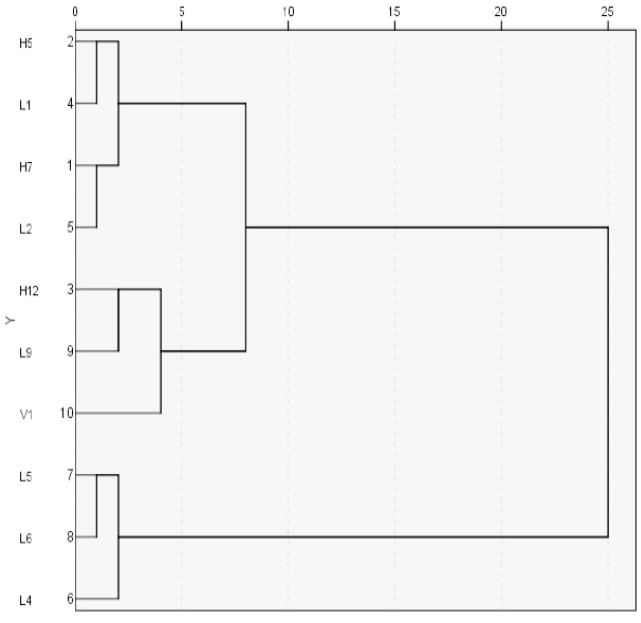

Fig. 3 - The hierarchical ascending classification oh the studied snake melon genotypes. L1, L2, L4, L5, L6 and L9 are the breeding lines H5 (L2 x L1), H7 (Mornagui x L5) and H12 (Mornagui x L6) are the hybrids, V1 is the traditional cv. Mornagui.

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