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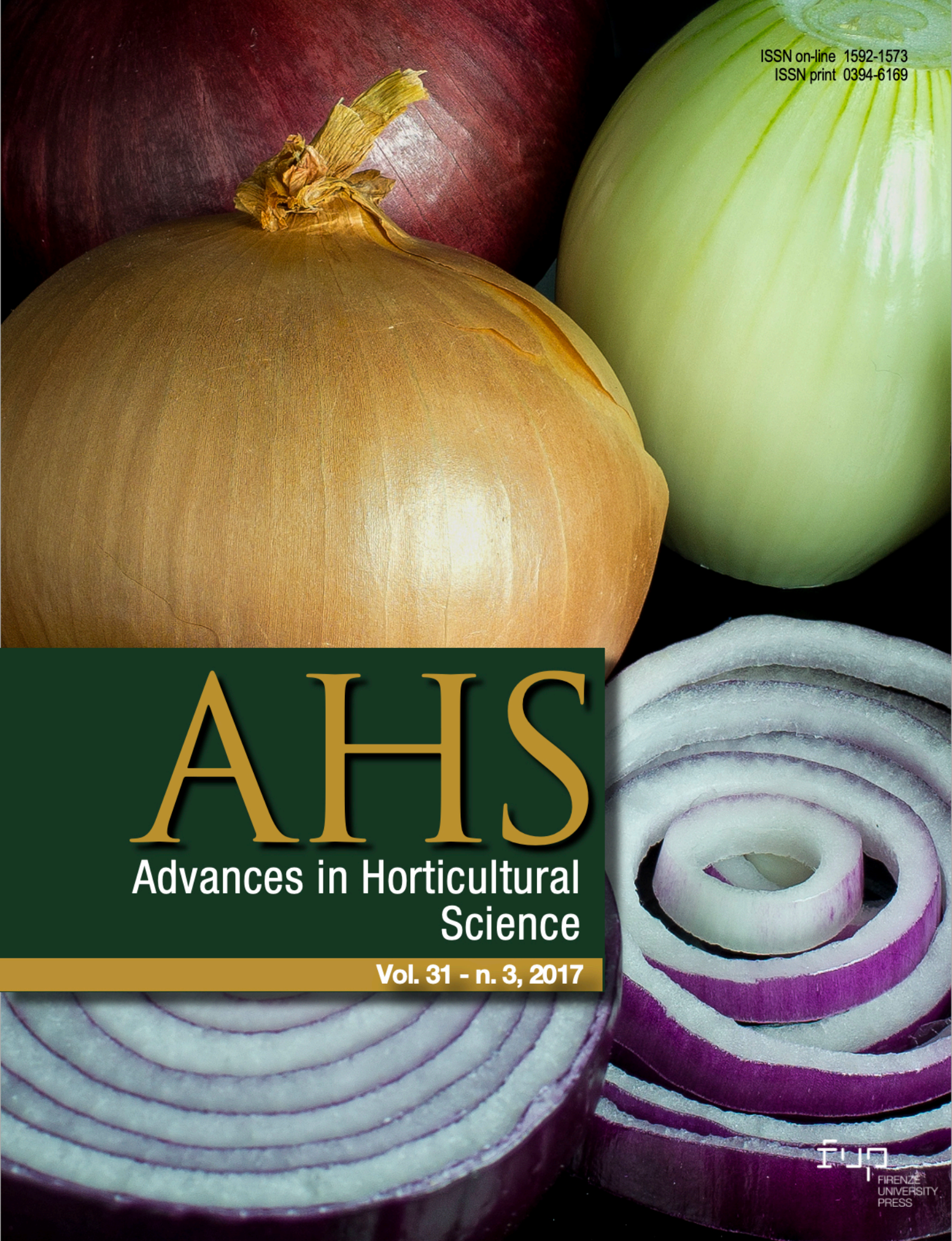

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Advances in Horticultural Science

Tuber yield and processing traits of potato advanced selections

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Key words: breeding, chipping ability, *Solanum tuberosum*, tuber specific gravity.

Abstract: World potato production continuously requires new cultivars to satisfy farmers' and consumers' demand. Tuber yield and quality are some of the main potato breeding targets. In this study, 27 advanced potato clones from 7 hybrid families were evaluated for yield, tuber specific gravity and chipping ability. Variability in tuber yield was found between families as well as between clones. Forty-eight percent of clones showed higher productivity compared to the best control (Agria, 1.1 Kg). Families displayed significant differences also in terms of tubers specific gravity, with about 70% of clones with a score higher than 1.080, which was considered the minimum acceptable value for processing. Chipping ability was evaluated at harvesting time and after cold storage with and without reconditioning. The majority of studied clones showed a good chipping ability score (<4.5) at harvest; five samples chipped well after cold storage with reconditioning, while good chippers were not identified after cold storage without reconditioning. The use of an arbitrary index calculated for each clone is proposed to assist the selection of materials with a good trait combination.

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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

Potato, *Solanum tuberosum*, is the most cultivated not cereal crop in the world, ranking fourth after rice, wheat and corn (FAO Statistics, 2012). It represents an important component of human diet, because tubers are able to supply several nutrients, such as essential amino acids, vitamins (as vitamin C) and minerals. It can be consumed either as fresh product or as processed ready-to-eat food (i.e. chips and French fries) (Carputo *et al.,* 2005). Overall, the number of potato varieties cultivated for processing purposes is increasing and more than 50% of potato yield is driven to food industries. In order to follow the food market trends, new varieties with a specific combination of processing-related traits are continuously requested. A traditional potato breeding program begins with sexual hybridization of tetraploid varieties or *élite* clones, in order to generate biparental families. Progenies are then evaluated for quality traits and the

most promising clones are then selected for further evaluations (MacKay, 2005; Rak and Palta, 2015; Melito *et al.*, 2017). Because of the autotetraploid nature of *S. tuberosum* and its heterozigosity level, the F1 presents trait segregation; as a consequence, vegetative propagation and field selection are needed for several years to identify superior clones (Haynes *et al*., 2012). Tuber traits considered by breeders in developing new varieties are different. Among them, tuber yield and tuber quality characteristics, including both external (skin color, tuber size and shape, eye depth) and internal (dry matter content, chip ability) traits (Carputo and Frusciante, 2011) are deeply evaluated. External traits play an important role in potato fresh market, because they influence consumers' choice. On the counterpart, internal traits mainly affect tuber processing. One of the principal goals of potato breeding programs is the selection of varieties with a good chipping ability following cold storage. Indeed, following harvest, potato are normally stored at low temperatures (<8°C) to keep an interrupted supply used by the processing industries, to prevent sprouting, to reduce bacteria soft rot attack and to contain the loss of dry matter (Malone *et al*., 2006; Zhao *et al.*, 2013). Unfortunately, in response to cold storage most potato varieties convert tuber starch to reducing sugars (glucose and fructose). This reaction is known as "cold-induced sweetening" and is recognized as a serious problem for potato processing industry (Dale and Bradshaw, 2003). The accumulated reducing sugars undergo the non enzymatic Maillard reaction with free amino acids when potatoes are fried in hot oil, resulting in dark unacceptable chips. Furthermore, Mottram *et al.* (2002) and Stadler *et al.* (2002) reported high level of acrylamide because of the Maillard reaction, which is potentially damaging for human health. Equally important for the potato processing industry is the availability of varieties with high tuber specific gravity. Tubers with specific gravity higher than 1.080 are generally considered suitable for processing.

The main aim of this study was to test the performance of 27 potato advanced clones belonging to 7 families with different genetic background. Tuber yield, specific gravity, chipping ability and earliness were determined to select the most promising clones. Finally, an evaluation index useful for selection of genotypes with a good combination of traits was calculated.

2. Materials and Methods

Genetic material and experimental design

The material used in this study derived from a conventional program of clonal selection started in 2009 in a single hill plot. From 2010 to 2013 selected clones were cultivated in larger unreplicated plots with spaced plants. Clones with undesired characteristics (i.e. long stolons, deep tuber eyes and tuber defects) were discarded and the number of seed tubers was increased. Three commercial varieties (Spunta, Adora and Agria,) were used as control. The field trials were conducted at Marigliano (district of Naples) (Lat. 40.927759°, Long. 14.451370°).

Tuber production and quality evaluation

Control varieties and experimental clones were planted in a randomized complete block design with 3 replications. For each clone, 10 tubers were planted in a single row with spacing of 30 cm between plants and 70 cm between rows. Plants were grown following the standard cultural practices of the geographic area. Tubers were planted in March and harvested in July, when plants start senescing (roughly 120 days after plantation). Total yield (TY) was evaluated at harvest. Chips were produced by frying 10 longitudinally cut tuber slices from the center of each tuber and 2 tubers for each clone were used. The chips color was evaluated in 3 consequent times: at harvest, at three months of cold storage (7°C) and at 2 weeks of reconditioning (20 - 24°C) after cold storage. To optimize the chipping test, slices were washed in water before frying in soybean oil. Tubers were considered completely fried when oil end to bubble. A colorimetric scale, from 1 (very light) to 10 (very dark) was used to determinate chipping ability. Based on Carputo *et al*. (2002), clones with a score ≤ 4.5 were considered suitable for chipping. The specific gravity of tubers (TSG) of each clone was estimated on 1 Kg of tuber sample, evaluating the ratio weight in air/weight in water (Woolfe, 1987). Foliage earliness was evaluated at 90 days after planting. An earliness score (from 1= very late to 5= very early) was associated to each clone comparing foliage senescence to the control variety Spunta (earliness score= 3).

Evaluation Index

To assist the selection of clones, an evaluation index (EI) was elaborated, associating an arbitrary score to each trait based on the value obtained: TY

(Kg/plant): 1= <0.5; 2= 0.51-1 ; 3= 1.1-1.5; 4= 1.51-2 ; 5= >2.1. TSG: 1= ≤1.080 (not suitable for processing); 2= 1.081-1.085; 3= 1.086-1.090; 4= >1.091.
Chipping color: 1= >4.5 at each test after cold storage; 2= <4.5 at least in one test after cold storage; 3= <4.5 at both tests after cold storage.
Average earliness score: 1= ≤1.0; 2= 1.1-2.0; 3= 2.1-3.0; 4=> 3.1.

The EI was calculated summing the scores for each trait: the higher index values, the more desirable genotypes. The EI was calculated only for clones for which all the evaluation data were available.

Statistical analysis

One-way ANOVA was run using JMP 7 software (SAS Institute, Cary, NC, USA). When a significant F was found ($P<0.05$), data were compared using Tukey's post hoc multiple comparison test. Each trait was used to compare the mean values among clones and varieties and to varieties individually.

3. Results and Discussion

The development of new potato varieties addressing production efficiency and sustainability requires a well-planned breeding program. The accumulation of multiple traits in a single variety is one of the main goal of potato breeding programs. Among the available different strategies, those based on sexual hybridization between tetraploid varieties or clones, followed by selection, still represent a successfully and widely used option. In this research, 27 advanced clones were selected and evaluated for yield and processing traits. They belong to seven families obtained from crosses involving ten cultivated varieties (Spunta, Victoria, Jenny, Blondy, Agria, Bolesta, Sandy, Majestic, Alcmaria, Primura,) and one breeding clone (MC 329). As reported in Table 1, clones under evaluation showed differences in terms of flesh color and tuber shape, whereas skin color, eye

Table 1 - Tuber characteristics of the potato clones under selection. For each clone, pedigree, tuber skin color (TSC), tuber flesh color (TFC), tuber shape (TS), eye and stolon characteristics are reported.

Families	Pedigree	TSC	TFC	TS	Eyes	Stolons
'Spunta' x 'Victoria'	S04-2-10	Yellow	Yellow	Oblong	Superficial	Short
	S04-2-17	Yellow	Yellow	Round	Superficial	Short
	S04-2-18	Yellow	Yellow	Long	Superficial	Short
	S04-2-28	Yellow	Yellow	Oblong	Superficial	Short
	S04-2-34	Yellow	Yellow	Oblong	Superficial	Short
	S04-2-40	Yellow	Yellow	Oblong	Superficial	Short
	S04-2-53	Yellow	Yellow	Long	Superficial	Short
	S04-2-55	Yellow	Yellow	Long	Superficial	Short
	S05-7-15	Yellow	Light yellow	Oblong	Superficial	Short
	S05-7-4	Yellow	Yellow	Long	Superficial	Short
'Jenny' x 'MC 329'	S04-7-2	Yellow	White	Round	Superficial	Short
	S04-7-6	Yellow	White	Round	Superficial	Short
	S04-7-27	Yellow	White	Round	Superficial	Short
'Blondy' x 'Victoria'	S05-1-2	Yellow	Yellow	Long	Superficial	Short
	S05-1-25	Yellow	White	Long	Superficial	Short
'Bolesta' x 'MC 329'	S05-2-10	Yellow	White	Round	Superficial	Short
	S05-2-11	Yellow	Light yellow	Round	Superficial	Short
	S05-2-15	Yellow	Yellow	Round	Hallowed	Short
	S05-2-18	Yellow	Yellow	Round	Superficial	Short
	S05-2-23	Yellow	Yellow	Round	Superficial	Short
	S05-2-3	Yellow	Yellow	Round	Superficial	Short
	S05-2-4	Yellow	Yellow	Oblong	Superficial	Short
	S05-8-5	Yellow	Yellow	Round	Superficial	Short
'Agria' x 'Sandy'	S04-5-32	Yellow	Yellow	Round	Superficial	Short
'Majestic' x 'Alcmaria'	S04-6-2	Yellow	White	Oblong	Superficial	Short
'Primura' x 'Alcmaria'	S05-4-2	Yellow	Yellow	Oblong	Superficial	Short
Solanum tuberosum	Adora	Light yellow	Yellow	Round	Superficial	Short
	Agria	Yellow	Yellow	Oblong	Superficial	Short
	Spunta	Light yellow	Yellow	Oblong	Superficial	Short

depth and stolon length were uniform. This is the result of the previous selection pressure aimed at discarding clones with undesired characteristics (e.g. deep eyes). Tuber yield, specific gravity, and chipping ability of material under evaluation are reported in Table 2. Data are summarized on a family basis. Overall, significant differences in TY were found among families. The average TY was 1.2 Kg/plant, ranging from 0.43 to 3.00 Kg, detected in a clone from 'Spunta' x 'Victoria' and 'Majestic' x 'Alcmaria', respectively. By contrast, the comparison between the mean TY of clones and that of control varieties revealed not significant difference (Table 3). However, analysis of ranges within each family revealed the presence of clones with good yield performances. In this research, 12 very promising clones for tuber yield were identified, belonging to 'Spunta' x 'Victoria' (5), 'Majestic' x 'Alcmaria' (1), 'Jenny' x 'MC 329' (1), 'Blondy' x 'Victoria' (1), 'Bolestra' x 'MC 329' (4) (not shown). This may outline the occurrence of allelic combinations providing satisfactory diversity, a prerequisite for heterosis in yield (Mendoza and Haynes, 1974).

Potato quality evaluation does not include only tuber yield, but also several qualitative and quantitative traits. Among them, tuber dry matter and chipping ability are fundamental for processing. In particular, the chipping ability is a quality parameter highly important for food industries because it influences not only yield of the processed product, but also oil absorption rate in fried products (Asmamaw *et al*., 2010). On the other hand, tuber specific gravity (TSG) is commonly accepted as measure of the dry matter content and it provides the suitability of potato varieties for processing (Kabira and Berga, 2003). Based on this finding, Fitzpatrick *et al*., (1964) identified three classes of TSG: low (less than 1.077), intermediate (between 1.077 and 1.086), and high (more than 1.086). Furthermore, Kabira and Berga (2003) reported that tuber should have a specific gravity higher

Table 2 - Tuber yield (Kg of tubers per plant) (TY), tuber specific gravity (TSG), chip category color and evaluation index (EI) of 27 potato advanced clones. Chip category color was evaluated at harvest and after 90 days of cold storage at 7°C, with and without reconditioning at room temperature for two weeks (respectively + Rec; - Rec) (see materials and methods). For each trait, the average family value (range) is reported

Material	No. of clones	TY *	TSG *	Chip category colour *			EI *
				Direct	Cold storage - Rec	Cold storage + Rec	
Families							
'Spunta' x 'Victoria'	10	1.09 (0.43-1.58) b	1.081 (1.073-1.088) bc	3.8 (2.0-7.0)	8.5 (7.0-10.0) b	6.1 (2.0-7.0) abc	10.1 (6.0-14.0) c
'Jenny' x 'MC 329'	3	0.95 (0.48-1.51) b	1.095 (1.086-1.104) a	2.0 (2.0-2.0)	10.0 (10.0-10.0 a	4.3 (2.0-7.0) bc	13.0 (11.0-15.0) ab
'Blondy' x 'Victoria'	2	1.20 (1.13-1.28) b	1.087 (1.086-1.089) ab	2.5 (2.0-3.0)	8.5 (8.0-9.0) ab	6.0 (5.0-7.0) abc	11.5 (11.0-12.0) abc
'Bolesta' x 'MC 329'	9	1.20 (0.98-1.45) b	1.086 (1.079-1.091) b	4.0 (7.0-2.0)	8.9 (7.0-10.0) ab	6.7 (3.0-8.0) a	10.6 (7.0-13.0) bc
'Agria' x 'Sandy'	1	0.76 b	1.080 bc	2	10.0 ab	8.0 ab	7.0 c
'Majestic' x 'Alcmaria'	1	3.00 a	1.079 bc	3	7.0 b	3.0 c	15.0 a
'Primura' x 'Alcmaria'	1	1.02 b	1.070 c	3	10.0 ab	8.0 ab	7.0 c
Varieties							
Adora	1	0.80 b	1.071 c	2	9.0 ab	5.0 abc	8.0 c
Agria	1	1.18 b	1.075 bc	5	10.0 ab	5.0 abc	11.0 abc
Spunta	1	1.10 b	1.078 bc	4	8.0 ab	7.0 abc	10.0 abc
F-ratio		13.52	8.92	NS	3.03	3.56	5.42
P-value		<0.0001	<0.0001		0.0036	0.0009	<0.0001

* Means comparison using Tukey's test. Levels not connected by the same letter are significantly different (P<0.05).
NS indicates not statistically significant data.

Table 3 - Means, ranges and comparisons between clones and control varieties (Spunta, Adora, Agria) obtained evaluating tuber yield (TY), tuber specific gravity (TSG), chipping ability (direct, after cold storage, ± Reconditioning, Rec) and the evaluation index (EI)

Family	TY (Kg) [z]	TSG [z]	Chip category color [z]			EI [z]
			Direct	Cold storage - Rec	Cold storage + Rec	
Selections	1.16 (0.43-3.00)	1.083 (1.070-1.104)	3.4 (2.0-7.0)	8.8 (7.0-10.0)	6.1 (2.0-8.0)	10.5 (6.0-15.0)
Cultivars	1.03 (0.80-1.18)	1.075 (1.071-1.078)	3.3 (2.0-4.0)	8.3 (8.0-9.0)	6.3 (5.0-7.0)	9.7 (8.0-11.0)
Comparisons						
Selection *vs* cultivar	NS	11 **	NS	NS	NS	NS
Selection *vs* Adora	NS	13 **	NS	NS	NS	NS
Selection *vs* Agria	NS	7 **	NS	NS	NS	NS
Selection *vs* Spunta	NS	4 **	NS	NS	NS	NS

[z] Number of clones with a significantly better score compared to the control.
NS, *, ** indicates that means are not different or statistically different at P<0.05 and P<0.01, respectively (LSD 0.05).

than 1.080 to ensure good processing ability. In this research, TSG significantly varied among families studied (Table 2). On average, the specific gravity was 1.083, higher than the three control varieties used (1.075). It ranged from 1.070 ('Primura' x 'Alcmaria') to 1.104 (a clone from 'Jenny' x 'MC 329'). Nineteen clones (70%) showed a specific gravity higher than 1.080; and 4 clones, belonging to 'Bolesta' x 'MC 329' (2) and 'Jenny' x 'MC 329' (2), revealed a TSG higher than 1.090 (Table 2). The mean specific gravity of the studied potato clones was significantly higher than that of the controls, with 11 clones showing a higher specific gravity than the mean of the controls (Table 3). The chipping ability of the selected clones was evaluated at three times (at harvest and 90 days of cold storage at 7°C, with and without reconditioning at room temperature), based on the requirement of the potato processing market. Indeed, cold storage allows potato industries to process tubers when fresh product is not available, preventing sprouting and diseases (Malone *et al.*, 2006). Meanwhile, cold storage induces degradation of starch, conversion of sucrose in glucose and fructose causing an accumulation of reducing sugars. This process is extremely disadvantageous for the potato processing industry because it induces browning of chips (Dale and Bradshaw, 2003). During frying, high temperatures on reducing sugars activate the Maillard reaction on chips, which became dark colored and bitter, and so not marketable (Kumar *et al*., 2004). Variability in chipping ability was found among families, with significant differences after cold storage with or without reconditioning (Table 2). By contrast, no significant contrast between the mean chipping value of clones and that of control varieties fried at harvest and after cold storage was found (Table 3). However, the analysis of the chipping score ranges of clones indicated that at harvest and after cold storage with reconditioning, 11 and 5 clones respectively, presented a chipping score lower than 4.5 (not shown). Therefore, they were all good chippers. By contrast, good chippers were not identified after chips were fried directly out of cold storage. These data were expected considering that during cold storage two principal phenomena occur. The first is called "reversion" and is caused by reducing sugar accumulation: potatoes that generally show a good chipping ability after harvest, give dark, not acceptable chips (Oltmans and Novy, 2002). The second event occurs when stored potatoes are subjected to a warm (room temperature) period. It is called "reconditioning" and it induces a decrease of reducing sugars in the tuber. This phenomenon happens because during the warming period about 80% of reducing sugars (glucose and fructose) are converted back to starch (Oltmans and Novy, 2002). Consequently, tuber cold storage may produce lighter colored chips compared to the chips produced without a warming period.

In order to support the selection of clones with interesting trait combination, an arbitrary evaluation index (EI) was estimated (Melito *et al.*, 2017) (Table 2). The average family EI was 10.90, ranging from 7 in 'Spunta' x 'Victoria' (1) and 'Primura' x 'Alcmaria' (1) to 16 in 'Majestic' x 'Alcmaria' (1). The best control variety was Agria, with an EI of 11. In general, high variability and significant differences in EI were observed among the seven families studied (Table 2). Analysis of ranges revealed that in almost all families clones with EI higher than that of control varieties were present. The development of a score to evaluate the performance of breeding materials can be used to summarize the analysis of multiple traits, providing a synthetic parameter to support and simplify the practical selection of potato: high EI could indicate interesting genotypes that can be further analyzed deeper for additional traits, while low EI could be discarded as inferior genotypes. Additional experiments will be carried out to evaluate the most promising genotypes for new traits, with the purpose to either produce new variety/s useful in Mediterranean environmental conditions or select parental lines for further breeding.

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Salicylic acid improves salinity-alkalinity tolerance in pepper (*Capsicum annuum* L.)

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Key words: alkali stress, pepper, salicylic acid, salt stress.

Abstract: Salinization and alkalization of soils are agricultural problems in arid and semiarid regions of the world such as Iran. In this experiment the effects of salicylic acid (SA) on resistance of pepper plants under salt stress (SS) and alkali stress (AS) were evaluated. Treatments include 0 and 150 mM of SS, 0, 50 and 100 mM of AS and 0, 0.75 and 1.5 mM SA. Results showed that SS and AS imposed negative effects on pepper plant growth and productivity. Reduction in growth and yield in SS was higher than AS and maximum reduction occurred in high mixed stresses. SA application improved growth parameters and increased yield, relative water content (RWC) and chlorophyll of plants subjected to SS and AS and provided significant protection against stress compared to non-SA-treated plants. For most traits, 0.75 mM of SA was more effective than 1.5 mM concentration. SA ameliorated the injury caused by SS and AS by increasing chlorophyll and RWC and inhibiting proline accumulation and leaf electrolyte leakage (EL). In general, results indicate that salinity and alkalinity have negative effects on growth and yield of pepper plants and these negative effects can be ameliorated by application of SA.

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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

Salinity and alkalinity of soil seriously affect about 932 million hectares of land globally, reducing productivity in about 100 million hectares in Asia (Rao *et al.*, 2008). In Iran 12.5% of the agricultural lands in arid and semi-arid areas are alkaline. While salt stress (SS) in a soil generally involves drought stress and ion-induced injury (Munns, 2002), alkali stress (AS) exerts the same salt stress influences with the added effects of high-pH stress (Shi and Yin, 1993). The high-pH caused by AS directly affects the mineral absorption and interferes with the re-establishment of ionic balance. It can strongly affect the absorption of inorganic anions such as

Cl^-, NO_3^- and $H_2PO_4^-$, and can disrupt the selective absorption of K^+-Na^+, and pH homeostasis in the plant tissues (Yang *et al*., 2007). To resist AS, plant not only have to regulate the intercellular pH to maintain ionic balance, but also have to spend material and energy to regulate pH in their root environment. Thus osmo-regulation and ion balance play key influence in plants SS and AS resistance (Yang *et al*., 2007). There has been considerable study of SS on plants growth and development, however, relatively little attention has been given to AS despite its importance. Like to other environmental adverse conditions, AS can lead to emerge reactive oxygen species (ROS), as a result of closing stomata and reducing CO_2 into cells and consequently blocking photosynthesis activities (Yan *et al.*, 2011). AS caused metabolic disturbance, lipid peroxidation and chlorophyll breakdown and proline accumulation in plant tissue (Gao *et al*., 2012).

Various techniques have been tested to improve saline-alkali soils, including chemistry, physics, biology and engineering improvements to increase soil fertility and crop yield (Li-Ping *et al.*, 2015). In recent studies a number of plant growth regulators (PGRs) have been under trial to alleviate the environmental stresses in plants. Salicylic acid (SA) is a phenolic compound which is considered as a PGR and plays an important role in defensive mechanisms against biotic and abiotic stresses in plants. Flowering induction, plant growth and development, synthesis of ethylene, opening and closure of stomata and respiration are some of the important roles of SA in plants (Raskin, 1992). SA protects plants from damages caused by oxidative stresses through increasing antioxidants enzymes activities (El-Tayeb, 2005; Idrees *et al.*, 2011). SA has received much attention due to its function in plants' responses to environmental stresses. Literature exists about some beneficial effects of SA on plants under drought (Jafari *et al.*, 2015), low temperature, high temperature (Wang and Li, 2006; Sayyari, 2012), salinity (Shakirova *et al*., 2003; El-Tayeb, 2005; Stevens *et al.*, 2006; Idrees *et al.*, 2011), heavy metal (Metwally *et al.*, 2003) and biotic stresses (Makandar *et al.*, 2012). However, no information exists on the effects of the mentioned compounds on salt-alkali stress defense mechanisms, until now. Thus the purpose of this experiment was to examine the possibility that application of SA would protect pepper plants from damaging effects of salt-alkali stresses. Specific objectives of this research were: (1) to compare SS and AS on pepper plant growth and development (2), to determine some physiological responses of pepper plant to SS and AS and (3) evaluate the protective effect of a pre-treatment with SA.

2. Materials and Methods

Plant material and growing conditions

Seeds of *Capsicum annuum* L. cv. Plenty were obtained from Pakan Bazr Co. (Isfahan, Iran) and cultured in bed to obtain seedlings for experiment. When the seedlings had 2-4 true leaves, the seedling with similar size were selected and transferred into plastic pots (20 cm height, and 23 cm diameter) which were filled with about 8 kg of 1:1:2 mixture of fine sand, leaf mound and garden soil. The pots were then transferred to the greenhouse with average temperature of 25.5/19.5°C (day/night) and natural light.

Salt stress, alkali stress and salicylic acid treatments

When the seedlings have been established in pots (4-6 true leaves), they were sprayed with 0 (as control), 0.75 and 1.5 mM SA until both sides of the leaves were completely wet. Three days later, plants were subjected to SS and AS treatments until end of each experiment. Two neutral salts (NaCl and Na_2SO_4) and two alkaline salts ($NaHCO_3$ and Na_2CO_3) were selected for salinity and alkalinity stresses imposition. The two neutral salts were mixed in a 9:1 molar ratio (NaCl:Na_2SO_4), and applied in 0 mM and 150 mM to the SS group, and two alkaline salts were also mixed in a 9:1 molar ratio ($NaHCO_3$:Na_2CO_3) in 0 mM, 50 mM and 100 mM, and applied to the AS group. SS and AS treatment were conducted with daily watering of plants by mentioned treatment. Control plants (0 mM SS and 0 mM AS) were watered with distilled water (Yang *et al.*, 2007; Rao *et al.*, 2008).

Chlorophyll determination

Chlorophyll was assessed by taking fresh leaf samples (0.1 g) of plants in each replicate from young and fully-developed leaves. The samples were homogenized with 5 ml of acetone (80% v/v) using pestle and mortar and centrifuged at 3000 rpm. The absorbance was measured with a UV/visible spectrophotometer at 663 and 645 nm and chlorophyll were calculated (mg/g FW) using the equations proposed by Strain and Svec (1966) given below:

$$\text{Total chlorophyll} = 20.2\times(A645)+8.02(A663).$$

Electrolyte leakage

Electrolyte leakage was determined according to Lutts *et al.* (1995) method. Ten leaf discs of randomly chosen plant were taken from the youngest fully-expanded leaf. The leaf discs were then placed in test tubes containing 10 ml of distilled water. These samples were incubated at 25°C on a shaker for 24 h. Electrical conductivity (EC) of bathing solution (EC1) was read after incubation. The same samples were then placed into a water bath (100°C) for 20 min and the second reading (EC2) was determined after cooling of the solution to room temperature. The EL was calculated as EC1/EC2 and expressed as a percentage.

Proline content determination

The proline was determined according to the method described by Bates *et al*. (1973). The amount of 0.5 g of samples were homogenized in 10 ml of 3% (w/v) sulfosalicylic acid. After centrifuging at 10000 rpm, 2 ml of the supernatant was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath (100°C) for 1 h. The reaction mixture was extracted with toluene and the chromophore-containing toluene was aspirated and cooled to 25°C. The absorbance was measured at 520 nm with a UV/visible spectrophotometer. Proline concentration was expressed as µg/g FW.

Measurement of relative water content

Relative water content was measured according to Costes *et al.* (2006). Leaves sample in each treatment was weighed (FW) and then immediately floated on distilled water for 5 h in the dark. Turgid weight (TW) of leaf disks was obtained after drying excess surface water with paper towels. Dry weight (DW) of disks was measured after drying at 75°C for 48 h. The RWC was calculated using the following formula:

$$\text{Relative water content} = (FW-DW)/(TW-DW)\times 100$$

Statistical analysis

Data were analyzed for significant differences using a factorial analysis of variance with SS and AS levels and SA concentrations as main factors with three replications and 5 seedlings per each. Statistical analysis was performed using SAS program and the means compared using the Duncan's Multiple Range Test at p=0.05.

3. Results and Discussion

Leaf area and total yield

The leaf area and pepper yield displayed a significant reduction in response to the increasing levels of SS and AS treatments. Among all stress treatments tested, 100 mM AS+SS exhibited the strongest reduction of leaf area and yield followed by 50 mM AS+SS. Foliar sprayed SA plants exhibited significant response to improve yield as compared to unsprayed ones in all stress levels (Fig. 1 and 2). The general effect of soil salinity in plants is to decrease the growth resulting in smaller leaves, shorter stature, and sometimes fewer leaves. The primary effect of salinity, especially at low to moderate levels, is due to its osmotic stress (Munns, 2002). The AS exerts the same stress factors as SS but with the added effect of high-pH stress. The effect of SS and AS in reducing the growth and yield of various plants was reported in (Yang *et al.,* 2007; Chen *et al*., 2011) studies that are in agreement with the findings of this study. However, in the present study, adverse effects of AS on yield and leaf area were lower than that of SS. This results implies not only that SS and AS are different stresses, but also that resistance of pepper plant to AS is stronger that to SS.

The treatment of SA caused a significant increasing in yield and leaf area in comparison to control plants at all stresses levels (Fig. 1 and 2). The positive effect of SA on plants under salinity stress have been reported (Shakirova *et al.*, 2003; Stevens *et al*., 2006; Idrees *et al.*, 2011) which their upshots are in agree-

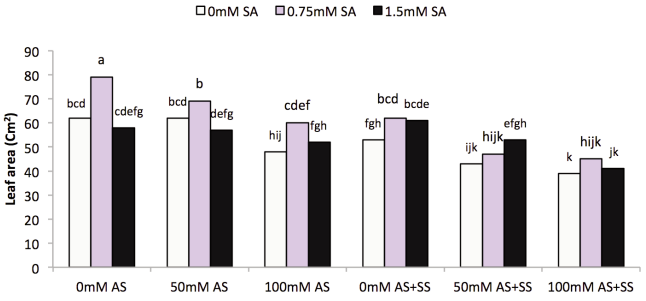

Fig. 1 - Effect of Salicylic acid (SA) on leaf area of pepper plant under salt stress (SS) and alkali stress (AS).

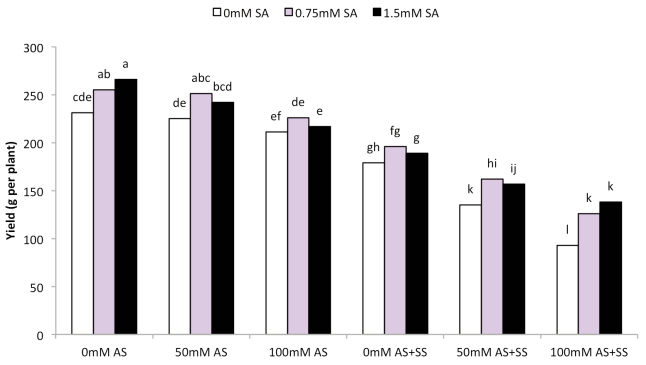

Fig. 2 - Effect of Salicylic acid (SA) on total yield of pepper plant under salt stress (SS) and alkali stress (AS).

ment with our findings. Application of SA improved wheat plants under water stress by activities of cell division in apical meristem (Shakirova *et al.*, 2003). Exogenous SA treatment also improved chlorophyll and photosynthesis rate and created stability of plant cell membrane, reduction of EL in barely plants and finally tolerance plants to water stress (El-Tayeb, 2005). The ability of SA to increase yield and growth parameters, ameliorating the adverse effects of stress, may have important implications in improving the plant growth and overcoming the growth barrier arising from SS and AS conditions.

Chlorophyll content

Some of the environmental stresses symptoms in the plants are reduction of chlorophyll content and this reduction depends upon the plant species (Colom and Vazzana, 2001). In this experiment, the reduction of chlorophyll was occurred due to SS and AS and 100 mM SS+AS treatment resulted in having the lowest rate of total chlorophyll (Fig. 3). In this respect, there are same reports over reducing chlorophyll due to exposing plants to SS and AS (Chen *et al*., 2011; Gao *et al.*, 2012). Environmental stresses leads to increase the ROS production in cells. These free radicals cause peroxidation and consequently destructing the photosynthesis pigments (Schütz and Fangmeier, 2001). Because of these events, the growth of plant will be affected and declined.

Treated plants with SA as foliar spray increased chlorophyll content in all concentrations, but 0.75 mM SA treated plants showed significant increases in chlorophyll compared with control and 1.5 mM SA (Fig. 3). These results are in agreement with those of Sayyari (2012) who found that SA foliar and soil applications increased chlorophyll content in cucumber plants following chilling stress.

Salicylic acid by eliminating of ROS may improve chlorophyll content in pepper plants under stressful conditions. Chen *et al.* (1993) showed that in response to environmental stresses SA accumulates

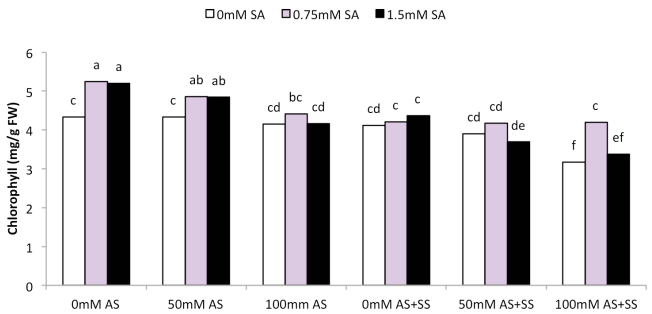

Fig. 3 - Effect of Salicylic acid (SA) on chlorophyll of pepper plant under salt stress (SS) and alkali stress (AS).

to high concentrations, and prevent antioxidant enzyme such as CAT activity, thereby leading to an enhancement in Hydrogen peroxide (H_2O_2) content, which could then induct the development of systemic acquired resistance (SAR), induce activity of ROS-detoxifying enzymes and antioxidant metabolites. Also, Idrees *et al.* (2011) reported that SA-induced salinity tolerance in periwinkle plants might be associated with an increase in the antioxidant activity. Therefore, impact of SA on plants chlorophyll under stresses condition may be related to its effect on the antioxidative enzyme activities and H_2O_2 metabolism (Idrees *et al*., 2011).

Proline content

Proline is sensitive physiological index of plants responding to some stresses. Salinity and alkalinity treatments had significant effects on proline contents in pepper plant leaves. Proline accumulation in leaves was markedly increased in salt and alkali stressed plants in comparison with that of the control plants. The results showed that mixed SS-AS can cause heavy accumulation of proline content compared with SS and AS single stresses (Fig. 4). In general, the accumulation of proline, relates closely with osmotic stress intensity. Osmo-regulation is a physiological phenomenon during which osmosis potential of stressed tissues are reduced due to the accumulation of some material such as elements, sugar, amino acids (proline) and organic acids. Thus, turgor pressure of the cells is kept well (Irigoyen *et al.*, 1992). Proline by osmosis control, maintaining enzymes

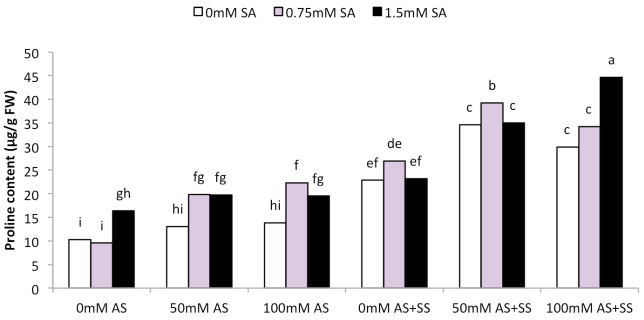

Fig. 4 - Effect of Salicylic acid (SA) on proline content of pepper plant under salt stress (SS) and alkali stress (AS).

activity and removal of hydroxyl radicals, increases the tolerance of the plants against stresses (Kuznetsov and Shevyakova 1999). The results showed that proline accumulation increased not only with increasing SS, but also when AS imposed (Fig. 4). This suggested that the proline accumulation, as the major osmolyte, correlates closely with the intensity of the osmotic stress induced by SS and AS.

Results showed that SA application increased proline content in pepper plants under SS and AS. A highest amount of proline (44.67 µM/gFW) was achieved in 1.5 mM SA treatment and 100 mM AS+SS, and lowest amounts (9.54 µM/gFW) were observed in 0.75 mM SA and 0 mM AS (Fig. 4). These results are in agreement with those of El-Tayeb (2005) who showed that SA treatment increases the proline content in the leaves of barely plant subjected to salinity stress. In the present study, SA induced an accumulation of proline in the leaves under SS and AS, and when SA was applied, a stress tolerance occurred in the pepper plant. Thus, osmo-regulation can be considered to be one of the important phenomenon involved in SA induced protective mechanism in pepper leaves in response to salt and alkali stresses.

Relative water content

One of the indices showed the water status of plants is RWC. Measuring of relative water content is an important physiological parameter in evaluation of plant response to environmental stresses (Nautiyal *et al.*, 2002). Results indicated that by increasing SS and AS, RWC in pepper leaves decreased slightly. Reduction under SS was greater than those under AS and greatest RWC reduction was achieved in highest mixed stress level (100 mM AS+SS) (Fig. 5). These results are in agreement with Yang *et al.* (2007) who found that salt-alkali stresses decreases the RWC in the leaves of *Kochia sieversiana*. Plants can reduce RWC as a quick and economical approach to osmotic adjustment in response to osmotic stress (Lissner *et al.*, 1999). Reduction of RWC of the plants due to stress is related to the reduction of soil humidity; in these conditions, plants close the stomata to avoid more water waste. The reason of stomata closure is ABA that is made in the root in stress conditions and is accumulated in stomata cells (Chaves *et al.*, 2002). Therefore, maintaining a high RWC might be a key characteristic of plants that allow it to absorption osmolytes with minimum energy consumption.

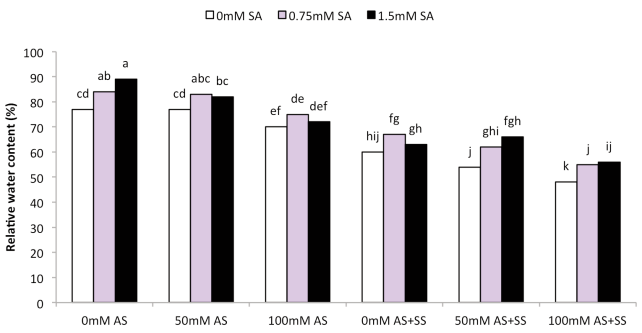

Fig. 5 - Effect of Salicylic acid (SA) on relative water content of pepper plant under salt stress (SS) and alkali stress (AS).

Results showed that SA treatment increased leaf RWC in pepper plants under SS and AS. A highest amount of RWC (89%) was achieved in 1.5 mM SA treatment and 0 mM AS, and lowest (54%) were observed in 0 mM SA and 100 mM AS+SS (Fig. 5). Parida and Das (2005) reported that the RWC, water potential and osmotic potential of plants under stress become more negative with an increase in salinity. This study showed that SA treatments induced an increase in RWC of plants as compared to the un-treated SA plant. Increases in RWC of plants treated with SA were also reported for other crops grown under stress including tomato (Stevens *et al.*, 2006) and barely (El-Tayeb, 2005). Increasing of RWC may be related to the role of SA in accumulation of compatible osmolytes in plants subjected to stress, as, this effect was observed in the results of proline.

Electrolyte leakage

In order to assess membrane permeability, electrolyte leakage (EL) was determined. Its relative conductivity can be used to evaluate the damage on structure and function of cell membranes under stresses. Results showed that EL significantly higher under SS than AS. On the other hand, both stresses increased the EL but the extent of the increment under SS was much greater than under AS and maximum of EL was achieved in highest mixed stress level (100 mM AS+SS) (Fig. 6). The results of the present study are in agreement with Gao *et al.* (2012) who determined that EL of oat (*Avena sativa* L.) was intensively increased by AS. Gao *et al.* (2012) showed that the EL of alfalfa seeds gradually increased with increasing salinity and alkalinity, this was attributed to the damage seeds cell membranes resulting from mixed salt-alkali stress. These results suggested cell membrane structure of pepper leaves under SS and AS received damage after treatment with salts.

Application of SA significantly decreased leaf EL in salt and salt × alkali stressed plants. Plants treated by SA foliar spray at 0.75 and 1.5 mM had shown significantly less EL than control plants (Fig. 6). These results indicated that, SA reversed the adverse effects of stress and caused a significant decrease in EL. The results of the present study are in agreement with Stevens *et al.* (2006) who showed that SA facilitated the maintenance of membrane functions in tomato under salinity stress. This effect could be attributed to the stimulation of antioxidant responses and elevated calcium absorption that protects the

plant from the oxidative damage (El-Tayeb, 2005). Also, Jafari *et al.* (2015) showed that exogenous application of SA in cucumber (*Cucumis sativus* L.) subjected to osmotic stress lead to a decrease in EL and induced drought tolerance. These results suggested that pepper leaves cell membrane structure in during salt and alkali stresses received less damage after application of SA.

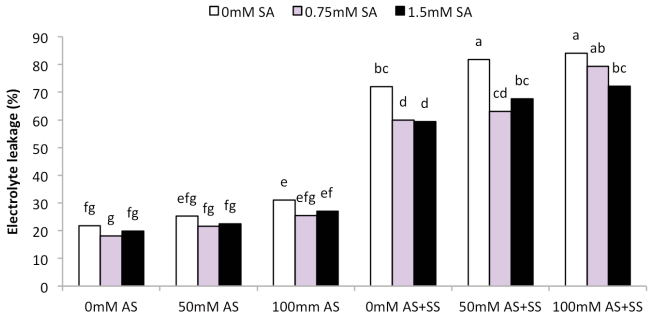

Fig. 6 - Effect of Salicylic acid (SA) on leaves electrolyte leakage of pepper plant under salt stress (SS) and alkali stress (AS).

4. Conclusions

In summary, our study clearly showed that saline and alkaline stresses as two types of abiotic stresses, have negative effects on pepper plant growth and productivity. On the other hand, the negative effect of mixed salt-alkali stress is more severe than that of only salt or alkali. The results of this research demonstrated that treating plants with 0 mM AS (control) is resulted in increasing plant growth parameters and RWC in comparison to other treatments. The harmful effect of saline stress on the pepper plants was significantly greater than that of alkaline; this harmful effect might have resulted from the higher concentration of salinity or higher sensitivity of pepper to SS compared to AS. SA pre-treatment of pepper seedlings via foliar spray was effective in saline and alkaline resistance. In most of evaluated treatments employed in this research, they did not show significant differences on SA concentrations. The best protection was obtained in plants treated with 0.75 mM SA, however 1.5 mM SA is more effective in 100 mM AS+SS in some trait for example proline content. This SA effect was associated with alter of physiological parameters such as increase of photosynthetic pigments and proline accumulation and decrease EL of plants by addition of SA. Further, the results indicate that SA can be considered as a potential growth regulator for improving plant growth and yield under SS and AS, and it may be recommended in arid and semiarid regions.

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Nuclear 2C DNA and genome size analysis in somatic embryo regenerated gladiolus plants using flow cytometry

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Key words: callus, embryo maturation and germination, genetic stability, PGR.

Abstract: Gladiolus is a valuable asexually propagated plant of horticultural importance. Here, in the present communication, *in vitro* embryogenesis protocol and 2C DNA content of embryo regenerated plant are described. Callus was first induced from corms on 2, 4-D and NAA + BAP amended MS medium and the frequency was maximum (75.12%) in 0.5 mg/l NAA + 0.5 mg/l BAP added medium. The callus differentiated into embryos on 2, 4-D at variable numbers (3.13-5.32/callus mass); the addition of 1.0 mg/l BAP and 0.25 mg/l NAA was found very efficient in proliferating embryos (7.99/callus mass). Direct somatic embryos were also formed on corm surfaces on 2, 4-D (0.5-1.0 mg/l) amended medium at varying numbers. The embryos did not progress to maturity in same induction medium, so other PGR treatments containing GA_3 and ABA were added. The amendment of GA_3 was more responsive compared to ABA and 0.5-1.0 mg/l of GA_3 was identified as best effective treatment. The embryos showed a maximum of 62.15% maturity in 0.5 mg/l GA_3 added medium after 8th of culture. On BAP containing medium the mature embryos converted into plantlets and highest germination (42.65%) was noticed on 0.5 mg/l BAP added medium. The 2C DNA content of regenerated plant was measured by flow cytometry and was noted to be 1.34 pg. The somatic embryo derived plantlets are true-to-type, stable and grew normally in outdoor conditions, genome size is identical to corm derived gladiolus plants. This is the first flow cytometric DNA analysis description in somatic embryo regenerated gladiolus plant.

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1. Introduction

Ornamental is a major group of plants, comprises of dicot, monocot, gymnosperm, fern and other lower groups, grown in indoor-, outdoor decoration and for aesthetic values. These plants are mostly annual and perennials. A large number of ornamentals are used in cut-flower industry, some common examples are carnation, rose, dahlia, lilies, amaryllis etc. (Sarkar, 2010). Gladiolus, often called as sword lily, is a perennial bulbous flowering plant, belongs to the family Iridaceae. It comprises of about 260 species, of which 250 are native to African continent and some

are to Eurasia (Ohri and Khoshoo, 1986). Beside its ornamental value, a few species are described to be wild, distributed in South Scandinavians Hill to the coast of Mediterranean sea. The *Gladiolus imbricatus,* one of the wilds, is known to be a rich source of vitamin C and minerals; the flowers are edible and its taste is very similar to lettuce (Elena *et al.,* 2012). The different species of *Gladioli* are also used as food plants by Lepidoptera larvae especially the Large Yellow Underwing. *Gladiolus* is primarily cultivated worldwide as cut flower because of its beautiful spike with moderate to long vase-life. The gladiolus flower spikes are long, one sided bearing variable flowers of attractive colours. The hybrid gladioli, in particular, demonstrate a wide range of colours that make the plant a success in cut flower market. The plant grow well in winter months, when temperature hovering around 10-25°C. The major gladiolus producing countries are Holland, Poland, Bulgaria, Romania, Israel, Australia, Denmark and US; in US, the annual sale of gladiolus is estimated to be around 370 million corms (Narain, 2004). In India, too, around 130.000 ha are under ornamental cultivation as the growth of floral market is high, upward and crossed 1000 crore mark, as per 2006 data (Singh, 2009, 2011). The gladiolus is predominantly distributed in Asia, South - and tropical Africa and in Europe (Goldblatt and Manning, 1998). Most species are diploid showing 2n = 30 chromosome number; a large number of species are tetraploid hybrids, exhibiting chromosome count of 60 (Krahulcová, 2003; Elena *et al.,* 2012). The various species of *Gladiolus* are propagated vegetatively by corms and cormlets separation, which is a very slow process while seeds are only used for raising new hybrid varieties (Memon, 2012). Beside conventional propagation, the *Gladiolus* is often multiplied by tissue culture in which leaf, nodal segment, apical shoot tip, inflorescence and corm pieces are cultured in various nutrient media (Xu *et al.,* 2009). Yelda and Bengi (2007) obtained profuse callus from leaf on 5.0 mg/l NAA added MS in dark condition. The MS amended with 1.0 mg/l BAP + 0.5 mg/l KIN + 0.5 mg/l NAA was found very effective in producing callus from cormlet (Pragya *et al.,* 2012). In *Gladiolus,* the plantlets are regenerated directly from cultured explants or plants arise de novo from callus following organogenesis and embryogenesis pathway (Stefaniak, 1994). Remotti (1995) obtained primary and secondary embryos in *Gladiolus x grandifloras* cv. Peter Pears from cell suspension, derived from cormlet embryogenic callus on 0.25 µM BAP or zeatin added MS medium. The incidences of somatic embryogenesis have also been reported in a number of other studies (Iantcheva *et al.,* 1999; Wu *et al.,* 2015) in which genetic transformation method was utilized to make the plant resistant to gray mold, dry rot, root rot and other plant diseases (Massey, 1928). Particle bombardment mediated genetic transformation was earlier attempted in improving traits such as shortening dormancy (Kamo and Joung, 2007; Kamo *et al.,* 2009). *Agrobacterium* mediated genetic transformation was also tried but was noted to be less efficient as fast regeneration protocols from transformed tissues are not fully optimized, which is a primary requirement for successful transformation studies (Wu *et al.,* 2015). Moreover, *Gladiolus* is a member of monocot, the tissue of monocotyledonous plant is noted to be less responsive/receptive in up taking foreign DNA during transformation mechanism (Smith and Hood, 1995), although there are some current exceptions (Zhao *et al.,* 2000; Zhang *et al.,* 2006). There are several factors, controlling the success of plant regeneration - the explant, medium and plant growth regulators (PGRs). The somatic embryogenesis way of plant regeneration has several advantages such as lower risk of somaclonal variation and higher rates of plant regeneration (Vergne *et al.,* 2010; Zhao *et al.,* 2013) and thus has been successfully practised in a variety of research programmes of biotechnological interest. Although *in vitro* embryogenesis has been reported in some specific gladiolus species, the plantlet recovery from embryos is still not too high. This is primarily because of non-synchronous embryo induction, and poor quality of embryos, preventing early maturation and subsequent germination in culture. Embryo (developed on explants directly) derived plantlets are true-to-type; genetic fidelity has often been maintained in somaclones (Jayanthi and Mandal, 2001). However, plants obtained from callus-mediated embryos demonstrate genetic and phenotypic variability and this instability increases with older culture of callus (Endemann *et al.,* 2001). The accumulation of higher levels of stress and the presence of PGRs are considered the signalling elements in inducing embryos and subsequent genetic variability in tissue culture raised plants (Clarindo *et al.,* 2008; Delporte *et al.,* 2013). Thus, there is an urge to check somaclones' fidelity in regenerated populations. A large number of conventional and modern techniques have been used recently to identify alteration in cultivating tissues and *in vitro* raised plants (Das *et al.,* 2013). Flow

cytometry, an important technique has frequently been used for measuring nuclear DNA content in plant cells and tissues (Zhang *et al.,* 2005). The technique offers simple, fast and precise way of determining 2C DNA in plant cells. It has several other fundamental and applied uses including the initiative of genome sequencing of known and unknown plant materials (Galbraith, 2009; Rewers *et al.,* 2012). In the present study, flow cytometric investigation was conducted to measure and compare the 2C DNA level of somatic embryo regenerated plantlets with naturally grown *Gladiolus,* which served as tissue source for *in vitro* study. The fast and large-scale plant regeneration was also described by studying the role of PGRs in developing callus and embryos and in the event of maturation and germination of embryo in gladiolus.

2. Materials and Methods

Plant material and cultural conditions

The gladiolus corms (*Gladiolus hybridus*) from Horticulture Society of India, Calcutta, were used as experimental material. First, the healthy gladiolus corms were selected and washed with cetrimide (a liquid detergent) for 10-15 min, followed by washing with running tap water for 30 min. This was followed by a treatment with 70% ethanol for 5 min, rinsed with distilled water for 3-4 times. Finally, the corms were surface sterilized with 0.05% $HgCl_2$ for 3 min, and washed 3-4 times with sterilized distilled water to remove the traces of mercuric chloride. The gladiolus corms were sliced into explants (8-10 mm), and were cultured in MS (Murashige and Skoog, 1962) amended with different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D), Indole acetic acid (IAA) and 1-naphthaleneacetic acid (NAA). The medium pH was adjusted to 5.7 before sterilization at 121°C. The cultures were kept at 25±2°C in a culture room under 16 h photoperiod provided by cool white fluorescent light of intensity of 40 Wm^{-2} S^{-1}.

Induction of callus and embryogenic tissue

Corm pieces were cultured on MS, amended with 3% sucrose, 100 mg/l inositol and 2, 4-D, IAA and NAA at different concentrations (0.25, 0.5, 1.0 and 2.0 mg/l) for callus induction. BAP (0.5 and 1.0 mg/l) was also used in combination with NAA (0.5, 1.0 mg/l) in separate experiments. Within 7-10 days, brown yellowish callus was formed from the cut end of corms, which later engulfed the whole surface. The same level of PGRs was also used for callus maintenance. The induced callus transformed into embryogenic callus, which showed small, globular or 'nearly globular' or elongated, cylindrical structures on callus surface. These embryo structures were visible with naked eye. The effectiveness of PGRs and their levels were optimized by scoring callus induction percentage. The ineffective PGR treatments were discontinued.

Somatic embryo differentiation and proliferation

Embryogenic callus (about 50 mg) was cultured on MS amended with 2, 4-D (0.5, 1.0 and 2.0 mg/l), NAA (0.5, 1.0 and 2.0 mg/l) and BAP (0.5, 1.0 and 2.0 mg/l) separately for embryo differentiation and proliferation. In other experiments, NAA (0.25, 0.5 mg/l) in combination with BAP (1.0, 2.0 mg/l) were also used. The embryo numbers increased with time and these cultures were maintained by regular sub-culturing at interval of 3-4 weeks. The callus showing embryogenesis (the percentage) and embryo numbers (number/callus mass) were recorded at periodic intervals.

Maturation of embryos

The developed embryos on callus were placed on a medium, containing all the essential components of MS, vitamins, inositol but without any PGRs for maturation of embryos. In other experiments, the MS was additionally added with abscisic acid (ABA) at 0.25, 0.5, 1.0 mg/l and gibberellic acid (GA_3) at 0.25, 0.5, 1.0 mg/l. In both GA_3 and ABA added medium, the somatic embryos transformed into green and elongated structures (maturing embryos), which germinated into plantlets later. The percentage of embryos showing maturation was recorded.

Embryo germination and plant recovery

Apparently green somatic embryos were separated and cultured on medium, added with various concentrations of BAP (0.5, 1.0, 2.0 mg/l) either alone or with NAA (0.25, 0.5 mg/l). In another set of experiments, the embryos were cultured on maturation medium for germination in which best maturation had noticed. A separate control medium (without PGR) was also prepared to make the comparison. The number of somatic embryos germinated and developed into plantlets (root and epicotyl development) were recorded. As the germinated plants had very little roots, plants were incubated in root promoting PGRs like Indole-3-butyric acid (IBA). Roots were formed at variable numbers soon after IBA treatment. Embryo regenerated plantlets were transferred to 250 ml conical flask containing ½ MS + 3% sucrose but without PGR and kept for a week for acclimatization.

Transplantation of plants

Finally, the plantlets were removed from the conical flasks, were transferred to small coffee cups filled with sterilized soil rite and sand (1:1), and wrapped with pored polythene bags for maintaining humidity. Plants were later transplanted to plastic pots (15 cm), kept for 3 weeks in incubation at room temperature of 25±2°C with 16 h photoperiod, provided by cool white fluorescent illumination. Finally, plants were transferred to plastic pots (15 cm) filled with soilrite and sand (1:1) in outdoor condition.

2C DNA and flow cytometry

Corm derived gladiolus plants, grown naturally in garden and somatic embryo regenerated plants were used for 2C DNA determination study. Leaf samples from both sources were processed according to the Dolezel *et al*. (2007) method. About 1.0 cm² of *Gladiolus* young leaf and *Pongamia pinnata*, 2C= 2.51 pg DNA (in which 2C DNA analysis study was optimized in the laboratories of our University) were harvested the day of analysis and chopped in 0.5 ml Otto I buffer (0.3% citric acid monohydrate, 0.05% NP-40), 50 µM propidium iodide and 100 µM RNAse (Sigma-Aldrich, USA) following Choudhury *et al.* (2014) method. The homogenate was filtered through a 100-µm nylon filter, and was analyzed by a (CFM) BD FACS Calibur (BD Biosciences, San Jose, CA, USA) flow cytometer. The 2C nuclear DNA level of this experimental material was determined as follows:

$$\text{2C DNA of } G.\,hybridus = \frac{2.51 \times \text{Mean position of } \frac{G0}{G1} \text{ peak of } G.\,hybridus}{\text{Mean position of G0/G1 peak of } P.\ pinnata}$$

Statistical analysis

The data on the effects of PGRs on callus induction, embryogenesis and embryo numbers were analysed and expressed as mean ± standard error. Each set of experiments consisted of three replicates and each experiment was repeated twice. The flow cytometry experiment was performed by randomly selecting regenerated plants. The presented mean values were separated using Duncan's Multiple Range Tests at $p \leq 0.05$.

3. Results

Induction of callus and callus biomass growth

Corms were used as explant on various auxin amended MS medium; the explant started to swell and callus was formed (Fig. 1 a) form the cut ends later. Three different auxins and their concentrations were tested of which 1.0 mg/l 2, 4-D showed highest callus induction percentage (72.66%), followed by the treatment with 0.5 mg/l 2, 4-D, in which 67.85% of cultured explants callused (Table 1). Other two

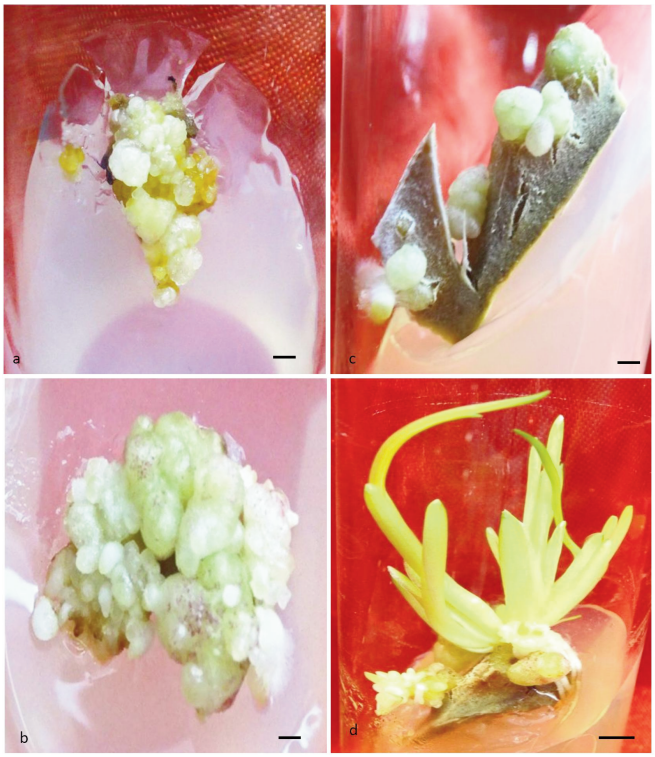

Fig. 1 - a) Corm callus induced in 2,4-D added MS medium; b) Numerous somatic embryos developed on callus; c) Directly induced somatic embryos on corm surface and d) Somatic embryo regenerated plantlets (bars: a-c: 2 mm; d: 0.5 cm).

Table 1 - Callus induction frequency in *Gladiolus*. Corm explants was cultured on MS medium, added with 2, 4-D, IAA, and NAA alone and BAP and NAA in below indicated combinations

2,4-D (mg/l)	IAA (mg/l)	NAA (mg/l)	Callus induction (%)
0.25	0.0	0.0	48.25±4.44 f
0.5	0.0	0.0	67.85±4.65 c
1.0	0.0	0.0	72.66±5.76 b
2.0	0.0	0.0	58.64±3.87 e
0.0	0.25	0.0	0.0 g
0.0	0.5	0.0	0.0 g
0.0	1.0	0.0	0.0 g
0.0	2.0	0.0	0.0 g
0.0	0.0	0.25	0.0 g
0.0	0.0	0.5	0.0 g
0.0	0.0	1.0	0.0 g
0.0	0.0	2.0	0.0 g
	BAP (mg/l)		
	0.5	0.5	75.12±5.55 a
	1.0	0.5	63.16±3.89 d
	0.5	1.0	64.66±4.85 d

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

auxins i.e. NAA and IAA were observed to be non-responsive when used alone. With BAP (0.5 mg/l), NAA was very active in producing callus, maximum efficient concentration being 0.5 mg/l NAA, followed by 1.0 mg/l NAA. Corm induced callus was cultured on a variety of 2,4-D added concentrations (0.25, 0.5, 1.0 and 2.0 mg/l) for fast growth of callus and the moderate level was observed to be very efficient in producing good callus biomass growth. At the end of 12th weeks culture, 1.12 g of callus biomass was observed in 1.0 mg/l 2,4-D added medium (Fig. 2), next effective concentration was 0.5 mg/l 2,4-D in which callus biomass was 0.91 g, little more of biomass (0.84 g) recorded at 2.0 mg/l 2,4-D.

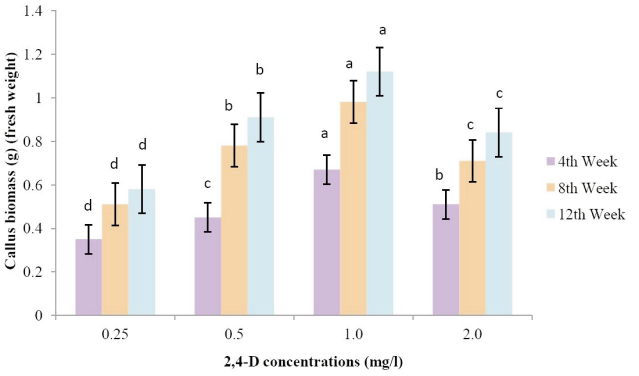

Fig. 2 - Callus biomass growth on MS medium, added with various concentrations of 2, 4-D. Values are means ± standard deviation of three replicates. Within each bar, means followed by the letter, are not significantly different at p≤0.05 according to DMRT.

Direct and indirect somatic embryogenesis

In order to study plant regeneration, hard compact callus was placed on a variety of PGR amended medium and within a few weeks of incubation, callus started to differentiate and produced somatic embryos at variable numbers (Fig. 1 b). Three different concentrations were tested, of which 1.0 mg/l 2, 4-D was observed to be very efficient in which 79.13 percentage embryogenesis with an average of 5.34 embryos/callus mass was noted. Equally effective 2,4-D concentration was 0.5 mg/l that showed good embryo numbers and embryogenesis percentage (Table 2). Unlike 2,4-D, NAA - another important auxin was noted to be inactive in inducing embryos. BAP, a member of cytokinin, was however, noted to be active in inducing embryo, although low in number with lower rate, most efficient concentration was 0.5 mg/l BAP in which 15.45 embryogenesis percentage and 2.93 embryo numbers were noted. The addition of NAA in BAP amended medium improved embryo numbers and embryogenesis percentage. The best treatment identified was 0.5 mg/l NAA and 2.0 mg/l BAP, which showed 69.18 embryogenesis percentage with 12.13 embryo numbers; next best treatment was 0.25 mg/l NAA and 1.0 mg/l BAP in which a mean of 7.99 embryo number/callus mass was noticed. The numbers of embryos however, increased with time. Beside callus induced embryos, direct embryo formation on corm surface (Fig. 1 c) was also noticed at lower frequency. The embryos were roundish, oval, elliptical in structure, often had white cotyledonary apex with yellowish radicle primordia at other ends.

Table 2 - Embryogenesis percentage and somatic embryo numbers in Gladiolus callus, MS was amended with below indicated PGR concentrations and combinations. Data were scored after 4 weeks of culture

2,4-D (mg/l)	NAA (mg/l)	BAP (mg/l)	Embryogenesis (%)	Embryo numbers/ callus mass
0	0.5	0	0.0 g	0.0 h
0	1	0	0.0 g	0.0 h
0	2	0	0.0 g	0.0 h
0	0	0.5	15.45±2.12 e	2.93±0.79 f
0	0	1	14.61±2.42 e	2.38±0.16 f
0	0	2	8.66±1.73 f	1.96±0.10 g
0	0.25	1	80.15±6.66 a	7.99±1.21 b
0	0.5	1	70.12±5.22 c	6.46±1.76 c
0	0.5	2	69.18±3.44 c	12.13±2.28 a
0.5	0	0	75.42±6.43 b	5.32±0.87 d
1	0	0	79.13±5.38 a	5.34±2.10 d
2	0	0	39.33±3.58 d	3.13±1.06 e

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

Somatic embryo maturation and germination, and the role of various PGRs

Embryos were cultured on medium amended with GA_3 and ABA at different concentrations for embryo maturation. The application of GA_3 was more efficient in comparison with ABA treatment (Table 3). In 0.5 mg/l GA_3 amended medium, 54.55% embryo maturation was noticed after 4 weeks, which increased to 62.15% after 8 of incubation. At the same level of ABA (0.5 mg/l), the embryo maturation percentage was low (18.75%) and with culture time the maturation percentage marginally improved i.e. 20.15% was noted after 8th weeks. Higher concentration of GA_3 and ABA reduced embryo maturation frequency quite sharply.

Well matured somatic embryos, grew well in maturation medium but the germination frequency was low. The best maturation conditions i.e. the medium amended with 0.5 mg/l GA_3 and 0.5 mg/l ABA

Table 3 - Influence of GA3 and ABA on somatic embryo maturation in Gladiolus. Data were scored after 4th and 8th weeks of culture

MS+ PGR (mg/l)	Maturation after 4 weeks (%)	Maturation after 8 weeks (%)
Control	4.21 f	6.30 f
GA_3 (mg/l)		
0.25	30.65±2.11 c	38.60±3.33 c
0.5	54.55±3.88 a	62.15±4.87 a
1	42.62±2.66 b	48.62±4.88 b
ABA (mg/l)		
0.25	12.15±1.86 e	16.65±2.11 e
0.5	18.75±2.01 d	20.15±1.89 d
1	13.12±1.12 e	15.34±1.78 e

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

showed very poor level of embryo germination (Table 4) i.e. 8.50 and 4.84% respectively. So other PGR combinations were tested for better and early plantlets recovery from somatic embryos. Three different concentrations of BAP were added in MS and the germination ability was observed (Table 4). In 0.5 mg/l BAP amended medium the maximum somatic embryo germination percentage (42.65%) was noticed, followed by a treatment with 1.0 mg/l BAP (31.85%), while higher level (2.0 mg/l) declined somatic embryo germination (18.16%) rate. The optimized BAP (0.5 mg/l) level with NAA was also similarly tested to improve embryo germination and a moderate germination percentage (33.25-33.25%) was noted in those PGR treatments. The somatic embryo regenerated plantlets (Fig. 1 d) grew well in culture, but did not have strong root systems always, so a medium amended with root promoting PGRs were added. Although in almost all treatments, roots were formed at variable numbers, IBA (0.5, 1.0, 2.0 mg/l) was observed to be very effective in inducing roots (data not shown). The plants were finally transferred to outdoor conditions.

Table 4 - Somatic embryo germination in various PGRs added medium in Gladiolus. Data were scored after 6 weeks of culture

MS+ PGR (mg/l)		germination (%)
Control		0.0 g
GA_3 (mg/l)		
0.5		8.50±0.89 e
1		6.66±1.11 f
ABA (mg/l)		
0.5		4.84±1.02 g
1		4.68±1.82 g
BAP (mg/l)		
0.5		42.65±2.67 a
1		31.85±1.89 c
2		18.16±1.22 d
BAP	NAP	
0.5	0.25	33.25±2.23 b
0.2	0.25	31.66±2.24 c

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

Genome size analysis of gladiolus

For the analysis of genome size, the 2C DNA content of somatic embryo regenerated plantlets was measured and was compared with naturally grown gladiolus using flow cytometric method. Nuclear homogenate of juvenile leaves from naturally grown and somatic embryo regenerated plants were used for flow cytometry and the obtained results are presented in figure 3. The somatic embryo regenerated plant of gladiolus showed 2C DNA content of 1.34 pg while corm grown plant obtained naturally had 2C DNA content of 1.29 pg. The 2C nuclear DNA content values of both the two sources are nearly the same that suggests no major alteration in genome size in embryo regenerated plants when compared with corm derived gladiolus. The genetic stability is thus maintained in the somatic embryo regenerated population.

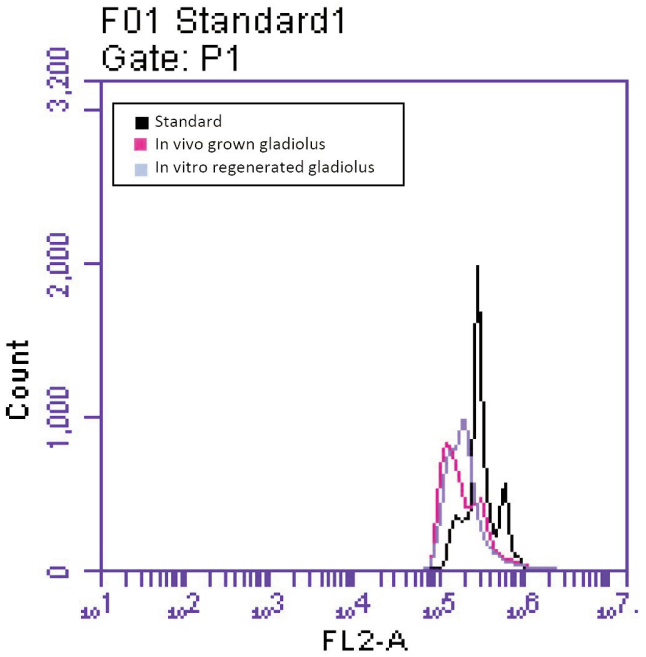

Fig. 3 - Histogram of relative 2C DNA content of corm grown (field) and somatic embryo regenerated gladiolus with standard *Pongamia pinnata* plant.

4. Discussion and Conclusions

In the present study, plant regeneration through somatic embryogenesis and 2C DNA content of regenerated plant were investigated in *Gladiolus*. Callus was first induced from corm on 2, 4-D added MS in which low to moderate doses were observed to be very effective. The amendment of auxins especially 2, 4-D was found very active in inducing callus in a number of investigated plants including gladiolus (Kamo and Joung, 2007; Su *et al.,* 2009; Feher, 2015). On 2, 4-D added medium, embryos were differentiated on callus, and thus the origin of embryos are indirect in gladiolus. This callus mediated embryo induction incidence has been noted in a number of investigated ornamentals of horticultural importance (Jiang and Zhang, 2007; Gow *et al.,* 2009).The process of in vitro embryogenesis is often controlled by a variety of PGRs and its analogues. The impact of 2, 4-D in inducing callus and somatic embryos are reported widely in literature (Gaj, 2004; Feher, 2015). At lower level, 2, 4-D activates *yucca* (*YUC)* gene, which helps in synthesizing IAA and its transport in developing embryos in cultivated tissues (Su *et al.,* 2009; Bai *et al.,* 2013). The application of 2, 4-D also induces stress in culture, which provokes vegetative cells to acquire embryogenic state (Gliwicka *et al.,* 2013), although the exact molecular mechanism influencing this transition is still not understood clearly.

Although the embryo formation was more frequent on 2, 4-D added medium, the addition of NAA and BAP at various levels was noted to be equally responsive in inducing callus and in promoting embryo numbers in gladiolus, this is in agreement with Stefaniak (1994) findings where NAA was identified to be more effective in inducing embryos compared to other auxins tested. The same level of NAA with BAP was earlier reported to be very effective in a number of bulbous/ tuberous ornamental monocots (Mujib *et al.,* 2006; 2008). Similar use of NAA with BAP was noted to be very beneficial for shoot formation in gladiolus, both during direct organogenesis and callus mediated regeneration (Ascough *et al.,* 2009; Memon, 2012). Wu *et al.* (2015) obtained globular somatic embryos on embryogenic callus on 3.0 mg/l TDZ and 0.2 mg/l BA amended MS without any requirement of NAA. Yelda and Bengi (2007) earlier reported somatic embryo induction on 0.1 mg/l BAP added MS medium, very similar to BAP induced embryo induction noted in our observations. Thus, for acquiring somatic embryo, the cytokinin signalling is also equally important, often sufficient as against common perception of auxins, which induce embryos in culture (Mujib *et al.,* 2016). Cytokinin induced somatic embryo formation has been observed in a number of plants and was reported (Iantcheva *et al.,* 1999). Although the molecular role of cytokinin in triggering *in vitro* embryogenesis is not fully elucidated, cytokinins induced *Wuschel* (*WUS*) gene expression and transcription factor synthesis at early embryogenesis and shoot apical meristem development time were reported in several studied organisms (Su *et al.,* 2009; Gordon *et al.,* 2009; Wang and Chong, 2016). The clear demarcation and establishment of shoot and root apical meristem are important steps in embryogenesis/somatic embryogenesis (Scheres, 2007). Although the role of auxin and auxin-cytokinin interaction in determining shoot apical meristem is known, cytokinin mediated root apical meristem development needs further investigation. Su *et al.* (2012) however, indicated that cytokinin induces the synthesis of *Arabidopsis response regulator*, ARR7 and ARR15, the two essential transcription factors, for root apical meristem development during somatic embryogenesis.

Induced embryos did not grow fast therefore other PGR treatments were added to improve embryo quality, which facilitates *in vitro* plant regeneration. In the present study, various concentrations of GA_3 and ABA were used, both influenced embryo maturation but the role of GA_3 was more profound compared to ABA. The observation is very similar to Vieitez findings (1995) in which poor role of ABA on embryo maturation was noted. ABA induced improved embryo maturation was, however, reported in other groups of plants including model *Arabidopsis* (Maruyama *et al.,* 2007; Bai *et al.,* 2013). In contrast, the incorporation of GA_3 facilitated embryo maturation by growing embryo size and by synthesizing more chlorophyll, which help in building protein, lipid, triglycerides and other energy reserves necessary during seed germination (Santos-Mendoza *et al.,* 2008). The combination of GA_3 and ABA has also been reported to activate *leafy cotyledon, LEC* and *FUS3* gene expression, help making energy reserves essential for seed/embryo germination (Braybook and Harada, 2008). Embryo maturation and germination steps are both very important in somatic embryo based plant propagation. Mature somatic embryos were transferred to a medium primarily amended with BAP or with NAA for plantlet formation. Within a few days, leaflets were coming out from coleoptiles and later roots were developed. The use of BAP alone or with NAA was earlier noted

to be very effective for conversion of plantlets from somatic embryos in other groups of plants (Mujib *et al.,* 2013). The regenerated shoots were transferred to IBA added medium for better induction of roots as the embryo derived shoots had with less developed roots. IBA promptly induced roots in gladiolus, like many other investigated materials (Mujib *et al.,* 2008). In gladiolus, it appears therefore that various PGRs control *in vitro* embryogenesis and plant regeneration and their requirement varies considerably. The embryo forming ability from callus in gladiolus was high and reproducible, which could efficiently be exploited in developing transgenics.

The plants obtained from callus through organogenesis and embryogenesis demonstrate genetic variation and these changes are identified by traditional and modern techniques (Das *et al.,* 2013). Here, in *G. hybridus*, plants were developed from callus via somatic embryogenesis pathway; the callus and the embryos are influenced by PGRs and stresses, both affect cell cycle/DNA synthesis. Thus, a study of regenerated plant status was indeed necessary. We analysed 2C DNA content in gladiolus plants, derived from corm and somatic embryo by flow cytometry. The level of 2C DNA of somatic embryo regenerated and corm obtained plants are the same in gladiolus. The amount of nuclear DNA was unchanged and the genetic fidelity was maintained. Due to its precision, the flow cytometric technique has widely been used in genetic stability studies (2C DNA analysis) in a number of investigated materials including *in vitro* regenerated plants (Loureiro *et al.,* 2007; Sliwinska and Thiem, 2007). The *in vitro* cultural condition, however, cause genetic irregularities in cultivating tissues (Giorgetti *et al.,* 2011); using flow cytometry this alteration can easily be identified (Dolezel *et al.,* 2007). Despite of the large number of applications and its ease, the 2C-DNA estimation has not been conducted widely, rather restricted to only about 2% of the flowering angiosperms (Galbraith, 2009). Thus, any attempt with obtained 2C value, even from *in vitro* raised culture, will significantly enrich C-value database in angiosperm. Similarly, the development of embryogenesis protocols discussed here can also be used in preparing synthetic seeds for clonal propagation and for conservation of important gladiolus germplasm.

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Uniform and virus-free citrus rootstocks production via nucellus culture

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Key words: citrus, nucellus culture, uniform rootstocks, virus-free.

Abstract: Prevalence of various virus and virus-like diseases is among the main reasons for the decrease in quality and quantity of citrus crops. These diseases are mainly spread through the propagation method in citrus which is budding. Using nucellus culture of bitter orange and Mexican lime seeds, uniform and virus-free rootstocks could be produced so that the diseases prevalence could be prevented. In order to generate adventitious shoots from nucellus culture in each of the two rootstocks, direct organogenesis method is used. In all conducted experiments, Murashige and Skoog (MS) medium were used. Two plant growth regulators of benzyl adenine (BA) in 0, 1, 1.5 and 2 mg l^{-1} concentration and gibberellins (GA) in 0, 1 and 2 mg l^{-1} concentration were used in the medium and the main effects of each plant growth regulator were studied separately and their interaction on shoot generation were also surveyed. Considering the retrieved data, it was determined that the interaction of BA and GA have a higher impact on shooting, comparing to the cases where each of the regulators is used alone. In Mexican lime rootstock, the best culture medium for generating shoots from nucellus culture is the culture medium containing 2 mg l^{-1} BA and 2 mg l^{-1} GA and in bitter orange rootstock, the highest shooting rate was attributed to the culture medium containing 2 mg l^{-1} GA and 1 mg l^{-1} BA. For the Mexican lime and bitter orange shoots rooting, indole butyric acid (IBA) was used. The concentrations of this plant growth regulator used in Mexican lime were 0, 0.5, 1 and 1.5 mg l^{-1} and for bitter orange were 0, 1 and 1.5 mg l^{-1}. The highest rooting rate for Mexican lime was in culture medium containing 0.5 mg l^{-1} IBA and for bitter orange, it was the culture medium containing 1 mg l^{-1} IBA. The obtained plantlets were gradually adapted with the external environment.

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1. Introduction

Citrus is among the most important fruit tree groups in tropical and subtropical regions in the world (Jajoo, 2010) and have a significant role in human diet as fruits which contain a high amount of vitamin C and other nutrients such as potassium. Similar to other fruit trees, citrus is commercially propagated through grafting a scion on a proper stock and a grafted tree is generated which retains the traits of stock and scion (Spreen, 2009). Citrus propagation through this method is among the most important limiting factors in generating them, since it leads to the

spread of various viral and virus-like diseases through generation of infected seedling (Rangan *et al.,* 1968). Choosing stock and scion suitable for each environment and then ensuring plants free of any disease play a great role in optimal generation of this crop (Shahsavar, 2005). Hence, it is required to generate healthy and virus-free stock and scion so that a healthy plant combination is generated. Generating healthy and virus-free scion is possible through shoot-tip-grafting (STG) (Murashige *et al.,* 1972; Shahsavar and Khosh-Khui, 1994; Shahsavar, 2005). However, virus-free rootstock production is not problematic since rootstocks are produced by seeds and seeds are not virus diseases vectors, even if the mother plant is infected (Altaf *et al.,* 2001; Singh *et al.,* 2006). Another issue which is important in rootstocks is their uniformity. Propagation from seeds could not lead to production of uniform rootstocks. Although some of the rootstocks are produced by the nucellar embryos which are uniform and similar to the mother plant, there are plants produced by natural embryos which are not completely similar to the mother plant and this could lead to the lack of uniformity among the produced rootstocks. Hence, in rootstock production, methods should ensure virus-free rootstocks, and uniformity, as well. The best method in reaching this objective is through planting nucellar embryos. Fortunately there are nucellar embryogenesis and polyembryonic in most citrus species which produce true to type plants (Wutscher, 1979). In 1958, Rangan carried out the initiation of nucellar embryos in one of citrus genotypes for the first time and micro propagation in these genotypes provided the possibility for production of uniform plant populations (Rangan Swamy, 1958). Nucellus culture is generally considered as an effective method for producing virus-free citrus and virus is not spread through nucellus cultured. Also, the produced plants through nucellus culture and somatic embryogenesis have the potentials to produce plants with the traits of the mother plant (Singh *et al.,* 2006). In 2001, Altaf reported that using nucellus tissue explants, they were able to produce virus-free plants showing minimum differences with the mother tissue. In the conducted research, the presence of cytokinin BA was necessary for shoot regeneration. However, the optimum concentration of BA is depending on explant genotype and other conditions. Rooting of the generated shoots have been reported differently considering used genotype, culture medium and IBA concentration (Chaturvedi and Mitra, 1974; Barlass and Skene, 1982; Duran-Vila *et al.*, 1989; Jakson and Looney, 1999).

The main objective of this research is to study Mexican lime and bitter orange micropropagation using generation of adventitious shoots through nucellus culture of these seeds and reaching a uniform and virus-free rootstock and also determining the effect of plant growth regulators in stimulation and growth of shoots and rooting of these shoots.

2. Materials and Methods

In this research two important citrus rootstocks including bitter orange (*Citrus aurantium* L.) and Mexican lime [*Citrus aurantifolia* (Christm.) Swingle] were used. Immature fruits (100-120 days after pollination) of these species were transferred from Darab Research Station to the laboratory of Horticultural Sciences Department at Shiraz University.

Initially, the fruits were washed with water and a dish washing detergent (Rika®) and rinsed. Subsequently, they were put in 40% Clorox® solution (regular commercial bleach which contains 5-6% sodium hypochlorite) for 15 minutes so that their surface was disinfected. The seeds of each fruit were separated and after washing the seed gel, they were transferred under laminar air flow cabinet. The seeds were left in Clorox® solution for 10 minutes and rinsed with sterilized distilled water so that their surfaces were disinfected. Subsequently, both seed shells were separated before planting. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with various plant growth regulators concentrations were used in this research. In order to study the impact of BA and GA and their interaction on shoot generation from nucellus tissue of Mexican lime and bitter orange, BA with concentrations of 0, 1, 1.5 and 2 mg l^{-1} and GA with concentrations of 0, 1 and 2 mg l^{-1} were used. Therefore, there were 4 treatments and 8 replications per treatment and one explant per replication, totally 32 tubes per treatment. Sucrose and agar concentration used in all culture media were 30 and 8 g l^{-1}, respectively. The pH of the culture medium was set to be 5.75±0.05 after putting in autoclave and disinfected for 15 minutes at 120°C and pressure of 1.5 kg cm^{-2}.

Culturing explants

After separating the area of the seeds which contained zygotic embryo, remaining tissue of the seed

which contained the nucellar embryo was cultured horizontally in 150×250 ml test tubes which had 20 ml of MS basal culture medium. One explant was sown per tube and exposed to 16-h daily of 2000-2500 lux illumination of fluorescent light at 27±1°C for 4 weeks.

Rooting of the generated adventitious shoots

For rooting of the generated adventitious shoots, MS basal culture medium along with IBA was used (0, 0.5, 1, 1.5 mg l^{-1} and 0, 1 and 1.5 mg l^{-1} of IBA for Mexican lime and bitter orange, respectively, in ten populations). The cultured shoots were kept in rooting culture medium for 20 days using day length of 16 h at 27±1°C.

Adaptation of rooted plantlets

For adaptation, the rooted plantlets were transferred to pots containing a mixture of 50% soil and 50% sand and plastic bags were put over them and kept at 25±3°C and at light intensity of 2,000 lux. The pots were initially irrigated with one eigth of the MS salts concentration for 10 days and they were irrigated by tap water subsequently and they gradually adapted with the external environment by forming holes in the plastic bags.

Experiment design and data analysis

At the end of regenerating shoots experiment, the number of shoots, the length of the shoots and the number of leaves were recorded for each explant. At the end of rooting experiment, the numbers of roots, the length of roots, roots fresh and dry weight were recorded. Experiments were conducted in factorial arrangement in a completely randomized design with several replications. The number of replications and the number of explants in each replication are presented at the bottom of each table. Data were analyzed by SPSS software and the means comparison was conducted by Duncan's multiple range test at probability level of 5%.

3. Results

Generating adventitious shoot from nucellus culture of Mexican lime

Explants of nucellar tissue began inflating after being placed on the culture medium in places of cuts and gradually adventitious seedlings and then shoots were emerged in the places of cuts. In this research, the highest number of adventitious shoots (Fig. 1) were retrieved in 2 mg l^{-1} BA and 2 mg l^{-1} GA with the value of 3.33 (Table 1) and there was no significant difference found on the generated adventitious shoots between the media which contained only BA or GA in various concentrations of these growth regulators. Considering the derived results, media containing both growth regulators of BA and GA had more impact on shoot regeneration, comparing to the media containing one of the growth regulators only (Table 1).

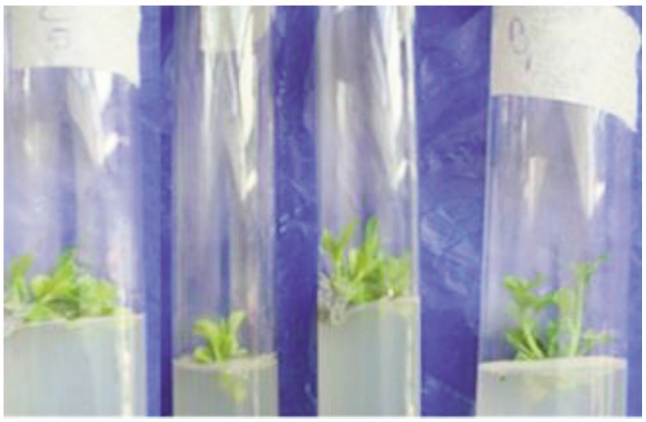

Fig. 1 - Proliferation of shoots of Mexican lime nucellus culture.

The average shoot length in various treatments was different (Table 1). The highest shoot length was derived in treatment of 2 mg l^{-1} of BA and 2 mg l^{-1} of GA with 6.33 cm which had significant difference with the control (0) treatment (Table 1).

The highest rate of leaf generation was 9 leaves in 2 mg l^{-1} of BA and 2 mg l^{-1} of GA treatment, which has a significant difference with the control treatment (Table 1).

Table 1 - The impact of BA and GA growth regulators and their interaction on the number of shoots, length of shoots and number of leaves derived from Mexican lime nucellus culture

Treatment	Number of shoots	Length of shoots	Number of leaves
GA0 mg l^{-1} +BA0 mg l^{-1}	0.0 c	0.0 e	0.0 d
GA 0 mg l^{-1}+BA1 mg l^{-1}	2.28 ab	4.4 abc	8.0 ab
GA 0 mg l^{-1} +BA1.5 mg l^{-1}	1.8 abc	3.20 bcd	7.0 ab
GA 0 mg l^{-1} +BA2 mg l^{-1}	1.5 abc	1.75 dec	4.5 bc
GA1 mg l^{-1} +BA0 mg l^{-1}	2.75 ab	5.125 ab	8.2 ab
GA 1 mg l^{-1} +BA1 mg l^{-1}	0.0 c	0.0 e	0.0 d
GA 1 mg l^{-1} +BA1.5 mg l^{-1}	2.0 ab	4.167 abcd	5.7 abc
GA 1 mg l^{-1} +BA2 mg l^{-1}	0.0 c	0.0e	0.0 d
GA2 mg l^{-1}+BA0 mg l^{-1}	1.0 bc	1.5 de	2.0 bc
GA 2 mg l^{-1} +BA1 mg l^{-1}	2.33 ab	3.167 bcd	5.1 abc
GA 2 mg l^{-1}+BA1.5 mg l^{-1}	0.0 c	0.0 e	0.0 d
GA 2 mg l^{-1}+BA2 mg l^{-1}	3.33 a	6.33 a	9.0 a

GA= Gibberellic acid;
BA= Benzyl adenine;
The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

Rooting of shoots derived from nucellus culture of Mexican lime

Table 2 presents the rooting of separated Mexican lime shoots in culture medium with all concentrations of IBA. The highest amount of roots was 4.3 which was achieved in 0.5 mg l^{-1} of IBA treatment, showing significant difference with the average number of roots in 1 and 1.5 mg l^{-1} and the control treatment. There was no significant difference found between the average root length in concentrations of 0.5, 1 and 1.5 mg l^{-1} and the average length of the control treatment. The highest fresh weight was 0.052 g in concentration of 0.5 mg l^{-1} of IBA that had a significant difference with the control and other treatments. The highest root dry weight was 0.011 g in concentration 0.5 mg l^{-1} of IBA that had no significant difference with the control and other treatments (Fig. 2).

Table 2 - The impact of IBA growth regulator on rooting of shoots derived from Mexican lime nucellus culture

Indole Butyric acid (IBA) mg l^{-1}	Root dry weight (g)	Root fresh weight (g)	Root length (cm)	Number of roots
0	0.0072 a	0.024 b	5.01 a	1.66 b
0.5	0.011 a	0.052 a	4.9 a	4.3 a
1	0.0094 a	0.024 b	6.62 a	2.0 b
1.5	0.007 a	0.018 b	4.93 a	2.28 b

The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

Fig. 2 - Rooting of the *Mexican lime* adventitious shoots.

Generating shoot from nucellus culture of bitter orange

The highest value of adventitious shoot regeneration was in the culture medium containing 1 mg l^{-1} BA and 2 mg l^{-1} GA which had a significant difference with the rest of treatments, including control treatment (Table 3). In this study, no shoot was generated in the culture medium which contained GA only. Also, in culture media which contained BA only, the shoot was generated in 1 mg l^{-1} growth regulator. Considering the retrieved results, culture media containing both growth regulators of BA and GA impact the shoot generation better in bitter orange, similar to Mexican lime (Table 3).

The highest generated shoot length average was in 1 mg l^{-1} of BA and 2 mg l^{-1} of GA treatment which had a significant different with the control treatment (Table 3).

The highest leaf generation was 5 leaves which was related to the 2 mg l^{-1} of BA and 1 mg l^{-1} of GA treatment that had a significant different with the control treatment (Table 3).

Rooting of shoots derived from nucellus culture of bitter orange

Table 4 presents the rooting of separated bitter orange shoots in culture medium with concentrations of 0, 0.5, 1 and 1.5 mg l^{-1} of IBA. The highest number of roots were 3 in 1 mg l^{-1} of IBA treatment, such result did not have any significant difference

Table 3 - The impact of BA and GA growth regulators and their interaction on the number of shoots, length of shoots and number of leaves derived from bitter orange nucellus culture

Treatment	Number of shoots	Length of shoots	Number of leaves
GA0 mg l^{-1} + BA0 mg l^{-1}	0 c	0 c	0 c
GA 0 mg l^{-1} + BA1 mg l^{-1}	1.33 b	2.6 ab	4 a
GA 0 mg l^{-1} + BA1.5 mg l^{-1}	0 c	0 c	0 c
GA 0 mg l^{-1} + BA2 mg l^{-1}	0 c	0 c	0 c
GA1 mg l^{-1} + BA0 mg l^{-1}	0 c	0 c	0 c
GA 1 mg l^{-1} + BA1 mg l^{-1}	0 c	0 c	0 c
GA 1 mg l^{-1} + BA1.5 mg l^{-1}	1 b	2 b	2 b
GA 1 mg l^{-1} + BA2 mg l^{-1}	1 b	4 a	5 a
GA2 mg l^{-1} + BA0 mg l^{-1}	0 c	0 c	0 c
GA 2 mg l^{-1} + BA1 mg l^{-1}	2.25 a	4 a	4 a
GA 2 mg l^{-1} + BA1.5 mg l^{-1}	1.5 b	3.5 a	4.5 a
GA 2 mg l^{-1} + BA2 mg l^{-1}	0 c	0 c	0 c

GA= Gibberellic acid;
BA= Benzyl adenine;
The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

with the average number of roots observed in 1.5 mg l^{-1} treatments, while it had a significant difference with the control. The root length average in each treatment was 10.30 and 8.51 cm respectively for concentrations of 1 and 1.5 mg l^{-1} which showed a significant difference with the control treatment. The highest fresh weight of 0.05 g and the highest dry weight of 0.012 g were observed using concentration of 1 mg l^{-1} of IBA, showing a significant difference with the control treatment.

Table 4 - The impact of IBA growth regulator on rooting of shoots derived from bitter orange nucellus culture

Indole butyric acid (IBA) mg l^{-1}	Root dry weight	Roots fresh weight	Root length	Number of roots
0	0.0 b	0.0 b	0.0 b	0.0 b
1	0.012 a	0.05 a	10.30 a	3.0 a
1.5	0.011 a	0.039 a	8.51 a	2.16 a

The results are based on eight replications and one explant per replication. In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range tests.

4. Discussion and Conclusions

Generating shoot from nucellus culture of Mexican lime and bitter orange

Some seedlings of some citrus cultivars were generated *in vitro* by culturing nucellus explants (Rangan *et al.,* 1968*,* 1969). However, this method has not been successful in all cases (Button and Kochba, 1977). The current study showed that nucellus in Mexican lime and bitter orange species is not capable of generating shoot without plant growth regulators, and application of plant growth regulators could increase their potentials for generating shoot. Usman *et al.* (2005) reported that the number of shoots induced in each explant depends on the citrus type. For instance, in Kinnow mandarin, there is more shoots generated comparing to orange during direct organogenesis. In this experiment, it was indicated that Mexican lime nucellus explants have higher potentials for generating shoot, comparing to bitter orange. Considering the role of plant growth regulators for generating shoot from nucellus, two growth regulators of BA and GA were used in this research. Results have suggested that BA is responsible for generating shoot from nucellus in both species, since in medium without the regulators, there was lower number of shoots in Mexican lime, and there was no shoot in bitter orange. External application of BA is necessary in culture medium (Raj-Bhansal and Arya, 1978). For direct organogenesis, Rattanpal *et al.* (2011) put epicotyl and hypocotyl explants of *Citrus jambhiri* Lush. in culture medium containing BA and this hormone led to generation of shoot in these explants. GA leads to increase the length of the generated shoots (Rattanpal *et al.,* 2011). In studying GA impact, without the presence of BA in the culture medium in this research, it could be claimed that this hormone has been effective on shoot length in both species and the results from this research are in accordance with the results from Saini *et al*. (2010). They reported that adding GA to the culture medium containing BA improves the number of elongated shoots (Saini *et al.,* 2010). Results from this research suggested that using a combination of various levels of BA and GA has been more effective in both species, comparing to the application one of these hormone only. For instance, the highest number of shoots, shoot length and number of leaves in Mexican lime was related to the treatment with 2 mgl^{-1} of BA and 2 mg l^{-1} of GA and in bitter orange, the highest number of shoots and shoot length was related to the use of 2 mg l^{-1} of GA and 1 mg l^{-1} of BA. There are various reports which show that the application of these two plant growth regulators have been effective on shoot generation, such as that of Gill and Gosal (2002) who generated a high rate of shoots in *C. depressa* by applying 1 mg l^{-1} of BA and 2 mg l^{-1} of GA. Also, the highest percentage of shoot generation in explants of *Poncirus trifoliate* was derived when 2 mg l^{-1} of GA and BA were used in culture medium (Usman *et al.,* 2005; Tzatzani *et al.,* 2009).

Rooting of shoots drived from nucellus culture of bitter orange and Mexican lime

The highest rate of rooting for bitter orange was obtained in the medium containing 1 mg l^{-1} of IBA. In Mexican lime the highest rate of rooting was obtained in the medium containing 0.5 mg l^{-1} of IBA. By increasing the concentration of this hormone, there was a decrease in the number of generated roots. Sandra and Morehart in 1998 (in orange) and Singh *et al.* in 2006 (in Mexican lime and tangerine) reported that by the increase in IBA concentration, the rooting rate decreases which are in accordance with the results from this experiment. IBA has been highly successful in rooting in many citrus species, including *Citrus aurantifolia* (Raj-Bhansal and Arya,

1978). In addition to the above mentioned, the results about the impact of IBA on rooting of nucellus shoots in Mexican lime and bitter orange were in accordance with the findings of Khalekuzzaman *et al*. (2008) on *Adhatoda vasica*, Wilson *et al.* (2010) on *Indoneesiella ecohides* and Purkayastha *et al.* (2008) on *Andrographis paniculata*. A great number of lateral roots were generated in Mexican lime in the concentration of 0.5 mg l^{-1} of IBA, while there was no lateral root observed in other treatments.

Transfer and adaptation

The plantlets adapted to the sterilized mixture of equal volume ratio of perlite and vermiculite in 2 months. Since the humidity is high in culture tubes, the humidity for the plantlets is provided by plastic bag during adaptation period and over time the humidity is decreased by forming holes in the plastic bags. All adapted plants were transferred to the greenhouse with 100% success and stayed alive. These results were in accordance with the findings from Dojam *et al*. (2001) in orange in and Jajo (2010) in *Citrus limonia* Osbeck.

Interaction of BA and GA has a higher impact on shooting in Mexican lime and bitter orange rootstocks. In Mexican lime the best culture medium for generating shoots containing 2 mg l^{-1} BA and 2 mg l^{-1} GA and in bitter orange rootstock, the highest shooting rate was attributed to the culture medium containing 2 mg l^{-1} GA and 1 mg l^{-1} BA. The highest rooting rate for Mexican lime was in culture medium containing 0.5 mg l^{-1} IBA and for bitter orange; it was the culture medium containing 1 mg l^{-1} IBA.

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Field evaluation of three biopesticides for control of the raspberry cane midge, *Resseliella theobaldi* (Barnes) in Bulgaria

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Key words: azadirachtin, *Bacillus subtilis*, biocontrol, efficacy, *Resseliella theobaldi,* spinosad.

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Abstract: The raspberry cane midge, *Resseliella theobaldi* is a key pest on red raspberry, *Rubus idaeus.* The larvae of the insect severely attack the raspberry canes, resulting in premature death of the plant canes. In the last decade, organic production of raspberry fruits has significantly increased in Bulgaria. At the same time there are few products of botanical or microbiological origin that might be used for control of this pest. In present study the effect of NeemAzal® T/C (azadirachtin A), Sineis 480 SC® (spinosad), and *Bacillus subtilis* on *R. theobaldi* was evaluated. The experiments were conducted in two raspberry fields at different altitude. In the field at lower altitude (196 m), the raspberry cane midge has developed four generation per year, while in the field at higher altitude (960 m) three generations of the pest have been completed. Lowest number of larvae in raspberry canes was observed after application of NeemAzal® T/C, and *B. subtilis* in both raspberry fields. Both products demonstrated highest efficacy at 7th day after treatment, when the number of larvae per splits was 67.1-82.5% for NeemAzal® T/C, and 75.1-81.2% for *B. subtilis* lower compared with the control at the two experimental sites.

1. Introduction

Among the small fruit crops grown in Bulgaria, the red raspberry (*Rubus idaeus* L.) is the most valuable. During the last five years, the total area of raspberry plantation has increased by 43% and the yield has reached 3620 kg^{-1} ha. At the same time approximately 54% of raspberry production is organic. In 2015 and 2016, about 75% of the total raspberry yield was exported mainly to western European countries, but also to several markets in Asia (Agrostatistics, 2015).

The most serious pest of raspberry is the raspberry cane midge, *Resseliella theobaldi* (Barnes) (Diptera: Cecidomyiidae), which causes premature death of the plant canes. In Bulgaria, the insect was first reported by Stoyanov in 1960. The author examined the life cycle of *R. theobaldi*

under different conditions and reported the development of three-five overlapping generations a year. The larvae of the insect attack the plant primocanes, both of those fruiting in June-July as well as those fruiting in August-September (Stoyanov, 1963). The larvae feed under the bark of canes and clearly define dark brown spots appearing on the green surface of the canes. Pitcher (1952) stated that damage to raspberry plants caused by *R. theobaldi* was usually associated with fungal pathogens such as *Botrytis cinerea*, *Fusarium avenaceum* and *Didymella applanta*. The fungi cause necrosis of vascular cylinder through the larval feeding sites (Williamson, 1987). The complex of damage involved the raspberry cane midge and mycoses is known as "midge blight" (Pitcher and Webb, 1952). As a result of usually present of midge blight, there is no established relationship between population level of *R. theobaldi* and degree of plant damage caused by the insect (Williamson and Hargreaves, 1979). According to Shternshis *et al*. (2002), the effect of the control treatments against the pest should be assessed by estimating the severity of midge blight including fungal lesions.

To date, the biological control of raspberry cane midge is still poorly investigated. There are few reports concerning alternatives to chemical control for *R. theobaldi*. Sex-pheromone-based strategies are promising techniques to control of many economically important pests. Pheromone traps for raspberry cane midge were used for the first time in the UK in 2005 (Milenković *et al*., 2006). Cross and Hall (2006) and Hall *et al.* (2009) identified 2-acetoxy-5-undecanone as a major component of *R. theobaldi* female sex pheromone. Over the past ten years, the sex pheromones have been tested for monitoring the male emergence (Cross *et al.*, 2008, Tanasković and Milenković, 2010, Sipos *et al*., 2012). Therefore, little information concerning the application of biopesticides for control of *R. theobaldi* in raspberry organic production is currently available. For instance, use of products based on entomopathogenic bacteria, *Bacillus thuringiensis* (Bt) and *Streptomyces avermitilis* against raspberry midge blight have been reported (Shternshis *et al*., 2002). Further, there are no publications on the biological control of this raspberry pathogen complex.

The objective of this study was to evaluate the possibility to control raspberry cane midge using two commercial biopesticides and one noncommercial bacterial strain under field conditions. In particular, I attempted to assess the role of used products in reducing the number of *R. theobaldi* larvae in raspberry canes.

2. Materials and Methods

Biopesticides and bacterium cultivation

Commercial formulations NeemAzal® T/C (azadirachtin A, Trifolio-M, Germany), and Sineis 480 SC® (spinosad, DowAgroSciences, Bulgaria), and a bacterium *Bacillus subtilis* were used against midge in raspberry fields.

The strain of *B. subtilis* was grown in the dark for 48 h at 24°C on tryptic soy broth agar (TSBA). For inoculum production a loop of the bacteria was transferred into 100 ml of TSB and allowed to multiply for 48 h on a rotary shaker (160 rpm) at the same temperature. Bacterial suspensions were centrifuged at 4000 rpm for 20 min, and the bacterial pellet was resuspended in sterile¼ strength Ringer's solution (Merck). The bacterial suspension was adjusted to a final concentration of 10^8 CFU ml^{-1} by dilution with Ringer's solution. The bacterial strain identification was determined by FAME Analysis, following by BIOLOG Analysis.

Experimental design

The trials were conducted in 2016 in two commercial raspberry plantations in the regions of Bogdanovo (196 m) and Samokov (960 m). The first location (Bogdanovo) has a flat topography, with small hills. The soil type is Leptosols (Bulgarian Soil Taxonomy), and the landscape is dominated by agricultural land use. According to the climatic data (National Institute of Meteorology and Hydrology, BAS), the average air temperature from April through October 2016 was 22.4°C. The average rainfall for the investigation period was 284 mm. The geographical coordinates of the raspberry field in Bogdanovo are 42°36' N, 26°00' E. The second location (Samokov) is a valley between two mountains - Rila and Verila. The soil type is Fluvisols, and the landscape is dominated by arable land. The average air temperature from April through October 2016 was 15.4°C. For the same period, the average rainfall was 531 mm. The geographical coordinates of the raspberry field in Samokov are 42°21' N, 23°34' E.

The cultivar Heritage (USA) was grown in both three years old fields. Plants were located in spacing of 50 cm within rows and 2.0 m between rows. Experimental plots were 10 m^2, each plot containing approximately 100-120 raspberry canes. The size of

the buffer zone between the plots was 4 m. The treatments were arranged in a completely randomized block design with four replications.

Treatment and application methods

The PheroNorm® standard large delta traps (Andermatt Biocontrol AG, Switzerland) were used to determine the population dynamic of the cane midge. The traps containing 10 µl cane midge sex pheromone lure per trap were mounted on bamboo sticks at height of 60 cm the 10th of April. Three traps were used in Bogdanovo (11 ha), and two in Samokov (7,5 ha). Two applications were made in Samokov, one against the first generation (on the 17th May), and one against the second generation (on the 14th July). Three applications were made in Bogdanovo on 9th May, 8th July and 13th August against the first, second and third generations of *R. theobaldi*, respectively. The timing of each treatment was chosen according to the number of males caught by the traps. The treatments were made during the period of midge oviposition and larvae hatching.

The test suspensions were: NeemAzal® T/C (0.2%), Sineis 480 SC® (0,025%), and *B. subtilis* (20 ml). The suspensions were applied at a volume application rate of 0.1 l m^{-2}, using a hand held sprayer. The plants in the control were treated with equal quantity (0.1 l m^{-2}) of water.

Data collection and analysis

The observations were made on the 3rd, 6th, and 12th day after treatments. Twenty canes per replicate, 20 canes were examined under stereomicroscope and the number of larvae in the natural splits was counted.

Obtained data were subjected to one-way analysis of variance (ANOVA) and the treatment means were compared with the control plants, according to the Duncan's test ($P<0.05$).

3. Results

In 2016, the raspberry midge cane flight pattern demonstrated four generations in Bogadonovo (196 m) and three generations in Samokov (960 m) (Fig. 1). In both commercial fields, the flight of midges started in the second half of April, a week later in Samokov (25.04) compared with Bogdanovo (18.04). At lower altitude, the first, the second, and the third generations of the pest showed three pronounced peaks of its flight dynamic. In the higher altitude, there were two peaks of midge cane flight - the first

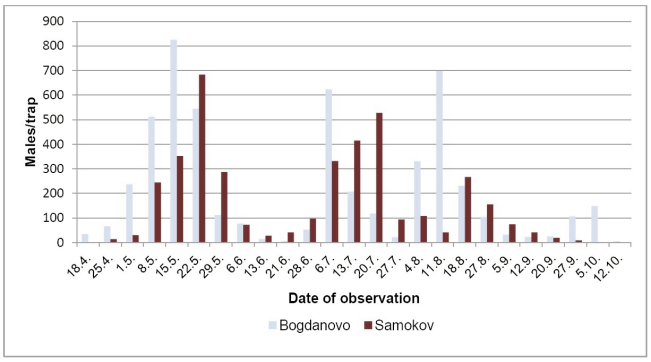

Fig. 1 - Trap catches of males of *Resseliella theobaldi* in the fields of Bogdanovo and Samokov during the spring, summer and autumn of 2016.

one between 19th and 25th of May, and the second one between 18th and 21th of July, for the first and second generations respectively (Fig. 1).

In general, the population density of the males was higher in Bogdanovo than at Samokov. The highest number of males was recorded during the intensive flight of the first generation of the midge in both Bogdanovo (824) and Samokov (683) sites (Fig. 1). Later in the season, the number of the males attracted by the traps in Bogdanovo was 623, 698 and 193 for the second, third and fourth generation, respectively. In Samokov, the number of the males was 528 and 267 for the second and third generation, respectively. The flight of the fourth generation of *R. theobaldi* continued until 12th of October in Bogdanovo, while the flight of the third generation of the midge in Samokov was completed on 27th of September (Fig. 1). The results obtained from this observation allowed finding the most appropriate date for treatments.

The evaluated bioinsecticides demonstrated biological activity against the raspberry midge cane applied during the period of pest oviposition and larvae hatching. Except for the treatment against the second generation of *R. theobaldi* in Samokov, in both raspberry fields, Bogdanovo and Samokov, the highest efficiency was found for the insecticide NeemAzal® T/C (Tables 1 and 2). This biopesticide also demonstrate the rapid initial effect against the penetration of larvae into raspberry canes. The number of midge larvae per split in raspberry canes treated with NeemAzal® T/C was significantly lower than the control at observations at 3rd, 7th and 12th day after applying the insecticide in Bogdanovo (Table 1, $P<0.05$). There was no significant difference between the efficacy demonstrated by NeemAzal® T/C and *B. subtilis*. After the treatment against the first generation the number of midge larvae per split varied

between 1.08 and 1.54 for NeemAzal® T/C, and between 1.55 and 2.07 for *B. subtilis*. Similar results were observed after the second and third treatments. The insecticide Sineis 480 SC® demonstrated the lowest efficacy compared with NeemAzal® T/C and *B. subtilis* (Table 1, P<0.05). At the observation at 3rd day after treatments against the first and third generation of raspberry midge in Bogdanovo, the number of larvae in plots treated with Sineis 480 SC® was not significantly different from the number of larvae in control plots. In the second generation there was a statistical difference between the variant with the bioinsecticide and the control variant. After the treatments at 7th and 12th day, the insecticide showed better effect, and the number of larvae into the canes was significantly lower than the control but still higher compared with NeemAzal® T/C and *B. subtilis* (Table 1, P<0.05).

In the experiments conducted in Samokov Sineis 480 SC® demonstrated higher efficacy against raspberry cane midge and the number of larvae per split was statistically different than the control at all three observations (Table 2, P<0.05). In this field, *B. subtilis* was more effective than NeemAzal® T/C and the number of larvae per split varied between 2.86 and 2.91 after first treatment. After the same treatment, the number of larvae into the canes treated with NeemAzal® T/C varied between 3.15 and 2.94. After the treatment against the second generation of *R. theobaldi*, *B. subtilis* showed rapid initial effect than NeemAzal® T/C. There was significant difference in number of larvae at 3rd day - 2.63 and 4.86 for *B. subtilis* and NeemAzal® T/C, respectively (Table 2, P<0.05).

Table 1 - Efficacy of three biopesticides using against raspberry midge cane in Bogdanovo

Treatments	Products/active ingredients	Number of midge larvae/split (day after treatment)		
		3rd (±SD)	7th (±SD)	12th (±SD)
First generation	NeemAzal® T/C (azadirachtin A)	1.54 (±0.18) a	1.12 (±0.07) a	1.08 (±0.37) a
	Sineis 480 SC® (spinosad)	4.25 (±0.45) b	2.93 (±1.04) b	3.41 (±1.35) b
	B. subtilis	2.07 (±0.67) a	1.35 (±0.11) a	1.55 (±0.64) a
	Control	5.62 (±1.02) b	6.28 (±1.22) c	6.78 (±0.56) c
Second generation	NeemAzal® T/C (azadirachtin A)	2.48 (±1.11) a	1.74 (±0.64) a	1.62 (±0.19) a
	Sineis 480 SC® (spinosad)	5.77 (±1.32) b	3.82 (±1.04) b	3.87 (±0.94) b
	B. subtilis	3.19 (±0.44) a	2.17 (±0.05) a	2.28 (±0.14) a
	Control	8.44 (±1.12) c	8.92 (±1.65) c	9.41 (±0.27) c
Third generation	NeemAzal® T/C (azadirachtin A)	1.24 (±0.72) a	0.92 (±0.08) a	0.98 (±0.34) a
	Sineis 480 SC® (spinosad)	3.48 (±1.13) b	2.14 (±0.22) a	2.21 (±0.75) a
	B. subtilis	1.37 (±0.18) a	1.22 (±0.31) a	1.43 (±0.86) a
	Control	4.05 (±0.93) b	4.89 (±1.16) b	5.12 (±0.99) b

Means within each column followed by the same letter are not significantly different, Duncan's test (p<0.05).

Table 2 - Efficacy of three biopesticides using against raspberry midge cane in Samokov

Treatments	Products/active ingredients	Number of midge larvae/split (day after treatment)		
		3rd (±SD)	7th (±SD)	12th (±SD)
First generation	NeemAzal® T/C (azadirachtin A)	3.15 (±1.11) a	2.13 (±0.82) a	2.94 (±0.54) a
	Sineis 480 SC® (spinosad)	6.05 (±1.32) b	4.74 (±1.08) b	5.38 (±0.67) b
	B. subtilis	2.92 (±0.97) a	2.39 (±0.17) a	2.86 (±0.91) a
	Control	10.21 (±1.24) c	12.17 (±1.98) c	14.71 (±1.15) c
Second generation	NeemAzal® T/C (azadirachtin A)	4.86 (±0.46) b	3.77 (±0.21) b	3.58 (±1.06) a
	Sineis 480 SC® (spinosad)	5.14 (±1.11) b	3.98 (±0.87) b	3.43 (±0.35) a
	B. subtilis	2.63 (±1.07) a	2.05 (±0.57) a	2.73 (±0.97) a
	Control	8.73 (±0.48) c	10.84 (±1.54) c	13.22 (±1.18) b

Means within each column followed by the same letter are not significantly different, Duncan's test (p<0.05).

4. Discussion and Conclusions

R. theobaldi was described by Theobald in 1920 (Barnes, 1926). Since then, almost a century later, it has become a pest of economic importance of raspberry crop throughout Europe. The midge cane is widely distributed in Bulgaria, Greece, Rumania, Italy, France, Ireland, UK, Sweden, Czech Republic, Slovakia, Hungary and Poland. In these countries the insect has been introduced mainly with infested planting materials and somewhere with infested soil. When establishing a new raspberry plantation, it is critical to choose the cultivar that is well adapted to local soil and climatic conditions and it is less susceptible to infestation by the raspberry cane midge, as well. Normally, infested raspberry plants have demonstrated the symptoms of dark brown, clearly defined spots in the canes 3 to 5 weeks after laying the eggs (Stoyanov, 1963). For this reason, it is important to determine the most appropriate timing for control of *R. theobaldi*. The treatments have to be done before the larval feeding sites become visible.

Organic production of raspberry in many countries, including Bulgaria, is a challenge because of lack of products for plant protection, which have nonchemical origin. Moreover, in Bulgaria there are no officially registered biopesticides or even chemical insecticides, that can be specifically used for control of the midge cane. Meanwhile, NeemAzal® T/C has been registered for control of tomato borer, *Tuta absoluta*, Meyr. Considering this need, the present study met its objective - the results showing the possibility of biological control of the *R. theobaldi*. The tested biologically based preparations demonstrated their efficacy against the larvae of midge cane. The pest had four generations in the plantation at lower altitude (Bogdanovo) and three generation in the plantation at higher altitude (Samokov). Further, the data from the pheromone traps showed that the first generation had the highest population density in both raspberry plantations. The forth and the third generations in both sites showed the lowest population density. According to this information, the time and the number of treatments were determined.

Among the tested biopesticides, NeemAzal® T/C and *B. subtilis* demonstrated the highest efficacy, causing up to 82.5% (the former) and 81.2% (the later) reduction of number of midge larvae in the splits compared to the control. Sineis 480 SC® demonstrated slower initial effect than NeemAzal® T/C and *B. subtilis*, but comparatively high efficacy, causing up to 63.26% reduction in number of larvae in the splits compared with the control.

In fact, the present evaluation is the second attempt to apply only environmentally safe products for control of *R. theobaldi*. The first one was made by Shternshis *et al.* (2002). The authors tested the preparations based on *Bt* (BACTICIDAE®), and *S. avermitilis* (PHYTOVERM®) for control of the raspberry midge blight and reported significant reduction of disease complex severity compared with the control variants. *S. avermitilis* is the base of spinosad, the active ingredient of Sineis 480 SC®. Spinosad as an active ingredient of the product Audienz®, was tested by Barrofio *et al.* (2011) against the raspberry midge cane. The result from this experiment is ambivalent, showing comparatively high efficacy against the midge larvae, but not significantly differ compared to the control. In this study spinosad showed to be less effective to the raspberry cane midge compared with both, azadirachtin A and *B. subtilis*. The result is interesting, because the spinosad penetrates translaminarly in plant tissues and this suggests higher efficacy compared with the other tested products. Deleva and Harizanova (2014) stated the rapid initial effect of spinosad against the larvae of tomato borer, *T. absoluta*. The authors reported 73.33% larval mortality in tomato leaves at 3rd day after treatment.

Neem-based products have been evaluated for their efficacy against the different pest on berry plants. Kim (2014) reported insufficient activity of azadirachtin against the rednecked cane borer, *Agrilus ruficollis* F. on blackberries in USA. Contrary, Aguilera *et al.* (2009) commented the high efficacy of Neem against the raspberry weevil, *Aegorhinus superciliosus* G. in Chile. The authors reported significant embryogenesis inhibition after applying the Neem. The lowest number of larvae in raspberry canes observed in this evaluation is probably due to affect of azidarachtin on both larvae and adult of raspberry cane midge.

The results obtained after application of, *B. subtilis* indicate that the bacterium might be considered as an effective agent for control of *R. theobaldi*. *B. subtilis* was originally isolated from the soil and has been tested as a biocontrol agent of root-knot nematode, *Meloidogyne arenaria* on tomato (Mohamedova and Samaliev, 2011). The bacterium is able to colonize successfully the rhizosphere of the plants and affect different pathogens in this zone. This suggests that *B. subtilis* could influence the pupae of the raspberry cane midge in the soil.

I have not observed any phytotoxicity or negative

influence of the three biopesticides on raspberry plants and beneficial insects. In several raspberry canes collected from the plantation in Samokov was observed parasitized 4th instar midge larvae of the third generation (Fig. 2). The midge larvae probably were infested by the parasitic larvae of *Aprostectus* genus.

Therefore the results of the present evaluation show the possibility of the tested biopesticides to control of the raspberry cane midge. These products are a good alternative to chemical insecticide and might be successfully integrated in the control strategies of *R. theobaldi* in both, conventional and organic raspberry production. Further research should focus on screening the pesticides and bioagents, which could be able to control the disease complex "midge blight", causing very often the dead of raspberry plants.

Fig. 2 - Parasitized and nonparasitized 4th instar larvae of *Resseliella theobaldi* in splits of raspberry canes collected from Samokov raspberry plantation (September, 2016)

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An examination into the effects of frozen storage of olive fruit on extracted olive oils

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Abstract: This study was achieved to examine the effects of freezing olive fruits of the Arbequina, Koroneiki and Mission cultivars (the most common olive oil producing cultivars in Iran) on the standard indices used for assessing virgin olive oil quality. Oil was obtained from olive fruits stored at -4˚C for 1 week and 3 weeks, and compared with oil obtained immediately after harvest (control). The quality indices of oils obtained from frozen fruit showed no significant degradation in quality compared with the control samples. In fact the peroxide value of the frozen fruits decreased compared to the control, which is considered to have a positive effect on oil quality. In addition, compositions of the main fatty acids are not altered by freezing which demonstrate frozen storage as a viable option. Oil derived from frozen olive fruit is not of inferior quality to non-frozen fruit in the production of olive oil.

1. Introduction

Conservation of foods prior to processing by means of cold and frozen storage has been a relatively recent technique coming to prominence over the last 50 years (Poerio *et al.,* 2008). Olive oil consumption is increasing throughout the world, even in countries that traditionally have not used olive oil. This trend has been promoted due mainly to the nutritional value of the Mediterranean diet (Patumi *et al.,* 2002). Olive fruits (*Olea europaea* L.) undergo some mechanical procedures (milling, malaxation and centrifugation) to extract extra virgin olive oil. The quality of virgin olive oil is intimately related to the characteristics and composition of the olive fruit at the time of its processing (Inarejos-Garcîa *et al.,* 2010). Highest quality extra virgin oils require optimum harvest stage, reduction in the time between harvest and milling, high quality oil extraction procedures and optimum storage conditions. The storage time between harvest and processing is one of the most important postharvest factors in oil quality. This becomes a significant problem when the volume of olive fruit exceeds the capacity of the mill plants. Olive fruits are often stacked

into large heaps at ambient temperature for several weeks prior to milling, which exceeds the storage limits (~48 hr) for the highest quality oil (Garcia *et al.,* 1996 a; Ranali *et al.,* 2000; Angerosa, 2002). During this period of time fermentation may also occur, and pressure and heat within the piles provide a medium for fungi and bacteria growth (Olias and Garcia, 1997). Anaerobic and aerobic processes take place inside and outside the piles of olive fruits, respectively, which causes deterioration of the fruit. This deterioration increases the acidity and reduces stability of the recovered oils (Garcia *et al.,* 1996 a). Increase in volatile acids (acetic and butyric acid) during decomposition results in an unpleasant musty smell (Olias and Garcia, 1997). Pigment content also decreases during this period, and additional refining needs to be done to clear all these unfavorable characteristics, increasing production cost and lowering market value (Gutierrez-Rosales *et al*., 1992). It is important to increase storage duration before milling the olive fruits to permit higher yields of better quality oil (Petruccioli and Parlati, 1987).

Studies have shown that cold storage can increase storage time without significantly affecting oil quality. Clovodeo *et al*. (2007) stored 'Coratina' olives used for oil production for 30 days at different temperatures and under different atmospheric conditions. They found that storage at 5°C, both under a flow of humidified air and a flow of 3% O_2 + 5% CO_2, produced oils that maintained their initial chemical qualities until the end of the experimentation. However, the olives stored at room temperature deteriorated after 15 days of storage and the extracted oil had significantly reduced quality.

Poerio *et al*. (2008) frozed olives at -18˚C for 24 hours and extracted the oil with and without thawing the fruits. Results were significantly different regarding peroxide value (PV), free fatty acids and polyphenols compared to control samples. Oils from frozen olives had lower free fatty acids and PVs and freezing reduced the oxidative stability of the oil.

The Iranian Government supports and subsidizes the expansion of olive cultivation to see about a six-fold increase in olive cultivated lands, i.e. from approximately 103,000 in 2014 to 600,000 ha by 2025, starting at 4,800 ha in 1993 at the launch of The Expansion of Olive Cultivation Plan (Asheri *et al.,* 2016). This has resulted in the spread of olive cultivation to areas where olive is not traditionally cultivated. Therefore, many of the olive groves produce small amounts of fruit and have no olive processing and oil extraction facilities, and the fruit must be transported to an extraction facility. The ability to reduce deterioration during the storage time between harvest and processing is important to maintain quality. Hence, it is worthwhile to discover ways to preserve olive fruits during this critical period.

The aim of this study is therefore, to find methods that allow olive fruits to be stored for longer periods of time, without compromising the quality of extracted oils. This study investigated the effect of freezing olive fruits at moderate temperatures (-4°C) on quality indices of extracted oil, to understand if it was possible to extract high quality oil from fruits frozen for extended periods of time. The study is aimed to examine how susceptible the oil quality of different cultivars is to freezing, and to establish if the fruit of a particular cultivar responds better to freezing. The study considered 3 freezing treatments for 3 cultivars: fresh samples (control), freezing for 1 week and 3 weeks.

2. Materials and Methods

The olive cultivars Mission, Arbequina, and Koroneiki were selected for study as they are commonly used for the production of olive oil in Iran. Fruits were hand-picked from Fadak Grove located near Qom, Iran (34° 30′ N, 51° 00′ E). The grove is located about 15 Km south of Qom, a city at 150 Km south of Tehran, in the central hyper-arid to arid parts of Iran. The fruits selected were at similar stages of ripening according to their ripening index which were 3.93, 3.84 and 4.23 for 'Arbequina', 'Koroneki' and 'Mission', respectively. The fruits were immediately transferred to the university lab, washed and de-leafed. Olives used to produce control samples were processed immediately, and the remaining olives stored in a freezer at -4°C.

Oil extraction

For oil extraction, only sound and undamaged fruits were used. Olives in the control samples were crushed using a hammer mill to form a paste. The paste was malaxed at 30˚C, then centrifuged at 4000 rpm for 10 minutes.

Olives used for treatment 1 were kept in freezer at -4˚C for one week and treatment 2 for 3 weeks at the same temperature. Frozen samples were crushed and malaxed until they reached 25˚C (due to not thawing the samples), and then centrifuged at 4000 rpm for 10 minutes. Extracted oil was collected using

a pipette and stored in dark glass jars in a refrigerator until analysis.

Determining oil content

Fifteen olive fruits from each replicate were placed in a Petri dish and then placed in an oven at 105°C for 48 h. The dried olives were used to determine oil content in three replicates. Olives were ground to a paste by mortar and pestle. The amount of 10 g of paste was placed in a Soxhlet cartridge and oil extracted with 150 ml hexane at 70°C. After 6 h of extraction, hexane was collected and oil content was calculated on dry mass basis (Agar *et al.,* 1998).

Peroxide value

The peroxide value of the oils was measured using the methods of Garcia *et al*. (1996 b). A 5 g sample of the extracted olive oil was placed in a 250 ml Erlenmeyer flask. The sample was shaken and then dissolved in 25 ml solution of acetic acid and chloroform (2:1, v/v). One milliliter of saturated potassium iodide (KI) solution was added. The mixture was placed in darkness for 5 min and then 75 ml of distilled water was added to stop the reaction. Half of one milliliter of freshly prepared starch indicator solution (0.5%) was added to each sample. Finally, the mixture was titrated with 0.01 N sodium thiosulfate until the blue indicator color disappeared. The peroxide value was expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2 kg^{-1}) (Agar *et al.,* 1998).

Coefficients of specific extinction values (K232 and K270)

Coefficients of specific extinction at 232 and 270 nm were measured by the methods reported in Regulation EEC/2568/91 of the European Union Commission (EEC, 1991). The amount of 1 g of oil sample was diluted in 100 ml isooctane. The sample was then homogenized using a vortex and the solution was transferred to a 10 mm cuvette. Absorbance at 232 and 270 nm was measured in a spectrophotometer using pure isooctane as a blank.

Fatty acid composition

Fatty acid composition in the oil was determined by the AOCS Official Method (1997). Methyl esters were prepared by vigorous shaking of a solution of oil dissolved in hexane (0.5 g in 7 ml) with 2 ml of 2N methalonic potash, and analyzed by gas chromatography. Chromatographic analysis was performed on a Trace GC (gas chromatograph), equipped with a flame ionization detector and split/splitless injector (Trace GC, ThermoFinnigan, Italy), using a silica capillary column, BPX-70 (30 m × 0.25 mm i.d. × 0.25 µm film thickness). The injector temperature was set at 250˚C and samples were injected manually (1 µL) with a split ratio of 1:80. The oven temperature was maintained at 175˚C for two min, and increased gradually to 230˚C at 3 ˚C/min and maintained for 10 min. Nitrogen was used as carrier gas at a flow rate of 0.8 ml/min. The detector temperature was maintained at 270˚C. Fatty acids were identified by comparing retention times with those of standard compounds. Oxidizability (Cox value) was calculated based on the fatty acid content of three unsaturated fatty acids [oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3)] using following relation (Fatemi and Hammond, 1980).

$$\text{Oxidizability} = [1 \times (C18{:}1\%) + 10.3 \times (C18{:}2\%) + 21.6 \times (C18{:}3\%)]$$

Determining oil chlorophyll and carotenoid content

Pigment contents were assayed using the spectrophotometric method of Minguez-Masquera *et al*. (1991). One gram of oil was dissolved in 10 ml of isooctane and the resulting solution transferred to a cuvette. Absorbance at 470 and 670 nm (for carotenoid and chlorophyll, respectively) was measured in a spectrophotometer (unico/2800 uv/VIS) using pure isooctane as a blank. The results are expressed as milligram of carotenoid or chlorophyll per kilogram of oil.

Statistical analysis

Experimental layout was factorial with three levels of freezing treatments and three olive cultivars. Data were analyzed by SPSS software. Comparison of means is performed using Duncan's multiple range test at a 95% confidence level and tables for analysis of variance (ANOVA) are provided.

3. Results and Discussion

Oil content

In fruit from the Koroneiki and Arbequina cultivars, there is no statistically significant difference in oil content between the control and the frozen samples (Table 1). However, the Mission cultivar fruit frozen for 1 and 3 weeks showed a significant decrease in mean oil content compared with the control under Duncan's multiple range test at 5%. Since the independent variables (i.e. cultivar and freezing) act independently, freezing had no significant overall effect on the content of extracted olive oil (Table 2). However in interpreting the results, it needs to be

considered that oil extraction of frozen treatments was performed at a different temperature (25°C) in respect to the control (30°C).

Peroxide value

The peroxide value (PV) is a measure of primary oxidation. Table 1 shows the measured mean PV (meq O_2 /kg) of the oils obtained from olives stored for the different time periods. Our data showed that freezing reduces the peroxide value. The reduction was significant when olive fruits were frozen for three weeks, but not significant when frozen for only 1 week. Cultivar's effect in reduction of PV was significant at a 95% statistical level, while freezing had a very significant effect at a 99% level (Table 2). No oil had peroxide values above the limit for extra virgin olive oil (20 meq O_2/kg).

Table 1 - Means of chemical characteristics of olive oils derived from fresh and frozen olive fruits and their comparison with Duncan's multiple range test at 5%

Samples	Oil content (% of dry matter)	PV (meq O_2 kg^{-1})	K232 nm	K270 nm	Chlorophyll (mg kg^{-1})	Carotenoid (mg kg^{-1})
M C	47.21 a	7.33 a	1.13 bc	0.08 c	2.22 a	1.84 a
M T1	43.08 b	6.63 ab	1.06 c	0.08 c	2.14 a	1.86 a
M T2	42.73 b	5.47 bcd	1.05 c	0.08 c	2.06 a	1.79 ab
K C	43.34 b	6.97 a	0.82 d	0.13 ab	2.20 a	1.53 bc
K T1	41.80 b	6.53 ab	0.80 d	0.12 b	2.03 a	1.50 bc
K T2	41.65 b	5.2 cd	0.76 d	0.12 b	2.02 a	1.43 c
A C	43.57 b	6.53 ab	1.33 a	0.15 a	2.12 a	1.52 bc
A T1	45.00 ab	5.63 bc	1.24 ab	0.14 ab	2.01 a	1.48 c
A T2	42.82 b	4.47 d	1.22 ab	0.14 ab	1.98 a	1.48 c

M= Mission; K= Koroneiki; A= Arbequina; C= control sample;
T1= 1 week freezing sample;
T2= 3 week freezing sample;
PV= peroxide value.
Means within a column with the same lowercase letters are not significantly different.

Poerio *et al*. (2008) and Gomez and Escoda (2010) also found reduced peroxide values in oil from olive paste and fruits that had been frozen. The lower peroxide value of oil from frozen fruit could be due to reduced enzymatic activity during crushing-malaxation due to the initial low temperature.

Specific extinction coefficients at 232 nm and 270 nm

The K_{232} and K_{270} values for all treatments were below the maximum permitted values for extra virgin olive oil [2.50 and 0.20 respectively, according to Regulation EEC (1991)]. Across all cultivars there was a net decrease in the K_{232} values due to freezing, but the difference was statistically insignificant. No change was detected in the K_{270} values between treatments (Table 2). Between cultivars however, the difference in the K_{232} and K_{270} values was significant with the Arbequina cultivar showing the highest values, 'Koroneiki' showing the lowest K_{232} and intermediate K_{270}, and 'Mission' showing intermediate K_{232} and the lowest K_{270} (Table 1). These differences were consistent with the variations between olive cultivars shown by Asheri *et al*. (2016). Gomez and Escoda (2010) also showed freezing to have no effect on the K_{270} and K_{232} values independent of cultivar.

Pigment content (chlorophyll and carotenoid)

The slight decrease in the pigment content observed between the frozen treatments and the control was not statistically significant (Tables 1, 2). Also different cultivars did not demonstrate different levels of chlorophyll content. Carotenoid content, on the other hand, was significantly different among the cultivars at 99% statistical level. Chlorophylls and carotenoids play crucial roles in health and also in the oxidative activity of processed food stuff, due to their antioxidant nature in the dark and pro-oxidant activi-

Table 2 - Results of ANOVA for chemical characteristics of olive oils derived from fresh and frozen olive fruits

Source	Mean square					
	Oil content	PV	K232	K270	Chlorophyll	Carotenoid
Treatments	8.83 NS	2.63 **	0.129 **	0.002 **	0.023 NS	0.091 *
Cultivar (A)	10.46 NS	2.11 *	0.50 **	0.009 **	0.024 NS	0.352 **
Freezing (B)	12.14 NS	8.35 **	0.16 NS	0.00004 NS	0.066 NS	0.008 NS
AB interaction (cv *fr)	6.37 NS	0.04 NS	0.001 NS	0.000004 NS	0.002 NS	0.002 NS
Error	3.56	0.39	0.004	0.00009	0.058	0.026

PV= Peroxide value.
NS= not significant; * significant at 5% level; ** significant at 1% level.

ty in the light (Fakourelis *et al.,* 1987). The higher the amount of these pigments, the higher the resistance to oil oxidation. Our results indicated that freezing did not change olive oil resistance to oxidation based on pigment contents.

Kiritsakis *et al*. (1998) found that storage at lower temperatures reduced the pigment content of the extracted oils and Morello *et al*. (2003) found slight decreases in chlorophyll and carotenoid concentration of oils obtained from frosted olives. These authors suggested that this could be due to the involvement of chlorophyllase and lypoxygenase. Amongst cultivars, the Mission cultivar shows significantly higher carotenoid content than 'Koroneiki' and 'Arbequina', as observed by Asheri *et al.* (2016).

Fatty acid composition

This paper reports 12 fatty acids detected in GC analysis including myristic acid (C14:0), palmitic acid (C 16:0), palmitoleic acid (C 16:1), heptadecanoic acid (C 17:0), heptadecenoic acid (C 17:1), stearic acid (C 18:0), oleic acid (C 18:1), linoleic acid (C 18:2), linolenic acid (C 18:3), arachidonic acid (C 20:4), arachidic acid (C 20:0), and erucic acid (C 22:1) (Table 3).

Analysis of variance (ANOVA) shows that freezing olive fruits did not have significant effects on the fatty acid composition of the oils, while cultivars had very significant influence on all the fatty acids except for myristic acid. In addition, freezing and cultivar variables acted independently on fatty acids level, except for myristic and palmitic acids in which the interaction of variables were very significant (at 99%) and significant (at 95%), respectively (Tables 4, 6).

Comparison of the means demonstrate that 'Koroneiki' and 'Mission' had significantly higher levels of oleic acid (74.85 and 72.96% in fresh state, respectively) than 'Arbequina' (60.76%). Oleic acid is the most prominent fatty acid in olive oil and is a monounsaturated fatty acid (MUFA) with demonstrated qualities in the stability of the oil. Oleic acid levels did not change significantly during the storage except for the 1 week freezing of 'Arbequina' (Table 5). The other predominant fatty acids are palmitic acid [a saturated fatty acid (SFA)] and linoleic acid [a polyunsaturated fatty acid (PUFA)]. 'Arbequina' had the highest amounts of palmitic and linoleic fatty acids. This indicates lower quality of its oil, especially when it is considered together with its lower oleic

Table 3 - Fatty acid (Myristic, Palmitoleic, Heptadecanoic, heptadecenoic, Stearic, Linolenic, Arachidic, arachidonic and Erucic acids) content (%) of olive oils derived from fresh and frozen olive fruits and comparison of the means with Duncan's multiple range test at 5%

Samples	Myristic acid	Palmitoleic acid	Heptadecanoic acid	Heptadecenoic acid	Stearic acid	Linolenic acid	Arachidic acid	Arachidonic acid	Erucic acid
M C	0.010 d	0.730 bc	0.0255 b	0.044 b	2.55 b	1.05 a	0.290 cd	0.409 a	0.068 c
M T1	0.014 d	0.701 bc	0.024 b	0.042 b	2.44 b	1.00 ab	0.281 d	0.387 ab	0.065 c
M T2	0.051 a	0.682 c	0.022 b	0.038 b	2.38 b	0.95 b	0.271 d	0.382 abc	0.063 c
K C	0.030 b	0.798 bc	0.031 b	0.050 b	3.26 a	0.76 c	0.465 a	0.379 abc	0.137 a
K T1	0.018 bcd	0.828 b	0.035 b	0.051 b	3.20 a	0.80 c	0.459 a	0.356 c	0.132 a
K T2	0.011 d	0.817 b	0.032 b	0.050 b	3.16 a	0.77 c	0.448 a	0.359 bc	0.136 a
A C	0.028 b	2.512 a	0.070 a	0.169 a	1.43 c	0.68 d	0.324 b	0.159 d	0.114 b
A T1	0.027 bc	2.515 a	0.070 a	0.170 a	1.45 c	0.68 d	0.319 bc	0.151 d	0.103 b
A T2	0.016 cd	2.579 a	0.072 a	0.169 a	1.44 c	0.69 d	0.319 bc	0.167 d	0.107 b

M= Mission; K= Koroneiki; A= Arbequina; C= control sample.
T1= 1 week freezing sample; T2= 3 week freezing sample.
Means within a column with the same lowercase letters are not significantly different.

Table 4 - Results of ANOVA for fatty acids content (reported in table 3) of olive oils derived from fresh and frozen olive fruits

Source	Mean square					
	Oil content	PV	K232	K270	Chlorophyll	Carotenoid
Treatments	8.83 NS	2.63 **	0.129 **	0.002 **	0.023 NS	0.091 *
Cultivar (A)	10.46 NS	2.11 *	0.50 **	0.009 **	0.024 NS	0.352 **
Freezing (B)	12.14 NS	8.35 **	0.16 NS	0.00004 NS	0.066 NS	0.008 NS
AB interaction (cv *fr)	6.37 NS	0.04 NS	0.001 NS	0.000004 NS	0.002 NS	0.002 NS
Error	3.56	0.39	0.004	0.00009	0.058	0.026

PV= Peroxide value.
NS= not significant; * significant at 5% level; ** significant at 1% level.

acid content. Palmitic and linoleic acids amounts in the Mission cultivar and linoleic acid in 'Koroneiki' show significant changes after three weeks of frozen storage (Table 5). Among the fatty acids of lower content, only linolenic and myristic acids demonstrate significant changes in their mean values (Table 3). Linolenic and myristic acid contents of the Mission cultivar show significant increases after three weeks of frozen storage. Myristic acid levels of 'Koroneiki' and 'Arbequina' decreased due to freezing.

Total SFAs, MUFAs and PUFAs did not demonstrate any statistically significant change under frozen storage, and all the change is due to cultivar (Table 6). Cultivar had very significant effect on the ratios of MUFA/PUFA and oleic/linoleic acids, and the effect of freezing on these ratios was significant at 95% confidence level. Also, the analysis indicated that cultivar and freezing have significant interaction during the storage of olive fruit on these ratios. Cox value, however was not influenced significantly by freezing (Table 6).

Mean total SFA levels for 'Koroneiki' and 'Arbequina' did not change, while 'Mission' showed a significant reduction in total SFA after 3 weeks of freezing. This change could be due to the reduction of palmitic acid. Means comparison of total MUFA content did not detect any significant changes. 'Koroneiki' shows a significant increase in the total PUFA content after 3 weeks of frozen storage, which leads to a reduction of mean MUFA/PUFA and oleic/linoleic acids ratios. Similar to MUFA/PUFA and oleic/linoleic acids ratios, Cox values did not change significantly for 'Mission' and 'Arbequina', while 'Koroneiki' experienced a significant decrease for 3 weeks freezing treatment. The higher the MUFA/PUFA and oleic/linoleic acids ratios and the lower the Cox values, the higher the oxidative stability of the oil. The implication of the reduction in MUFA/PUFA and oleic/linoleic acids ratios and increase in Cox values after 3 weeks freezing for 'Koroneiki' is that longer periods of freezing storage could reduce oxidative stability of its oil. However, it is important to note that despite the changes after 3 weeks of storage, 'Koroneiki' oil still possessed signif-

Table 5 - Fatty acid (Palmitic acid, Oleic acid, Linoleic acid, ∑SFA, ∑PUFA, MUFA/PUFA, Oleic/Linoleic and cox value) compositions and comparison of the means with Duncan's multiple range test at 5%

Samples	Palmitic acid	Oleic acid	Linoleic acid	ΣSFA	ΣMUFA	ΣPUFA	MUFA/PUFA	Oleic/linoleic acid	Cox value
M C	11.11 c	72.96 c	10.47 c	14.25 c	73.80 c	11.93 b	6.19 c	6.97 c	2.03 b
M T1	10.73 c	73.49 bc	10.53 c	13.76	74.30 c	11.91 b	6.24 c	6.98 c	2.03 b
M T2	10.23 d	73.60 bc	11.00 b	13.24 d	74.39 bc	12.34 b	6.03 c	6.69 c	2.07 b
K C	12.48 b	74.85 a	6.51 e	16.51 b	75.83 a	7.65 d	9.92 a	11.52 a	1.58 d
K T1	12.48 b	74.74 a	6.62 e	16.43 b	75.75 a	7.78 cd	9.74 a	11.29 a	1.60 cd
K T2	12.47 b	74.15 ab	7.09 d	16.37 b	75.15 ab	8.22 c	9.14 b	10.46 b	1.64 c
A C	16.63 a	60.76 e	15.96 a	18.73 a	63.56 d	16.80 a	3.78 d	3.81 d	2.40 a
A T1	16.54 a	61.76 d	15.94 a	18.65 a	64.55 d	16.77 a	3.85 d	3.87 d	2.41 a
A T2	16.96 a	61.53 de	15.68 a	19.05 a	64.39 d	16.54 a	3.89 d	3.92 d	2.38 a

M= Mission; K= Koroneiki; A= Arbequina; C= control sample.
T1= 1 week freezing sample; T2= 3 week freezing sample.
SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acid; Cox value: calculated oxidizability.
Means within a column with the same lowercase letters are not significantly different.

Table 6 - Results of ANOVA for fatty acids (reported in table 5) composition of olive oils derived from fresh and frozen olive fruits

Source	Mean square								
	Palmitic acid	Oleic acid	Linoleic acid	ΣSFA	ΣMUFA	ΣPUFA	MUFA/PUFA	Oleic/linoleic acid	Cox value
Treatments	14.46**	80.35 **	31.51 **	9.76 **	58.30 **	29.31 **	12.70 **	19.90 **	0.23 **
Cultivar (A)	57.37 **	320.35 **	126.63 **	38.45 **	232.15 **	116.90 **	50.43 **	78.93 **	0.93 **
Freezing (B)	0.06 NS	0.338 NS	0.13 NS	0.13 NS	0.33 NS	0.10 NS	0.14 *	0.30 *	0.001 NS
AB interaction (cv*fr)	0.21 *	0.365 NS	0.14 NS	0.24 *	0.36 NS	0.12 NS	0.11 *	0.19 *	0.001 NS
Error	0.03	0.142	0.04	0.05	0.12	0.05	0.03	0.04	0.0004

SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acid.
Cox value: calculated oxidizability;
NS= not significant; * significant at 5% level; ** significant at 1% level.

icantly higher MUFA/PUFA and oleic/linoleic acid ratios and lower Cox values than the other cultivars, and therefore indicated the highest resistance to oxidative stability among these three cultivars.

4. Conclusions

The oils obtained from olive fruits stored at -4˚C are demonstrated to maintain the same characteristics of the control.

Among the biochemical indices, the decrease in PV was the only factor that varied significantly from the control samples. This decrease is received positively, as less oxidation occurs. Pigment content remained stable during freezing period which is considered to be good to maintain quality oil. No differences in characteristics evaluated in this study were observed between cultivars, suggesting that freezing the olive fruits of all three cultivars in this research did not have any negative effect on the studied characteristics of the extracted oil. Based on this study, freezing could be a suitable method of preserving olive fruits in the waiting period between harvesting and processing, which could assist in the maintenance of characteristics evaluated in this study during olive oil production. However, it may be worthwhile extending this experiment with different cultivars and to test characteristics such as sensory quality evaluation and oxidative stability. Nonetheless, our results suggest that olive fruit may be stored frozen before processing into oil. So the products could be harvested at the optimal stage of ripening and preserved frozen while shipping them to the mill plant stations. This preservation system could be extended to the pre-processing waiting period at the mill plant, so that the processing of olives could be optimized.

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Aggressiveness of four Fusarium head blight species on wheat cultivars

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Key words: disease development, diseased-head severity, *Fusarium* species, soft dough stage, Syrian wheat cultivars.

Abstract: Aggressiveness of four Fusarium head blight species (*F. culmorum*, *F. solani*, *F. verticillioides* and *F. equiesti*) was studied on six Syrian wheat cultivars under controlled conditions. Two aggressiveness criteria: diseased-head severity (DHS, Fusarium infection) and disease development (DD, Fusarium spread) were visually estimated as percentage of heads showing Fusarium symptoms in wheat cultivars at the soft dough stage. Results showed significant differences among fungal isolates and wheat cultivars for the two tested criteria. The mean values of DHS evaluations ranged from 33.27 to 45.49% among fungal isolates, and from 29.62 to 42.22% among tested cultivars. The mean DD rating varied from 25.58 to 35.43% among fungal isolates, and from 25.33 to 34.01% among tested cultivars. Results in the current research highlighted that the level of resistance in Syrian cultivars to *Fusarium* species is characterized with low to moderate DHS and DD evaluations (%). Also, the results were comparable with those previously obtained using the same fungal isolates and wheat cultivars *in vitro*. The current study confirmed the suitability of *in vitro* method to be used as fast and reliable test to analyze aggressiveness in *Fusarium* species.

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1. Introduction

Fusarium head blight is one of the most destructive global diseases of wheat. In infected plants, it leads to kill the developing seed (prematurely bleached spikes) within moist conditions and moderate temperatures prevail during flowering. Since it was identified in 1884, severe epidemic outbreaks caused quantitative losses in yield of up to 50-75% (Parry *et al.,* 1995; McMullen *et al.,* 2012). It also reduces grain quality due to contamination of harvest with large amount of mycotoxins that cause toxicities to human and livestock (Maresca, 2013). At least seventeen *Fusarium* species with several habitats and types of mycotoxins produced have been associated with *Fusarium* species (Parry *et al.,* 1995). *Fusarium graminearum* is the main causal agent of this disease and has been subdivided into at least 11 cryptic species (O'Donnell *et al.,* 2004). Other species can cause Fusarium disease on a lesser scale such as *F. avenaceum*, *F. culmorum*, *F. solani*, *F. equiseti*, *F. verticillioides* and *F. poae*

(Xu *et al.,* 2008).

Understanding the interaction between wheat plants and Fusarium populations requires more detailed knowledge about the variation of aggressiveness (Wu *et al.,* 2005). Van der Plank (1968) defined aggressiveness as a quantitative ability of an isolate to cause disease on a susceptible host plant in a non-race-specific pathosystem. Aggressiveness is an important factor determining the potential ability of Fusarium isolates to cause Fusarium epidemics. Variability of quantitative component of pathogenicity in *F. graminearum* has been the subject of several studies (Parry *et al.,* 1995; Leonard and Bushnell, 2003; Wu *et al.,* 2005; McMullen *et al.,* 2012). However, other Fusarium species have attracted less pathogenic analyses (Xu *et al.,* 2008; Bakri *et al.,* 2012; Sakr, 2017). Fusarium resistance in wheat plants is conferred by quantitative trait loci (QTL) detected on all chromosomes (Loffler *et al.,* 2009). It is necessary to combine type I (resistance to initial infection) and type II (resistance to spreading) to get Fusarium resistant wheat plants (Loffler *et al.,* 2009).

In Syria, about 1.7 million hectares were sown to wheat, with an annual production of 3.9 million tons in 2011. Host-pathogen interactions were evaluated for several local wheat cultivars inoculated with fungal isolates of different species associated with *Fusarium* species, and differential reactions on cultivars were detected (Alazem, 2007; Talas *et al.,* 2011; Bakri *et al.,* 2012). Recently, Sakr (2017) analyzed aggressiveness of four Fusarium species *in vitro*, and significant differences were detected between pathogen isolates and wheat genotype. In order to underline pathogenic variation for *Fusarium* species collected from Ghab Plain, one of the principal Syrian wheat production areas, the objectives of the current study were to (1) evaluate aggressiveness of four isolates [F2 (*F. culmorum*), F27 (*F. verticillioides*), F35 (*F. solani*), and F43 (*F. equiesti*)] on six wheat Syrian cultivars under controlled conditions, and (2) compare results previously obtained by Sakr (2017) *in vitro* with the current data from floret inoculation under controlled conditions.

2. Materials and Methods

Fungal isolates and inoculum production

The fungal isolates of four *Fusarium* species [*F. culmorum* (F2), *F. verticillioides* (F27), *F. solani* (F35), and *F. equiesti* (F43)] were collected in 2015 from naturally wheat spikes exhibiting Fusarium symptoms from different locations of Ghab Plain in 2015. Isolates were identified morphologically according to Nelson *et al.,* (1983). The cultures were maintained in sterile distilled water at 4°C and freezing at -16°C until needed.

For inoculum preparation, four to six agar plugs out of the stored isolates were put over the surface of PDA in 9-cm Petri dishes and incubated for 10 days, at 22°C in the dark to allow mycelial growth and sporulation. Ten ml of sterile distilled water were added to each dish, and the resulting spore suspensions were adjusted to 5×10^4 spores/ml for inoculation following a count in a hemacytometer (Bakri *et al.,* 2012).

Wheat cultivars and growth chamber conditions

In the current study, aggressiveness for the four Fusarium head blight isolates was measured on six wheat cultivars previously analyzed *in vitro* ('Cham1', 'Cham7', 'Acsad65', 'Cham4', 'Cham6' and 'Douma4', most cultivated in different Syrian areas) under controlled conditions.

Wheat seeds were surface-sterilized with 5% sodium hypochlorite solution for 8 min and then washed six times in sterile distilled water (Purahong *et al.,* 2012). They were sown into plastic pots (15-cm) filled with 2 kg of sterilized soil (ten seeds per pot), and arranged in a complete randomized design with three replicates. Three plots per replicate were left non-inoculated as control treatment. Pots were placed in a growth chamber operated at 20°C during day and night with an 16-h photoperiod. Following emergence, plants were thinned to three per pot and nitrogen fertilizer was applied twice at two dates: emergence and tillering.

Aggressiveness tests

At 10-14 days after heading, spore suspensions of the four Fusarium head blight isolates or sterile distilled water (control) were sprayed one time into flowering spikes. Six flowering spikes were randomly selected within each replicate of the six cultivars. After the inoculum dried for 30 min, inoculated spikes were then kept covered for 48 h using polythene bags to ensure 100% RH.

Head blight symptoms were evaluated as percentage of spikes showing Fusarium symptoms after 7, 14, and 21 days, when plants were at the soft dough stage. Fusarium disease severity was visually estimated *in situ* for each inoculated spike using the Xue's *et al.,* (2004) scale. This scale includes nine levels of incidence expressed in percentage of bleached spike area on a head: 0 (no visible Fusarium symptoms) to

9 (severely diseased, spike dead). Each head was assessed separately in all experiments.

The values of diseased-head severity as percentage of infected spikes measured 21 days after inoculation (DAI) were considered, for each cultivar, a parameter to determine initial infection. The values of disease development calculated by the means of each evaluation; 7, 14 and 21 DAI over the estimation time were considered, for each cultivar, a parameter to determine pathogen spreading.

Statistical analyses

Statistical analyses of aggressiveness data were performed using StatView, 4.57® Abacus Concepts, Berkley, Canada. Before statistical analysis, the percentages were transformed using the Arcsines function. A complete randomized design with two factors (Fusarium isolate and wheat genotype) and 3 replications was used for aggressiveness analysis. Fisher's LSD test was used to compare the means at $P < 0.05$.

3. Results and Discussion

Understanding the interaction between Fusarium head blight species and wheat plants requires knowledge of the variation of quantitative component of pathogenicity (Wu *et al.,* 2005). With this in mind, aggressiveness variability for four local *Fusarium* species was analyzed by using a floret inoculation in a growth chamber on six wheat cultivars most cultivated in different Syrian areas.

Differences in aggressiveness of four *Fusarium* species (*F. culmorum*, *F. verticillioides*, *F. solani*, and *F. equiesti*) are indicated when isolates vary in the amount of damage that they cause in wheat plants. The results demonstrated that none of the six tested cultivars was immune from disease. However, typical Fusarium symptoms induced by the four isolates (F2, F27, F35 and F43) were clear and easy to score in the inoculated spikes, while no symptoms were present in the control (Fig. 1). The mean values of diseased-head severity (DHS) ranged from 33.27% to 45.49% as compared with 0% for the control treatment (Table 1). There were significant differences among four isolates (F isolates=4.376; Probability=0.0084). The mean values of disease development (DD) varied from 25.58% to 35.43% (Table 1). Significant differences among four isolates (F isolates=4.257; Probability=0.0096) were detected. Results in the current study showed that there was no interaction between Fusarium isolates and host plant for the two parameters. This indicates to non-race-specific interaction described for this pathosystem (Loffler *et al.,* 2009).

Fig. 1 - Fusarium head blight symptoms on spike of Syrian wheat cultivar Cham4 inoculated with isolate F35 (*Fusarium solani*) compare with control (water).

High diseased-head severity and disease development values represent high aggressiveness (Parry *et al.,* 1995; Wu *et al.,* 2005; Xu *et al.,* 2008). Results shown in Table 1 indicate that the isolate F35 of *F. solani* was the most aggressive with a mean value of DHS of 45.49% and DD of 35.43%; followed by other tested isolates. These results are in accordance with previous analysis on the aggressiveness of theses isolates *in vitro*; Sakr (2017) observed that the isolate F35 was the most aggressive one, followed by other analyzed isolates. The current study confirmed the suitability of *in vitro* modified Petri-dish method to be used as fast and reliable test to analyze aggressiveness in Fusarium species. Results in the current study are comparable with those found by Alazem (2007) and Bakri *et al.,* (2012) for *F. culmorum*, *F. solani*, *F. verticillioides* and *F. equiesti* in which significant differences were detected for aggressiveness among fungal isolates in each Fusarium head blight species in a growth chamber.

The mean value of DHS and DD rating for six

Table 1 - Diseased-head severity and disease development scores in % among isolates of four Fusarium head blight species measured on six Syrian wheat cultivars

Isolate	'Cham1'	Cham7'	Acsad65'	Cham4'	Cham6'	Douma4'	Mean
Diseased-head severity scores (%)							
F2	25.92	25.92	29.62	37.03	44.44	44.44	35.24
F27	29.62	29.62	37.03	33.33	37.04	36.66	33.27
F35	33.33	33.33	40.74	58.88	47.77	47.77	45.49
F43	29.62	29.62	44.44	40.00	37.04	40	37.40
Mean	29.62	29.62	37.96	42.31	41.57	42.22	
	F isolates=4.376; Probability=0.0084						
	F cultivars=2.907; Probability=0.0226						
	F interactions=0.601 NS; Probability=0.8594						
Diseased development scores (%)							
F2	23.56	24.69	21.16	30.86	40.4	29.62	28.38
F27	24.69	23.56	28.49	25.64	24.69	26.45	25.58
F35	30.30	31.74	29.10	45.58	32.09	43.77	35.43
F43	22.79	25.64	37.03	33.95	26.45	29.10	29.16
Mean	25.33	26.41	28.94	34.01	30.91	32.23	
	F isolates=4.257; Probability=0.0096						
	F cultivars=1.870; Probability=0.1171						
	F interactions=1.199 NS; Probability=0.3051						

Fusarium head blight incidence scores were evaluated as percentage of spikes showing *Fusarium* species symptoms using the Xue's *et al*., (2004) scale.
F tests (P<0.05), NS= not significant.

wheat cultivars (Table 1) reflects the ability of the same isolate of the pathogen (F2, F27, F35 and F43) to distinguish different levels of resistance as observed for the same pathosystem (Alazem, 2007; Talas *et al.,* 2011). Also, the resistance of a given wheat cultivar is not related to a certain Fusarium species (Table 1). Significant differences were underlined for DHS (F cultivars=2.907; Probability=0.0226) and DD (F cultivars=1.870; Probability=0.1171) criteria among wheat cultivars (Table 1). The mean values of DHS evaluations ranged from 29.62 to 42.22% among tested cultivars. The mean DD rating varied from 25.33 to 34.01% among tested cultivars. Quantitative resistant wheat cultivars are identified by low DHS and DD values of the fungus compared with the susceptible one (Parry *et al.,* 1995). Results in the current research highlighted that the level of resistance in Syrian cultivars to Fusarium species is characterized with low to moderate DHS and DD evaluations (%). These results are in accordance with previous analysis on the comportment of local wheat cultivars in which differential reactions on cultivars were detected (Alazem, 2007; Bakri *et al.,* 2012; Talas *et al.,* 2011). However, Fusarium resistance scores ranged one fold and half between resistant and susceptible cultivars for the two tested parameters (Table 1). Thus our observation suggests that in resistant wheat cultivars, the development of the pathogen was slowed, and may be due to resistance mechanisms expressed by accumulation of QTL in host cultivars (Alazem, 2007; Talas *et al.,* 2011). Results in the current study showed that the level of quantitative resistance in the six wheat cultivars made it possible to detect significant differences between isolates of four *Fusarium* species. These results are in accordance with our previous analysis on the behavior of theses cultivars *in vitro* (Sakr, 2017). The variability of resistance for the Syrian cultivars is interesting and promising for ecological framing/breeding and also for improving resistance of wheat cultivars.

For *F. graminearum*, Purahong *et al.,* (2012) validated the modified Petri-dish method (used by Sakr, 2017) by highly significant correlation with the data from floret inoculation in adult plants in a growth chamber. Results indicted that the Petri-dish aggressiveness test conducted on other Fusarium species (*F. culmorum*, *F. verticillioides*, *F. solani*, and *F. equiesti*) is repeatable and stable with the six wheat cultivars in a growth chamber (Table 1). It will be necessary to analyze the pathogenic variation in a large number of Fusarium isolates on several wheat cultivars under controlled and field conditions to screen Fusarium resistance in Syrian wheat cultivars.

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Effect of different physio-chemical factors on sex expression and fruit yield in greenhouse cucumber

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Abstract: Male flower expression is considered an important aim in greenhouse cucumber breeding for creating paternal lines as a base for hybrid progeny. The study was carried out to evaluate the effects of different treatments on sex expression and fruit yield of cucumber in two different season (autumn-winter and spring-summer), in particular this research focuses on the influence of 1) usage of two chemical agents: silver thiosulphate [$Ag(S_2O_3)_2^{-3}$] and silver nitrate ($AgNO_3$) at different concentration, respectively 200 and 500 ppm and 100, 200 and 300 ppm 2) plant development stages at the moment of the treatment (5, 10, and 15-leaf growth stages) and 3) application of single or double sprayings. Analysis of variance showed that season, chemical applications and number of spaying had significant effect on the induction of a higher number of male flowers. A positive significant effect of season suggested that longer days and higher temperature promote the formation of male flowers in cucumber. This study showed that male flower production was induced by all concentrations of silver ions, especially high concentrations. Important traits related to change of sex expression such as the number of days to male flowering and the number of male flowers are more affected by different interactions of studied factors in contrast to vegetative and yield related traits. Also, the quadruple interaction effects indicated that silver ion could change sex expression at higher temperatures and longer days (second season in summer) with high concentration when applied in 15 leaf stage for $AgNO_3$ and 5 and 15 leaf growth stage for $Ag(S_2O_3)_2^{-3}$ with double spraying. Consequently, female lines can be induced to male flowering with silver ions, thus increasing the feasibility of large scale seed production of gynoecious × gynoecious cucumber hybrid.

1. Introduction

Commercial cucumber (*Cucumis sativus* L., 2n= 2x= 14) is a member of cucurbitaceae that is indigenous to India (Renner *et al.*, 2007). It is one of

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the most economically important cucurbit vegetable plants (Tatlioglu, 1993, Robinson and Decker-Walters, 1997) and among the most widely-grown vegetable crop in the world, after tomato, onion and cabbage (Plader *et al.*, 2007). Cucumber is used in different types (Harvesting, slicing and fresh eating) that are used as fresh or processed vegetable (Shetty and Wehner, 2002). The cucumber is a thermophilic and frost- susceptible crop, usually cultivated in fields during the spring-summer period. Its high demand has also made it an important crop to be widely grown in glasshouses or plastic houses (Sarkar and Sirohi, 2011). Sex expression is an important factor that has a positive effect on yield and that constitutes a major component of cucumber improvement programs (Yamasaki *et al.*, 2003). In gynoecious cultivars, all flowers are female, so, male flowers are required for new line production via female flowers crossing. The sex expression of *Cucumis sativus* L. is determined by genetics as well as environment (e.g. photoperiod, temperature) (Yamasaki *et al.*, 2003; Bano and Khokhar, 2009). Change from vegetative growth to reproductive stages is a complex process regulated by many factors, and could be influenced by the application of plant growth regulators (Ainsworth, 1999; Sure *et al.*, 2013). Growth regulators have tremendous effects on sex modification and flowering in cucurbits that leading to either suppression of male flowers or an increase in the number of female flowers (Al-Masoum and Al-Masri, 1999). Some researchers have already reported the effects of plant growth regulators on modification of sex expression in cucumber flowers (Vadigeri *et al.*, 2001; Rafee kher *et al*., 2002; Bano and Khokhar, 2009).

Line production is important in breeding programs, because many cultivars in cucumber are hybrids (Golabadi *et al.*, 2015). In gynoecious cucumbers, male flower induction is necessary for production of F_1 hybrid seeds (Yamasaki *et al.*, 2003; Wang *et al.*, 2011). Therefore, change of sex expression in gynoecious cucumber is necessary.

In general, auxin, ethylene and cytokinins promote female sex expression in various monoecious and dioecious systems (Mohan Ram and Sett, 1982). Exogenous application of plant growth regulators could alter the sex ratio if applied at the two- or four-leaf stage, which is the critical stage at which the suppression or proportion of either sex is possible (Hossain *et al.*, 2006). The effect of ethylene can be antagonized by specific ethylene inhibitors (Hirayama and Alonso, 2000). Silver ions (Ag^+) restrain the physiological effect of the ethylene by blocking the ethylene receptors (Hirayama and Alonso, 2000). It is known that silver ions (Ag^+) applied as silver nitrate ($AgNO_3$) or as silver thiosulfate [$Ag(S_2O_3)_2^{-3}$] replace copper ions (Cu^+) which is a part of the ethylene receptor preventing the receptor from responding to ethylene (Abeles *et al.*, 1992). Another inhibitor of the ethylene action, $AgNO_3$, suppresses the development of female flowers and induces the male ones (Kumar *et al.*, 2009; Stankovic and Prodanovic, 2002). Male flower formation increased proportionally with the concentration of $AgNO_3$ applied (Stankovic and Prodanovic, 2002).

According to previous reports, $AgNO_3$ induced more male flowers than GA_3 on gynoecious breeding lines of cucumber (Kalloo and Franken, 1978) and summer squash (Yongan *et al.*, 2002). The positive effects of $AgNO_3$ on male flower production in cucumber have reported by Karakaya and Padem (2011). Thappa *et al*. (2011) used different plant growth regulators (ethephon, naphthalene acetic acid and maleic hydrazide) at the two, four and six-leaf and full-bloom stages in cucumber to induce male flowers. Law *et al*. (2002) reported that silver thiosulfate, an ethylene inhibitor, enhanced stamen development in female white campion (*Silene latifolia*). However, little effort seems to have been directed toward the study of the effects of silver ions on male flower production sprayed at different growth stages and if the numbers of spray applications could improve the effectiveness of the induction. On the other hand, it seems that there are few studies that correlate the effect of environmental conditions, especially season, to the number of spraying and leaf-stage growth on male flower induction of plant treated with silver ions.

Although silver is a toxicity ion, however the amount of its toxicity depends on species, silver concentration, times of application and plant growth stage spraying and exposure media. For exmple Fuente *et al*. (2014) applied different concentrations of $AgNO_3$ (0, 30, 60, 90, 200 mg l^{-1}) at intervals of 8 days throughout the crop cycle (90 days) in watermelon. Their results showed that silver accumulate in root more than other parts of plants and antioxidant in fruits increased in plants exposed to 30 mg l^{-1} $AgNO_3$, but lycopene content decreased.

Therefore the main objectives of this study was to investigate the effects of chemical application [$AgNO_3$ and $Ag(S_2O_3)_2^{-3}$], different leaf growth stages and number of spraying on flower and fruit characteristics of greenhouse cucumber in two different

seasons to identify the best condition and the optimal level of chemical application to boost the formation of male flowers. Our findings will help to develop practical recommendations on these issues for improve male flowers production in cucumber breeding programs. Furthermore, this work aimed to evaluate the stability of sex expression of a gynoecious cultivar Adrian in different seasons. The present investigation was done as a first step to a hybridization program of cucumber.

2. Materials and Methods

Planting material and field evaluation

The seeds of cv. Adrian as a gynoecious genotype were sown at the mid autumn of 2014 and mid spring of 2015 at Research Greenhouse of Agriculture Department at Islamic Azad University Isfahan Branch, Isfahan, Iran (51° 36´ longitude and 32°63 latitude). The soil used was loam with pH 7.7 and Electrical conductivity (EC) of 4.1 (ds/m). Seeds were planted to soil directly and covered with peat moss and coco peat. Plants were disposed in two rows, where the spaces between cucumber seedling was 50 cm and within the rows were 90 cm respectively, and 180 cm was left between every couple rows. The greenhouse air temperature at the growing period was maximum 29°C/18°C (day/night) in first season (autumn-winter) and 34°C/20°C (day/night) in second season (spring-summer) with a relative humidity of about 55% and 60%, respectively. Nutrient levels in the irrigation solution water were N:216 (mg l^{-1}), P:58 (mg l^{-1}), K:286 (mg l^{-1}), Ca:185 (mg l^{-1}), Mg:185 (mg l^{-1}), S:43 (mg l^{-1}), Fe:5.59 (mg l^{-1}), Mn:1.97 (mg l^{-1}), B:0.7 (mg l^{-1}), Zn:0.2 (mg l^{-1}), Cu:0.07 (mg l^{-1}) and Mo:0.05 (mg l^{-1}). Different fertilizers were used based on soil analysis that included: potassium nitrate, ammonium nitrate, magnesium nitrate, iron and other mineral elements such as sulphate dissolved in the irrigation water. Dichlorvos, Trigard, Abamectin and organic neem oil were applied for insect control. The same fertilizers and pest management were used for all plants in the same time. Drip irrigation was applied when needed. The source of water was urban water with electrical conductivity (EC) of 0.4 (ds/m). The pH of the irrigation water was adjusted to 6.5 by adding nitric acid.

Silver application

Different chemical treatments including $AgNO_3$ [h_1=100 ppm (0.59 mM), h_2=200 ppm (1.18 mM) and h_3=300 ppm (1.77 mM)], and $Ag(S_2O_3)_2^{-3}$ [h_4=200 ppm (0.6 mM) and h_5=500 ppm (1.5 mM)] and control (h_6) were applied to induce changes in sex expression. $AgNO_3$ was purchased from Merck Chemical Company and $Ag(S_2O_3)_2^{-3}$ was synthesized according to Law *et al.* (2002). The solutions were applied as a spray on the whole plant. Water was sprayed on the control plants. Spraying was accomplished at 5, 10, and 15-leaf growth stages (five leaves stage: LS_1, ten leaves stage: LS_2, and fifteen leaves stage: LS_3). Chemical treatments were employed in single or double spraying at one week interval. $AgNO_3$ and $Ag(S_2O_3)_2^{-3}$ applications were conducted early in the morning (before sunrise) to avoid plant sunburn. Each treatment was applied with adequate amounts of the solutions to assure that all the leaves were completely wetted in each spray event. The total time of experiment in every season was about 4 months after sowing and measurement of traits were done in this period.

Studied traits

The traits of interest were measured on eight plants per replication and included: days to male flower expression (days to first male flower appearance after sowing) (DMF); the node number that the first male flower was appear (NNMF); the mean of internode length (the mean of five internode from node number 15 to 20) (IL); the number of male flower (total male flower that opened in whole plant) (NMF), male flowering period (days from observation the first male flowering until the last male flowering observation (MFP); male flower diameter (was recorded at the widest points) (MFD); single fruit weight (from dividing total fruit weight to total fruit number in every harvesting) (SFH); fruit number per harvesting (FNH); fruit weight per harvesting (FWH) (the numbers and the weights of all the fruits harvested in each plot in each harvesting).

Statistical analysis

The experiment was conducted as a combined analysis of variance with three different factors (chemical treatments, leaf growth stage, and number of spraying) under two different seasons. The data collected were subjected to analysis of variance (ANOVA) based on a completely randomized block design with three replications using the general linear model (GLM). Statistical analysis system program (SAS Ver. 9) was used for data analysis. The differences between applications were decided on the basis of the Least Significant Difference (LSD) test

(P<0.05) according to their importance at the 0.05 confidence level.

3. Results and Discussion

The result of analysis of variance based on a factorial experiment is presented in Table 1. According to Table 1, the season (S) had significant effect on all of the studied traits, except for single fruit weight. This result is logical, since phenotypic expression of sex is strongly modified by environmental and hormonal factors (Mohan Ram and Sett, 1982). Long days, high temperature, and silver ion promote formation of male flowers, whereas short days and low temperature promote the formation of female flowers (Perl-Treves, 1999).

Clearly, chemical treatments (CH) had significant effects on all the traits studied, except for fruit number per harvesting (Table 1). Number of spraying (single or double) also had significant effects on the days to male flowering, the node number of first male flower, the mean of internode length, the number of male flower and male flowering period (Table 1). Hallidri (2004) reported that increased number of spraying events had significant effects on male flower production. He suggested that doses of 400 and 500 ppm of $AgNO_3$ produced the highest number of male flowers. The leaf growth stage (LGS) of spraying showed significant differences on days to male flowering, the node number of first male flower, male flowering period, male flower diameter and fruit weight per harvesting (Table 1).

The interaction effect of environment × silver application had a significant effect on all traits with the exception of male flower period and fruit number per harvesting (Table 1). Interaction of season × leaf stage was significant for days to male flowering, the node number of first male flower, the number of male flower and male flower diameter. Interaction of number of spray events × chemical application had a significant effect on days to male flowering, the node number of first male flower, the number of male flower and single fruit weight (Table 1). Interaction of chemical application × leaf stage of spraying showed significant differences on days to male flowering and the node number of first male flower (Table 1). The interaction of environment × chemical application × leaf stage of spraying showed significant difference on days to male flowering, the node number of first male flower, the number of male flower and period of male flowering. Study of these interactions

Table 1 - Analysis of variance for single and combined effects of different studied traits in greenhouse cucumber

Source of variation	Mean squares of studied traits									
	DF	DMF	NNMF	IL	NMF	MFP	MFD	SFW	FNP	FNH
Season (S)	1	**	**	**	*	**	**	NS	**	**
Replication (season)	4	NS	NS	**	NS	NS	NS	**	NS	NS
Chemical treatments (CH)	5	**	**	**	**	**	**	**	NS	**
Number of spraying (S0)	1	**	*	**	**	**		NS	NS	NS
Leaf growth stage (LGS)	2	**	9**	NS	NS	**	**	NS	NS	NS**
SP × CH	5	**	**	*	**	NS	**	**	NS	NS
S×SP	1	NS	NS	NS	*	NS	*	NS	NS	**
S×LGS	2	**	**	NS	*	NS	**	NS	NS	NS
CH×SP	5	*	*	NS	**	NS	NS	*	NS	NS
CH ×LGS	10	*	**	NS	NS	NS	NS	NS	NS	NS
SP×LGS	2	NS	NS	NS	NS	*	NS	NS	NS	NS
S×CH×SP	5	NS	NS	NS	NS	NS	NS	NS	NS	NS
S×CH×LGS	10	**	**	NS	**	*	NS	NS	NS	NS
CH×SP×LGS	10	NS	NS	NS	NS	NS	NS	*	NS	NS
S×SP×LGS	2	NS	NS	NS	NS	*	NS	NS	NS	NS
S×CHSP×LGS	10	NS	NS	NS	NS	NS	NS	NS	NS	NS
Residual	140	NS	NS	NS	NS	NS	NS	NS	NS	NS

DF= Degree of freedom; S= season; CH= Chemical treatments [Silver nitrate ($AgNO_3$) and Silver thiosulphate $Ag(S_2O_3)_2^{-3}$); SP= number of spraying (single or double); LGS= Leaf growth stage (5, 10 and 15).
DMF= days to male flowering; NNMF= the node number of first male flower; IL= the mean of internode length (cm); NMF= the number of male flower; MFP= male flowering period; MFD= male flower diameter (cm); SFW= single fruit weight (g); FNH= fruit number per harvesting; FWH= fruit weight per harvesting (g).
NS= non significant. *, ** significant at P<0.05 and P<0.01, respectively.

showed that important traits related to change of sex expression such as days to male flowering, node number of first male flower, and number of male flower more affected by different interactions in contrast to vegetative and yield related traits. Therefore selection of best treatments should be done based on other treatments. For example numbers of male flower are affected by silver application, leaf stage, number of spraying and all their interactions. There was no significant difference for other triple interactions and environment × chemical application × leaf stage of spraying × number of spray events interaction in all of the studied traits.

Effects of season on the studied traits

The comparison between two seasons showed that the first season had superior mean on days to male flowering, the node number of first male flower, male flower diameter and single fruit weight. It could be concluded that in the first season male flowers expressed later than second season and therefore DMF and NNMF were increased (Table 2). On the other hand, in second season the number of male flower was increased (from 36 to 100 male flowers) and also the time of male flowering was earlier that is related to high temperature and long days. This result showed that environmental conditions, especially low temperature, could retard male flower formation and confirmed also that cucumber is a thermophilic plant. Accordingly, Stankovic and Prodanovic (2002) reported that sowing season affected the number of male flowers. The interaction effect of season × chemical application × leaf stage was significant for number of male flower, days to male flowering and period of male flowering that all of them are related to sex expression, even though this interaction was not significant for vegetative related traits (Table 2).

Effects of silver application on the studied traits

In this study, different doses of chemical treatments [$AgNO_3$ and $Ag(S_2O_3)_2^{-3}$] were used. The mean comparisons showed that chemical application increased the mean values of days to male flowering (Table 3). There was a narrow variation in days to male flowering between different chemical treatments. The least day to male flowering was at a high concentrations of $AgNO_3$ and $Ag(S_2O_3)_2^{-3}$ (Table 3). This result showed that high concentrations of different chemicals agent caused earlier male flowering.

Table 2 - The mean comparison of different studied traits in cucumber in different seasons

Season	Traits								
	DMF	NNMF	IL	NMF	MFP	MFD	SFW	FNH	FWH
S1	23.08 a	20.17 a	7.13 b	36.24 b	12.20 b	5.46 a	73.17 a	1.93 b	138.80 b
S2	19.89 b	11.96 b	7.63 a	99.78 a	13.35 a	4	74.95 a	3.60 a	272.96 a

DMF= days to male flowering; NNMF= the node number of first male flower; IL= the mean of internode length (cm); NMF= the number of male flower; MFP= male flowering period; MFD= male flower diameter (cm); SFW= single fruit weight (g); FNH= fruit number per harvesting; FWH= Fruit weight per harvesting (g).
Means followed by the same letter in each column were not significantly different at 0.05 level using LSD test.

Table 3 - The effects of various chemical treatments application on studied traits in cucumber

Traits	Control	Chemical treatments				
		$AgNO_3$			$Ag(S_2O_3)_2^{-3}$	
		100 ppm	200 ppm	300 ppm	200 ppm	500 ppm
Days to male flowering	0.23 c	26.41 a	26.08 a	25.04 b	26.02 a	25.17 b
Node number of male flower	0.13 b	20.18 a	19.11 a	19.23 a	19.08 a	18.68 a
The mean of internode length	6.25 b	7.38 a	7.8 a	7.64 a	7.28 a	7.65 a
The number of male flower	0.18 d	20.89 cd	68.07 b	129.76 a	44.79 bc	144.4 a
Male flowering period	0.074 e	10.16 d	16.13 bc	17.366 ab	14.71 c	18.21 a
Male flower diameter	3.29 d	5.36 c	5.45 bc	5.65 ab	5.36 c	5.71 a
Single fruit weight	84.7 a	73.9 b	70.95 b	72.15 b	70.68 b	71.89 b
Fruit number per harvesting	2.69 a	2.71 a	2.65 a	2.88 a	2.84 a	2.84 a
Fruit weight per harvesting	219.74 a	205.88 a	190.87 a	212.9 a	199.72 a	206.22 a

Means followed by the same letter in each row were not significantly different at 0.05 level using LSD test.

The highest value for male flower diameter observed at 500 (ppm) of $Ag(S_2O_3)_2^{-3}$ and 300 (ppm) of $AgNO_3$ (Table 3). Therefore these concentrations of $Ag(S_2O_3)_2^{-3}$ and $AgNO_3$ could affect both vegetative and reproductive growth of flower. However there was no significant difference between all treatments for internode length. Hallidri (2004) reported that the greatest number of staminate nodes was produced on plants sprayed two or three times with 400 to 500 (ppm) of $AgNO_3$. Rafee Kher *et al*. (2002) reported that 200 (ppm) of GA increased the length of internode in cucumber. According our results the highest value for the number of male flower was denoted at 300 (ppm) of $AgNO_3$ and 500 (ppm) of $Ag(S_2O_3)_2^{-3}$ about 130 and 144 male flower, respectively. Law *et al.* (2002) reported that application of $Ag_2S_2O_3$ produced stamens in female flowers of white campion (*Silene Latifolia)* with longer filaments and larger anther locules. Yongan *et al*. (2002) reported that $AgNO_3$ had more significant effects than GA_3 on male flower production in summer squash. Stankovic and Prodanovic (2002) reported that increasing concentrations of $AgNO_3$ from 0.01% to 0.04% enhanced the number of male flowers in gynoecious lines, which is similar with the results obtained in the present study. Jadav *et al.* (2010) reported that ethrel (200 ppm) had the greatest effect on male flower production among the hormones they investigated (GA_3, ethrel, naphthalene acetic acid, and absisic acid). Jutamanee *et al.* (1994) reported that the effects of different doses of GA_3 and $AgNO_3$ depended on the genotype and photoperiod. Similar to their results, $AgNO_3$ in the present study induced the formation of male flowers in all concentration. Hallidri (2004) reported the concentration of 100 (ppm) of $AgNO_3$ was ineffective on male flower induction. Kalloo and Franklen (1978) reported that different doses of $AgNO_3$ (50, 200, and 500 mg l^{-1}) led to non-significant effects on male flower production, fruit weight and fruit number. They showed similar trends in all the treatments. However in this study all concentrations of $AgNO_3$ (100, 200, 500 ppm) produced male flower with significant differences. One explanation which might account for disparities observed between experiments was the difference in average quanta of solar radiation received by the plants during two different environmental conditions in two seasons. This result confirms the inducing effects of different chemical compounds used on male flower production. The highest value for single fruit weight was observed at control. Mean comparison showed that chemical application has reduced the mean of single fruit weight in comparison to control, because production of male flower in every treated plant will reduce female flower number in contrast to control. There were no significant differences between all treatment for fruit number per Harvesting and fruit weight per harvesting (Table 3). So, this result could demonstrated that chemical application has only effects on flower number and its sex expression.

Effects of number of sprays on the traits studied

Number of sprays (single or double) showed significant effects on days to male flowering, the node number of first male flower, the number of male flower and period of male flowering (Table 4). According to Table 4, double spraying treatment gave rise to higher mean values for days to male flowering, the node number of first male flower, the number of male flower and period of male flowering. On the other hand, double spray treatment was found to affect only sex expression while it had no effect on the traits related to morphology and fruit yield.

Effects of leaf stage on the studied traits

The comparison of the mean values for the studied traits at different stages of leaf growth [Five leaf

Table 4 - The effects of single and double spraying on studied traits in cucumber

Number of Spraying	Traits								
	DMF	NNMF	IL	NMF	MFP	MFD	SFW	FNH	FWH
Single spraying (SP)	20.85 b	15.49 b	7.31 a	49.15 b	11.61 b	5.11 a	74.04 a	2.76 a	205.96 a
Double spraying (DP)	22.13 a	16.64 a	7.44 a	86.86 a	13.94 a	5.16 a	74.08 a	2.77 a	205.82 a

DMF= days to male flowering; NNMF= the node number of first male flower; IL= the mean of internode length (cm); NMF= the number of male flower; MFP= male flowering period; MFD= male flower diameter (cm); SFW= single fruit weight (g); FNH= fruit number per harvesting; FWH= fruit weight per harvesting (g).
Means followed by the same letter in each column were not significantly different at 0.05 level using LSD test.

stage: LS_1, Ten leaf stage: LS_2, and Fifteen leaf stage: LS_3] is presented at Table 5. The highest mean for DMF and NNMF were observed at LS_3 stage (Table 5).This logical result is resulted from the most distance (days) between planting time to fifteen leaf stage. On the other hand, the highest DMF at 15 leaf growth stage belonged to 200 ppm $Ag(S_2O_3)_2^{-3}$ treatment (Fig. 1). El-Ghamriny *et al.* (1988) confirmed that sex differentiation in cucumber takes place at the 2-true leaf stage and that this was the best time for studying the effects of growth regulators on sex expression. There was no-significant difference between three leaf growth stages for number of male flower (Table 5). Therefore use of silver ions at different three growth stages had similar effects on modification of flower sex type in cucumber. Therefore, if it is necessary to separate monoecious or androecious from gynodioecious plant types in breeding programs (for example in line production), breeder could apply these chemical agents at 15 leaf stage, when sex form of plant has been expressed. Raymond (2004) proposed that 1000 ppm of GA_3 at the 2-leaf stage had the best effect on male flower production with 3 hormone applications at 2-week intervals in cucumber. Also, the highest values for period of male flowering and male flower diameter were obtained at the five leaf stage; that is logical, since the period between male flower observation and end of male flowering in LS_1 was higher than LS_2 and LS_3. As already mentioned, male flower diameter was greater at the 5-leaf stage, which might be caused by the higher production of pollen and the increased capacity for crossing in the breeding programs.

Effect of different factor interactions on number of male flower

As the number of male flower trait is the most important trait related to male flower induction in cucumber, the different significant interaction effects

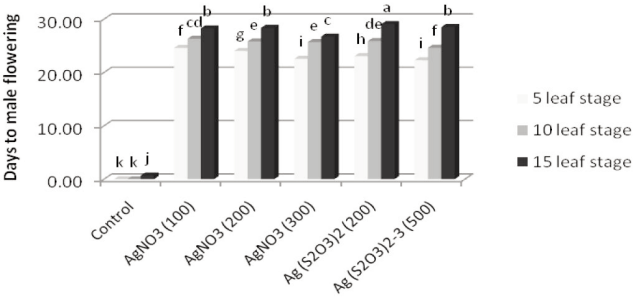

Fig. 1 - The interaction effect of chemical treatment × leaf growth stage on the days to male flowering in cucumber

in this trait was assayed (Table 2). The highest number of male flower was recorded at double spraying in 500 ppm of AgS_2O_3 and double spraying at 300 ppm of $AgNO_3$, respectively of 187.36 and 179 flowers (Fig. 2 a). In the two chemical treatments at higher concentration, double spraying caused increasing of male flower number, although differences between one and two spraying in low concentrations of chemical agents was not significant. The highest (119.2) and the least (24) number of male flower was observed at second season and the first season at fifteen leaf stage, respectively (Fig. 2 b). In all stages, high number of male flower was observed in second season and pointed out significant differences with first season. Also, the most number of male flowers was obtained in high concentration of chemical treatments at the second season, however in all chemical treatments the number of male flowers was more than at second season (Fig. 2 c). Again, the second season showed the highest number of male flower with double spraying in contrast to first season, although double spraying in two seasons was more than single spraying (Fig. 2 d). The triple interaction effects of season, leaf growth stage and chemical treatment on male flower number showed that male flower number in all three leaf growth stages and all chemical treatments under second season were more than first season, specially high concentration

Table 5 - The mean comparison for some of the studied traits in different leaf growth stages in cucumber

Developmental stages	Traits								
	DMF	NNMF	IL	NMF	MFP	MFD	SFW	FNH	FWH
Five leaf stage (LS1)	19.44 c	8.73 c	7.55 a	68.94 a	14.21 a	5.40 a	74.63 a	2.88 a	216.06 a
Ten leaf stage (LS2)	21.40 b	17.10 b	7.40 a	63.47 a	12.40 b	5.05 b	73.00 a	2.69 a	195.85 a
Fifteen leaf stage (LS3)	23.62 a	22.38 a	7.18 a	71.60 a	11.71 b	4.95 b	74.55 a	2.73 a	205.76 a

DMF= days to male flowering; NNMF= the node number of first male flower; IL= the mean of internode length (cm); NMF= the number of male flower; MFP= male flowering period; MFD= male flower diameter (cm); SFW= single fruit weight (g); FNH= fruit number per harvesting; FWH= fruit weight per harvesting (g).
Means followed by the same letter in each column were not significantly different at 0.05 level using LSD test.

of chemical treatments in fifteen leaf growth stage under second season (Table 6). These results demonstrated that the second season had progressive effect on induction and development of male flower and environmental condition could be having a significant effect on this phenomenon. As mentioned before, in second season the temperature of greenhouse was higher than first season and also days were longer. These two reasons changed sex expression in second season more than first season.

4. Conclusions

Phenotypic expression of sex determining loci in cucumber is strongly modified by environmental, chemical and hormonal factors. The results of the experiment revealed that $AgNO_3$ and $Ag(S_2O_3)_2^{-3}$ have similar and positive effects on the male expression of cucumber. This study showed that male flower induction was induced by all concentrations of silver ions. These two chemical agents could induce a high number of male flowers in 300 and 500 ppm concentration [$AgNO_3$ and $Ag(S_2O_3)_2^{-3}$], respectively. Chemical treatments with silver ions as $AgNO_3$ and $Ag(S_2O_3)_2^{-3}$ application can, therefore, be recommended for enhancing male flower production in cucumber, if further study will confirm the accumulations of silver in the fruit is negligible for the human health. As the time of $AgNO_3$ spraying in this experiment was only three times, so the toxicity of this ion was low in a short time of period. On the other hand, as this chemical agent only was applied in experimental greenhouse, therefore the fruits of treated

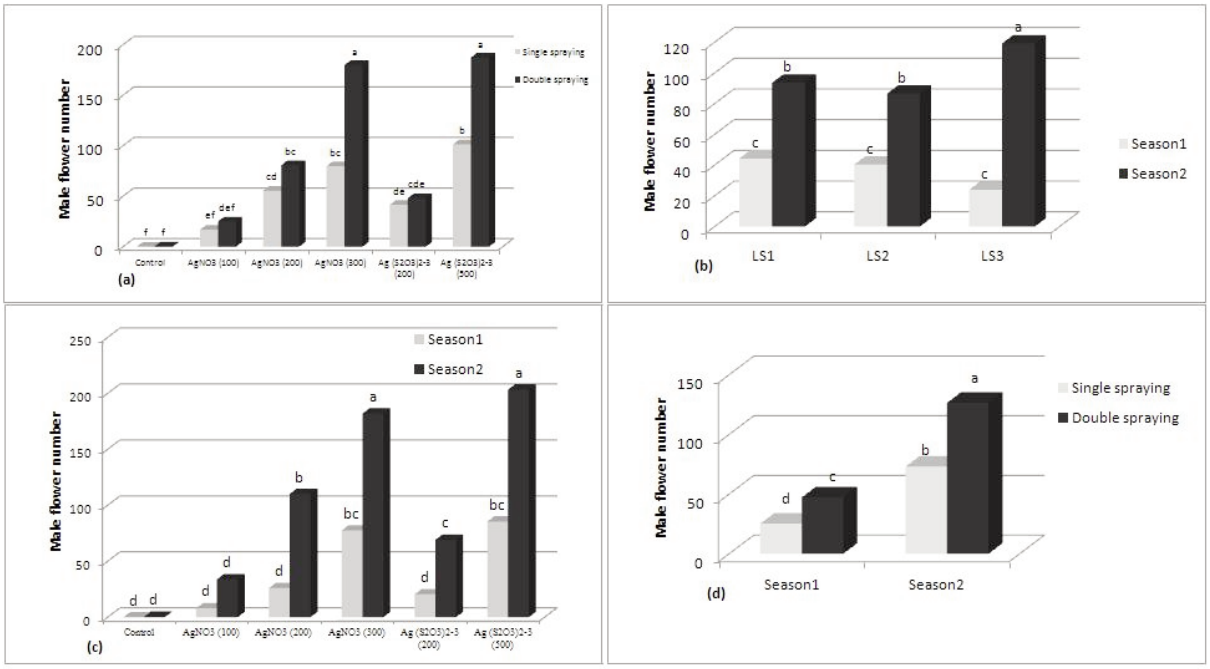

Fig. 2 - The effect of different interactions (a, b, c and d) on the number of male flower in cucumber.

Table 6 - The effects of interaction between chemical treatment, seasons and leaf growth stage on male flower number in cucumber

Chemical treatment	Seasons					
	Season 1			Season 2		
	LS1	LS2	LS3	LS1	LS2	LS3
Control	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n	0.00 n
$AgNO_3$ (100 ppm)	6.83 mn	8.00 mn	9.17 mn	42.42 i-n	31.75 j-n	27.17 k-n
$AgNO_3$ (200 ppm)	43.00 i-n	20.33 lmn	14.67 mn	64.33 h-m	123.33 d-h	142.75 d-g
$AgNO_3$ (300 ppm)	122.33 d-h	91.00 e-j	19.33 lmn	145.33 def	147.42 cde	253.17 a
$Ag(S_2O_3)_2^{-3}$ (200 ppm)	16.331 mn	26.00 k-n	19.00 lmn	84.58 f-k	38.00 i-n	84.67 f-k
$Ag(S_2O_3)_2^{-3}$ (500 ppm)	77.33 h-l	97.17 e-i	81.83 g-k	224.83 ab	178.75 bcd	206.5 abc

LS1= Five leaf growth stage; LS2= Ten leaf growth stage; LS3= Fifteen leaf growth stage.
Means followed by the same letter were not significantly different at 0.05 level using LSD test.

plants were disposed from toxic effects of Ag ion. Also, the morphologic appearance of the cucumbers showed no abnormality after ion application. Overall, this result indicates that silver ion could change sex expression in higher temperatures and longer days with high concentration and when applied in 15 leaf stage for $AgNO_3$ and in 5 and 15 leaf stage for $Ag(S_2O_3)_2^{-3}$, although this ion is able to modify sex form in lower concentration and earlier growth stage in cucumber. Although double spraying led to late male flowering, however this treatment could increase number of male flower significantly. Therefore, the double spray treatment is proposed for male flower production in greenhouse conditions. This can be beneficial for breeding programs that need high numbers of male flowers and flowering periods, especially for parents that have different periods of growth stage.

The interaction effect showed that the highest number of male flowers was induced by applying $Ag(S_2O_3)_2^{-3}$ (500 ppm) in 5 and 15 leaf stage in high temperature about maximum 35°C at day and minimum 20˚C at night. Another finding of this study was that the number of male flowers showed significant increase with increasing doses of $AgNO_3$ from 100 ppm to 300 ppm and $Ag(S_2O_3)_2^{-3}$ from 200 ppm to 500 ppm. Therefore, high dosages of $AgNO_3$ and $Ag(S_2O_3)_2^{-3}$ should be selected for inducing male flowers in cucumber. The highest dosage of chemical agents led to decrease in days to male flowering and the mean of internode length that is related to the effect of silver ion on sex form earlier than low dosage of this ion. Leaf stage of the spray event was found to have a toxic effect on fruit. Over all, this result indicates that high concentration of silver ion could change sex expression at higher temperatures and longer days in 15 leaf stage for $AgNO_3$ and in 5 and 15 leaf stage for $Ag(S_2O_3)_2^{-3}$. Because the highest level of number of male flower and male flower period and the lowest level of days to male flowering were obtained in these treatments. Finally, male flowering started about 3 weeks after treatment and lasted for a period of up to 3 weeks thereafter. Plants treated with silver ions did not elongate more than normal plants and grew normally; effective concentrations of $AgNO_3$ and $Ag(S_2O_3)_2^{-3}$ did not proved phytotoxic effects in growing conditions.

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Commercial advantages on basil architecture by ultraviolet-B irradiation

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Abstract: Sweet basil (*Ocimum basilicum* L.) is one of the most important herbs widely used for its medicinal properties and as food ingredient. The marketing of this product highlights the problem that these plants have long and slender stems, which are easy to break off and thus making difficult their market distribution. In this work, two cultivars of basil (Genovese and Profumo) at the adequate development stage for sale were used. We evaluated the effect of supplemental ultraviolet (UV)-B irradiation (15 W m^{-2}; 3 h day^{-1}) on plant growth and market quality. Both cultivars of basil plants under UV-B irradiation resulted in increased leaf size and biomass, and decreased shoot length in comparison to that of under control growth conditions. These results indicate that the application of UV-B irradiation beneficially influenced plant architecture in basil improving their greenhouse production for fresh market.

1. Introduction

Many species in the genus *Ocimum* (Labiatae) are ranked among the most important herbs for their medicinal properties, that are associated to high content of secondary metabolites including essential oils and caffeic acid derivatives (Gülçin *et al.*, 2007). The most significant species of the genus is sweet basil (*Ocimum basilicum* L.), which originated from tropical areas, such as India, Africa and southern Asia. Sweet basil is an annual herbaceous species that is usually cultivated as an aromatic plant. The minimum temperature for the growth of sweet basil has been determined to be 10.9°C (Vågen *et al*., 2003; Chang, 2004). It is typically used in Italian and Asian cuisines because of the pronounced scent of its leaves, which depends on genotype (Chang *et al*., 2009), nitrogen nutrition (Sifola and Barbieri, 2006) and harvesting system (May *et al*., 2008). Sweet basil is not only cultivated for the use of aroma additives in food but also for other house-hold purposes, pharmaceuticals, cosmetics and folk medicine. Numerous different chemo-types exist in both wild and cultivated

basil. For instance, sweet basil contains high levels of phenylpropanoids, e.g. eugenol and methyleugenol, and terpenoids e.g. linalool and 1,8-cineole (Lachowicz *et al*., 1997).

In recent years, the consumption of fresh basil has been expanded in supermarkets where young seedlings are directly sold in pots obtained from greenhouses. These plants are intended for family use, which after to be transplanted, are placed on balconies, vegetable gardens or gardens. The marketing of fresh basil highlights the problem that these plants have long and slender stems, which are easy to break off and thus making difficult their market distributions. Therefore, the objective of this study was to facilitate the sale of fresh basil in pots resulting not only in plants more compact and resistant to movements along the supply chain, but also healthy products in the market because of the avoidance of chemicals to control the plant height (Körner and Van Straten, 2008; Nagashima *et al*., 2011).

The interest on the effects of ultraviolet (UV) irradiation on plants has considerably increased in the last ten years due to the continuous depletion of the ozone layer (Ballaré *et al*., 2011). The stratospheric ozone layer completely absorbs solar UV-C (200-280 nm) which is extremely active and biologically lethal. UV-B (280-320 nm) is the most susceptible to ozone layer depletion because it is efficiently absorbed although small proportion is transmitted to the Earth surface. UV-A (320-400 nm) is hardly absorbed by ozone and thus passes almost unaltered through the stratospheric layer reaching the Earth surface (Houghton *et al*., 2001; Solomon *et al*., 2007). Despite the small proportion of UV-B in the natural daylight and higher energy than UV-A, UV-B has substantive effects on plant growth and metabolism (Kolb *et al*., 2001). Moreover, plants are differently sensitive to UV-B levels which strongly depend on the latitude, hours of direct sunlight and variation in the thickness of the ozone layer (Ballaré *et al*., 2011). For instance, plant species grown in Mediterranean and Tropical environments and/or at high altitudes have developed defensive mechanisms to protect themselves against UV radiation (Zheljazkov *et al*., 2008).

Many studies found that UV-B irradiation significantly affects secondary compounds such as the biosynthetic pathway of phenylpropanoids, which are antioxidant agents (Korkina, 2007) that also act as protection against UV (Johnson *et al*., 1999; Ioannidis *et al*., 2002). Previous studies reported that UV-B irradiation also increases essential oils and total phenolic compounds content in plants (Kumari *et al*., 2009; Kumari and Agrawal, 2011). Moreover, it has been demonstrated that the UV-B irradiation stimulates the production of volatiles organic compounds in fresh herbs which usually are depleted when plants grown under glass or plastic greenhouses conditions in the absence of natural levels of UV-B (Johnson *et al*., 1999; Ioannidis *et al*., 2002). In the case of plant morphology, several studies have been demonstrated the role of UV-B in controlling the growth of various plants such as *Lycopersicon esculentum* Mill. and *Salvia splendens* L. (Garner and Björkman, 1996; Giannini *et al*., 1996; Del Corso and Lercari, 1997). Additional techniques to control plant growth have been proposed: temperature manipulation, induction of mild water or mechanical stress, and the use of chemical compounds, however all these methods present many drawbacks (Moe and Mortensen, 1992; Garner and Björkman, 1996; Barreiro *et al*. 2006; Sun *et al*., 2008). Therefore, we focused on the role of UV-B as a growth regulator of potted basil intended for fresh consumption.

2. Materials and Methods

Plant material and growth conditions

Two cultivars of basil were used in this study, Genovese and Profumo. Basil seeds were sowed into peat-based growing medium in 8 cm diameter pots (10 seeds each pot). Pots were covered with non-woven fabric and incubated in a growth chamber at 23±1°C, relative humidity 60-65%, 12 h light (150 µmol m^{-2} s^{-1}). After one week of incubation, the coverage was removed and half of the pots were kept at the same conditions while the other half were under supplementary UV-B irradiation (fluorescent tubes with UV-B 10%, 15 W m^{-2}, *Exo Terra*). UV-B was applied for 3 h starting 1 h before darkness, and lamps were placed at 0.5 m above plants. Basil plants were regularly irrigated. Control and UV-B treated plants were sampled 11 and 22 days after the onset of UV treatment.

Morphological analysis

Leaf length and width, shoot length, fresh and dry weight of shoot and leaves were determined upon treatment completion. All measurements were the mean of three independent experiments.

Analysis of pigments

Pigments were extracted and analysed from full expanded leaves as previously described (Pompeiano *et al*., 2013). All the analyses were conducted in trip-

licate.

Statistical analysis

The statistical analyses of biometric and physiologic traits were subjected to an analysis of variance (ANOVA). Differences between treatments were assessed using the F-test, and the least significant difference (LSD) was calculated at $P \leq 0.05$. All computations were performed with R 2.14.2 R Development Core Team 2012.

3. Results

In this study, we analyzed the plant architecture and photosynthetic pigments content of two basil cultivars, Profumo and Genovese, at time zero (T0) and after treatment. Two different time points of treatment were analyzed: 11 and 22 days after UV-B treatment (UV) and their respective control (C) conditions.

Morphological analysis was determined on leaves and shoots of both basil cultivars (Fig. 1, 2). Length and width of cotiledonary leaves did not show differences between cultivars at T0, and also between treated and control plants during all the experimental time (Fig. 1). The first true leaves, which appeared at the first time point of the experiment, showed a significant increased size from 11 to 22 days at control conditions in both cultivars (Fig. 1). It was also observed that UV treated plants significantly increase, about two times, the leaf expansion in both cultivars in comparison with their respective control (Fig. 1). Interestingly, only 22 days UV treated seedlings showed the second leaves pair without morphological differences between the cultivars (Fig. 1). In the case of shoot length evaluation, no differences were observed between cultivars at T0, although 'Genovese' showed longer shoot than 'Profumo' at 11 and 22 days control conditions (Fig. 2). Moreover, the UV irradiated plants showed a considerably shorter shoots than that of control plants at both 11 and 22 days after treatment, and in all cases 'Genovese' still longer than 'Profumo' (Fig. 2).

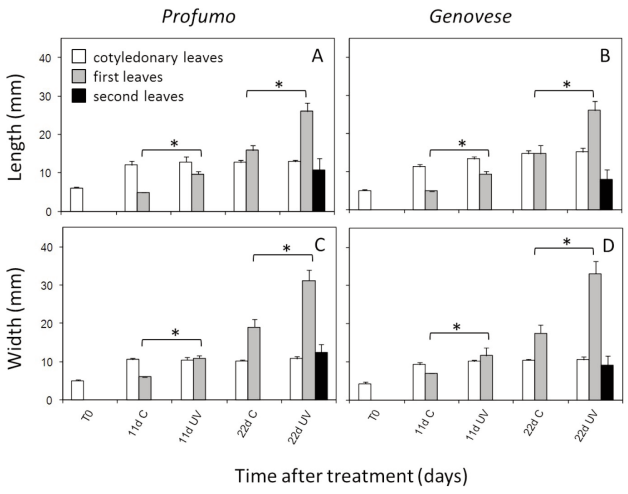

Fig. 1 - Effect of UV-B radiation on the morphology of basil leaves. Two cultivars of basil were use: (A, C) cv. Profumo and (B, D) cv. Genovese. (A, B) Length and (C, D) width of basil leaves were measured at time zero (7 days old basil seedlings, T0) and after treatment. Two different time points of treatment were evaluated: 11 and 22 days (d) after UV-B treatment (UV) or control (C) conditions. Cotyledonary (white bars), first (grey bars) and second leaves (black bars) were measured separately. Each value is the mean ± SD of three independent experiments. Asterisk indicates significant differences among treatments ($P \leq 0.05$).

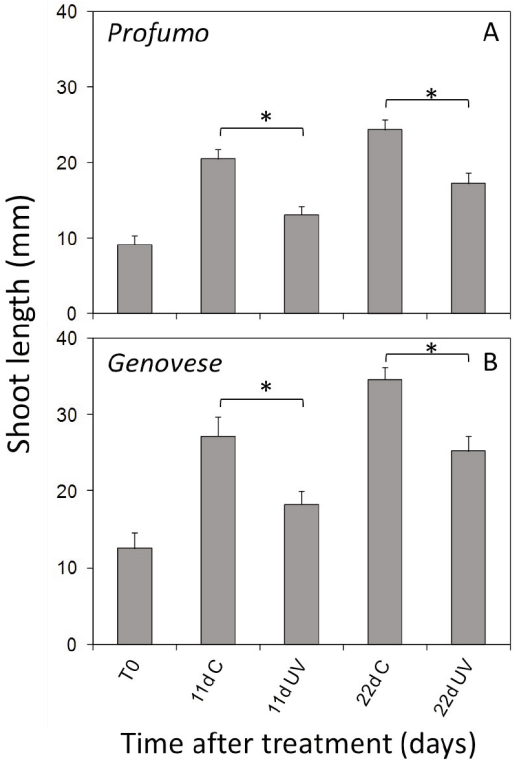

Fig. 2 - Effect of UV-B radiation on the length of basil shoots. Two cultivars of basil were use: (A) cv. Profumo and (B) cv. Genovese. Shoot length of basil cultivars was measured at time zero (7 days old basil seedlings, T0) and after treatment. Two different time points of treatment were evaluated: 11 and 22 days (d) after UV-B treatment (UV) or control (C) conditions. Each value is the mean ± SD of three independent experiments. Asterisk indicates significant differences among treatments ($P \leq 0.05$).

The effect of UV irradiation on basil biomass of both cultivars was also analyzed (Fig. 3). At T0, no differences were exhibited in fresh weight (FW) and dry weight (DW) leaves (only cotyledon) between cultivars, while FW and DW shoots were almost double in 'Genovese' in comparison with 'Profumo' (Fig. 3 A-B).

At 11 days after treatment, no differences were reported in FW and DW of shoot or leaves (cotyledon plus first pair) between cultivars (Fig. 3 C-D). Both cultivars treated with UV irradiation showed significant increase of FW and DW leaves in comparison with that of the control conditions, while no differences were reported in shoots (Fig. 3 C-D). At 22 days after treatment the biomass in both cultivars was increased in comparison with that of 11 d, while similar pattern was maintained when it was compared the biomass before and after UV irradiation (Fig. 3 E-F).

The photosynthetic pigments analyzed in both cultivars include chlorophyll *a*, chlorophyll *b* and carotenoids (Fig. 4). All pigments were detected at T0 without differences among cultivars. Then, the amount was dramatically reduced after 11 days under control conditions as well as UV irradiation, showing no statistical differences even between cultivars (Fig. 4). At 22 days after treatment, the level of chlorophyll *b* was maintained similar to that of 11 days and no differences were registered between control and UV conditions (Fig. 4). Chlorophyll *a* and carotenoids levels at 22 days under control conditions were increased in respect to that of 11 days, and no differences were detected when compared with that of after UV treatment (Fig. 4).

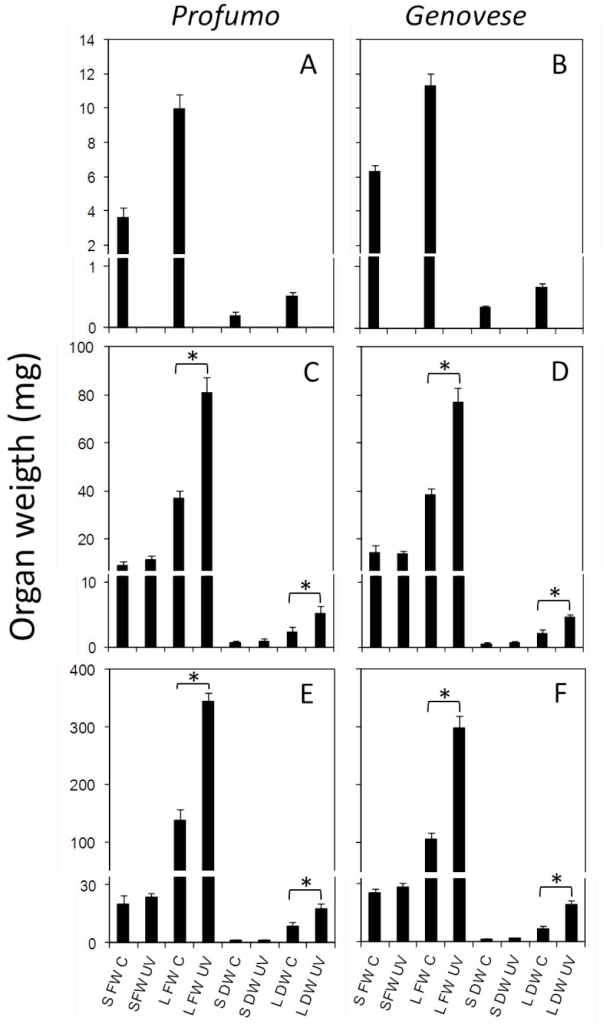

Fig. 3 - Effect of UV-B radiation on fresh and dry biomass of basil. Two cultivars of basil were use: (A, C, E) cv. Profumo and (B, D, F) cv. Genovese. Measurements were monitored at (A, B) time zero (7 days old basil seedlings) and after treatment. Two different time points of treatment were evaluated: (C, D) 11 days and (E, F) 22 days after UV-B treatment (UV) or control (C) conditions. S: shoot, L: leaf, FW: fresh weight, DW: dry weight. Each value is the mean ± SD of three independent experiments. Asterisk indicates significant differences among treatments ($P \leq 0.05$).

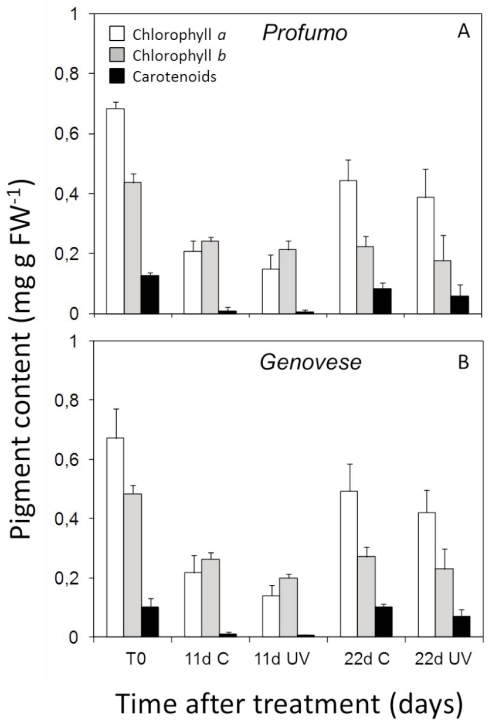

Fig. 4 - Effect of UV-B radiation on photosynthetic pigments in basil leaves. Two cultivars of basil were use: (A) cv. Profumo and (B) cv. Genovese. Measurements were monitored at time zero (7 days old basil seedlings, T0) and after treatment. Two different time points of treatment were evaluated: 11 and 22 days (d) after UV-B treatment (UV) or control (C) conditions. Chlorophyll a (white bars), chlorophyll b (grey bars) and carotenoids (black bars) content are expressed as mg g FW^{-1}. Each value is the mean ± SD of three independent experiments

4. Discussion and Conclusions

Results of the present study pointed towards a positive impact of supplementary UV-B irradiation on basil architecture, resulting on the reduction of stem elongation, increase of leaf length and width, increase of biomass, induction of second leaf sprout-

ing and with a similar trend of photosynthetic pigments content in comparison to that of plants under control conditions. These UV-B effects on plants are in accordance with previous studies (Giannini *et al*., 1996; Del Corso and Lercari, 1997).

The reduction of stem elongation found in our research was also reported previously in other species such as wheat (Yuan *et al*., 1998). This phenomenon could be related to the impact of UV-B radiation on phytohormones metabolism, such as photo-oxidation of indole-3-acetic acid which plays an important role in stem elongation and lateral shoots (Ros and Tevini, 1995; Mark and Tevini, 1996). In addition, effects of UV-B radiation on leaf thickening, leaf elongation and biomass accumulation are highly dependent on UV-B dose and source, experimental parameters and species of study (Kakani *et al*., 2003). Therefore, in this study, we used growth chamber instead of open fields and greenhouses in order to control all the environmental conditions, making this condition replicable in any situation and avoiding the influence of environmental and seasonal variations.

Our results showed also an increase of leaf thickening, leaf elongation and biomass after UV-B irradiation, which could be related to a morphological architecture strategy to protect plants to deleterious effects of UV-B radiation (Maffei and Scannerini, 2000; Jansen, 2002; Santos *et al*., 2004; Chang *et al*., 2009). Moreover, the generation of second leaves in basil plants was earlier promoted after UV-B treatment which may also involve phytohormones metabolism changes; this effect was also previously reported (Barnes *et al*., 1988). Thus, structural changes on leaves in response to the applied UV-B were observed, although no changes on the content of chloroplast pigments was registered between UV treated and control plants. Photosynthetic pigments are useful indicators of UV-B tolerance or sensitivity (Kataria *et al*., 2014). Lower pigments content was obtained at 11 days of treatment in comparison with T0, which could be due to the stress arising from the removal of the non-woven fabric that protected plants from excessive light. However, this phenomenon was recovered in a tendency to the start point T0, suggesting the acclimatization of plants, as observed similarly in previous report (Teramura and Sullivan, 1994; Radyukina *et al*., 2012). Therefore, our data suggest that Genovese and Profumo basil cultivars are tolerant to the supplementary UV-B irradiation (15 W m^{-2}; 3 h day^{-1}) at chlorophyll level, but producing other strategy to prevent UV-B penetration to the mesophyll cell at plant architecture level.

Although the impacts of UV-B radiation on plant growth and development have been widely studied (Strid *et al*., 1994; Ballaré *et al*., 1995; Giannini *et al*., 1996; Del Corso and Lercari, 1997; Santos *et al*., 2004; Zu *et al*., 2004; Körner and Van Straten, 2008), the detailed mechanism of how UV-B radiation affects plant morphogenesis is still unclear. However, previous evidences found that UV-induced morphological changes are associated with the induction of the phenylpropanoids pathway resulting in accumulation of flavonoids (Jansen *et al*., 1998). Moreover, it has been demonstrated that flavonoids regulate auxin transport affecting plant architecture (Jansen, 2002; Robson *et al*., 2015).

In conclusion, the results of this work have practical implications for greenhouse production of pot basil. In fact, supplementary UV-B radiation reduced plant height but increased biomass and leaf number, width and length with no significant effect on photosynthetic pigments. Therefore, the application of UV lamps could replace chemical growth retardants and/or other techniques adopted for growth control in the production of potted basil for fresh market.

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