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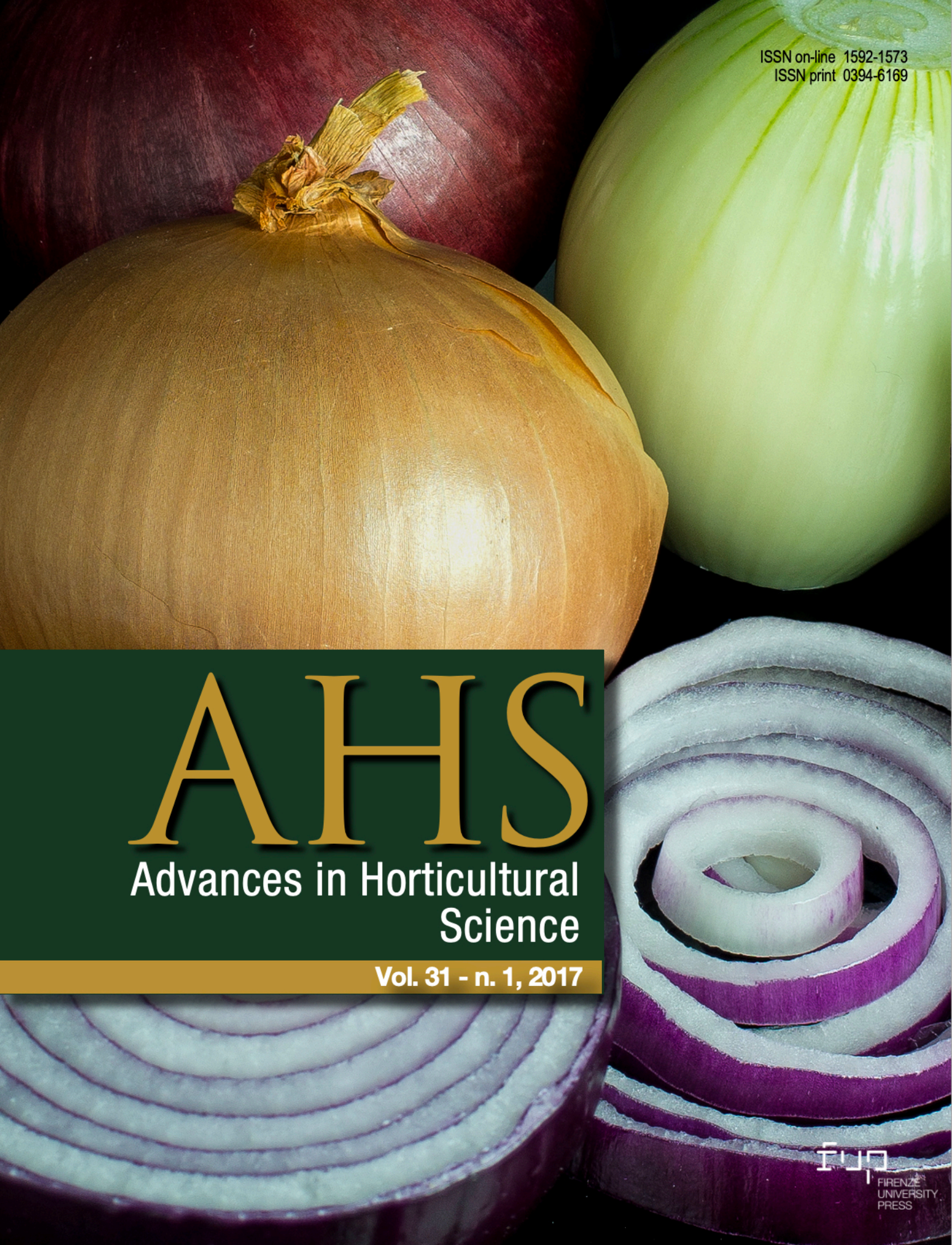

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A calcium lactate treatment at harvest, growing system and refrigerated modified atmosphere can affect strawberry's ´Camarosa´ postharvest quality?

M. Harris (*), M.C. Llorens, D. Frezza
Universidad de Buenos Aires, Facultad de Agronomía, Departamento de Producción Vegetal, Cátedra de Horticultura, Avenida San Martin 4453 (1417) Ciudad Autónoma de Buenos Aires, Argentina.

Key words: nutritional quality, organoleptic quality, perlite, postharvest behaviour, soil, storage temperature.

Abstract: The aim of this work was to evaluate the effect of a calcium lactate treatment on postharvest behaviour, organoleptic and nutritional quality of strawberries (*Fragaria x ananassa* Duch., cv. Camarosa) grown in different growing systems and stored in refrigerated modified atmosphere. Strawberry grown in perlite and soil in greenhouse and soil in open field was harvested and dipped in a calcium lactate 1% solution. Fruits were packed in modified atmosphere at 1°C and 8°C. At 1, 3 and 7 days of storage postharvest behaviour, organoleptic and nutritional quality was evaluated. Calcium had a positive effect on fruit firmness and no differences were observed between storage temperatures in calcium treated fruits. Organoleptic quality (except visual quality) was better in fruits grown in open field soil, regardless calcium treatment and storage temperature. Nutritional quality was better in untreated fruits and stored at 1°C.

1. Introduction

Strawberry (*Fragaria x annanassa* Duch.) is one of the most important berries produced in the world. In Argentina, 33000 tons are produced, mainly cultivar Camarosa (Gómez Riera *et al.*, 2013). It is highly accepted by consumers firstly because of its physical aspect and organoleptic and nutritional properties (Shin *et al.*, 2008; Garriga *et al.*, 2015). Nevertheless, its postharvest quality rapidly declines due to the soft texture, high metabolic activity and susceptibility to bacterial and fungal rots. Usually, there is quality decay during transport and commercialization (Dotto *et al*., 2011).

Cell wall degradation is one of the principal causes of strawberry´s postharvest quality decay. As pectine synthesis occurs along the fruit maturation, they are less firmly attached to the cell wall. Additionally, middle lamella´s debilitation and solubilization during fruit ripening diminishes cell cohesion (Lara *et al.*, 2004).

Calcium plays a preferential role on permeability and cell integrity and has a direct influence on fruit firmness and storage time (Fernández *et al.*, 2006). Calcium functions as an intracellular cement because it forms calcium-pectine complexes that give firmness to vegetable tissues. Calcium´s presence also favours pectic material insolubilization and inhibits its degradation by polygalacturonase enzyme (Alonso, 1995).

An immersion in calcium at harvest could increase calcium´s content in strawberry, increasing fruit firmness (Galetto *et al.*, 2010) and thereby storage period. However, the key factor for quality maintenance is temperature - optimum for strawberry is 0°C (Mitcham *et al*., 2015). Could an immersion in calcium allow an increase in storage temperature, maintaining fruit quality?

Other factors, such as growing system and substrate, can influence strawberry´s quality. Greenhouse production improves organoleptic quality of fruits and vegetables because they are not exposed directly to air conditions as in open field (Gruda, 2009). Soilless production, moreover, diminishes incidence of diseases and pests (Urresterazu, 2004).

(*) Corresponding author: mharris@agro.uba.ar
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The aim of this work was to evaluate the effect of a calcium lactate treatment on postharvest behaviour, organoleptic and nutritional quality of strawberries grown in three growing systems and stored at two temperatures in modified atmosphere during 7 days of storage.

2. Materials and Methods

Plant material and growth conditions

The experiment was conducted in the Horticulture experimental field, School of Agriculture, University of Buenos Aires (latitude 45° S, longitude 58° 31´ W, altitude 26 m asl). Strawberry seedlings (commercial variety 'Camarosa') were planted in three growing systems: soil in open field, soil and perlite in greenhouse. Plant density was 7 plants m^{-2}. Soil treatments were covered with a black plastic mulching. All treatments were fertirrigated: nutrient solutions were formulated according to strawberry´s requirements (Table 1). Hourly intervals of temperature (°C), relative humidity (%) and radiation ($W \cdot m^{-2}$) were measured in open field and greenhouse with datalogger HOBO.

Table 1 - Nutrient solution formulation

Macroelements		Microelements	
Element	Concentration (mg l^{-1})	Element	Concentration (mg l^{-1})
Nitrogen	64	Iron	2.8
Phosphorus	31	Sodium	1.2
Potassium	200	Manganese	0.5
Sulfur	64	Boron	0.5
Magnesium	48	Copper	0.02
--		Zinc	0.05
		Molybdenum	0.01

Harvest

Mature strawberry fruits (at least 75% red colour) were harvested and immediately submerged in cold water to decrease fruit temperature. Half of the fruits of each growing system were treated with a calcium lactate 1% solution during one minute. Although calcium chloride is most commonly used, calcium lactate was used as a firming agent (Codex Alimentarius - World Health Organization, 2015) because chloride can give a bitter taste to fruits (Oms-Oliu *et al.*, 2010). Sixty-five grams (g) of fruit were packed in a modified atmosphere (medium density polyethylene semi rigid container). Modified atmosphere increases postharvest life in fruits and vegetables by reducing respiratory rate (Sandhya, 2010). Containers were stored at storage chambers at 1°C and 8°C.

Postharvest

At 1, 3 and 7 days of storage, quality characteristics of fruits were evaluated as follows:

Postharvest behaviour

- Oxygen and carbon dioxide concentration in the container was measured with Dansensor gas analyzer and expressed as percentage.
- Fresh weight loss. Fruits were weighted and weight loss was expressed as percentage relative to the initial value.

Organoleptic quality

- Visual quality. The overall visual and sanitary quality was determined by scoring each strawberry using a 1-10 hedonic scale, being 10 excellent and 6 the commercialization limit.
- Colour was measured with Minolta Chromameter CR300. L, a, b, c, h parameters were determined in four spots in equatorial zone of three fruits per treatment.
- Firmness was measured in equatorial zone with Ludwig penetrometer fitted with a 3 mm diameter round probe.
- Total soluble solids were determined with Atago refractometer and expressed as °Brix.

Nutritional quality

- Ascorbic and dehidroascorbic acid was determined by liquid chromatography and expressed as mg of ascorbic acid 100 g^{-1} of fresh weight.
- Antioxidant capacity. Antioxidant capacity was estimated by determining the free-radical scavenging capacity evaluated with the stable radical DPPH (adapted from Brand Williams *et al.*, 1995 and Leong and Shui, 2002). Two g of edible portion of the fruit was homogenized using a blender and inserted into a 50 ml centrifuge tube. Twenty ml of 50% aqueous ethanol was added (1:10 w/v) and mixed in a vortex mixer for 15-30 seconds. The extract was centrifuged at 2000 g for 5 min al 4°C. The supernatant was filtered before using. A 25 mg solution of DPPH (1,1 diphenyl-2-picrylhydrazyl) was prepared in methanol. For calibration curve, aliquots of ascorbic acid (0, 25, 50, 75, and 100 µl) solved in aqueous ethanol 50% (0.1 ml ml^{-1}) were placed in tubes with in 3 ml DPPH. Absorbance at 517 was measured at 1, 10, 30, 60, 90 and 120 minutes. An aliquot of 50 ml of an antioxidant/fruit extract solution was added to 3 ml of the DPPH solution. The decrease in absorbance at 517

nm was measured at 0, 1, 5 and then every 10 minutes until the reaction reached a *plateau*. The decreased absorbance of DPPH remaining at the steady-state was calculated and expressed as mg of ascorbic acid (AA) equivalents per 100 g of homogenate (AEAC). The AEAC was calculated using the following equation:

$$AEAC=\Delta A \times f \times V \times 100 \times 1/W$$

where Δ A is the change of absorbance after addition of fruit extract, f is the inverse of the calibration curve slope, V is the volume of filtrate (ml) and W is the weight of homogenate used for extraction (g).

Statistical analysis

A completely randomized factorial design with 3 replicates per treatment was used. The results were analyzed by multivariate analysis of variance repeated in time with a 5% significance level. Tukey test to compare means was used (Kuehl, 2001). Infostat software was used (Di Rienzo *et al.*, 2015).

3. Results and Discussion

Oxygen and carbon dioxide concentration in the container

Temperature, as expected, generated the most significant differences: strawberries stored at 1°C respired less than those stored at 8°C (Fig. 1). Nevertheless, in all cases, at equal temperatures, strawberries treated with calcium decreased the respiration.

On the other hand, an interaction between growing systems and calcium treatment was observed ($p<0,0001$) both in oxygen and carbon dioxide levels: strawberries grown in soil systems (both open field and greenhouse) and without calcium treatment showed larger differences between storage temperatures: those stored at 8°C showed a high oxygen decrease and carbon dioxide increase, especially at the 7th day of storage. Treated fruits, instead, showed similar behaviour during the seven days of storage. Thus, calcium treatment could be considered as a regulator because differences between temperatures in treated strawberries were smaller. Waghmare and Annapure (2013) observed that a combination of modified atmosphere, calcium chloride and nitric acid treatment in chopped papayas stored at 5°C had a significant decrease of oxygen and increase of carbon dioxide compared to fruits only stored in modified atmosphere.

Equilibrium modified atmosphere was not established, especially those stored at low temperatures. This could be because medium density polyethylene was used and this material has low permeability to gases: 2600 cm^3/m^2.d.atm for oxygen and 7600 cm^3/m^2.d.atm for carbon dioxide (Sandhya, 2010). Additionally, as temperature increases, material permeability increases as well. Thus, containers stored at 8°C had a higher permeability (Oliveira *et al.*, 2015).

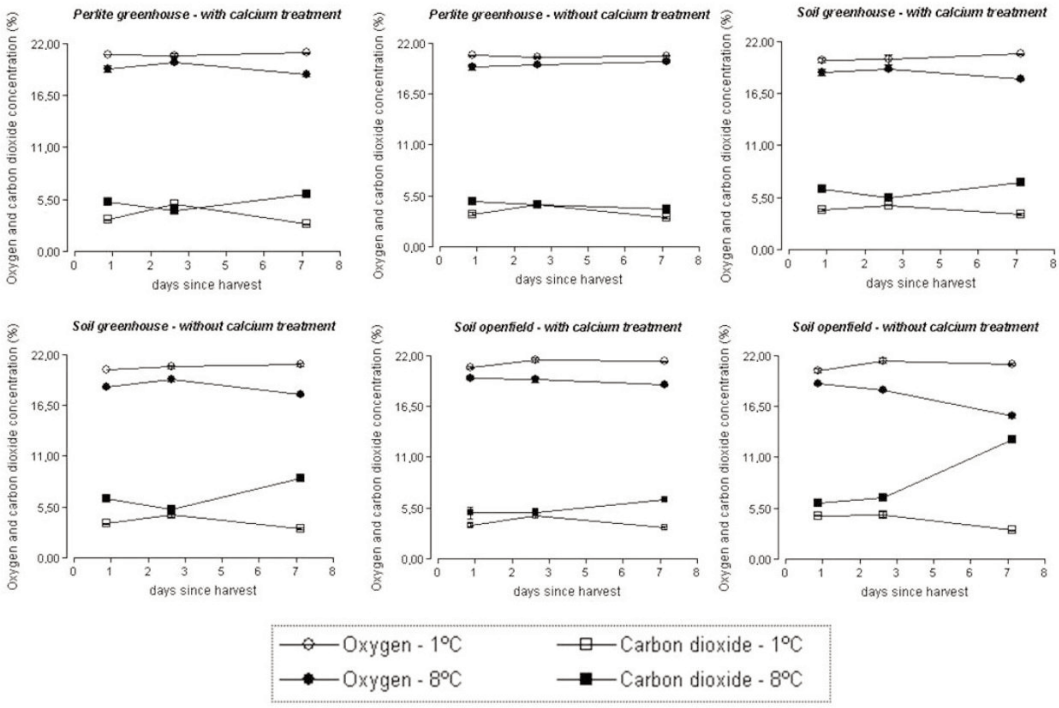

Fig. 1 - Oxygen and carbon dioxide concentration (%) in containers at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures.

Fresh weight loss

Maximum allowable weight loss in strawberry is 6% (Laurin *et al.*, 2003). All treatments, except one case, were below those values (Fig. 2). The smaller weight loss was observed in open field fruits ($p<0.0001$), independently storage temperature and calcium treatment. Preharvest temperatures can affect postharvest shelf life. For example, fruits grown at high temperatures can exhibit water soaking (Benkeblia *et al.*, 2011). In this work, differences between greenhouse and open field temperatures reached 7°C. Fruits harvested in greenhouse soil and soilless systems had surely higher temperature, what determined higher weight loss in those systems.

Visual quality

Fruits grown in perlite had better quality: 7.7% more than open field soil and 20% compared to greenhouse soil fruits (Fig. 3). At the 7th day of postharvest, all the strawberries stored at 1°C pre-

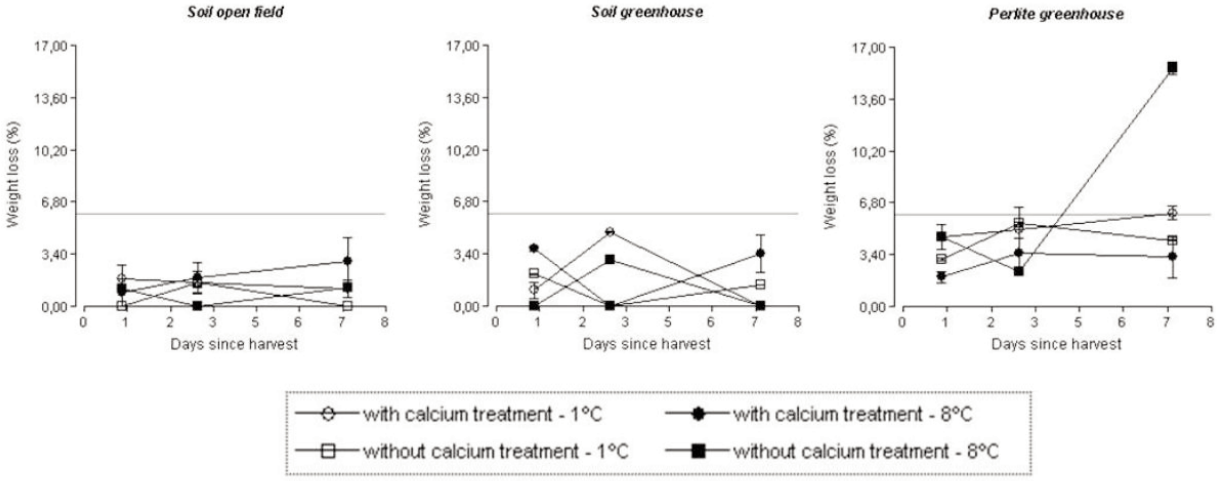

Fig. 2 - Fresh weight loss (%) at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures. Maximum allowable weight loss (6%) is indicated.

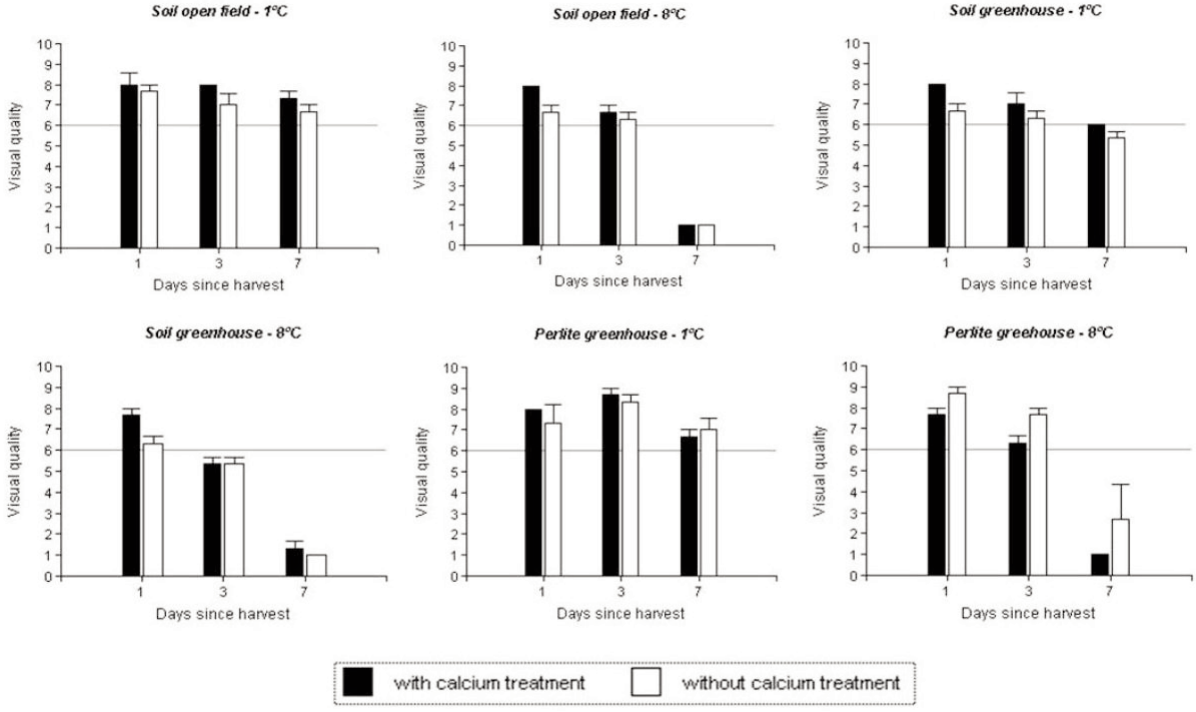

Fig. 3 - Visual quality at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures. Each graph represents a combination of growing system and storage temperature. Commercialization limit (6) is indicated.

sented visual and sanitary quality above commercialization level. Fruits stored at 8°C, instead, were below that level and in most cases they had *Botrytis cinerea* symptoms. Temperature management is a key factor to minimize postharvest deterioration in strawberry. At high storage temperatures, fruits have higher respiration rates, and consequently, shorter postharvest shelf life (Shin *et al.*, 2008).

Colour

Significant differences were not observed for all colour parameters. Shin *et al.* (2008) did not find colour changes during 7 days of storage of L c and h° values.

Firmness

An interaction was observed between growing system and calcium treatment (p=0,0203): treated strawberries grown in soil systems, both open field and greenhouse had 20% more firmness than others. In perlite, difference between treated and untreated fruit was not significant (Fig. 4).

Firmness increased in fruits stored at 1°C along the storage time. Shin *et al.* (2007) also observed an increase in firmness along 4 days of storage at high (10.5°C) and low (0.5°C) temperatures. The same authors investigated firmness during 12 days of storage and observed a positive tendency in fruits stored at 3°C and a decrease in those stored at 10°C (Shin *et al.*, 2008). Firmness increase in low storage temperatures is due to physical changes in cell wall: cold produces an increase in pectin viscosity, which impacts positively in fruit firmness (Lara *et al.*, 2004).

Total soluble solids

Total soluble solid content (Table 2) in all cases was above the minimum content recommended (7°Brix) for the postharvest quality maintenance (Mitcham *et al.*, 2015). A significant interaction was found between temperature and growing system (p<0,0001): fruits stored at 8°C and grown in open

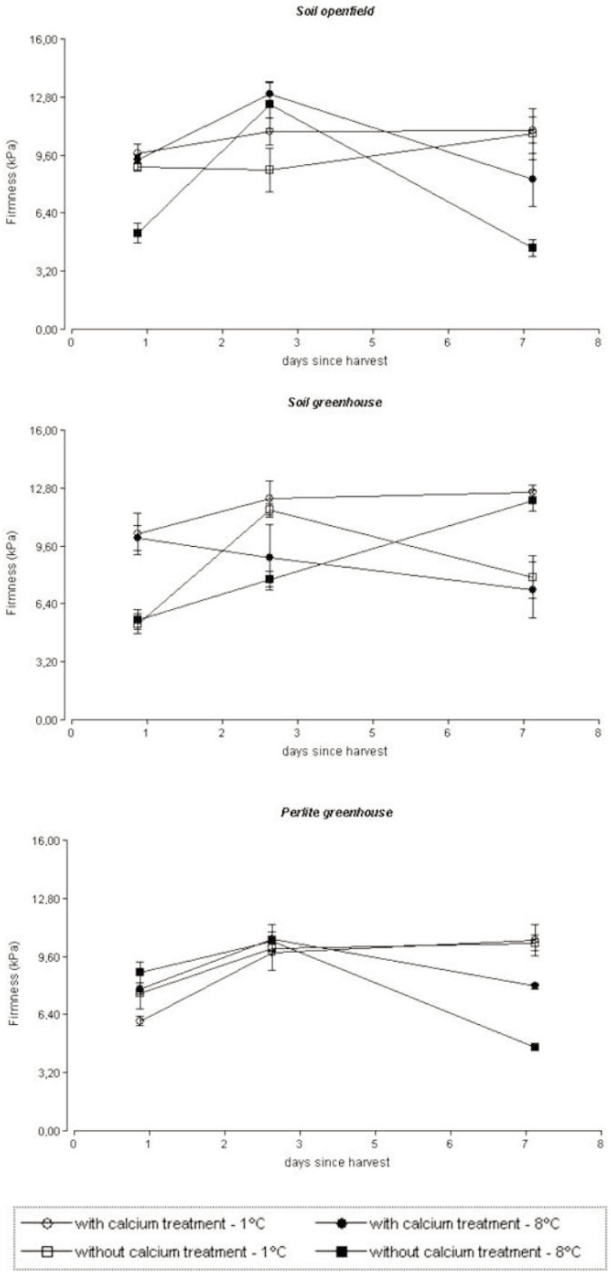

Fig. 4 - Firmness (kPa) at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures.

Table 2 - Total soluble solids (°Brix) at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures

Growing system	Storage 1°C						Storage 8°C					
	1 day		3 days		7 days		1 day		3 days		7 days	
	Ca with	Ca without	Ca with	Ca without	Ca with	Ca without	Ca with	Ca without	Ca with	Ca without	Ca with	Ca without
Soil open field	8 A bc	10 A d	7.63 AB ab	7.83 AB bc	8 A bc	6.33 A a	8.50 A bc	7.17 A a	9.17 AB cd	12 A e	8 A ab	9 AB cd
Soil greenhouse	8 A bc	7.33 B a	7.75 AB ab	8.67 BC cd	8.17 AB bc	9.33 AB de	8.33 A cd	8.17 BC bc	8.33 B cd	7.33 BC ab	6 B a	8.83 B e
Perlite greenhouse	8 A a	9.5 A bc	8 A a	9.17 CD ab	8 A a	8.83 BC ab	9 AB cd	6.5 AB a	8 BC ab	8.33 CD bc	10.5 C de	7.67 BC ab

Different capital letters indicate differences between row and different small letters indicate differences between columns (p<0.05).

field had higher content of soluble solids. It was only in the open field system that a difference between storage temperatures was found: those stored at 8°C had a 12.5% higher content of total soluble solids.

Calcium lactate immersion affected negatively the total soluble solid content. Similar results were found by other authors with different calcium sources. Singh *et al.* (2007) observed that weekly foliar applications of calcium chloride since flowering decreased total soluble solid content in 10% at harvest. Dunn and Able (2006), as well, found that a calcium deficiency during growth stage increased significantly soluble solids content in fruits.

Ascorbic acid content

In almost all cases, ascorbic acid content (Fig. 5) decreased during storage period. Phillips *et al.* (2016) also observed a decrease in strawberries stored at -1.5°C during 7 days. Only the fruits stored at freezers (-10 to -20°C) and ultra freezers (-55°C) maintained ascorbic acid contents similar to those observed at harvest time. Low temperature storage is a key factor to maintain ascorbic acid content during postharvest (Lee and Kader, 2000).

An interaction was observed between calcium treatment and storage temperature (p=0.0026). Untreated fruits stored at 1°C had 26% more ascorbic acid compared to the others. Many authors recognize that a calcium treatment at harvest enhances ascorbic acid content in fruit mainly due to an increase in fruit firmness (Aghdam *et al.*, 2013). Nevertheless, other authors did not find differences between calcium treatment and the control (Shaffie *et al.*, 2010). Regardless the calcium treatment, temperature was the key factor to maintain ascorbic acid content in strawberries.

Fruits grown in perlite had a 49% higher content compared to those grown at soil (both open field and greenhouse). These results are in agreement with de data reported by Treftz and Omaye (2015), who observed that soilless grown strawberries had a 74% higher content of ascorbic acid compared to soil grown fruits. For other fruits, the results were contradictory: Isabelle *et al.* (2010) observed higher ascorbic acid content in pepper and Özcelik and Akilli (1999) in tomato. However, Gruda (2005) didn´t find differences in strawberry.

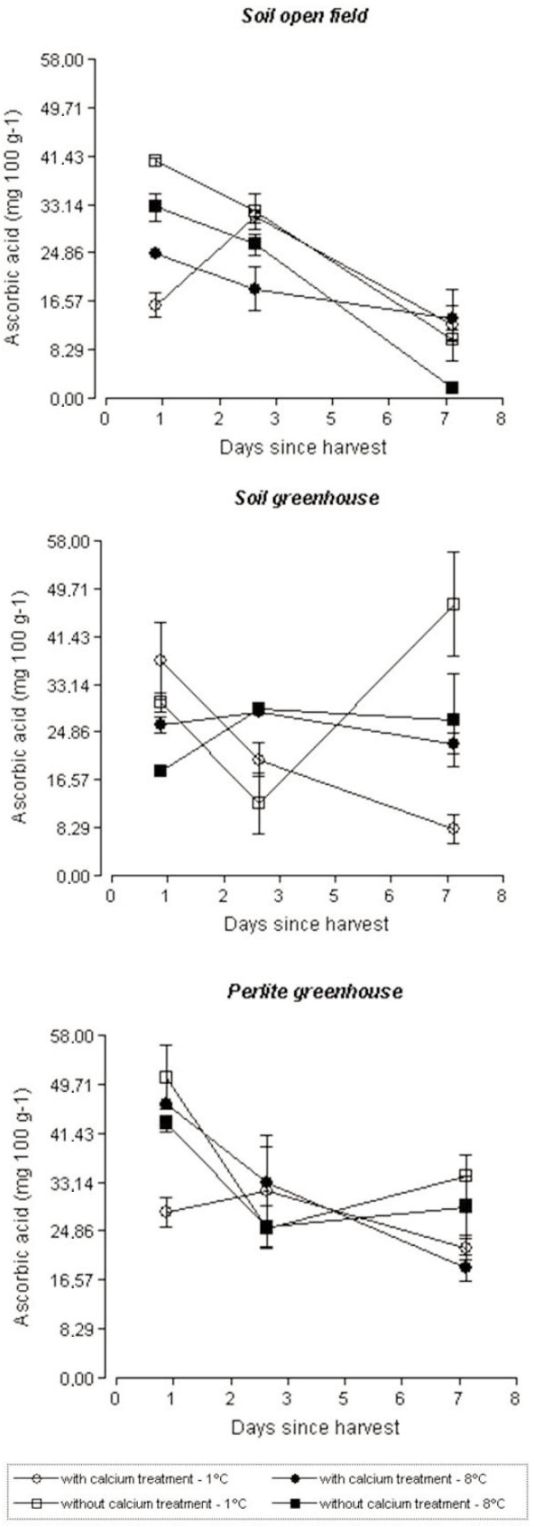

Fig. 5 - Ascorbic acid content (mg 100 g^{-1}) at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures.

Antioxidant capacity

Several interactions were observed for antioxidant capacity. Untreated and greenhouse soil grown fruits presented 13% higher antioxidant capacity (p=0,0003) (Table 3). Wang and Zheng (2001) observed that strawberries grown under higher temperature (day and night) had higher antioxidant capacity, as it was observed in this work, where differences of temperatures between greenhouse and

Table 3 - Antioxidant capacity (mg of ascorbic acid 100 g^{-1} of fresh weight) at 1, 3 and 7 days of postharvest of strawberries grown in different growing systems, calcium lactate treatment and two storage temperatures

Growing system	Storage 1°C				Storage 8°C			
	1 day		7 days		1 day		7 days	
	Ca with	Ca without	Ca with	Ca without	Ca with	Ca without	Ca with	Ca without
Soil open field	150.6 A a	213.1 B ab	232.2 C bc	173.85 AB ab	176.2 AB ab	173.65 AB ab	193.1 AB ab	175.8 AB ab
Soil greenhouse	170.55 AB ab	230.1 C bc	171.05 AB ab	234.65 C bc	157.65 A a	146.7 A a	190.3 AB ab	189.8 AB ab
Perlite	173.15 AB ab	220.55 B b	174.5 AB ab	148.3 A a	162.55 AB ab	174.55 AB ab	201.3 B ab	146.75 A a

Different capital letters indicate differences between row and different small letters indicate differences between columns ($p<0.05$).

open field reached 7°C.

As well, untreated fruits stored at 1°C had 10% higher antioxidant capacity ($p=0.0295$). Many authors, as described in 3.7, explain that a calcium treatment enhances ascorbic acid content (and consequently antioxidant capacity) due to an increase in cell wall firmness. Nevertheless, other authors express that a disruption in cell wall composition increases antioxidant capacity, but this increase is different depending the product (Reyes *et al.*, 2006). With respect to storage temperature, Shin *et al.* (2008) found significant differences since the 12th day.

Different capital letters indicate differences between columns and different small letters indicate differences between rows ($p< 0.05$).

Yield in the growing systems

Although yield was not an objective of this work, we observed that the perlite system had 35% and 17% higher yield than soil in greenhouse and soil in open field systems, respectively.

4. Conclusions

Strawberry is highly accepted by consumers but its postharvest quality rapidly declines. A calcium lactate treatment can increase principally strawberry's firmness. This, in addition to storage temperature and growing system can increase postharvest shelf life of fruits.

In this research, the calcium lactate treatment had a positive effect on fruit firmness, especially in those grown in open field and greenhouse soil. Furthermore, treated fruits had less respiration during storage time and there was no significant difference between storage temperatures in fruits treated with calcium lactate. Equilibrium modified atmosphere was not established.

The other variables were not affected or had a negative response to calcium lactate treatment. Weight loss and organoleptic quality (except visual quality) were better in fruits grown in open field soil, regardless calcium treatment and storage temperature. On the other hand, nutritional quality was better in untreated fruits and stored at 1°C.

In conclusion, even though temperature is substantial to maintain fruit quality at postharvest, a calcium lactate treatment could be useful to improve strawberry (cv. Camarosa) quality during transport and commercialization and decrease incidence of postharvest diseases.

Acknowledgements

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Effect of pre-harvest putrescine treatment on quality and postharvest life of pear cv. Spadona

M.S. Hosseini (*), Z. Fakhar, M. Babalar, M.A. Askari
Department of Horticulture Science, College of Agriculture and Natural Resources, University of Tehran, 31587 Karaj, Iran.

Key words: cold storage, color, fruits, quality assessment.

Abstract: The study was conducted to determine the effect of pre-harvest foliar spraying with putrescine (at 0.5, 1 and 2 mM) on quality and postharvest life of *Pyrus communis* cv. Spadona during cold storage. Fruit quality assessment such as weight loss, firmness, total soluble solids (TSS), titratable acidity (TA), flavor index, skin color (L^*, hue angle), vitamin C total phenol (TP), and total antioxidant activity (TAA) were made at harvest and after 3, 6, 9, 12, 15, 18 and 21 weeks of storage at 0±1°C, 80-85% relative humidity. Weight loss, fruit softening, TSS and pH increased during storage but the rate of changes was significantly lower in fruit treated with putrescine at 1 and 2 mM. Putrescine application maintained higher levels of TA, vitamin C, TP, TAA, L^*, hue angle and reduced decay incidence compared to control. Furthermore, higher doses of putrescine were effective in terms of prolonging the storage and marketability of fruits more than 127-142 days. In conclusion, pre-harvest application of putrescine could be an effective means for extending the postharvest life of pear cv. Spadona.

1. Introduction

The polyamines as natural compounds are present ubiquitously in almost all living organisms. The main polyamines in significant amounts are putrescine, spermidine, and spermine which are crucial for the growth and development of plant and fruit as well as stress responses (Valero and Serrano, 2010). They are known as anti-senescent agents that decrease the rate of fruit softening and senescence by suppression of ethylene production (Kramer *et al.*, 1991). Reduced values of polyamines have been attributed with enhanced ethylene production and vice versa (Walden *et al.*, 1997). This mechanism is correlated to a competition between polyamine and ethylene for the common precursor *S*-adenosyl methionine (SAM) (Pandey *et al.*, 2000). The use of polyamines has been claimed to decrease ethylene synthesis in a wide range of plants by decreasing ACC synthase (ACS) and ACC oxidase (ACO) enzymes activities (Ke and Romani, 1988; Kakkar and Rai, 1993; Lee *et al.*, 1997; Martinez-Romero *et al.*, 2001; Bregoli *et al.*, 2002; Perez-Vicente *et al.*, 2002; Serrano *et al.*, 2003; Petkou *et al.*, 2004; Malik and Singh, 2005; De Dios *et al.*, 2006; Khan *et al.*, 2007).

In several investigations putrescine applied exogenously have been reported to increase storage life and quality attributes of mango (Razzaq *et al.*, 2014), pear (Franco-Mora *et al.*, 2005), apricot (Martinez-Romero *et al.*, 2002), strawberry (Zokaee Khosroshahi *et al.*, 2007), plum (Abu-Kpawoh *et al.*, 2002; Pérez-Vicente *et al.*, 2002), grapes (Harindra Champa *et al.*, 2015; Mirdehghan and Rahimi, 2016), pomegranate (Mirdehghan *et al.*, 2007; Barman *et al.*, 2011) and litchi (Jiang and Chen, 1995).

Therefore, the aim of this study was to investigate the role of preharvest putrescine treatment on maintaining postharvest quality of pear fruit cv. Spadona.

2. Materials and Methods

The experiments were conducted on pear trees (*P. communis* cv. Spadona) in the center of horticultural research of the University of Tehran, Karaj, Iran. Eighteen 16-year-old trees (250 cm height) were selected in terms of uniformity in size and fruit load then sprayed with putrescine at 0.5, 1 and 2 mM (3.5

(*) Corresponding author: m.hosseini79@yahoo.com
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L per tree) at different stages of fruit development in May, June, and July. Six trees sprayed with water (3.5 L per tree) were used as control. Fruits were harvested manually and transported to the postharvest laboratory, and selected for absence of visual symptoms of disease or blemishes, then stored (5 fruits per basket) at 0±1°C, 80-85% RH for 21 weeks. Quality attributes were measured in five fruits of each replicate at harvest and after 3, 6, 9, 12, 15, 18 and 21 weeks of cold storage.

Fruit quality assessments

Fruit color changes were calculated at two opposite sides of fruit with a Minolta Chroma Meter CR-400 (Osaka, Japan). The values of L^*(0 - black; 100 - white), a^* (green to red), b^* (blue to yellow) and hue angle ($h°=180 + \tan^{-1} b^*/a^*$, if $a^* < 0$) were recorded (Fernando *et al.*, 2007; Pek *et al.*, 2010).

The percentage of weight loss was recorded by using following equation:

$$\% \text{ weight loss} = (A-B)/B \times 100$$

in which A was the initial fruit weight and B was the final fruit weight. Fruit firmness was determined using a penetrometer FT327 (GFFECI, Italy) fitted with an 8 mm tip on the equatorial position of fruit. The results were expressed in newton (N).

Total soluble solids (TSS) in the extracted juice of each treatment were measured by a refractometer (Atago N1, Japan) at 20°C and the result was recorded as percentage. Five ml of diluted juice titrated against 0.1 N NaOH to pH 8.2 to assess TA. Phenolphthalein was used as an indicator. The TA was expressed as malic acid percentage (Saini *et al.*, 2001). The pH of fruit juice was calculated using a MTT65 (Japan) pH meter calibrated by pH 4 and 7 buffer solutions. Flavor index was estimated by dividing TSS with the corresponding TA value. Vitamin C was measured using the procedures of Tian *et al.* (2002).

Total phenol (TP) content and total antioxidant activity (TAA)

TP and TAA were assessed according to Koushesh Saba *et al.* (2012).

Decay incidence determination

Fruit decay was determined based on the procedure of Khademi and Ershadi (2013). Scales from 1 to 5 were given to individual treatment group whereas: 1= normal (without decay), 2= (up to 5 % decay), 3= (5-20 % decay), 4= (20-50% decay) and 5= (more than 50% of fruits skin was decayed).

Statistical analysis

This experiment was conducted in a randomized experimental design with three levels of putrescine (0.5, 1 and 2 mM), using plants sprayed with water as control in three replications and two trees in each experimental unit. To estimate storability of pear fruit cv. Spadona, a factorial design in completely randomized were carried out and the experimental data analyzed using SAS statistical software package 9.4 for windows and mean comparisons were conducted using Duncan's multiple range tests.

3. Results and Discussion

Color

A high rate of color changes was observed in control fruits and 0.5 mM putrescine treated fruits, whereas, they exhibited lower L^* and hue angel than others during storage (Fig. 1 A and B). Therefore, the conversion rate of green to yellow and degradation

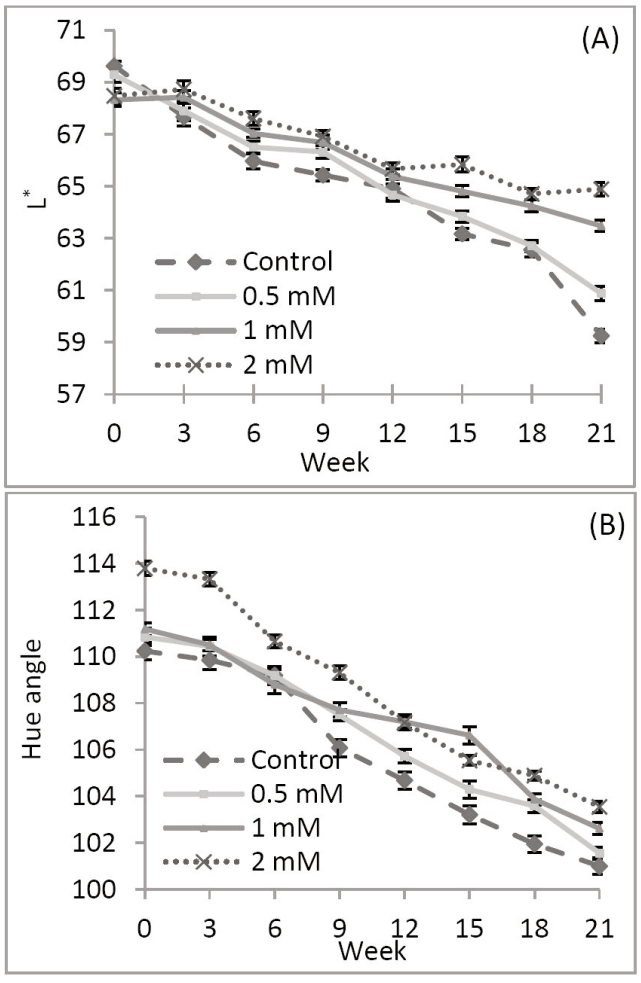

Fig. 1 - The effect of putrescine at different concentrations on L* (a) and hue angle (b) of pear cv. Spadona along the storage. Values are the mean ± SE.

of chlorophyll were shown slower in putrescine treated fruits by 1 and 2 mM. The effect of putrescine in retarding skin color changes throughout the storage by decreasing senescence rate has also been reported in table grape (Harindra Champa *et al.*, 2015), and pomegranate (Barman *et al.*, 2011).

Weight loss and firmness

The weight loss increased in all fruit samples during the 21 weeks cold storage. As shown in figure 2 A, putrescine at 1 and 2 mM reduced the weight loss value than control at the end of storage. However, fruit treated with 2 mM putrescine showed inferior weight loss which started at the third sampling date (6th week), while it was not seen in those treated with 1 mM before the fifth sampling date (12th week). Reduction of weight loss in putrescine treated fruits can be ascribed to conjugation of polyamines to the cell membrane phospholipids and consequently stabilization as well as consolidation of both cell integrity and permeability (Barman *et al.*, 2011; Mirdehghan and Rahimi, 2016). Irrespective of treatments, fruit firmness decreased with the advancement of storage but putrescine treatment at 1 and 2 mM maintained highest fruit firmness compared to control (Fig. 2B). It is suggested that polyamines maintain fruit firmness by their cross-linkage to the pectin substances carboxyl groups in the cell wall and lead to strengthening of cell wall and consequently decreasing cell wall degrading enzymes activities of pectin methyl esterase (PME), pectin esterase (PE) and polygalactouronase (PG) (Valero *et al.*, 2002). The role of putrescine in reducing weight loss and maintaining fruit firmness has been reported for peach (Zokaee Khosroshahi and Esna-Ashari, 2008) and pear (Franco-Mora *et al.*, 2005).

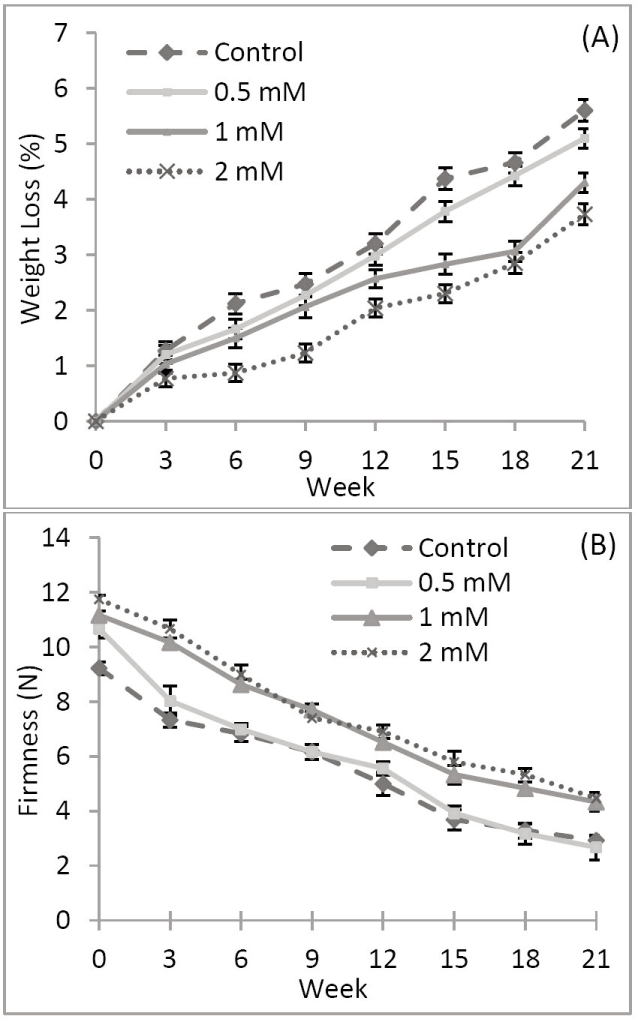

Fig. 2 - The effect of putrescine at different concentrations on weight loss (a) and firmness (b) of pear cv. Spadona along the storage. Values are the mean ± SE.

TSS, TA, pH and flavor index

The contents of TSS (in the first 12 weeks of storage), pH and flavor index increased in all treated and untreated fruits while TA showed reverse trend along the storage. However, the lowest TSS, pH and flavor index were observed in treated fruits by 1 and 2 mM (Fig. 3 A, B, C and D). The role of putrescine on maintaining TSS, TA and pH in treated fruits would be attributed to the reduction of respiration rate (Valero *et al.*, 2002), ethylene synthesis (Barman *et al.*, 2011) and subsequently retarding the ripening process. Similar results have been reported in peach (Zokaee Khosroshahi and Esna-Ashari, 2008) and apricot (Enas *et al.*, 2010).

Vitamin C

Vitamin C significantly declined as the storage advanced. However, this trend was slower in 1 and 2 putrescine treated fruits (Fig. 4). This effect can be associated with the property of putrescine on reducing or delaying the activity of ascorbate oxidase and consequently maintaining vitamin C (ascorbic acid) content (Ishaq *et al.*, 2009). Similar results have been reported in mango (Razzaq *et al.*, 2014) and apricot (Davarynejad *et al.*, 2013).

Total phenol (TP) and total antioxidant activity (TAA) measurement

Irrespective of treatments, total phenolic content and total antioxidant activity decreased at the end of storage; while these decreases were significantly higher at 1 and 2 mM putrescine treated fruits (Fig. 5 A and B). In spite of TAA, the TP changes were not constant during storage, it reached the highest value at the 9th week in fruits treated with 1 and 2 mM with the maximal values of 28 and 31 mg of GAE/100 g of FW at the 9th week respectively, then followed by reducing TP during the rest of storage period.

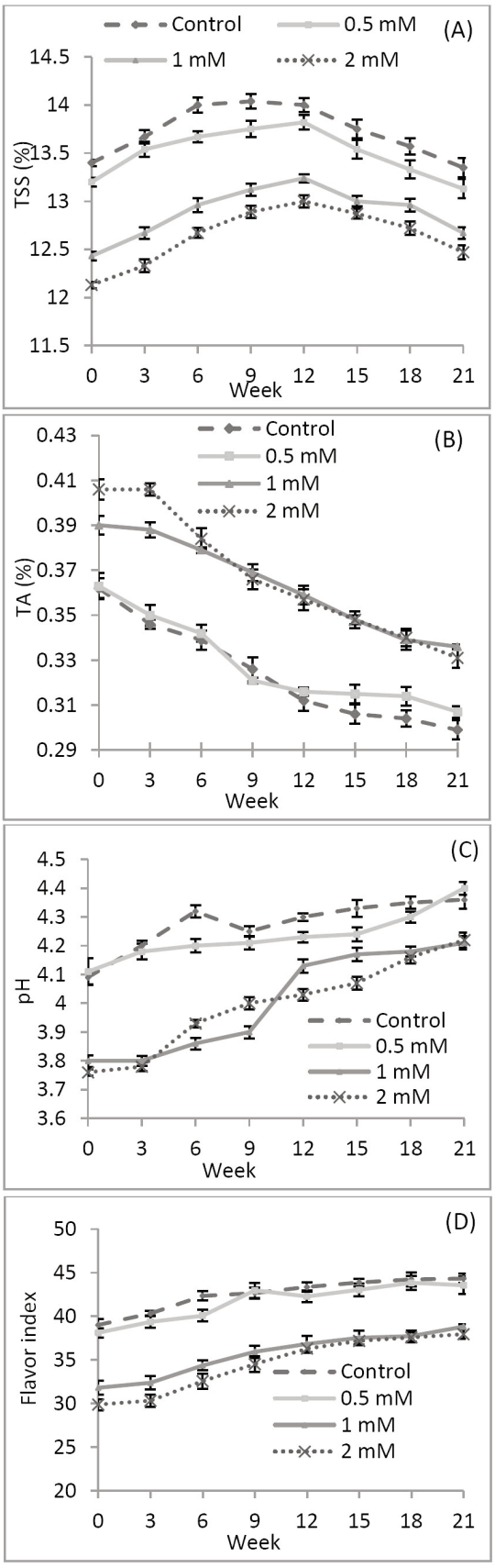

Fig. 3 - The effect of putrescine at different concentrations on TSS (a), TA (b), pH (c) and flavor index (d) of pear cv. Spadona along the storage. Values are the mean ± SE.

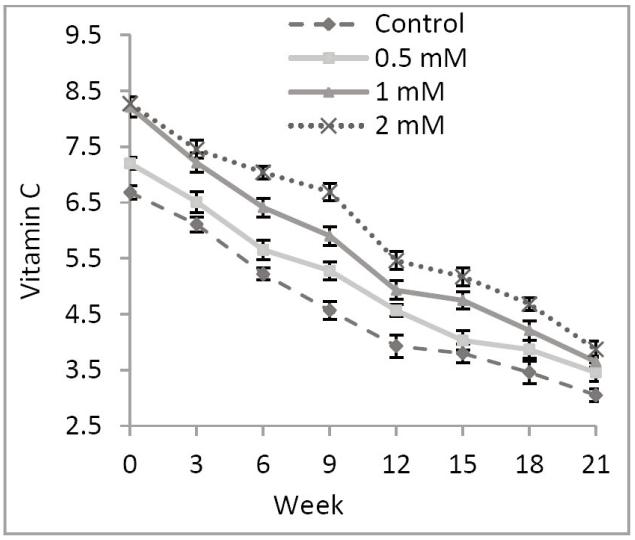

Fig. 4 - The effect of putrescine at different concentrations on vitamin C of pear cv. Spadona along the storage. Values are the mean ± SE.

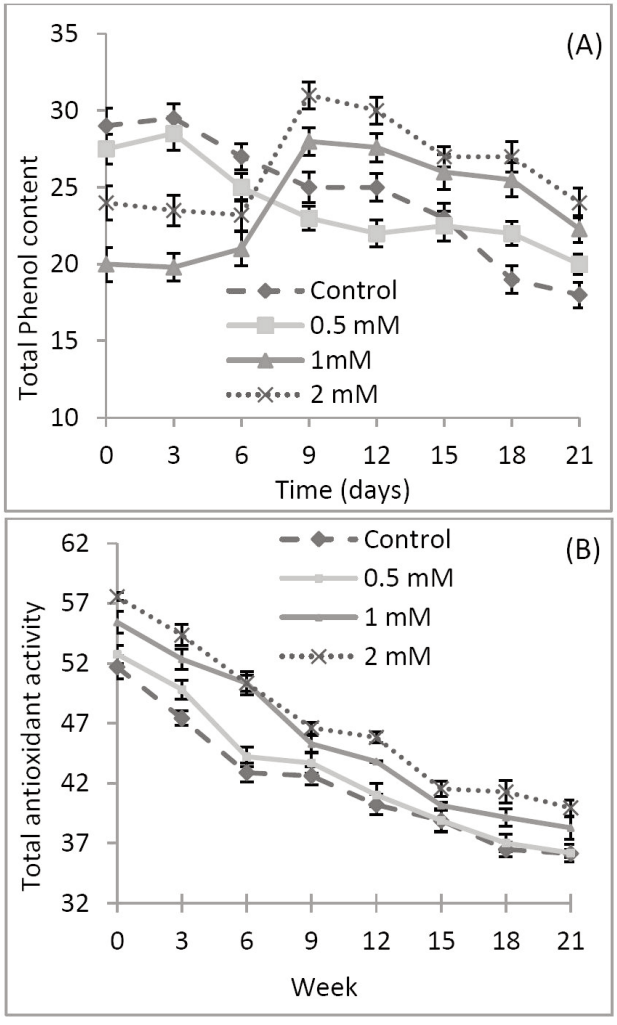

Fig. 5 - The effect of putrescine at different concentrations (0.5, 1 and 2 mM) on total phenol content (A) and total antioxidant activity (B) of pear cv. Spadona along the storage. Values are the mean ± SE.

The changes in the level of TP content may be associated to the breakdown of cell structure and subsequently senescence (Ghasemnezhad *et al.*, 2010). The role of putrescine treatment to maintain TP could be ascribed to the delay of senescence process (Arora *et al.*, 2002; Razzaq *et al.*, 2014).

As shown in figure 5, the value of TAA decreased along with a decrease of TP during storage. It may be ascribed to a direct correlation among TP content and TAA (Razzaq *et al.*, 2014). However, putrescine treatment at 1 and 2 mM maintained TAA compared to control during storage. Similar results demonstrated a positive correlation among TP and TAA in mango (Palafox-Carlos *et al.*, 2012) and apricot (Ghasemnezhad *et al.*, 2010).

Decay incidence

The lowest rate of fruit decay percentage was observed in fruits treated with 1 and 2 mM putrescine contrary to control at the end of storage (Fig. 6). It is suggested that polyamines have all requirements of an alternative approach for management of postharvest decay (Romanazzi *et al.*, 2012).

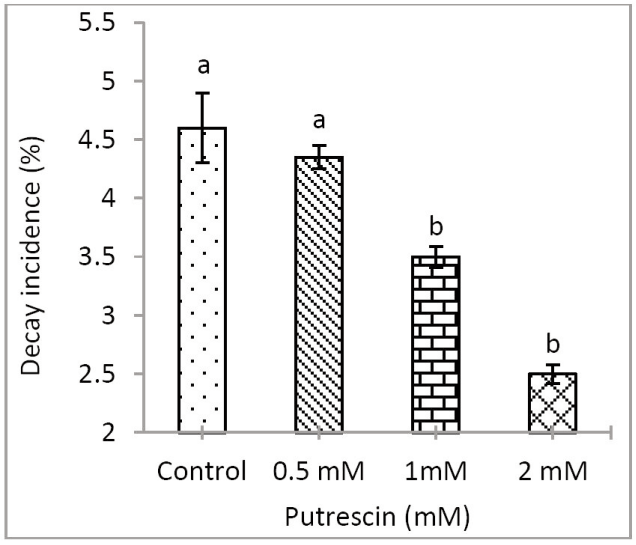

Fig. 6 - The effect of putrescine at different concentrations (0.5, 1 and 2 mM) on decay incidence of pear cv. Spadona at the end of storage. Values are the mean ± SE.

Storage life

The application of putrescine at higher doses (1 and 2 Mm) extended storage life of pear fruits, and consequently they were suitable to be exposed in the market more than 127-142 days after the beginning of storage in comparison to control (109 days) (Fig. 7).

4. Conclusions

The pre-harvest application of 1 and 2 mM putrescine treatment maintained the postharvest life of pear cv. Spadona by reducing weight loss, fruit softening, color changes as well as retarding the degradation rate of TSS, TA, pH, vitamin C, total phenol and total antioxidant in pear fruit during storage. Moreover, the storage life and marketability of putrescine treated fruits were prolonged by decreasing decay incidence. Thus, pre-harvest application of putrescine can be an effective means for extending the postharvest life of pear cv. Spadona.

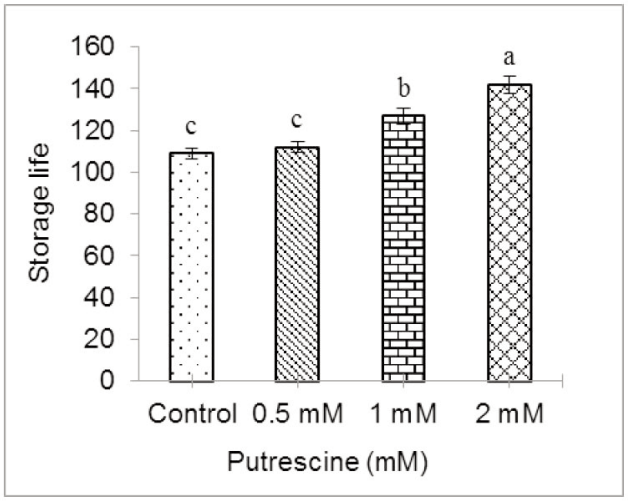

Fig. 7 - The effect of putrescine at different concentrations (0.5, 1 and 2 mM) on storage life of pear cv. Spadona. Values are the mean ± SE.

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Improving oil and flavonoid contents of milk thistle under water stress by salicylic acid

K. Ghassemi-Golezani*, S. Ghassemi, I. Yaghoubian
Department of Plant Eco-physiology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.

Key words: foliar application, plant biomass, seed yield, *Silybum marianum* L., water deficit.

Abstract: Adverse environmental conditions such as water deficit can limit production. However, some of these adverse effects may be overcome by application of plant growth regulators including salicylic acid (SA). Thus, a field experiment was conducted in 2015 to evaluate the effects of SA (0 and 1 mM l^{-1}) on yield components, seed yield and oil and flavonoid contents of milk thistle (*Silybum marianum* L.) under different irrigation treatments (I_1, I_2, I_3 and I_4: irrigation after 70, 110, 150 and 190 mm evaporation from class A pan, respectively). The experiment was arranged as split-plot based on randomized complete block (RCB) design in three replicates. Irrigation treatments and SA levels were located in the main and sub plots, respectively. The results indicated that plant biomass, seeds per plant, 1000 seed weight, seed yield per unit area and harvest index of milk thistle decreased as a consequence of water stress. Oil percentage and yield were also reduced, but flavonoid content enhanced with increasing water deficit. All these traits were considerably augmented by foliar application of SA under non-stress and stressful conditions. Therefore, it was conclude that SA can be used to improve field performance of milk thistle under different environmental conditions.

1. Introduction

Milk thistle (*Silybum marianum* L.) is one of the most important medicinal plants in the pharmaceutical industry worldwide, and is used in the production of flavonoids of the silymarin group (silybin, silidianin and silychristine) which are important in the modern pharmaceutical industry (Ghavami and Ramin, 2007). The origin of this plant has been reported to be the East Mediterranean region (Keville, 1991). Water availability may influence physiological and biochemical properties and seed yield of this medicinal plant.

Water stress severely limits growth and yield of plants by reducing ground green cover (Ghassemi-Golezani and Ghasssemi, 2013), chlorophyll content of leaves, photochemical efficiency of photosystem II (Ghassemi-Golezani and Lotfi, 2012) and photosynthesis (Munns *et al*., 2006). Water stress during vegetative stages largely reduces plant height and biomass, while during reproductive stages it has the greatest negative impact on seed yield (Ghassemi-Golezani *et al*., 2008). Reports in oil crops indicated that water stress decreases oil and increases protein percentages of seeds. However, both oil and protein yields per unit area are decreased as a result of large reduction in seed yield per unit area due to water limitation (Ghassemi-Golezani and Lotfi, 2013; Ghassemi-Golezani *et al*., 2015 b). Some of the deleterious effects of environmental stresses on plant performance could be alleviated by foliar application of growth regulators such as salicylic acid (SA) (Ghassemi-Golezani *et al*., 2015 a).

It has been reported that the exogenous application of SA induces plant tolerance to several abiotic stresses including drought tolerance in wheat (Singh and Usha, 2003), salinity tolerance in safflower (Ghassemi-Golezani and Hosseinzadeh-Mahootchi, 2015) and mung bean (Ghassemi-Golezani *et al*., 2015 a), heat tolerance in mustard (Dat *et al*., 1998) and chilling tolerance in maize (Janda *et al*., 1999). These studies suggest that SA may enhance the multiple types of stress tolerance in plants by interactive effects on several functional molecules. Hayat *et al*. (2008) found that there was a significant increase in photosynthetic parameters, chlorophyll and proline contents, and antioxidant enzyme activities in SA treated tomato plants. Loutfy *et al*. (2012) reported that SA induced drought tolerance and increased plant biomass, leaf relative water content, and the solute contents in four wheat cultivars. Moreover,

(*) Corresponding author: golezani@gmail.com
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foliar spray of SA decreased the inhibitory effects of drought on *Phillyrea angustifolia* (Munné-Bosch and Penuelas, 2003). Abd el-Lateef Gharib (2006) stated that oil content of Basil and Marjoram significantly increased with application of SA. However, the responses of some medicinal plants to SA treatment in stressful conditions were not documented so far. Thus, this research was undertaken to evaluate the effects of foliar application of SA on seed yield and oil and flavonoid contents of milk thistle under different irrigation intervals.

2. Materials and Methods

Seeds of milk thistle (*Silybum marianum* L.) were obtained from Pakan bazr, Isfahan, Iran. The experiment was conducted in 2015 at the Research Farm of the Faculty of Agriculture, University of Tabriz, Iran (Latitude 38° 05′ N, Longitude 46° 17′ E, Altitude 1360 m above sea level). The climate is characterized by mean annual precipitation of 245.75 mm per year, mean annual maximum temperature of 16.6°C and mean annual minimum temperature of 4.2°C. The field experiment was arranged as split-plot based on randomized complete block design in three replications, with irrigation intervals (I_1, I_2, I_3, I_4: irrigation after 70, 110, 150 and 190 mm evaporation from class A pan, respectively) in main plots and two levels of salicylic acid (SA; 0 and 1 mM l^{-1}) in sub-plots. Seeds of milk thistle were treated with 3.3 g/kg Benomyl and then were sown by hand on 28 May 2015 in 3 cm depth of a sandy loam soil. Each plot consisted of 6 rows of 3 m length, spaced 25 cm apart. All plots were regularly irrigated up to seedling establishment, but thereafter irrigations were carried out according to treatments. Weeds were frequently controlled by hand during crop growth and development. Salicylic acid (SA; 0 and 1 mM) was sprayed at vegetative and flowering stages.

Plant biomass and seed yield

At maturity, plants in 1 m^2 (8 plants) of the middle part of each plot were harvested and seeds per plant, 1000 seed weight and seed yield per unit area were determined. Then above ground biomass was oven-dried at 80°C for 48 hours and weighed and subsequently harvest index was calculated.

Oil extraction

Oil was extracted from 3 g mature seeds of each plot in petroleum ether for 5 hours using a Soxhlet system according to the AOCS method (AOCS, 1993). Oil content was determined as a percentage for each sample and then oil yield per unit area was calculated as:

$$\text{Oil yield} = \text{Seed yield} \times \text{Oil percentage}$$

Flavonoid extraction

Powdered air-dried mature seeds (1 g) were extracted in a Soxhelt extractor with 100 ml ethanol for an hour and the extract filtered. Three ml of the extract was placed in a 15 ml volumetric flask. Then 0.3 ml $NaNO_2$ (1:20) and after 5 minutes 3 ml $AlCl_3$ (1:10) and 6 minutes later 2 ml of 1 mol $litre^{-1}$ NaOH were added and the total was made up to 10 ml with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm with a HALO DB-20 spectrophotometer (Zhuang *et al*., 1992). The flavonoid content was calculated using the following linear equation:

$$A = 0.01069C - 0.001163$$

Where A is the absorbance and C is the flavonoid content in µg/g.

Analysis of variance

Analysis of variance of the data appropriate to the experimental design and comparison of means at $p \leq 0.05$ were carried out, using GenStat 12 and MSTATC softwares. Excel software was used to draw figures.

3. Results

Analysis of variance (Table 1) showed that plant biomass, seeds per plant, 1000 seeds weight, seed yield per unit area and harvest index were significantly affected by water limitation and SA, but the interaction of irrigation × SA was only significant for 1000 seed weight and harvest index.

Table 1 - Analysis of variance of the data for plant biomass, yield components and seed yield of milk thistle affected by irrigation treatments and salicylic acid (SA) treatments

Source of variation	df	Mean square				
		Plant biomass	Seeds per plant	1000 seeds weight	Seed yield	Harvest Index
Replication	2	1198	2162	0.1163	61.7	0.0129
Irrigation (I)	3	38104 **	56241 **	4.6906 *	3121.7 **	4.1715 **
Error	6	2795	4424	0.2785	140	0.1257
SA	1	837851 **	1718420 **	15.0417 **	71195.6 **	48.4504 **
I × SA	3	2633 NS	7585 NS	0.9128 **	262 NS	3.3993 **
Error	8	3987	7553	0.1212	259.2	0.1058
CV (%)	-	8.8	9.2	1.5	9.2	1.4

NS, * and ** No significant and significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Plant biomass, seeds per plant and seed yield per unit area decreased with decreasing water availability, but all these traits were considerably enhanced by foliar application of SA. Reduction in seeds per plant was only significant under severe water stress (I_4), with no significant difference among I_1, I_2 and I_3 treatments. Differences in plant biomass and seed yield between I_1 and I_2 and also between I_2 and I_3 were not statistically significant. Application of SA improved plant biomass, seeds per plant and seed yield by about 71%, 79% and 91%, respectively (Table 2).

Table 2 - Means of plant biomass, seeds per plant and seed yield of milk thistle for irrigation and salicylic acid (SA) treatments

Treatments	Plant biomas (g/m^2)	Seeds per plant	Seed yield (g/m^2)
Irrigation			
I_1	797.2 a	1034.8 a	197.0 a
I_2	746.2 ab	984.0 a	185.2 ab
I_3	705.0 b	939.7 a	172.5 b
I_4	609.0 c	808.8 b	143.9 c
Salicylic acid			
Irrigation	528 b	674 b	120.2 b
SA1	901 a	1209 a	229.1 a

Different letters in each column indicate significant difference at p≤ 0.05.

One thousand seeds weight (Fig. 1A) and harvest index (Fig. 1B) of untreated plants with SA gradually decreased as water stress increased. But, these reductions were only significant under severe water deficit. In contrast, SA treated plants did not show significant reduction in seed weight and harvest index due to water limitation.

Oil percentage, oil yield and flavonoid content were significantly affected by irrigation and SA treatments, but the interaction of irrigation × SA was not significant for these traits (Table 3). Oil percentage and yield of milk thistle decreased, but flavonoid content increased as a result of water stress. Seed oil percentage and yield and flavonoid content were significantly enhanced by foliar spray of SA. This superiority was more pronounced for oil yield per unit area (Table 4).

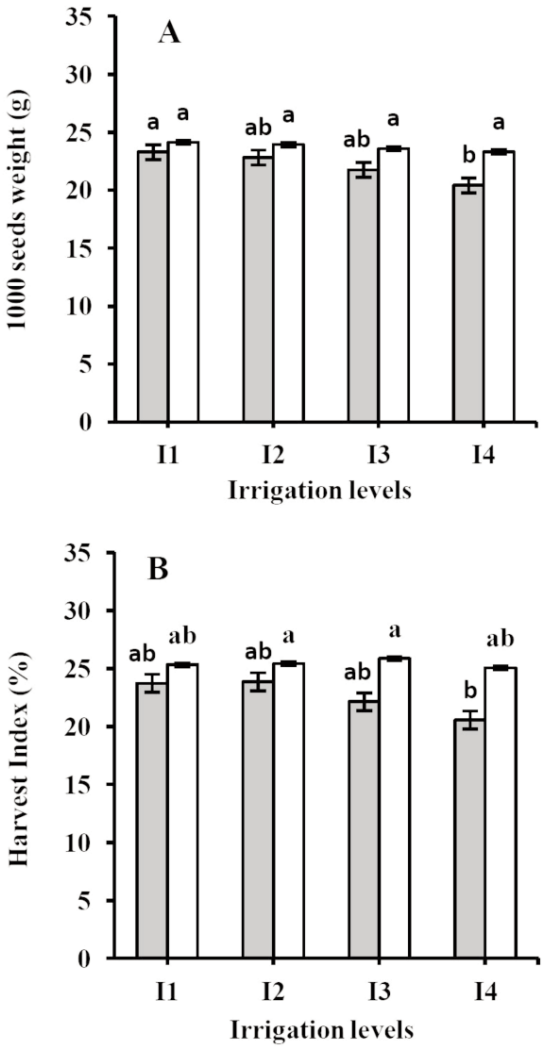

Fig. 1 - Mean seed weight (A) and harvest index (B) of milk thistle affected by irrigation and SA treatments.
I_1, I_2, I_3, I_4= Irrigation after 70, 110, 150 and 190 mm evaporation, respectively.
SA0, SA1= 0 and 1 mM salicylic acid, respectively.
Different letters in each column indicate significant difference at p≤0.05.

Table 3 - Analysis of variance of oil percentage and yield and flavonoid content of milk thistle affected by irrigation and SA treatments

Source of Variation	df	Oil content	Oil yield	Flavonoid content
Replication	2	2.2604	23.12	1.0208
Irrigation (I)	3	24.2749 **	367.88 **	20.0397 **
Error	6	0.6199	5.49	15046
Salicylic acid (SA)	1	5.9004 **	3294.13 **	26.8182 **
I × SA	3	0.0849 NS	0.59 NS	0.2903 NS
Error	8	0.1625	10.8	0.3488
CV (%)	-	2	9.2	1.2

NS, * and ** No significant and significant at p≤0.05 and p≤0.01, respectively.

4. Discussion and Conclusions

Reduction in plant biomass due to water stress (Table 2) was associated with diminishing leaf area expansion and plant growth during vegetative stages (Ghassemi-Golezani *et al*., 2009) and also with early leaf senescence (Hugh and Richard, 2003). Drought

Table 4 - Means of oil percentage and yield and flavonoid content of milk thistle for irrigation and SA treatments

Treatments	Oil content (%)	Oil yield (g/m^2)	Flavonoid content (μg/g)
Irrigation			
I_1	22.13 a	43.60 a	48.05 c
I_2	21.33 a	39.50 b	49.12 bc
I_3	19.35 b	33.38 c	50.04 b
I_4	17.67 c	25.43 d	52.34 a
Salicylic acid			
SA0	19.63 b	23.60 b	48.83 b
SA1	20.62 a	47.24 a	50.95 a

Different letters in each column indicate significant difference at p≤0.05.

stress decreases water potential of plant, leading to stomata closure and reduction in photosynthesis rate and leaf growth (Ozturk, 1999), which ultimately decreases plant biomass. This reduction in plant biomass resulted in decreasing the number of seeds per plant (Table 2), 1000 seeds weight (Fig. 1A) and consequently seed yield (Table 2) and harvest index (Fig. 1B). The losses in plant biomass and seed yield due to water deficit have also been reported for sesame (Kim *et al*., 2007), dill (Ghassemi-Golezani *et al*., 2008), maize (Ghassemi-Golezani and Dalil, 2011) and safflower (Ghassemi-Golezani *et al*., 2016).

Application of SA largely improved seed yield of milk thistle by enhancing plant biomass, seeds per plant (Table 2), 1000 seeds weight and harvest index (Fig. 1). SA influences a wide variety of plant processes, including stomatal regulation, chlorophyll content and photosynthesis (Yildirim *et al*., 2008). Ghassemi-Golezani and Lotfi (2015) found that exogenous application of SA enhances maximum quantum efficiency of PSII (Fv/Fm) and performance index (PI) in mung bean plants. In another report, Ghassemi-Golezani and Hosseinzadeh-Mahootchi (2015) stated that chlorophyll content index (CCI), photosystem II efficiency (Fv/Fm), relative water content (RWC), leaf area index (LAI) and finally seed yield of safflower were augmented by foliar application of SA.

The low oil percentage due to water deficit (Table 4) may be resulted from the short seed filling duration (Ghassemi-Golezani and Lotfi, 2013) and low seed weight (Fig. 1A). Adequate irrigation during plant growth and development can likely increase seed weight and oil storage. Decreasing oil yield per unit area as a consequence of water limitation (Table 4) strongly related with reduction in seed yield under stressful condition (Table 2). It was similarly reported that water limitation significantly decreases seed and oil yields of sunflower (Soleimanzadeh *et al*., 2010) and maize (Ghassemi-Golezani *et al*., 2015 b). Increasing seed yield (Table 2) and oil percentage by application of SA resulted in considerably higher oil yield of milk thistle (Table 4). Similar pattern of oil yield improvement by foliar spray of SA was observed in *Ocimum basilicum* and *Origanium hortensis* plants (Abd el-Lateef Gharib, 2006).

With decreasing photosynthesis rate under water stress, carbons from the photosynthesis cycle shift to the shikimic acid pathway in order to produce higher flavonoid content (Table 4). It was found that phenolics and flavonoids are able to regulate plant growth and improve the physiological efficiency and can enhance effective partitioning of accumulates from the sources to the sinks in plants (Ghasemzadeh *et al*., 2010). Stimulation of flavonoid accumulation by SA treatment (Table 4) may protect plants from certain biotic and abiotic stresses (Dučaiová *et al*., 2013). An increase in secondary metabolites content was also detected in chamomile (*Matricaria chamomilla* L.) plants as a result of SA spray (Dučaiová *et al*., 2013). This result suggests that flavonoid accumulation is a biochemical response to water stress, and SA can induce flavonoid synthesis, providing an effective protection of milk thistle plants from stress.

Water stress reduced plant biomass, seeds per plant, 1000 seeds weight, seed yield, harvest index, oil percentage and consequently oil yield of milk thistle. However, flavonoid content of seeds increased with decreasing water availability. All these traits were considerably enhanced by foliar spray of SA under different irrigation intervals. This suggests that exogenous application of SA could be an effective way for improving field production of milk thistle under different environmental conditions.

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Effects of nutritive solution electrical conductivity and plant density on growth, yield and quality of sweet basil grown in gullies by subirrigation

Morano G. [1], **Amalfitano C.** [1], **Sellitto M.** [2], **Cuciniello A.** [1], **Maiello R.** [1], **Caruso G.** [1] (*)

[1] *Dipartimento di Scienze Agrarie, Università degli studi di Napoli Federico II, via Università, 100, 80055 Portici (NA), Italy.*

[2] *Microspore S.p.A., 86035 Larino, CB, Italy.*

Key words: leaves quality, nutrient uptake, *Ocimum basilicum* L., plants number per pot, production, soilless.

Abstract: The increasing demand for basil in the last decade has arisen from consumer tendency towards high nourishing produce. Soilless growing of this crop is a current farm strategy and the quality targets are affected by nutritive solution as well as by plants density per pot. Research was carried out with the aim of assessing plant growth, yield and leaves quality of basil (*Ocimum basilicum* L., cv. Gecom FT) grown in pots (peat-lapil) and fed by subirrigation inside plastic gullies, under a heated greenhouse. Comparisons were made of four electrical conductivities (EC: 2.2, 2.5, 2.8, 3.1 mS·cm^{-1}) in factorial combination with four plant densities (9, 12, 15, 18 plants per pot) and a split plot design was arranged with three replicates. The 2.8 mS·cm^{-1} EC resulted in the best yield, growth indexes and biometrical parameters values. Water absorption was highest under the 2.8 mS·cm^{-1} EC, whereas the highest nutrient consumptions as well as the best quality indicators and chemical composition corresponded to the 2.8 to 3.1 mS·cm^{-1} EC range. The 12 plants per pot density gave the best results, in terms of yield, growth indexes and biometrical parameters, also showing the highest plant water and nutrient uptakes. The leaves quality attributes and chemical composition always displayed decreasing trends as a function of the plant density increase, the highest values corresponding to 9 and 12 plants per pot; only the nitrates concentration showed an opposite trend compared to the other nutrients. In conclusion, the 2.8 mS.cm^{-1} nutritive solution and the 12 plants per pot density resulted in the best yield and leaves quality. Further enhancement of both experimental factors level even caused the reduction of water and nutrient efficiency use.

1. Introduction

The increasing demand for basil resulted in cropping area extension by 66% since 2001 in Italy (www.istat.it), with the frequent use of local ecotypes (Zecchinelli, 1999; Tesi and Lenzi, 2002). Basil (*Ocimum basilicum* L.) is a high marketable value vegetable, which is consumed both as a fresh aromatic ingredient and combined with pasta in a cooked dish (pesto). As today's consumer choices are oriented towards high quality produce, not only from a sensorial point of view but also in terms of nutritional properties, a particular emphasis is given to this product. In this direction, soilless growing could represent an effective crop management in order to enhance product quality attributes (Sgherri *et al.*, 2010), which mainly depend on variety (Tesi *et al.*, 1991) but they are also affected by crop system (Tesi *et al.*, 1997). Plant growing in pots sown with several seeds per pot, to be sold when the plants set reaches a scheduled size, is one of the current farm strategies and interesting market perspectives mainly arise from the winter crop cycle. In this season, light intensity is sufficient for carrying out efficient crop cycles (Beaman *et al.*, 2009), but it is necessary ensuring the adequate minimum temperature, which is also positively correlated with basil flavour (Chang *et al.*, 2007). Moreover, the nutritive solution supplied to plants plays a crucial role as it significantly affects yield (Bekhradi *et al.*, 2015) and plant features, such as stem height and dry weight (Adler *et al.*, 1989; Bione *et al.*, 2014); in this respect, basil is considered a moderately tolerant species (Herrera, 2005). The plants density is also of primary importance for the

(*) Corresponding author: gcaruso@unina.it
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produce quality (Bohme and Pinker, 2014), as the individual space available has a major impact on the balance among the different plant parts.

Due to literature shortage on the above mentioned topics, research was carried out on soilless pot-grown basil in Naples (southern Italy), with the aim of evaluating the effects of nutritive solution electrical conductivity and pot plants density on crop growth, yield and leaves quality.

2. Materials and Methods

Research was carried out on basil (*Ocimum basilicum* L. cv. Gecom FT) at the experimental site of Naples University Federico II in Portici (Naples, southern Italy, 40°49' N, 14°20' E, 63 m a.s.l.), in a Mediterranean or Csa climate (Peel *et al.*, 2007), during winter season in 2007 and 2008. The crops were soilless grown in pots, placed in gullies and fed by sub-irrigation, under plastic (IR-PE) tunnels equipped with an air heating system set to 16°C. Comparisons were made of four electrical conductivities (EC: 2.2, 2.5, 2.8, 3.1 $mS \cdot cm^{-1}$) in factorial combination with four plant densities (9, 12, 15, 18 plants per pot) and a split plot design was arranged with three replications; each treatment included 30 pots. The hydroponic equipment consisted of: a) 48 rigid PVC gullies (each 12 cm wide and deep, 300 cm long) supported by plastic elements at 70 cm above ground level, according to 1% slope; b) 12 plastic tanks holding 220 l; c) 12 submerged pumps of 90 watt unit power; d) 24 delivery and return overhead lines. The sowing was performed on 15 January in pots (Ø = 10 cm) filled with peat and lapil (1:1 in volume), placed in the gullies through a pierced white PE film and spaced 10 cm along and between the rows (18 pots per m^2). They were fed by nutritive solutions (Table 1), with 1-6 interventions per day of 5 minutes each, which were never adjusted but they were completely changed at the end of each weekly cycle. Besides, during the crop, an insecticide application against aphids was practiced.

The crop cycles ended when each plants set reached the scheduled size for pot commercialization, i.e. the plants had four fully expanded leaves couples, and at that time the following determinations were made on plant samples obtained by 8 pots per plot: fresh and dry weight of whole plants and of leaves; leaf area; stems thickness and height. Moreover, water consumption was calculated assessing the volume of nutritive solution in each tank at the beginning and at the end of each weekly cycle. Concurrently, nutritive solution samples were collected in order to assess nutrient consumption through laboratory analyses of nitrogen, phosphorus, potassium, calcium, magnesium and iron, using the same methods as described below for leaf cation and anion determinations.

At the crop cycles end, leaves samples were also collected from 8 pots per plot, in order to perform laboratory analyses. In this respect, two hundred grams of leaves per plot were homogenized in a 1.0 L Waring blender (Waring Laboratory, Torrington, CT, USA) and aliquots of this raw homogenate were used for the analyses of cations. The raw homogenate was centrifuged at 10,000 x g for 30 min at 6°C in an 5810R Eppendorf refrigerated centrifuge (Eppendorf HQ, Hamburg, Germany). The resulting supernatant was passed through a 0.45 µm Acrodisc filter (Gelman Sciences, MI, USA). Samples of this filtered leaves extract were used for assessing anion, sugar and ascorbic acid contents.

The laboratory determinations were performed as follows:

- the dry residue was assessed in an oven at 70°C with a vacuum;
- the soluble solids content or SSC (in °Brix) was measured at 20°C with a Bellingham & Stanley, model RFM 81 digital refractometer on the supernatant obtained from raw homogenate centrifugation;
- anions, sugars and ascorbic acid were determined by high performance liquid chromatography (HPLC) as previously described (Caruso *et al*., 2011);

Table 1 - Chemical composition of the soilless nutrient solutions

Nutritive solution EC ($mS \cdot cm^{-1}$)	macronutrients ($mmol \cdot L^{-1}$)							micronutrients ($\mu mol \cdot L^{-1}$)					
	N	P	K	Ca	Mg	S	Cl	Fe	Cu	Mn	Zn	B	Mo
2.2	12.3	1.6	5.3	3.8	2.2	1.9	1	35	1	15	5	35	1
2.5	13.8	1.7	5.9	4.3	2.5	2.5	1	35	1	15	5	35	1
2.8	15.7	2.0	6.7	4.8	2.8	2.8	1	35	1	15	5	35	1
3.1	17.5	2.2	7.6	5.4	3.0	3.3	1	35	1	15	5	35	1

For all treatments pH was adjusted to 6.0 and NH_4/NO_3 ratio was 1/9.

- titratable acidity of the leaves homogenate was determined as previously described (Caruso *et al*., 2014) and it was expressed as grams of anhydrous citric acid per 100 g of leaf fresh weight;
- cations (Ca, Mg, K) content in the leaves homogenate was determined by atomic adsorption spectrophotometry as previously described (Caruso *et al*., 2011).

Data statistical processing was performed by analysis of variance using the SPSS software version 21, referring to 0.05 probability level, and Duncan multiple test was used for mean separation.

3. Results and Discussion

From the data statistical processing, the year of research resulted to have no significant effect either as a main effect or as an interaction with the two experimental factors, therefore in the following tables the mean values of the experimental data of the years 2007 and 2008 are reported.

As for yield results relevant to the comparison among the nutritive solution electrical conductivities (Table 2), the 2.8 $mS \cdot cm^{-1}$ EC resulted in the highest basil yield, both as whole plants and of leaves, and in the shortest crop cycle; the lowest nutrient solution strength (2.2 $mS \cdot cm^{-1}$) showed the worst performances.

Table 2 - Basil yield results

Nutritive solution EC ($mS \cdot cm^{-1}$)	Yield			Crop cycle duration (days)
	Whole plants ($g \cdot m^{-2}$)	Leaves ($g \cdot m^{-2}$)	Leaves/ Plant (%)	
2.2	579.1 d	418.7 d	72.3 c	64.0 a
2.5	733.9 c	538.7 c	73.4 b	62.7 b
2.8	958.9 a	713.4 a	74.4 a	61.3 c
3.1	845.5 b	629.9 b	74.5 a	61.0 c
No. plants per pot				
9	761.9 b	570.7 b	74.9 a	61.2 c
12	931.1 a	695.5 a	74.7 a	61.4 c
15	776.3 b	569.8 b	73.4 a	62.6 b
18	648.5 c	465.6 c	71.8 c	63.9 a

Among the plant densities, the 12 plants per pot treatment resulted in the highest yields and both the 9 and 12 plants per pot led to the shortest crop cycle (Table 2).

In terms of growth indexes and biometrical parameters (Table 3), the 2.8 $mS \cdot cm^{-1}$ EC also produced the highest values of plant dry matter, leaf area and stem thickness, though the latter was not statistically different from that obtained with the highest EC level

Table 3 - Basil growth and biometrical parameters

Nutritive solution EC ($mS \cdot cm^{-1}$)	Plant dry matter ($g \cdot m^{-2}$)	LAI ($m^2 \cdot m^{-2}$)	Leaf area ($cm^2 \cdot pt^{-1}$)	Plant height (cm)	Stem thickness (mm)
2.2	45.0 d	0.66 c	28.5 c	17.2 a	2.76 b
2.5	64.8 c	0.92 b	39.3 b	17.0 a	2.90 b
2.8	90.0 a	1.11 a	47.1 a	16.7 b	3.11 a
3.1	82.8 b	0.98 b	41.6 b	16.6 b	3.16 a
No. plants per pot					
9	73.6 b	0.86 c	51.9 a	16.8 b	3.33 a
12	88.2 a	1.06 a	47.6 b	16.9 ab	3.12 b
15	67.9 b	0.93 b	33.1 c	16.9 ab	2.85 c
18	52.7 c	0.83 c	24.3 d	17.1 a	2.60 d

(3.1 $mS \cdot cm^{-1}$); conversely, nutritive solution dilution caused the internodes extension and, indeed, the two lowest electrical conductivities enhanced plant height.

With regard to plant density (Table 3), the 12 plants per pot treatment resulted in the highest dry weight and LAI, whereas the lowest density produced the thickest stems; the 15 and 18 plants per pot densities proved excessive and, in particular, the highest one caused the most unbalanced growth of plants. In fact, the plants grown under the 18 plants per pot treatment showed thinner stems and smaller leaves compared to the other experimental treatments, and they were also taller than the most spaced ones.

In contrast with our findings, in previous research (Tesi *et al.*, 1995) the 1.6 $mS \cdot cm^{-1}$ EC showed the best effect on basil yield. Moreover, other authors reported that doubling the nutrient availability did not affect basil yield (Raimondi *et al.*, 2006), but it led to the increase of leaf dry matter percentage and LAI (Chen *et al.*, 2004). In our research, the depressing effects of salt stress caused by the 3.1 $mS \cdot cm^{-1}$ EC on plant vegetative growth and in particular on leaf area corresponds to the rapid plant adaptation to water deficit (Munns, 2002). Moreover, in our investigation, the density increase presumably caused the light conditions worsening within the canopy and, accordingly, the reduction of plant photosynthetic efficiency. Chang *et al.* (2007) also reported that basil plant weight is adversely correlated with canopy shading. Consistently, Tesi *et al.* (1995) recorded the plant weight increase per soil unit area up to a critical density value, over which a decrease occurred; however, they also found that a doubled density, compared to our best treatment of 12 plants per pot provided with the best results using 10 cm diameter pots. Moreover, Raimondi *et al.* (2006) found that plant density increase from 66 to 100 plants per m^2 results in total yield increase. Further, in studies on canopy dynamics simulation (Van Oosteron *et al.*, 2001), leaf

area index showed increasing trend with the plant density enhancement.

The highest water and nutrient absorptions were recorded in the last crops week, when plant leaf area reached the highest expansion and the greenhouse temperature showed the highest value of 26.4°C (as an average of the two research years). As reported in Table 4, plant water consumption showed a similar trend to the yield one, with the highest values corresponding to 2.8 mS·cm^{-1} EC, whereas the highest nutrients consumption was assessed under the 2.8-3.1 mS·cm^{-1} EC range; the most diluted nutritive solution always resulted in the lowest absorption rates.

As for the comparison among the plant densities (Table 4), the highest daily values of both water and nutrient absorption occurred in the 12 plants per pot treatment, which also resulted in the best yield (Table 2); the highest plant density (18 plants per pot) always showed the lowest consumptions.

Compared to our research findings, a similar plant response to water deficit was recorded in previous investigations (Savvas *et al.*, 2007), where the increase of the nutrient solution strength caused the reduction of plant water absorption. The latter represents a salinity adaptation mechanism, consisting of leaf area and stomata decrease which in turn contributes to reducing transpiration and increasing water use efficiency (Chartzoulakis and Klapaki, 2000).

The quality indicators were significantly affected by the nutritive solution strength (Table 5), as their trends were always increasing with the electrical conductivity raise from 2.2 to 2.8 mS·cm^{-1}, whereas no further increases were recorded in the last 0.3 mS·cm^{-1} rise.

The quality parameters showed decreasing trends as a function of the plant density increase (Table 5), with the 9 and 12 plants per pot treatments generally attaining the highest values and the 18 plants per pot treatment displaying the worst performances.

In previous investigation (Adams and Ho, 1989), an increase in sugar content and titratable acidity was reported as a consequence of salinity increase or water deficit. Moreover, Raimondi *et al.* (2006) found that nutrient solution EC interacted with the cultivars in modifying leaf antioxidant content: i.e. Napoletano leaves showed an ascorbate increase with the EC enhancement, whereas Genovese displayed opposite trend. The same authors also recorded that the plant density increase from 66 to 100 plants per m^2 did not

Table 4 - Basil water and nutrient absorptions

Nutritive solution EC (mS·cm^{-1})	Maximum daily absorptions						
	Water (L·m^{-2})	Nitrogen (g·m^{-2})	Phosphorus (g·m^{-2})	Potassium (g·m^{-2})	Calcium (g·m^{-2})	Magnesium (g·m^{-2})	Iron (mg·m^{-2})
2.2	1.6 d	0.35 c	0.11 c	0.42 c	0.29 c	0.11 c	5.76 c
2.5	2.0 c	0.51 b	0.15 b	0.61 b	0.42 b	0.16 b	7.20 b
2.8	2.6 a	0.76 a	0.21 a	0.92 a	0.64 a	0.23 a	9.00 a
3.1	2.4 b	0.78 a	0.22 a	0.93 a	0.65 a	0.24 a	8.46 a
No. plants per pot							
9	2.2 b	0.62 b	0.19 b	0.74 b	0.53 b	0.19 b	7.56 b
12	2.6 a	0.73 a	0.21 a	0.90 a	0.58 a	0.23 a	9.00 a
15	2.1 b	0.58 b	0.17 c	0.70 b	0.49 c	0.17 c	7.38 b
18	1.7 c	0.47 c	0.13 d	0.54 c	0.40 d	0.15 d	5.94 c

Table 5 - Basil leaves quality indicators

Nutritive solution EC (mS·cm^{-1})	Dry residue (%)	Soluble solids (°Brix)	Titratable acidity (g · 100 g^{-1} d.w.)	Glucose (g · 100 g^{-1} d.w.)	Fructose (g · 100 g^{-1} d.w.)	Sucrose (g · 100 g^{-1} d.w.)	Ascorbic acid (mg·100 g^{-1} d.w.)
2.2	9.5 c	3.2 c	0.76 c	1.80 c	2.25 c	0.40 c	508.4 c
2.5	9.7 bc	3.4 bc	0.84 b	2.12 b	2.52 b	0.52 b	585.7 b
2.8	10.0 ab	3.6 ab	0.96 a	2.34 a	2.83 a	0.63 a	703.8 a
3.1	10.0 a	3.7 a	1.02 a	2.47 a	2.98 a	0.67 a	744.6 a
No. plants per pot							
9	10.0 a	3.6 a	0.98 a	2.30 a	2.86 a	0.64 a	708 a
12	10.0 a	3.6 a	0.96 a	2.24 a	2.78 a	0.62 a	689.5 a
15	9.8 ab	3.4 ab	0.88 b	2.14 ab	2.54 b	0.52 b	617.3 b
18	9.6 b	3.3 b	0.78 c	2.02 b	2.40 b	0.45 c	527.4 c

affect fresh produce quality, but it just lowered the soluble solids content.

As reported in Table 6, mineral nutrient concentrations were significantly affected by the nutritive solution strength, as their trends were always increasing with the electrical conductivity raise from 2.2 to 2.8 $mS \cdot cm^{-1}$, whereas no further increases were recorded in the last 0.3 $mS \cdot cm^{-1}$ rise.

With regard to plant density (Table 6), the 9 and 12 plants per pot treatments always resulted in the highest nutrient accumulation in the leaves, except for the nitrates which showed an opposite trend, increasing from the lowest to the highest density. The decreasing trend of the leaves mineral ion concentrations as a function of the pot plant density increase resulted in relation with the plant nutrient absorptions (Table 4).

Notably, the increase of mineral cations concentration in the plant tissues is caused by salt ion accumulation in the rizhosphere (Sonneveld, 2002) as a consequence of the plant active exclusion in response to salt occurrence in the external solution (Bethke and Drew, 1992). Moreover, the nitrate concentration increase in response to nutritive solution strength raise recorded in our research is consistent with the reports of previous investigations (Tesi *et al.*, 1997; Raimondi *et al.*, 2006). As for plant density, the increasing nitrate accumulation corresponding to the enhancement of the plants number per pot was presumably caused by the gradual light conditions worsening. Contrastingly, in previous research (Tesi *et al.*, 1995) a decreasing trend of nitrate accumulation as a function of plant density increase was reported. Interestingly, in our research the nitrate concentration was always very low and, accordingly, basil leaves consumption not exceeding 563 g per day complies with the Acceptable Daily Intake for nitrate (222 $mg \cdot d^{-1}$ for 60 kg adult) (Authority EFS, 2008).

Table 6 - Basil leaves chemical composition

Nutritive solution EC ($mS \cdot cm^{-1}$)	Nitrates ($g \cdot kg^{-1}$ d.w.)	Phosphates ($g \cdot kg^{-1}$ d.w.)	Sulphates ($g \cdot kg^{-1}$ d.w.)	Calcium ($g \cdot kg^{-1}$ d.w.)	Magnesium ($g \cdot kg^{-1}$ d.w.)	Potassium ($g \cdot kg^{-1}$ d.w.)
2.2	3.2 c	3.6 c	1.5 c	5.9 c	3.3 c	45.3 c
2.5	3.7 b	4.1 b	1.8 b	6.4 b	3.8 b	48.8 b
2.8	4.4 a	5.0 a	2.2 a	7.1 a	4.3 a	53.4 a
3.1	4.8 a	5.3 a	2.3 a	7.3 a	4.4 a	55.0 a
No. plants per pot						
9	3.4 d	4.8 a	2.1 a	7.1 a	4.2 a	54.2 a
12	3.8 c	4.7 ab	2.1 a	7.0 ab	4.1 ab	52.4 ab
15	4.2 b	4.4 bc	1.9 ab	6.5 bc	3.8 bc	49.7 bc
18	4.7 a	4.1 c	1.7 b	6.1 c	3.6 c	46.2 c

4. Conclusions

From research carried out in southern Italy on soilless pot-grown basil, it can be inferred that the 2.8 $mS \cdot cm^{-1}$ nutritive solution resulted in the best product yield and quality, whereas a further increase to 3.1 $mS \cdot cm^{-1}$ caused the reduction of the water and nutrient efficiency use. Moreover, the 12 plants per pot density showed the optimal compromise between the individual plants and the pot plants set performances, providing with the highest production and leaves quality. Indeed, density intensification to 15 and further to 18 plants per pot caused the reduced efficiency use of water and nutrients and accordingly the plant growth worsening, as well as the crop cycle extension up to 2.7 days.

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Effect of pre- and post-harvest salicylic acid treatments on quality and antioxidant properties of 'Red Delicious' apples during cold storage

M. Hadian-Deljou, M. Esna-Ashari*, H. Sarikhani
Department of Horticultural Sciences, Bu-Ali Sina University, Hamedan, Iran.

Key words: anthocyanin, firmness, phenolic compound, soluble solid content, weight loss.

Abstract: Salicylic acid is a natural phenolic compound known as a plant hormone having positive effect on storage life and quality of fruits. This study aimed to investigate the effects of pre- and post-harvest application of salicylic acid on antioxidant properties and quality of 'Red Delicious' apples during 193 days cold (0±0.5°C) storage. Both pre- and post-harvest salicylic acid treatments did not affect soluble solid content, titratable acidity and fruit firmness, with the exception of 1 mM at pre-harvest application for titratable acidity. Fruit juice pH was reduced in all fruits at the end of storage, while it was not quite uniform during storage. Although there was no significant difference between the concentrations of salicylic acid in terms of fruit weight loss, but the highest amount of weight loss was observed in post-harvest treatments. Salicylic acid application increased total phenolics and antioxidant activity at the earlier stages of storage showing the highest capacity with 2 mM followed by 1 and 4 mM salicylic acid concentrations, while 1 mM concentration belonged to the highest antioxidant capacity at the end of storage. Anthocyanin content showed a gradual increase during storage until day 60, then decreased right afterwards. The highest amounts of anthocyanin were obtained from the concentrations of 1 and 2 mM salicylic acid in pre-harvest treatments, while 4 mM treatment was not encouraging. Overall, salicylic acid treatments could increase apple storage life and quality for a short period of time only.

1. Introduction

Apple (*Malus domestica* Borkh) is one of the most important horticultural crops considered as the third major fruit in the world (Garming, 2014). The main proportion (½ million tons) of apple production in Iran is used for fresh consumption (Iranian Ministry of Agriculture, 2016), while some problems such as tissue softening during storage, tissue browning due to physical damages at post-harvest handling, high water loss and some physiological disorders such as bitter pit, water core, scald and internal browning are the most dominant post-harvest restriction factors (Esna-Ashari and Zokaee Khosroshahi, 2011). Generally, quality apple must be mature, firm, crispy and juicy with a good flavor composition and free from mechanical damage, physiological disorders, and pathological diseases (Baldwin, 2002). However, reducing consumer acceptance or nutritional value usually happens in the period after harvesting. Apples contain several health-promoting compounds functioning as antioxidants, or modulators of enzyme activity. Peel of red apples contains higher antioxidant than their flesh. Meanwhile, Drougoudi *et al*. (2008) discovered a positive correlation between phenolic content and antioxidant capacity in both flesh and peel of apple. Color is also another important factor regarding fruit evaluation. There is a close correlation between color and overall quality of fruits (Ritenour and Khemira, 2007).

Salicylic acid (SA) is a natural compound that functions as plant growth regulator. SA carries a high potential of controlling post-harvest losses of horticultural crops. It has been discovered that the SA is associated with a delay in fruit ripening, (Srivastava and Dwivedi, 2000; Zhang *et al*., 2003; Mo *et al*., 2008), induction of disease resistance (Shafiee *et al*., 2010), increasing antioxidant and phenolic compounds (Peng and Jiang, 2006; Geransayeh *et al*., 2015), maintenance of post-harvest quality (Srivastava and Dwivedi, 2000; Zhang *et al*., 2003; Wang *et al*., 2006; Mo *et al*., 2008; Harindra *et al*.,

(*) Corresponding author: m.esnaashari@basu.ac.ir
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2015) and increasing storage life of horticultural crops. Hence, SA is suggested to be utilized in post-harvest handling of fruits and vegetables (Peng and Jiang, 2006) which necessitates further investigation into know-how of its application. Wen *et al*. (2008) found that treatment of grape berries with SA could induce an increase in phenylpropanoid and phenylalanine ammonia-lyase (PAL). Qin *et al*. (2003) reported a significant increase in polyphenoloxidase, PAL and β-1, 3-glucanase activity in cherries fruit through SA treatment. Exogenous application of SA on tomato green mature fruits has shown a delay in biosynthesis of some biochemicals including carotenoids, lycopene, ascorbic acid, total phenolics, free amino acids and γ-amino butyric acid that led to increase in the keeping quality of fruits (Kant *et al*., 2016). Pre-treatment of SA combined with lower storage temperature could provide a useful means of maintaining beneficial antioxidant activity during storage of navel orange (Huang *et al*., 2008). Exogenous application of SA enabled grape leaves to maintain relatively higher activities of antioxidant enzymes under normal temperature, heat, or cold stress. The effect of SA on alleviating chilling injury of peaches during cold storage may be attributed to its ability to induce antioxidant systems (Wang *et al*., 2006). The aim of this study was to maintain apple quality through the application of SA controlling physiological damages as well as investigating content of phenolic compounds, anthocyanin and antioxidant activity of fruits during cold storage.

2. Materials and Methods

Plant materials and salicylic acid treatments

Apples (*Malus domestica* Borkh cv. Red Delicious) were provided from a 20 years old orchard in Horticultural Research Center, Bu-Ali Sina University, Hamedan, Iran. This study was conducted as a factorial experiment based on a complete randomized block design with three replications. First stage of experiment launched on September when apples' green skin had just started to turn reddish. Four concentrations of SA including 0 (control), 1, 2 and 4 mM were sprayed on previously-selected trees, branches and fruits. SA concentrations were made by dissolving powdered SA (Merck, Germany) in hot water. In the second stage of experiment, non-treated fruits were first harvested according to the maturity index (starch test) using Cornell Starch-Iodine Chart in late September. Starch test is a standard method of determining apple maturity to estimate optimum harvest dates well before picking fruit (Blanpide and Silsby, 1992). The harvested fruits then dipped in the same concentrations of SA solution for approximately three minutes at room temperature (25±1ºC). Previously-treated apples were also harvested at this time. In this experiment, 15 apple fruit were used in each replicate for a totally of 45 fruit for each treatment. Two apples per each replicate were used for the measurments of all parameters (except for the weight loss) at any time of determination (totally 6 times). Three remaining apples from each replicate were kept to weigh at any time of determination for the evaluation of weight loss. For the packaging of the samples, each group of five apples was packed in a cubic plastic container with two small pores (2.5 mm in diameter) in each side, and stored at 0±0.5°C with 90% relative humidity and kept up to 193 days. Measurements of all parameters started at the beginning of the storage and then continued until the day of 90 with 30 days intervals. Two other measurements were taken 157 and 193 days of storage.

Soluble solid content, titratable acidity and juice pH

Apple juice was first prepared using a domestic electric apple juice maker available in the local shops. Soluble solid content (SSC) was determined by measuring refractive index of the juice with a handhold refractometer (N1, Atago Co., Tokyo, Japan) at room temperature (25±1°C) and the results were expressed as °Brix. Titratable acidity (TA) was measured by titration with a calibrated titrator using a solution of the juice and water (2/10 ratio) with 0.1 N NaOH to pH 8.2±0.1 and converted to malic acid percentage.

Fruit juice pH was determined using an Aqualitic digital pH meter (model AL10 pH) with a gel electrode.

Fruit firmness and weight loss

Firmness values of each individual apple were measured at three points of their equatorial region after which the peel was removed by using a manual penetrometer (FDK; Wagner Instruments, Greenwich, CT, USA) with a 2 mm diameter flat probe (Zhang *et al*., 2009).

Total phenolic content

Total phenolic content (TPC) of treated fruits were determined through a slightly modified version of Folin-Ciocalteu's method as suggested by Slinkard and Singleton (1977). Chlorogenic acid was used as standard phenolic compound. Extraction was performed by homogenizing 0.5 g flesh tissue powder in 3 ml of 85% MeOH and the extract was filtered with

No.1 Whatman filter paper. Sample extract (300 µl) was mixed with 1500 µl of Folin-Ciocalteu's reagent. After 5 min, 1200 µL of sodium carbonate solution (7.0%, w/v) was added, and the mixture vortexed and allowed to stand at room temperature (25±1°C) in the dark for 90 min. The absorbance was read at 765 nm in a UV/vis spectrophotometer (Carry 100, Varian Analytical Instruments, Walnut Creek, CA, USA), and the total phenolic concentration was calculated from a calibration curve, using chlorogenic acid as the standard. Results were expressed as mg L^{-1} chlorogenic acid equivalents.

Total anthocyanin content

For the assessment of anthocyanin contents of the apples, only their peels were used through applying a pH differential protocol (Giusti and Wrolstad, 2003). The absorbance was measured at 510 and 700 nm with a UV/vis spectrophotometer (Carry 100, Varian Analytical Instruments, Walnut Creek, CA, USA). Anthocyanin content was then calculated using the following equation and expressed as mg cyanidin 3-galactoside equivalent per gram of fresh weight.

$$\text{mg Cya-3-gal /g FW} = [(A_{510} - A_{700})_{pH1} - (A_{510-} A_{700})_{pH4.5}]\times MW\times F\times 1000/\varepsilon\times d$$

where: A= absorbance, MW= molecular weight of cyanidin 3-galactoside = 445.2 [g/mol], F= dilution factor = 10, d= cell pathlengths [cm], ε= molar absorbance of cyanidin 3-galactoside = 34300 [L/mol×cm] and 1000= Factor for mg.

Total antioxidant activity

Antioxidant activity was estimated using the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical (Arnous *et al*., 2001) with slight modifications. The absorbance was read at *t* = 0 and *t* = 30 min with a spectrophotometer (Carry 100, Varian Analytical Instruments, Walnut Creek, CA, USA).

For all the above assessments (except the anthocyanin measurement), the apple flesh was used.

Statistical analysis

Statistical analysis (analysis of variance) of the data was performed with SAS software (version 9.1, 2002-2003, SAS Institute Inc., Cary, NC, USA). Means were compared with Duncan's Multiple Range Test. Differences at *p*=0.05 were considered as significant.

3. Results and Discussion

Soluble solids content, titratable acidity and juice pH

Soluble solids content increased gradually during storage with no significant differences between the treatments (Fig. 1). With pre-harvest application of 1 mM SA, TA slightly increased until the 60th day of storage, when reached its maximum value being significant with the other treatments, but decreased gradually afterwards (Fig. 2). Fruit juice pH was reduced in all fruits at the end of storage, while it was not quite uniform during storage (Table 1). SSC may increase during fruit ripening due to the action of sucrose-phosphate synthase, a key enzyme in sucrose biosynthesis. This enzyme is activated by ethylene and the ripening process itself during storage. Because organic acids are substrates of respiration, their levels decrease during ripening. Utilization of these compounds over post-harvest period is the main reason of increasing sweetness in originally

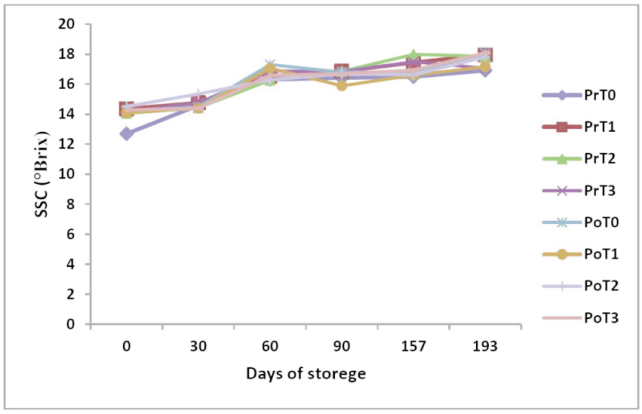

Fig. 1 - Effect of pre- and post-harvest salicylic acid treatments on SSC of 'Red Delicious' apples stored at 0±0.5°C for 193 days.
Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

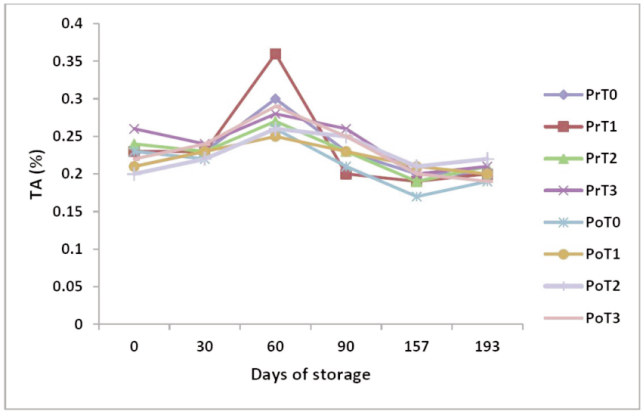

Fig. 2 - Effect of pre- and post-harvest salicylic acid treatments on TA of 'Red Delicious' apples stored at 0±0.5°C for 193 days.
Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

Table 1 - Effect of pre- and post-harvest salicylic acid treatments on pH of 'Red Delicious' apples stored at 0±0.5°C for 193 days

Treatment	Days of storage					
	0	30	60	90	157	193
Pr	4.55 a	4.34 b	4.36 a	4.39 a	4.28 a	4.18 a
Po	4.56 a	4.54 a	4.34 a	4.43 a	4.31 a	4.10 b
T0	4.52 a	4.39 c	4.35 a	4.41 a	4.27 a	4.13 a
T1	4.54 a	4.42 bc	4.35 a	4.42 a	4.31 a	4.14 a
T2	4.58 a	4.49 a	4.33 a	4.39 a	4.28 a	4.13 a
T3	4.55 a	4.46 ab	4.37 a	4.42 a	4.33 a	4.15 a
PrT_0	4.53 a	4.30 d	4.34 a	4.36 a	4.26 a	4.15 abc
PrT_1	4.57 a	4.34 cd	4.36 a	4.41 a	4.30 a	4.25 a
PrT_2	4.52 a	4.36 cd	4.34 a	4.38 a	4.26 a	4.19 ab
PrT_3	4.59 a	4.37 c	4.40 a	4.40 a	4.32 a	4.15 abc
PoT_0	4.40 a	4.49 b	4.36 a	4.46 a	4.28 a	4.12 bc
PoT_1	4.53 a	4.50 b	4.34 a	4.43 a	4.32 a	4.03 c
PoT_2	4.46 a	4.62 a	4.32 a	4.40 a	4.31 a	4.08 bc
PoT_3	4.51 a	4.55 b	4.34 a	4.43 a	4.34 a	4.16 ab

The same letters in any column show no significant difference between the data. Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

high-sugar, high-acid apples or insipidity and blandness of fruit when sugar and acid concentrations are initially low (Jackson, 2003). Similar to our founding have been reported by Sayyari *et al*. (2011) in which SSC increased during storage in both control and treated fruits. As previously reported, in climacteric fruits such as apples, starch turns into sucrose during post-harvest storage. Mo *et al*. (2008) studies showed SSC in treated fruits to be lower than the control, suggesting that SA slowed starch degradation. This could be a possible reason why SSC increased in this study. However, dehydration of fruits during storage could be mentioned as another reason. Organic acids are consumed in the process of respiration during post-harvest storage. It could be concluded that, the apples' respiration rate in this study was possibly higher at the first two months of storage, and for this reason, the TA values were increased over this period, then lowered afterwards. Srivastava and Dwivedi (2000) stated that SA treatment has made TA fixed in banana. Similar results have been reported while investigating chestnut by Peng and Jiang (2006). As the contents of organic acids in fruit are mainly dependent to the activities of their synthetic and hydrolytic enzymes, SA treatment was probably regulated the activities of related enzymes (Ding *et al*., 2007).

Effects of salicylic acid on fruit softening

Fruits showed signs of softening during storage (Fig. 3). Firmness of apples was initially decreased in both control and SA-treated fruits, and then

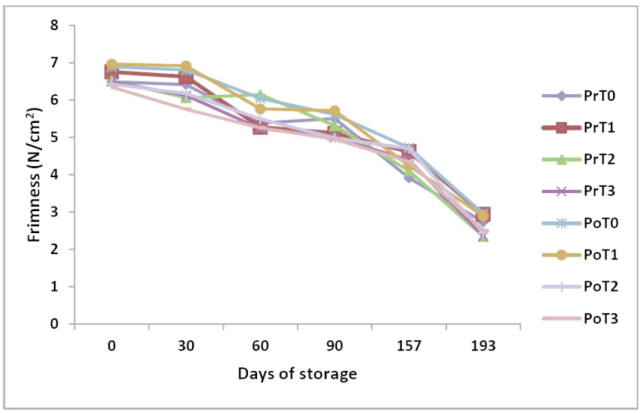

Fig. 3 - Effect of pre- and post-harvest salicylic acid treatments on firmness of 'Red Delicious' apples stored at 0±0.5°C for 193 days.
Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

appeared rapidly. Similar results was found by Ding *et al*. (2007) and Sayyari *et al*. (2011) who reported that fruit firmness decreased during storage. A close relationship between the change at endogenous SA level and the rate of fruit ripening and softening in kiwifruit was observed by Zhang *et al*. (2003). Softening could be the result of turgor loss, starch degradation, and most importantly cell wall degradation associated with weakening intercellular cohesive forces (Jackson, 2003). SA decreases production of ethylene and inhibits activation of cell wall and membrane degrading enzymes such as polygalacturonase, lipoxygenase, cellulose and pectinemethylesterase resulting in the reduction of softening (Srivastava and Dwivedi, 2000; Zhang *et al*., 2003). Adding SA to the nutrient solution could induce firmness and delay the softening process in strawberry and apple fruits (Shafiee *et al*., 2010; Kazemi *et al*., 2011).

Effects of SA on weight loss

SA-treated fruits demonstrated controlled weight loss during initial stages of post-harvest. The lowest amount of weight loss was observed in 2 mM concentration of SA- pre-harvest-treated fruits. Higher concentrations of SA could induce fruit weight loss, but did not control it (Fig. 4). It was observed that the apples kept their quality until the 157 days of storage, so that they were still suitable to be transferred to the market, but they gradually started to show visible defects on the peel losing their quality afterwards. Therefore, we recommend our method to be suitable for the storage of Red Delicious apples for the period of five months.

Weight loss is caused by both dehydration and consumption of soluble solids during respiration. SA

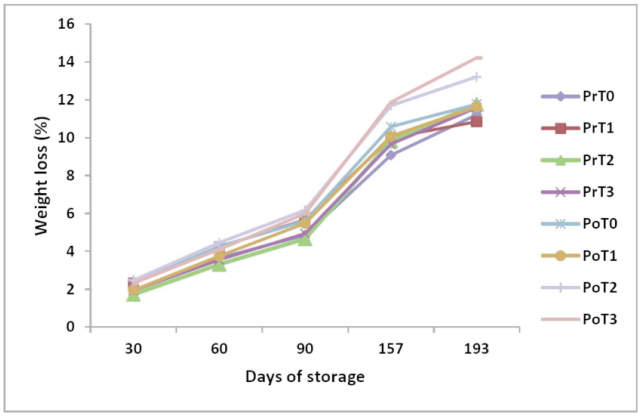

Fig. 4 - Effect of pre- and post-harvest salicylic acid treatments on weight loss of 'Red Delicious' apples stored at 0±0.5°C for 193 days.
Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

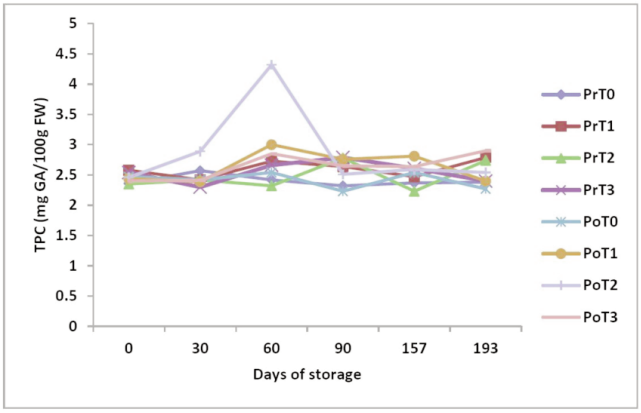

Fig. 5 - Effect of pre- and post-harvest salicylic acid treatments on TPC of 'Red Delicious' apples stored at 0±0.5°C for 193 days.
Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

could decrease water loss and respiration rate through controlling degradation of cell wall and reducing ethylene biosynthesis (Srivastava and Dwivedi, 2000), respectively. Jonagold apples immersed in SA have shown a significant decrease in weight loss during cold storage (Kazemi *et al*., 2011). In pear fruits, ratio of weight loss has been decreased at 0.5 mM SA after 48 h of treatment (Imran *et al*., 2007). Strawberries dipped in SA solution demonstrated less weight loss as well (Shafiee *et al*., 2010). It seems that high concentrations of SA could induce aggregation of intoxicated materials such as certain polyphenols in later stages of storage, which is probably why plant tissues degrade followed by increasing weight loss. Further investigations are needed to clarify this.

Effects of salicylic acid on accumulation of phenolic compounds

Application of SA partially induced accumulation of phenolic compounds and these effects were significant only from the 60th day until the end of storage (Fig. 5). Since then, SA-treated fruits contained higher quantities of phenols than the control. These results are in agreement with the findings of Harindra *et al*. (2015) in grapes and Geransayeh *et al*. (2015) in strawberries. In contrast with the results of this work, Kant *et al*. (2016) reported a delay in the biosynthesis of total phenolics in tomato green mature fruits dipped in SA solution resulting in an increase in fruit quality that is possibly related to the different physiological status of tomato green mature fruits during storage. Apples contain many bioactive compounds including phenols. SA may act as modulator of phenylpropanoid metabolism leading probably to the accumulation of phenolics correlated with the induction of enzymes which are involved in general phenylpropanoid metabolism, e.g. PAL (Godoy-Hernandez and Loyola-Vargas, 1997). It has shown that PAL activity could be induced by SA elicitation in citrus (Lafuente *et al*., 2001) and grapes (Chen *et al*., 2006), which would result in the accumulation of plant secondary metabolites.

Effects of salicylic acid on anthocyanin content

Anthocyanin content of apples revealed an increasing trend until the 60th day of storage while decreased right afterwards (Table 2). Initially, effects

Table 2 - Effect of pre- and post-harvest salicylic acid treatments on anthocyanin content (mg/g FW) of 'Red Delicious' apples stored at 0±0.5°C for 193 days

Treatment	Days of storage					
	0	30	60	90	157	193
Pr	5.57 a	7.39 a	12.41 a	9.15 a	4.91 a	2.54 a
Po	3.74 a	5.53 b	10.92 a	9.10 a	5.13 a	2.22 a
T0	5.75 a	6.32 ab	12.08 a	7.84 b	4.59 a	2.83 a
T_1	7.37 a	8.29 a	13.08 a	11.62 a	5.07 a	1.54 a
T_2	4.33 a	6.46 ab	11.63 a	10.02 ab	5.44 a	2.22 a
T_3	3.29 a	4.68 b	10.06 a	7.29 b	4.85 a	2.91 a
PrT_0	5.26 a	5.66 bc	12.50 a	7.65 b	4.04 a	2.88 ab
PrT_1	5.68 a	8.54 ab	15.39 a	12.98 a	5.24 a	1.35 b
PrT_2	7.56 a	10.13 a	12.02 a	9.43 ab	5.94 a	2.47 ab
PrT_3	4.46 a	5.38 bc	9.69 a	6.46 b	3.93 a	3.64 a
PoT_0	5.17 a	7.14 abc	11.53 a	8.00 b	5.14 a	2.79 ab
PoT_1	5.67 a	7.98 ab	10.78 a	10.27 ab	4.96 a	1.70 ab
PoT_2	2.75 a	3.52 c	11.23 a	10.74 ab	5.02 a	2.03 ab
PoT_3	2.10 a	3.97 c	10.43 a	7.96 b	5.54 a	2.36 ab

The same letters in any column show no significant difference between the data. Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

of SA on anthocyanin content were significant. The highest contents of anthocyanin were obtained in the concentrations of 1 and 2 mM at pre-harvest treatments with no significant differences between one and with 1mM of post-harvest treatment. Similar result has been reported by Jamali *et al*. (2013) who sprayed strawberry plants by SA together with nickel sulfate and found higher amounts of anthocyanin in strawberry fruits. Anthocyanins degrade by polyphenol oxidase during post-harvest, and this might be the main reason behind the reduction of anthocyanin compounds. The role of SA on anthocyanin production is unknown, one may hypothesize that SA could activate the key enzyme (Chalcone synthase) in the anthocyanin biosynthetic pathway (Godoy-Hernandez and Loyola-Vargas, 1997). Obinata *et al*. (2003) reported that SA could markedly increase the production of procyanidin in grape. Total anthocyanin has also been increased with storage period in both control and treated pomegranates (Sayyari *et al*., 2011). Our results agree with findings of Obinata *et al*. (2003) and Sayyari *et al*. (2011).

Effects of salicylic acid on antioxidant activity

Antioxidant activity increased in all treatments during storage (Table 3). Antioxidant activity was increased in SA-treated fruits, but this process was not uniform, i.e. changes antioxidant activity was not followed from fixed pattern during storage. Antioxidative potential of apple, however, is known to depend on the concentration and composition of phenolics. Numerous researchers have indicated that SA and its functional analogs have inhibitory effects on CAT and POD activities or serve as substrates for POD. Knorzer *et al*. (1999) reported, when applied exogenously at suitable concentrations, SA was found to enhance the efficiency of antioxidant system in plants. Imran *et al*. (2007) and Sayyari *et al*. (2011) found that SA have the capacity of increasing antioxidant, the result that confirms the present study. Kazemi *et al*. (2011) also observed the higher ascorbic acid (vitamin C) content as well as peroxidase and superoxide dismutase activities in apples treated with SA exogenously. SA is possibly effective through the activation of responsible genes for producing antioxidant compounds (Wang *et al*., 2006) and increasing activity of the antioxidant enzymes such as superoxide dismutase, peroxidase, catalase and ascorbate peroxidase (Mo *et al*., 2008).

Table 3 - Effect of pre- and post-harvest salicylic acid treatments on antioxidant activity (% of DPPH inhibition) of 'Red Delicious' apples stored at 0±0.5°C for 193 days

Treatment	Days of storage					
	0	30	60	90	157	193
Pr	60.20 a	74.34 a	62.00 b	73.67 b	78.67 a	82.18 a
Po	47.40 a	67.35 b	84.25 a	81.53 a	76.79 a	82.49 a
T_0	59.29 a	62.43 c	55.62 b	68.22 b	72.50 c	78.76 b
T_1	50.14 a	69.88 b	79.16 a	78.26 a	83.93 a	83.67 ab
T_2	61.03 a	81.16 a	81.52 a	81.93 a	79.84 ab	80.36 b
T_3	58.24 a	70.27 b	79.68 a	81.42 a	74.55 bc	86.33 a
PrT_0	58.46 a	70.85 cd	50.37 d	70.33 cd	82.55 a	63.54 c
PrT_1	61.94 a	68.59 d	68.09 b	73.09 bcd	84.57 a	77.94 b
PrT_2	56.35 a	78.56 b	60.33 c	75.35 bcd	81.73 a	87.23 a
PrT_3	60.13 a	77.82 bc	66.23 b	76.76 abcd	61.65 b	91.00 a
PoT_0	49.36 a	54.01 e	59.82 c	64.71 d	60.44 b	90.17 a
PoT_1	52.22 a	71.48 bcd	92.99 a	82.39 abc	83.29 a	87.49 a
PoT_2	63.47 a	86.35 a	92.12 a	88.52 a	78.25 a	71.20 bc
PoT_3	45.92 a	63.99 d	90.90 a	85.15 ab	84.87 a	78.54 b

The same letters in any column show no significant difference between the data. Comparison of the means was conducted through the Duncans' Multiple Range Test (P<0.05).
Pr= pre-harvest; Po= post-harvest; T0 to T3= control, 1, 2 and 4 mM SA treatment respectively.

4. Conclusions

This study showed the effectiveness of pre- and post-harvest treatment of SA on the quality of 'Red Delicious' apples. Application of SA affected on juice pH and anthocyanin content until the middle of storage, maintaining phenolic compounds and increasing antioxidant activity. No significant difference between SA treatments was seen in terms of SSC, TA and fruit firmness. The lowest weight loss was observed on pre-harvest-SA treatment when sprayed, and the highest concentration of phenolic compounds was observed in post-harvest treatment when dipped. Anthocyanin content was also increased during storage up to 60 days, but by the day 90, it was gradually decreased until the end of storage when reached to the lower level. As a conclusion, treatment of 'Red Delicious' apples with SA could properly maintain the quality of fruits and increase their post-harvest life and antioxidant capacity for at least 2 months.

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Flower anatomy related to blooming development of *Berberis microphylla* G. Forst (*Berberidaceae*)

S. Radice, M. Arena
Department of Plant Physiology, Facultad de Agronomía y Ciencias Agroalimentarias UM-CONICET, Machado 914, Lab. 501, B1708EOH, Morón. Prov. de Buenos Aires, Argentina.

Key words: anthesis, barberry, nectar, Patagonia, pollen grain, stigma.

Abstract: *Berberis microphylla* G. Forst. is a Patagonian native shrub commonly named "calafate", which has a growing economic potential due to its dark blue berries that are consumed fresh, as jams and preserves, and are used for the production of soft drinks and ice cream. Moreover, the fruits have a high content of carbohydrates, phenols and antioxidants. The objective of this work was to show the changes observed in the flower from the emergence in relation to the floral phases and the importance that they have on pollination and fertilization. During the anthesis, the nectar is excreted inside and outside of the petal through the epidermis of the secretory tissue. The epidermis of the stigma is papillae with cells of greater length in the periphery of this structure simulating an additional ring. Secretory tissue is also present on the area of the fusion carpel. During anthesis, the epidermis glands of the stigma showed active secretion and these conditions favor pollen grain germination. Germinated pollen grains were observed after 12 hours of pollination and ten days later the pollen tube reached the ovule area. Pollen tube grew surrounded the ovules and probably some of them already accomplished the fertilization.

1. Introduction

Berberis microphylla G. Forst. is a Patagonian native shrub commonly named "calafate", with a large distribution from Neuquén (37° SL) to Tierra del Fuego (54° 8´ SL) (Orsi, 1984). This species has a growing economic potential due to the production of fruits as a non-timber forest product (Tacón Clavaín, 2004). In fact, its dark blue berries are consumed fresh, as jams and preserves, and are used for the production of soft drinks and ice cream. Moreover, the fruits have a high content of carbohydrates, phenols and antioxidants (Arena and Curvetto, 2008; Arena *et al*., 2011, 2012, 2013 b). The genetic and morphological analysis of spontaneous accessions in natural populations of *B. microphylla* grown on Tierra del Fuego (Giordani *et al*., 2016), as well as the changes in form and leaf anatomy due to weather conditions (Radice and Arena, 2015) were recently studied.

The study of flower anatomy related to blooming is an important step for programs of genetic resource conservation and improvement, complementing basic studies of floral biology (de Castro Nunes *et al*., 2012; Wetzstein *et al*., 2014). Flower structure and floral biology was well described by Arena *et al*. (2011), like the phenological stages (Arena *et al*., 2013 a) and flower bud differentiation (Arena and Radice, 2014). More recently, a comprehensive study of pollen grain was published (Radice and Arena, 2016 a).

Nevertheless, pollination was not clear until now. Fertilization of Patagonian *Berberis* has been classified as cross-pollination by Orsi (1984). However, Hegi (1958) and Romeo *et al*. (2005) referred the *Berberis* species as autogamous due to the absence of visiting insects together with extreme climatic conditions prevalent in most areas of Patagonia. However, during flowering, the activity of different *Syrphidae* was observed (Radice *et al*., 2016). On the other hand, floral movements have been appointed as mechanisms to facilitate self-pollination (Darwin, 1862). Stamen movement has been documented in a few plant families, among them *Berberidaceae* (Lechowski and Bialczyk, 1992). However, results of controlled treatments of self- and cross-pollination compared with those of open-pollination performed during three different periods in *B. microphylla* do

(*) Corresponding author: siradice@yahoo.com
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not support this hypothesis (Radice and Arena, 2016 b). Thus, self-pollination resulted only in pollen germination on the stigmas but the pollen tubes were not able to reach the ovules. According to these antecedents, anatomical studies are necessary for a better understanding of the physiological processes during floral biology and pollination that interact with *Syrphidae* activity. The objective of this work was to show the changes observed in the flower from its emergence in relation to the floral phases and the importance that they have on pollination and fertilization.

2. Materials and Methods

Plant material

Flowers of *Berberis microphylla* G. Forst. (*n = 20*) on growth stages ranging from 53 to 68 on the BBCH scale proposed for *B. buxifolia* (Arena *et al*., 2013 a) were collected on plants grown near Ushuaia city, Tierra del Fuego (54° 48' SL, 68° 19' WL and 30 m asl), and were fixed in FAA (formaldehyde, 100 mL; ethyl alcohol, 500 mL; acetic acid, 50 mL; distilled water, 350 mL).

Light microscopy

Button flowers were dehydrated in an ethanol series and embedded in Spurr's resin. Thin sections (75-90 nm thick) were stained with uranyl acetate and lead citrate.

Fluorescent microscopy

Flowers fixed in FAA were washed with distilled water and softened with NaOH (8N) as described by Martin (1959). Then, they were stained with aniline blue to study pollen tube growth. Squash material was observed by a Leica microscope (DM 2500) with DAPI filter.

Scanning electron microscopy (SEM)

Button flowers fixed in FAA were dehydrated in an ethanol series and critical point drying technique was employed. Samples were sputter coated with 20 nm gold and observed with a Philips XL 30 SEM.

Ovules and seeds relation

The number of ovules on button flowers (*n= 130*) and the number of seeds on formed fruits (*n=100*) were counted.

3. Results

Pistil of *B. microphylla* is similar to a bottle (Fig. 1A). Flowers before anthesis (growth stage 54) showed anthers with microspores at an advanced stage of development and underdeveloped ovules (Figs. 1A-D). In effect, microsporangia contain tapetal cells metabolized, i.e. a thick portion was deposited on the wall of the microspore and inside it is possible to observe vegetative and generative cells that are surrounded by a thin delicate wall (Fig. 1D). On the other hand, ovules are rudimentary with an active cellular proliferation on the nucellus and integuments (Fig. 1C).

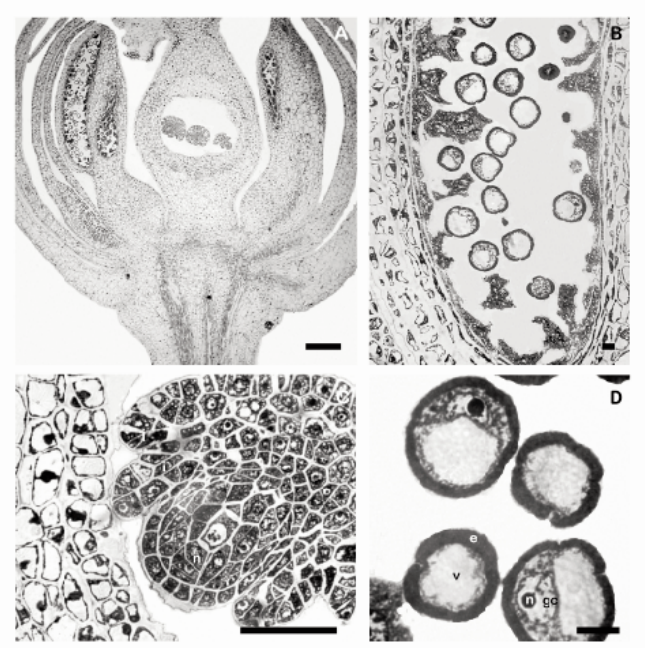

Fig. 1 - Bottom flower of *B. microphylla* G. Forst (light micrograph). A, longitudinal section of a flower on stage 54. B, anther with mature pollen grains; C, rudimentary ovule with nucellus (n) and integuments (i) in development; D, detail of pollen grain with exine (e), Vacuole (v) and generative cell (gc) contained into the vegetative cell; n= nucleus. Bars: A = 200 µm; B, D = 10 µm; C = 50 µm.

The epidermis of the stigma is covered by secretory cells (Figs. 2 A, C, E) and the short style is also recovered by glands (Fig. 2B). Petals have two thick nectaries on the basal position (Fig. 3A). Both petal epidermises are also formed by glandular cells (Figs. 3B, D, E, F). On a later flower phase (growth stage 59), the epidermis and nectariferous cells of the nectar have dense cytoplasm and conspicuous nucleus (Figs. 3C). Nectar is abundant in the intercellular spaces (Fig. 3C). Flowers on the anthesis phase (growth stage 60) showed nectariferous cells with a gradual diminution of staining density (Fig. 3G). Total production of nectar per flower is poor; it is secreted through the gland cells that are present on the epidermis of the nectaries (Figs. 3C, G) and two epider-

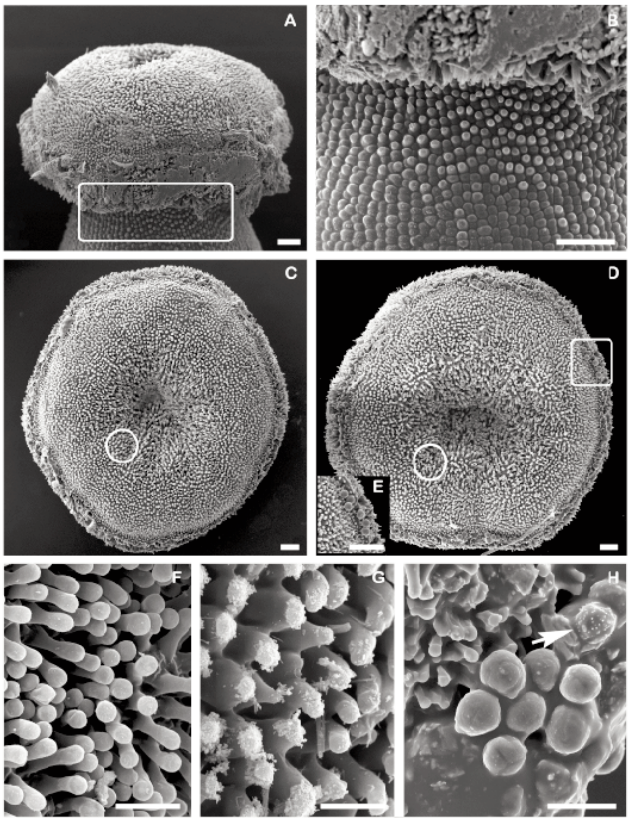

Fig. 2 - SEM micrograph of the pistil of *B. microphylla* G. Forst. A, stigma of a flower on stage 59; B, detail of box in the picture A, periphery of the upper end of the ovary with glands; C, stigma front view shown in A; D, stigma front view shown of a flower on stage 60; E, detail of box in the picture D, long hairs peripheral stigma with pollen grains attached; F, detail of circle in the picture C, epidermal cells of stigma; G, detail of circle in the picture D, active secretions glands; H, mixture of pollen grains wrapped in stigmatic mucilage, arrow points to a grain of foreign pollen. Bars: A-E =100 µm; F-H= 50 µm.

mal layers of petals (Figs. 3E,F). Nectar is exuded through the cuticle with rupture of its outer layer. Greater presence of vacuoles is observed in both nectar tissues (Fig. 3G).

Flowers on growth stage 59 showed a monocarpellary pistil with a clavate shaped stigma (Fig. 4A). Epidermis of the stigma is papillae (Figs. 4B, C, E) with cells of greater length in the periphery of this structure simulating an additional ring (Fig. 4E). These cells secrete a sticky substance that keeps always the stigma hydrated during the anthesis phase (Fig. 2F). Secretory tissue is also present on the area of the fusion carpel (Figs. 4B, D). On the other hand, stigmas on growth stage 59 are receptive, i.e. they can promote pollen germination while stigma on less developed growth phases is not receptive (Fig. 2E).

Flowers on anthesis phase (growth stage 60)

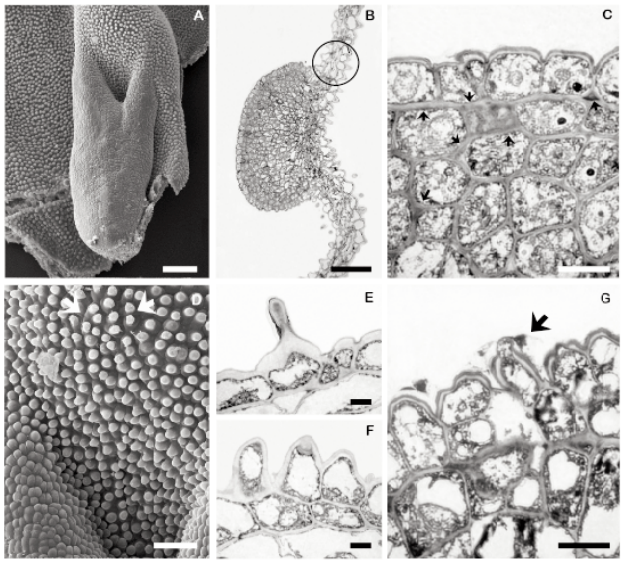

Fig. 3 - Nectary of *B. microphylla* G. Forst. A-D SEM micrograph. B-C, E-G light micrograph. A, nectary view at the base of petal; B, section of a petal and a nectariferous area; C, detail of tissue of nectariferous area of a flower on stage 59; intercellular space with nectar (arrows); D, detail of circle in the picture A, epidermis with glands, arrows show secretory glands. E, detail of circle in the picture B, petal outer epidermis; F, detail of circle in the picture B, petal inner epidermis; G, detail of tissue of nectariferous area of a flower on stage 60, arrow indicates an epidermal cell in active secretion. Bars: A-B= 100 µm; C= 10 µm; D= 50 µm; E-G= 10 µm.

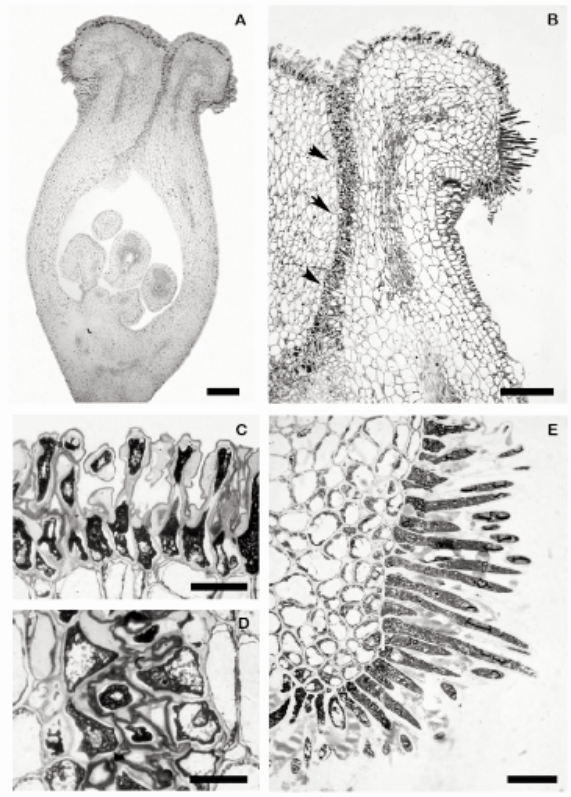

Fig. 4 - Light micrograph of the pistil of *B. microphylla* G. Forst. A, longitudinal section of a flower on stage 59.B, view of stigma and the upper end of the ovary; C, glandular cells of the flat part of stigma; D, detail of glandular cells of fusion area of the carpel; E, detail of hairs surrounding the stigma. Bars: A= 100 µm; B= 200 µm; C-E= 50 µm.

showed mature pollen grain with a well-formed external wall and the cytoplasm of the vegetative cell rich of starch (Fig. 5E). In this phase anthers are dehiscent. On the contrary, ovules are externally coated by the integuments and attached to the ovary by the funiculus on basal placentation (Fig. 5D). Internally, the ovules present some delay in comparison to the pollen grain development. Pollinated flowers show ovules with the embryo sac with egg cell, synergists, antipodes and polar nuclei cells developed (Figs. 5 A-C); nevertheless, no pollinated pistils show ovules with megaspore mother cells without development.

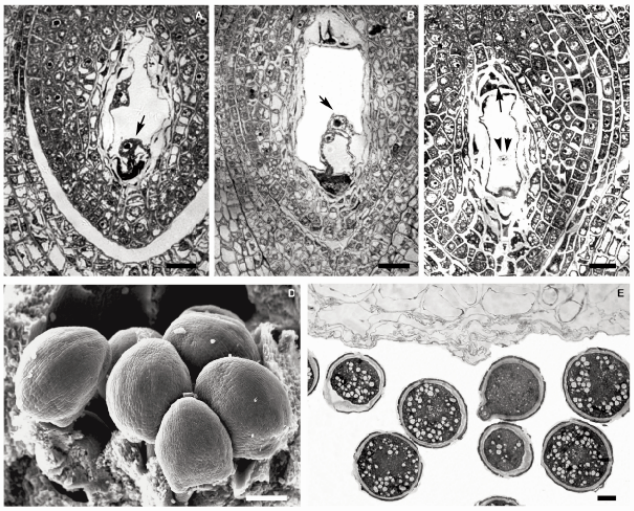

Fig. 5 - Details of the ovary and pollen grains of a flower on anthesis stage of *B.* G. Forst. A-C, embryo sac with egg cell (A, arrow), synergids (B, arrow) and antipodes (C, arrow) and polar nuclei (C, arrows head); D, SEM micrograph of ovules on the basal insertion of ovary; E, mature pollen grains with cytoplasm rich in starch grains. Bars: A-C= 200 µm; D= 100 µm; E= 10 µm.

Pollen is deposited on the stigma mainly between the secretory cells that surround this structure (Figs. 2D, G). Germinated pollen grains were observed after 12 hours from pollination (Fig. 6B). After 24 hours pollen tube crosses the stigma (Fig. 6A) and ten days later the pollen tube reached the ovule area. Pollen tube grew surrounded the ovules and probably some of them accomplished the fertilization (Figs. 7A, B).

Subsequently seed growth is observed (Fig. 7C) but this process is not given in all the ovules. In effect, on a selected natural population the ovules and seeds were counted, and an average of 8.95 (ranged from 6.9 to 10.0) and 5.28 (ranged from 3.3 to 7.2), respectively, were registered, i.e. 40.97% of all ovules produced aborted.

4. Discussion and Conclusions

Flowering plants are associated with a broad spectrum of animal pollinators, among these bees constitute an important but not exclusive one (Dötterl and Vereecken, 2010). In effect, it has already been demonstrated that *B. microphylla* is not self-fertile so it depends on insect pollination (Radice and Arena, 2016 b). Tierra del Fuego (Argentina) offers an extreme climatic situation where the bees cannot prosper; accordingly calafate flowers are visited by different syrphids (Radice *et al.,* 2016). Pollination by insects, including flies, is commonly a mutualistic interaction, in which both the plant and the insect benefit; thus, anatomical organization and pollination strategies developed on the flowers must be adapted to the environmental conditions.

Calafate shrubs bloom with abundant yellow flowers that produce aromatic nectar (Radice *et al.,* 2016), that exudates inside the flower as well as the outside through the petals mainly in their insertion

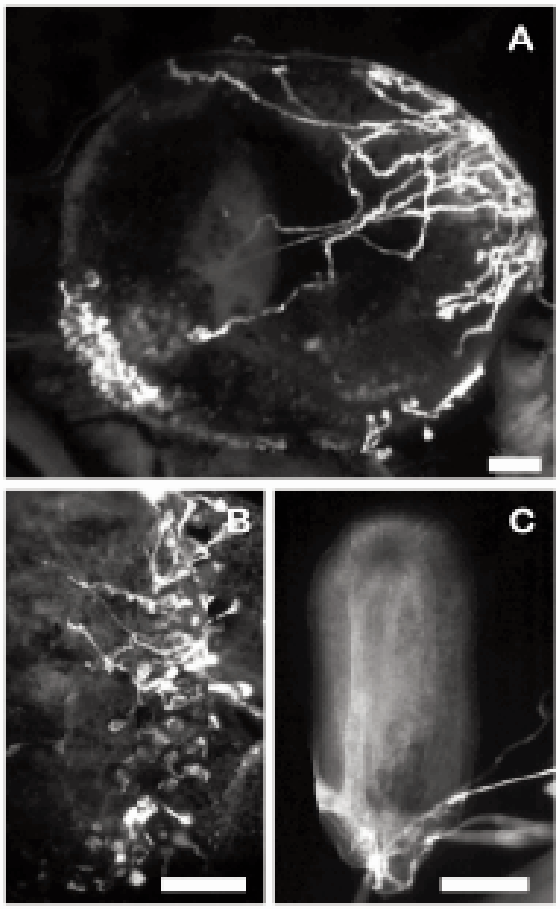

Fig. 6 - Fluorescent light micrograph of pollen tube germinated on pistils of *B. microphylla* G. Forst. A, view of stigma with pollen grain germinated and pollen tubes inserted in the ovary; B, pollen tubes on the first stage of growth; C, pollen tube penetrating the ovule. Bars: A-C= 100 µm.

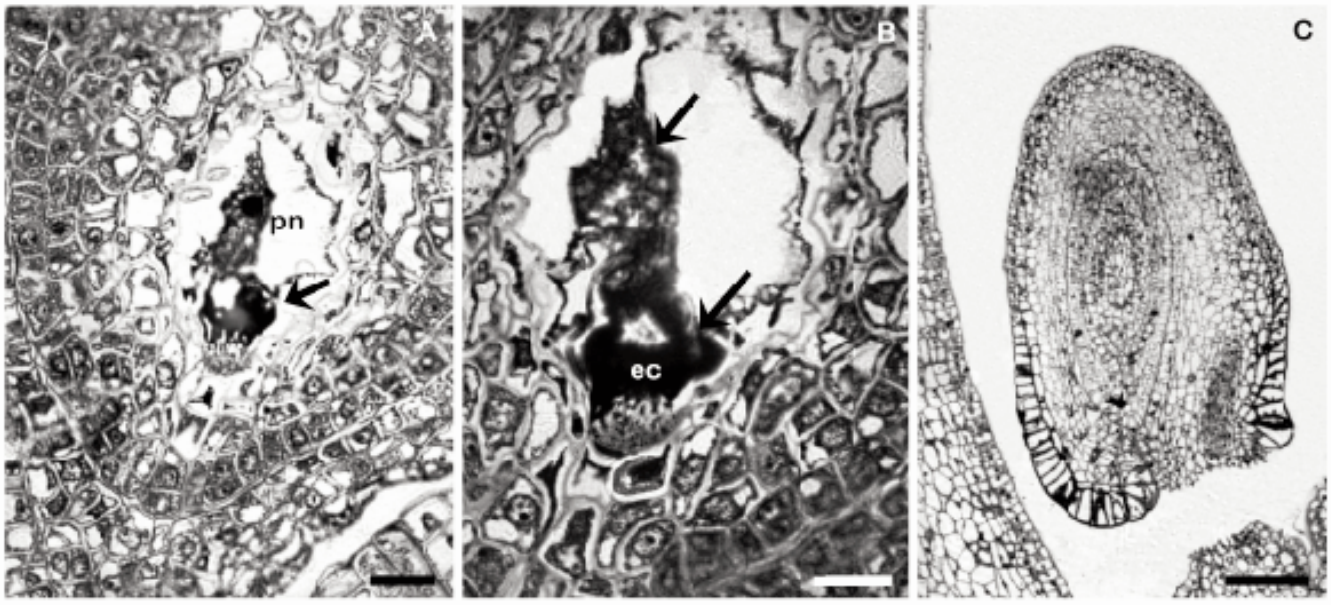

Fig. 7 - Fertility of *B. microphylla* G. Forst. A-B, time of discharge of sperm nuclei (arrows) into the egg cell (ec) and polar nuclei (pn), C, growing seed. Bars: A-B= 200 µm; C= 100 µm.

area. This particularity is very important because the fluorescent emission of nectar attracts pollinating insects. *B. microphylla* have perigonial nectaries type "1a" according to the topographic classification of Fahn (1982). Unlike what was found on the nectar tissue of *Berberis corymbosa* by Bernardello *et al*. (2000), no stomata were found on this species either on histological sections or through SEM observations. In effect, nectar is exuded through the epidermis as cited by Bernardello (2007). *Berberis* produce small amounts of nectar per flower; effectively it was registered less than 1 µl on *B. corymbosa* (Bernardello *et al*., 2000) and 1.57 µl on *B. microphylla* (Radice *al*., 2016). Nectar concentration was considered as intermediate for *B. buxifolia* (31.2±14.8%) (Chalcoff *et al*., 2006). This result is in coincidence with Radice *et al*. (2016) who measured 36.28 °Brix in nectar of the *Berberis* population studied.

Stigma epidermis is covered by hairs that secrete a stigmatic fluid to promote pollen germination. Surface hairs on the stigma can be seen in others species like *Papaver rhoeas*, or *Lupinus luteus* (Fahn, 1982). Once overcome the stigma, the pollen tubes grow through the carpel wall. In effect the margin of the carpel is covered by glands that nourish the germinated pollen. The *Berberidaceae* are generally considered to have originated in some part of the ranalian complex (Chapman, 1936), i.e., it is belonging to the group of the oldest dicotyledons which is confirmed by its structure devoid of carpelar style (Fahn, 1982).

There are three important elements to attract the pollinating insect on *B. microphylla* such as color, scent and nectar. It has long been known that bees utilize not only visual but also olfactory flower cues for finding suitable host plants (Dötterl and Vereecken, 2010). This pollination strategies present in calafate could be useful in other growth areas of the species. On the other hand, young flies in the presence of generic floral scent respond more strongly to a uniformly yellow cue than to any other uniform color cue (green, white, black, blue, red) except for ultraviolet (Brodie *et al*., 2015). Nectar is the most commonly sought reward by flower-visiting flies (Woodcock *et al*., 2014) because carbohydrates contained in the nectar provide short-term energy supply. So the abundant number of yellow flowers plus the fluorescent emitted cue by nectar must constitute a strong attraction for syrphids.

Acknowledgements

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Development of pollination and *in vitro* germination techniques to improve the hybridization in *Hydrangea* spp.

G.A. Venturieri [1], B. Nesi [2], S. Lazzereschi [2], S. Pecchioli [2], G. Burchi [2]

[1] *UFSC/CCA-FIT, Federal University of Santa Catarina, Campus Itacorubi, Rod. Admar Gonzaga, 1346 Itacorubi, CEP 88034-000, Florianopolis (SC), Brazil.*

[2] *Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Unità di Ricerca per il Vivaismo e la Gestione del Verde Ambientale ed Ornamentale (CREA-VIV), Via dei Fiori, 8, 51017 Pescia (PT), Italy.*

Key words: breeding, fertilization barriers, fruit cut system, hortense, seed disinfection, sowing system.

Abstract: *Hydrangea* is a genus of ornamental plants which is gaining new markets mainly as a fresh or dried cut flower, but it is also important as a pot plant and for landscaping. To expand its market, new hybrids should be developed. To increase the hybridization efficiency, some techniques were developed and tested: i) evaluation of two pollination systems; ii) comparison among fruit-cut systems before *in vitro* cultivation to develop embryos and to allow the growth of new genotypes; iii) evaluation of seed disinfection systems for *in vitro* germination; iv) sowing systems using seeds and fruits from stocks cultivated in two environments. To increase inter- and intra-specific hybridization, pollination by dispersion of previously collected pollen on the top of a corymb by a brush was more effective than pollination using the corymb itself as a brush. A longitudinal cut system can be considered the best treatment to be applied on fruits before *in vitro* cultivation to allow growth of seedlings. Sterilization of seeds can be done by immersion in a solution of commercial bleach for 5 minutes on MS culture medium with PPM®. When stocks are cultivated in greenhouses, *in vitro* contamination is lower and seeds have a better rate of germination. The results of these experiments were applied in a breeding program on *Hydrangea* using sexual crosses.

1. Introduction

The genus *Hydrangea* L. includes 23 species, mainly distributed in the American and Asiatic continents (McClintock, 1957). It is a very popular ornamental plant for both garden and interior design and has recently been commercialized as a high value fresh or dried cut flower.

Interest in *Hydrangeas* is mainly due to the striking coloration of its inflorescences (corymbs or panicles), that range from pink, blue, white, to light purple or dark purple. Flowers are produced from early spring to late autumn and have two inflorescence morphologies: 'mophead' - with large flowers forming spherical flower heads; and 'lacecap' that resemble round, flat flower heads with a center core of subdued, fertile flowers surrounded by outer rings of showy, sterile flowers.

To further increase their popularity, new hybrids and cultivars should be developed. In flowering plants, the main objective of selective breeding is to increase genetic variability in ornamental traits such as flower color, flower shape and plant form. To achieve this objective, intra- and inter-specific hybridizations have been widely used in breeding programs. Hybrids between *H. macrophylla* (Thunb.) Ser. and *H. paniculata* Sieb. were produced using embryo rescue, but the resulting plants were sterile and lacked vigor (Reed *et al.*, 2001; Reed, 2004). *In vitro* embryo rescue procedures have been used to facilitate the recovery of interspecific hybrids of many genera (Bridgen, 1994; Sharma *et al.*, 1996), and have recently been used to recover a putative *H. macrophylla* (Thunb.) Ser. x *H. arborescens* L. hybrid (Kudo and Niimi, 1999 a, b). Hybrid embryos often resume growth and develop into normal plants when removed from ovules and placed on an aseptic nutrient media. This procedure is known as in ovule

(*) Corresponding author: giorgini.venturieri@ufsc.br
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embryo culture (Reed, 2000).

In vitro germination of seeds is a common technique used to overcome incompatibility barriers in ornamental plant hybrids (Eeckhaut *et al.*, 2006 a, b; Lazzereschi *et al.*, 2012; Nesi *et al.*, 2012). Since most *Hydrangea* seeds are so small (diameter of 0.5 mm) and hybrid seeds production is usually low (0-5 seeds/fruit), so, the development of a successful *in vitro* method for *Hydrangea* seed germination would be an important tool for breeding programs.

To increase seed germination, Greer and Rinehart (2010) have developed an *in vitro* method for cultivation and assay of *H. macrophylla* (Thunb.) Ser. and *H. paniculata* Sieb. seeds, through germination on solid media in conjunction with Plant Preservative Mixture (PPM®, Plant Cell Technology, Inc., Washington, DC, USA), and by sterilizing seed with trichloro-s-triazinetrione (Trichlor).

The objective of the present study was to develop and test several techniques for the generation and recovery of hybrids in *Hydrangea*. These included: two pollination systems (using a brush or an inflorescence on the top of the corymb); different ovary cut systems, aimed at embryo and seed rescue; different seed disinfection systems for *in vitro* germination (evaluating the time of immersion in a bleach solution and the addition of Plant Preservative Mixture - PPM® in media); comparison among three sowing systems using seeds and immature fruits in aseptic conditions, and in compost in climatized beds, from stocks cultivated in two environments (in a greenhouse and under shading net). The outcomes of these experiments were used to determine best practices for the hybridization of *Hydrangea* species using sexual propagation.

2. Materials and Methods

The experiments were carried out at the Council for Agricultural Research and Economics - Landscaping Plants and Nursery Research Unit (CREA-VIV) in Pescia (PT) (43° 49' 00" N; 10° 48' 00" E), Italy. At CREA-VIV, a germplasm collection of *Hydrangea*, composed of 66 genotypes belonging to *H. macrophylla* ssp. *macrophylla* (Hortensia and Lacecap Group), *H. paniculata, H. serrata, H. villosa, H. quercifolia, H. anomala* ssp. *petiolaris, H. arborescens, H. heteromalla, H. involucrata, H. aspera* and three genotypes of *Schizophragma hydrangeoides*, was maintained. Some trials were also arranged in Sanremo (IM) (43°49' N; 7°47' E) with some genotypes replicated from the CREA-VIV collection. Selected cultivars of this collection were used for hybridizations. During the summer of 2014, crosses among different genotypes, belonging to *H. macrophylla* ssp. *macrophylla* (38 cultivars), *H. macrophylla* ssp. *serrata* and *H. paniculata* (5 cultivars each); *H. arborescens*, *H. aspera*, *H. quercifolia* and *H. involucrata* (one cultivar for each species) were made (Table 1). Then, the capsules obtained from controlled pollinations were collected and used as starting material in the experiments, as described below.

Table 1 - Plant material used for intra- and inter-specific crosses between different genotypes of *Hydrangea* spp.

Species	Cultivars
H. macrophylla ssp. *macrophylla*	'Alberta', 'Alpen Gluhen', 'Ayesha', 'Benxi', 'Bianca Ceriana', 'Dienemann', 'Elbatal', 'Endless Summer', 'Etoille Violette', 'Europa', 'First Red', 'Grattino', 'Green Shadow', 'Hanaby', 'Harlequin', 'Intermezzo', 'Lake San Markos', 'Lanarth White', 'Lemon Wave', 'Libelle', 'Magical Coral', 'Magical Garnet', 'Magical Jade', 'Magical Noblesse', 'Masja', 'Myharayama Yae', 'Nymphe', 'Paris', 'Red Beauty', 'Renate Wate', 'San Baronto', 'Schnball', 'Seour Therese', 'Sibilla', 'Tricolor', 'White First', 'Zorro' and 'Kardinal'
H. macrophylla ssp. *serrata*	'Acuminata', 'Blue Bird', 'Miranda', 'Preziosa' and 'Yae-no-amacha'
H. paniculata	Limelight', 'Phanthom', 'Pink Diamond', 'Unique', and 'Vanilla Fraise'
H. arborescens	'Annabelle'
H. aspera	'Rowellane'
H. involucrata	'Yorakutama'
H. quercifolia	'Snow Queen'

Pollination systems

Twelve crosses, involving *H. macrophylla*, *H. arborescens* and *H. quercifolia,* randomly distributed in a germplasm collection, were subjected to two pollination systems (treatments). Before pollination, sterile flowers and all extremely immature fertile were removed from inflorescences to be used as females prior to opening of the fertile flowers. After, the petals and anthers of all remaining fertile flowers were removed and the inflorescence was covered with a breathable plastic bag. Inflorescences to be used as males were also bagged prior to flower opening. Pollination experiments were performed 1 to 4 days following emasculation.

The two pollination treatments included: a) pollination by the dispersion of previously collected pollen on the top of a corymb, aided by a brush; b) a simple dispersion of pollen using the corymb as a brush, where anthers presenting freshly-dehisced pollen were touched with the exposed stigmas of the

emasculated flowers. After pollination, inflorescences were covered again with the bags which remained on the plants until fruit collection. Effect of treatment was evaluated by the number of developing fruits on bagged female flowers. A *t* test was used to compare averages between treatments (Sokal and Rohlf, 1981).

In total, one hundred and thirty-five hand pollinations were carried out in Pescia, and 33 in Sanremo, using a different subset of *Hydrangea* species. Each species cross was repeated 3 times.

Embryo and seed rescue from immature fruits

Approximately 90 days after pollination (DAP), well-developed fruits (n = 444), obtained from the breeding program were used to investigate rates of embryo and seed rescue from immature fruits.

Fruits were sterilized in a solution of commercial bleach (5% of active chlorine), in distilled water (1:2, v/v) plus 2 µl/100 ml Tween20® (Sigma, St. Louise, MO, USA) for 10 minutes. Subsequently four ovary cut systems (treatments) were applied: a) stigma off - stigmatic branches were removed; b) longitudinal cut - stigmatic branches were removed and a longitudinal cut, from the top of the fruit up to approximately to peduncle insertion, was made; c) equatorial cut - stigmatic branches were removed and a transversal cut at the larger diameter of the fruit was made (both sections were cultivated); d) top cut - 1/3 of the distal part of the fruit was removed (Fig. 1 a-d).

Fruits were cultured in a one-half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg/L of naphthalene acetic acid (NAA), 1 ml/L of PPM®, 30% sucrose, 6 g/L of agar and pH was adjusted to 5.8. Fruits were individually placed in test tubes. Six replication were used by cross. All vials were cultivated in a chamber maintained at 23±1°C under 16 h/day photoperiod provided by fluorescent tubes at 35 µmol/m·s.

Ovaries that were considered as contaminated or dead were counted 7 days after, and their proportion (*p*) was a square root of transformed arcsine (Ayres *et al*., 2007) and evaluated using Analysis of Variance. Averages were compared by Fisher LSD test of significance for α= 0.05 (Ayres *et al*., 2007). Values were expressed as a percentage. Physical attributes of germinated seedlings were described for each treatment.

Disinfection of seeds for in vitro *germination*

Fruits considered as mature (130±10 DAP) were collected and left to dry on a laboratory bench, and then grinded carefully using a mortar and pestle to liberate seeds from capsules. The obtained mass was passed through 0.71 mm mesh of a soil gradation sieve (Grade 25, Giuliani Tecnologie, Scientific Instrument, Torino, IT) to remove large debris, and used in two disinfection experiments as described below.

Sterilization as a function of time immersion in bleach solution. About 0.025 g of the mass of seeds and debris was placed in a piece of TNT envelope and submerged for the disinfection process (Fig. 2 a). The envelopes were dipped in 70% ethanol for 30 sec, followed by sterilization in a commercial bleach solution, as already as described above, for 5, 10, 20, and 30 minutes and rinsed in sterilized distilled water twice (Fig. 2 b). Then the envelopes were opened (Fig. 2 c) and their contents were laid on a one-half MS culture medium supplemented with 1 mg/L of NAA, 1 ml/L of PPM®, and 30% sucrose, and adjusting pH to 5.8. TNT was used to cover petri dishes (Fig. 2 d), and then cultures were placed in the chamber as described above. Germinated seeds and contamination of petri dishes were evaluated after 15 days. Frequency of contaminated and uncontaminated petri dishes that contained germinated seeds was used to evaluate the effect of contamination on ger-

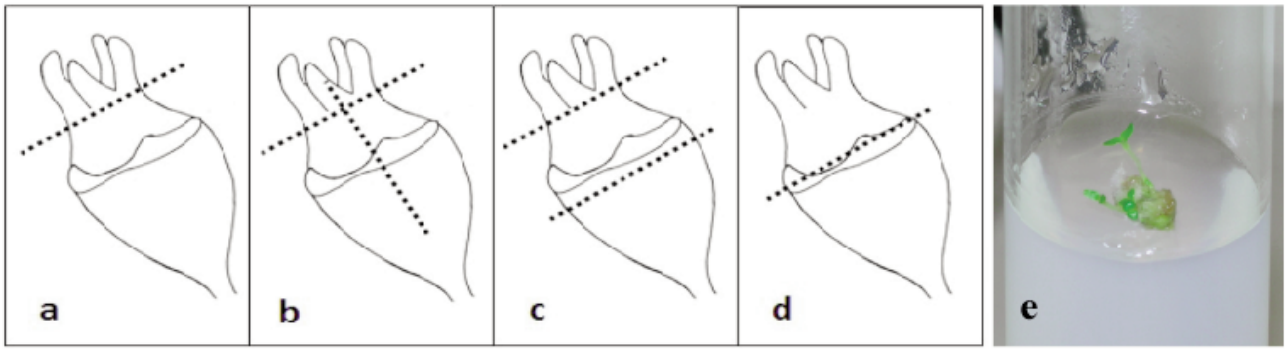

Fig. 1 - Graphic representation of the different cuts applied to the immature fruits of Hydrangea: a) stigma off; b) longitudinal cut; c) equatorial cut; d) top cut (Fig. 1 a-d); e) development of new plantlet from immature fruits *in vitro* cultured.

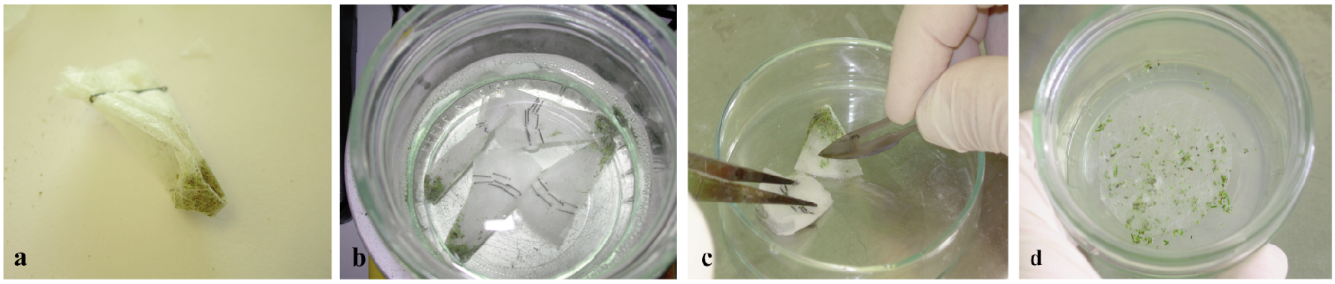

Fig. 2 - Disinfection process of seeds obtained from mature capsules harvested: (a) TNT envelops with seeds plus debris inside; (b) Envelops dipped in the disinfection solution; (c) Staples were taken off and envelop opened; (d) Seeds plus debris were layered on medium and TNT was left to cover seeds.

mination by Fisher's test. Spearman rank order correlation was calculated between number of germinated seeds and disinfection time (Ayres *et al*., 2007).

Sterilization as a function of duration in bleach solution and PPM® concentration in the media. In this experiment, culture medium and *in vitro* sowing system were the same as described above, but using a factorial design of two variables: PPM® in the medium at 3 different concentrations (2, 4 and 6 ml/L) and four disinfection times (5, 10, 20 and 30 minutes). About 0.025 g of the mass of seeds was utilized to the disinfection process. Each treatment was repeated 3 times. The number of germinated seeds per petri dish and the proportion of contaminated petri dishes were used as parameters. A factorial analysis of variance was performed and averages were compared by Fisher LSD test of significance for α=0.05.

Sowing systems using seeds and fruits from stocks cultivated in two environments

Seeds were sown in Pescia, using material from two environments (Pescia and Sanremo), following three different systems. From 130 different crosses made in Pescia, seeds from 33 crosses were sown *in vitro,* well developed but still immature fruits, from 22 crosses were cultivated *in vitro* and seeds from 130 crosses were sown in organic compost (peat and perlite, 1:2, v/v) on artificially climatized beds, inside a greenhouse. From 34 different crosses made in Sanremo, seeds from 19 crosses were sown *in vitro*, well developed but still immature fruits from 24 crosses were cultivated *in vitro* and seeds from 34 crosses were sown in the same compost and conditions as described above. About 0.025 g of the mass of seeds was utilized to *in vitro* sowing for each combination of cross. Three ovaries of each cross were *in vitro* cultivated, and of the total mass of seeds were sown in organic compost. Each sowing system was repeated 3 times.

All *in vitro* germinations were carried out in fall-winter of 2013 and compost germination in summer of 2014.

Due to the difficulty in counting the number of seeds sown inside a fruit or inside a mass of sieved seeds and debris, the comparison was based in number of germination events that happened per treatment. A nonparametric χ^2 statistical analysis was applied, and where significant intergroup differences were found, multiple comparisons were conducted using the partitioning χ^2 test to differentiate between treatments, both for α=0.05 (Ayres *et al.*, 2007).

3. Results

Pollination systems

According to the *t*-test, pollination using a brush with pollen collected from the corymb (average of 7.6 fruits per inflorescence cross), showed a mean significantly higher than using a corymb as a brush (average of 4.8 fruits per inflorescence cross) (p= 0.001). Although, the time needed for pollination using a brush is considerably greater than that using only a corymb.

Embryo and seed rescue from immature fruits

No statistical significance (p=0.07) was observed in the rate of contamination between different cut treatments. A lower percentage of contamination was observed in the treatment "stigmas off" (Fig. 1 a), probably due to less damage in the fruit tissues compared to the other cutting systems (Table 2). The number of dead fruits was not affected by the different fruit cut systems applied (p=0.50). Cut system were further differentiated based on variation in germination behavior. In the treatments "stigmas off" and "top cut", fruits swelled but seedlings did not emerge from fruit, suggesting poor germination. Furthermore, some seedlings were confined inside the fruit and did not develop and grow outside of the fruit. In the "equatorial cut" treatment, two portions of the fruit were grown: seeds were able to germi-

nate and develop new seedlings from the lower portion, but not from the upper portion. This suggests a potential loss of seedlings. Conversely, the treatment "longitudinal cut" (Fig. 1 b) allowed the seeds to germinate and seedlings promptly grew and developed upright (Fig. 1 e).

Table 2 - Proportions of contaminated and dead fruits, by ovary cut system, and behaviour of eventual germinated seeds from survived fruits

Cutting systems	Sample size (N)	Contamination (%)	Death (%)	Behaviors of germinated seedlings
Stigma off	150	31 a [z]	31.3 a	Fruits swelled but seedlings could not emerge from fruit and develop
Longitudinal cut	150	34 a	38.0 a	Seeds germinated promptly, grew up and develop upright
Equatorial cut	72	38 a	29.2 a	Germinated seed developed but none from the top slices, suggesting potential seedlings loses
Top cut	72	49 a	31.9 a	Some seedlings were confined inside the fruit and did not emerged

(z) Values followed by the same letter in each column do not differ statistically for α=0.05.

Disinfection of seeds for in vitro *germination*

Sterilization as a function of time immersion in bleach solution. No statistical differences were observed in the number of contaminated petri dishes between genotypes (p=0.37) or duration of disinfection period (p=0.22). Seed germination was lower in contaminated petri dishes only, where sterilization time was positively correlated with the number of germinating seeds (r_s of Spearman=0.51; p=0.01) (Table 3).

Sterilization as a function of duration in bleach solution and PPM® concentration in the media. Seed immersion duration in bleach solution did not significantly affect the rate of contamination (p=1.00); however, a significant difference was observed for PPM® concentration (p≈0.00). No interaction between these two variables was observed (p=1.00). Therefore, seeds surface sterilization with bleach solution was not enough to prevent contamination, but sterilization was achieved only with the addition of PPM®. Contamination occurred only when 2 ml/L of PPM® were added to the culture medium; when a higher concentration of PPM® was used, no contamination was observed at any time of immersion in the solution of bleach (Table 4).

So, to sterilize seeds for *in vitro* germination, the use of a culture media with 4 ml/L PPM® and a sterilization of 5 minutes is recommended.

Table 3 - Effect of application of bleach solution in function of duration of disinfection by different *Hydrangea* genotypes

Genotype	Duration of disinfection (minutes)				Contaminated Petri dishes/ genotype
	5	10	20	30	
H. macrophylla spp. *macrophylla* Proc. Izu Ohoshima	○[z]	○	○	○	0
H. macrophylla spp. *macrophylla* Proc.Takeoka Chiba	○	○	○	○	0
H. involucrata Proc. Yamamae Yoko Tama	○	○	○	○	0
H. macrophylla 'Libelle' x *H. paniculata* 'Limelight'	●	●	●(♣)	●(♣♣♣♣♣)	4
H. involucrata 'Myharayama kokonoe Tama' x *H. macrophyll*a 'Alberta'	●	●	●	●(♣♣♣)	4
H. macrophylla 'Libelle' x *H. macrophylla* 'Europa'	●(♣)	●(♣)	●	●	4
Total of contaminated Petri dishes/duration of disinfection	3	3	3	3	

(z) Symbols: ○ uncontaminated petri dishes; ● contaminated petri dishes; ♣ number of germinated seed.

Table 4 - Evaluation of PPM® concentration on the number of *Hydrangea* spp. germinated seeds/per petri dishes, and proportion of contaminated petri dishes

PPM® concentration (ml/L)	Germinated seeds/petri dishes (N)	Contaminated petri dishes (%)
2	5.5 a [z]	66.7 a
4	3.8 a	0 b
6	4.0 a	0 b

(z) Averages and percentage (by column) followed by the same letter do not differ at α>0.001.

Sowing systems using seeds and fruits from stocks cultivated in two environments

The average percentage of germination in Pescia was 30.5%; and in Sanremo, 55.8%. Inside the environment "Pescia", no statistical differences were observed between sowing systems (p=0.8, d.f.=2), but in "Sanremo" there were differences (p≈0.00 d.f.=2). Partitioning χ^2 tests revealed that treatments in the Sanremo environment were all statistically different from each other (p<0.007, d.f.= 1), with immature fruits cultivated *in vitro* demonstrating the highest percentage of germination among treatments (Fig. 3).

4. Discussion and Conclusion

In the present paper, we use several experiments to define work strategies and priorities to hybridize

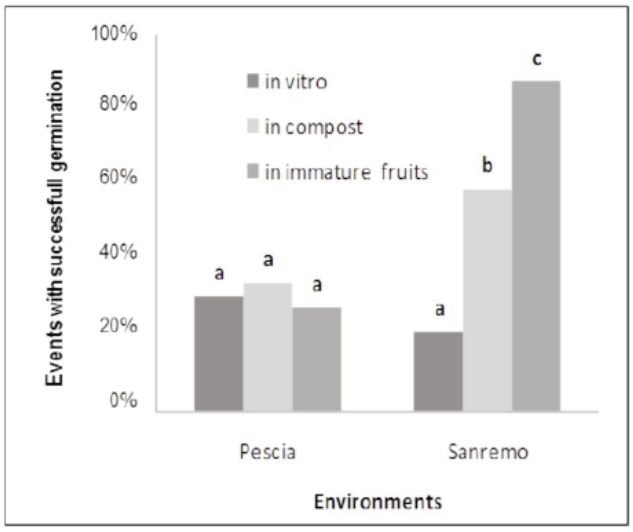

Fig. 3 - Events with successful germination of potential *Hydrangea* spp. hybrids by different sowing systems, in two environments. Columns, by environment, followed by the same letter do not differ statistically for α=0.05.

Hydrangea species using sexual propagation. The aim of the project was to hybridize all species involved in a breeding program, therefore pollination and *in vitro* germination techniques had to be developed to maximize efficiency.

To efficiently produce fruits of inter- and intraspecific hybrids, pollination by the dispersion of previously collected pollen on the top of a corymb aided by a brush resulted in better pollination than using the corymb itself as a brush. Nevertheless, pollination using only the corymb was used for the purpose of the present project because it was faster and more convenient. However, the hybrid origin should be certified using molecular markers when seedlings are established or at flowering, based on morphological characters.

Based on the germination behaviour, the "longitudinal cut" system applied to fruits prior to their transfer to the culture medium is recommended to promote vigorous growth of seedlings. It is considered as the best system because seeds readily germinated and developing seedlings promptly grew upright. Similar responses were also observed in the production of interspecific hybrids of *Lilium longiflorum* Thunb. and *L.* × *elegans* (Roh *et al*., 1996).

Seed surface sterilization for *in vitro* germination with bleach solution was not enough to prevent contamination, but sterilization was achieved with the addition of PPM®. In some plant species, germination is favoured by the presence of microorganisms, usually attributed to *Rhizobacteria* (Saharan and Nehra, 2011), but seldom adapted to *in vitro* germination of ornamental plants except in orchids (Tsavkelova *et al*., 2007); this probably could be an explanation of what happened in the case of *Hydrangea* seeds. For the objectives of the present study, however, it would not be an advantage because almost all culture media were completely covered by fungal mycelia causing seedlings to collapse within the first week following germination.

Germination in fruits showed a higher number of successful germinations than the other two systems. The highest proportion of germination success, observed for "*in vitro* inside fruit with a longitudinal cut for embryo and seed rescue" system, could be due to supplementary nutrients, given by the medium, that ensured development of hybrid seeds without endosperm (Eeckhaut *et al*., 2006 a and b). Nevertheless, specific studies of endosperm lacking in seeds from *Hydrangea* hybrid crosses are unknown.

The number of germinated seeds in Pescia was lower than in Sanremo: in Pescia the stock plants were cultivated under shading net while in Sanremo in greenhouse conditions. In fact, the contamination of explants cultivated *in vitro* can be associated to management of stock plants.

Pollination system and *in vitro* germination techniques have allowed us to obtain a number of new individuals with potential ornamental traits. Currently this material is under selection, providing the basis for the development of a *Hydrangea* breeding program in course at CREA-VIV at Pescia - Italy.

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Physiological responses of olive cultivars to salinity stress

M. Rahemi [1 (*)], S. Karimi [2], S. Sedaghat [1], A. Ali Rostami [1]

[1] *Department of Horticultural Science, College of Agriculture, Shiraz University, Shiraz, Iran.*

[2] *Department of Horticultural Science, College of Abouraihan, University of Tehran, Tehran, Iran.*

Key words: electrolyte conductivity, osmoregulation, proline, soluble carbohydrates.

Abstract: The aim of this study was to evaluate the tolerance of seven promising olive cultivars for southern parts of Iran ('Amigdalilolia', 'Dakal', 'Zard', 'Dezful', 'Tokhm-e-Kabki', 'Shiraz', and 'Conservalia') against salinity stress. Biochemical and physiological responses of the cultivars irrigated with saline water application (control, 4, 8, and 12 dS m^{-1}) were evaluated and the tolerant cultivars were identified. In contrast to the tolerant cultivars, the sensitive ones continue to grow with lower rate and died under salinity stress. In general, growth indices of olive cultivars were reduced with increasing salinity stress and the lowest growth indices were obtained under 12 dS m^{-1} treatment. Results indicated that the accumulation of higher levels of soluble carbohydrates and proline in the leaves of the tolerant cultivars helps them to deal with salinity stress. The results showed that saline waters up to 4 dS m^{-1} for irrigation can be used for olive cultivars, however, based on the result of this study, it is not recommended to use water sources with higher electric conductivities to irrigate sensitive olive cultivars. We concluded that the tolerant cultivars stopped growth and used their energy to defend against the salinity stress.

1. Introduction

Salinity stress is dependent on environmental condition (Kozlowski and Pallardy, 1997), farming, water management and genotype (Kozlowski and Pallardy, 1997). Olive (*Olea europea* L.) is one of the most valuable and widespread fruit trees in the Mediterranean area. Its cultivation is continuously being extended to irrigated land. Furthermore, in Mediterranean area salinity is becoming a major problem due to high rates of evaporation (Kozlowski and Pallardy, 1997). Olive is considered a moderately salt tolerant plant (Ayers and Westcot, 1976; Aragues *et al*., 2005; Weissbein *et al*., 2008). In comparison with other Mediterranean-grown tree crops, olive is more tolerant than citrus but less tolerant than date palm (Ayers and Westcot, 1976). The tolerance of olive cultivars are different to salinity stress (Therios and Misopolinos, 1988; Perica *et al*., 2004; Chartzoulakis, 2005).

The relationship between saline water and olive cultivation has been intensively studied for many years and significant progress has been made in the understanding of this topic (Ayers and Westcot, 1976; Wiesman *et al*., 2004). It is generally well established that saline conditions limit the vegetative and reproductive development of olives mainly as a result of interference with the osmotic balance in the root system zone and detrimental effects caused by specific toxic accumulation of chloride and sodium ions in the leaves (Weissbein *et al*., 2008). Salt stress reduces water availability in soil solution as a result of an increased osmotic potential, inducing the generation of reactive oxygen species (ROS) (Zhu, 2001; Melloni *et al*., 2003), the reduction of hormonal signals generated by the roots (Munns, 2002), altered carbohydrate metabolism (Gao *et al*., 1998), reduced the activity of certain enzymes (Munns, 1993; Chartzoulakis, 2005) and ultimately impaired photosynthesis (Chartzoulakis, 2005). Therefore, these physiological changes result reduced growth in either reduced cell division, expansion or promoting cell death (Hasegawa *et al*., 2000). Furthermore these criteria make plant reduce growth rate and yield, chlorophyll destruction which lead to leaf senescence. The plant response to salinity stress is depen-

(*) Corresponding author: rahemi@shirazu.ac.ir
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dent on environmental condition, farming, water management and plant genotype.

The aim of this study was to screen for the tolerance of seven olive cultivars from the Southern parts of Iran, against salinity stress. Tolerance was evaluated over several biochemical (proline content, stored carbohydrate, total chlorophyll and starch concentration), and physiological (Cell Membrane Injury) responses of these cultivars under salinity stress.

2. Materials and Methods

Experiment was carried out in the Department of Horticultural Sciences, Shiraz University during the growing season in 2012, using one year old cuttings of seven olive cultivars: 'Dakal', 'Zard', 'Shiraz', 'Tokhm-e-Kabki', 'Dezful', 'Amigdalilolia', and 'Konservalia' with four replicates for each cultivar. The cuttings were transplanted into 15 kg pots containing soil mixture (1:1:1) of soil (pure soil), sand and leaf mould. The physicochemical characteristics of the soil are shown in Table 1.

Table 1 - The physiochemical characteristics of the soil used

Characteristics	
Zn (ppm)	1.5
Fe (ppm)	7.6
Mn (ppm)	21.14
Cu (ppm)	1.76
K (ppm)	400
P (ppm)	23.8
Total Nitrogen (%)	0.094
OC (%)	1.54
Ph (%)	7.9
EC (%)	1.93
Clay (%)	34.4
Silt (%)	44.2
Sand (%)	21.4

During the establishment phase in greenhouse, olive cultivars were pruned uniformly in order to produce a single stem. The salinity stress treatments were applied by sub irrigation with different salinity levels [control (1.1), 4,8,12 ds/m]. In order to prevent salinity shock, the concentration of salts was gradually increased to reach a given level. The day and night temperature of the greenhouse was 35°C and 25°C, respectively. The saline water was prepared by dissolving sodium chloride (control, 4, 8, 12 ds/m) in the water. The pots were irrigated with saline water for 90 days. They were irrigated with saline water to the Field Capacity (FC) level, which was equivalent 20% of the dry weight of the soil of pot.

The total shoot length of olive cultivars was measured at the beginning and at the end of the experiment. Additionally, the number of fully expanded leaves and branches of each cultivar were recorded. At the end of the experiment, the average length of new shoot was measured. Using the data collected at the start and the end of salinity stress treatments, the rate of these changes was calculated.

Total chlorophyll measurement

Total chlorophyll content was determined by spectrophotometer (Saini *et al.*, 2001). Briefly, chlorophyll a and b contents were obtained by extraction in 85% acetone solution and measuring their absorbances using Camp spec M501 Single Beam UV/vis Spectrophotometer at λ= 663 nm and λ = 645 nm. The concentration of chlorophylls and carotenoids were calculated according to the following formula:

$$\text{Total chlorophylls (mg /g fw)} = [(20.2\times OD_{645\ nm} + 8.02\times OD_{663\ nm}) \times V] /(fw \times 1000)$$

where OD is optical density, V is the final solution volume in mL and fw is tissue fresh weight in mg. V is the final solution volume in mL and fw is tissue fresh weight in mg.

Proline measurement

Free proline was extracted from 0.5 g samples of fully expanded and young leaves with 3%, sulfuric acid and estimated by using ninhydrin reagent, according to the protocol described by Bates *et al*. (1973). The absorbance of the fraction with toluene was determined at 520 nm, using a spectrophotometer (Model UV-120-20, Japan).

Cell membrane injury (CMI)

Cell membrane injury was calculated according to the method of Blum and Ebercon (1981). For the CMI, 20 samples of stressed and unstressed young leaves were washed with distilled water to remove the dust and injured cells from samples. The samples were then immersed in 20 ml distilled water at room temperature. After 24 h the conductivity of the solutions was read. The samples were autoclaved for 15 min, cooled to room temperature and the conductivity of the solutions was read again. The electrolyte leakage was measured with a conduct meter (644 Conduct meter, Metrohm, Herisau, Switzerland). CMI was estimated from the formula:

$$\text{Id (Drought injury index)} = 1 - (1 - T1/T2)/(1 - C1/C2) \times 100$$

where T1 and T2 are the first and second measurement of the conductivity of the solutions in which the

treated samples were immersed and C1 and C2 are the respective values for the conductivity of the solutions.

Soluble carbohydrate extraction

To determine soluble carbohydrate concentration, 150 mg of dried leaf samples was extracted twice with 80% ethanol. The sample was centrifuged at 3500 rpm for 10 min and the volume of the supernatant was adjusted to 25 ml Soluble carbohydrate concentration was measured according to the method of Buysee and Merckx (1993). In summary, 1 ml of supernatant was transferred to a test tube and 1 ml phenol 18% and 5 ml sulfuric acid were added. The mixture was shaken immediately and its absorption was recorded at 490 nm using a spectrophotometer (Model UV-120-20, Japan).

Starch concentration

Starch concentration in the leaf samples was measured using anthron reagent (McCready, 1950). In this method, 5 ml of water (0°C) and 6.5 ml perchloric acid (52%) were added to the pellet used for sugar analysis and mixed for 15 min. About 20 ml water was then added and the sample was centrifuged. The supernatant was separated and the same procedure was repeated with the pellet for each leaf samples. The supernatants were combined and left for 30 min at 0°C. After filtration, the supernatant volume was adjusted to 100 ml. About 2.5 ml of cold 2% anthron solution was added, and the sample was heated at 100°C for 7.5 min. It was then transferred immediately to an ice bath and cooled to room temperature. Absorption at 630 nm was recorded using a spectrophotometer (Model UV-120-20, Japan).

Statistical analysis

The experiment was conducted as a complete randomized design with factorial arrangements. Analysis of variance was performed using the SPSS software package and significant differences among mean values were compared by Duncan Multiple Range Test (DMRT) ($P<0.05$).

3. Results

Effect of salinity on plant growth

After 90 days of salinity treatment plant growth (total shoot length, number of branches and leaf number) significantly reduced in all cultivars (Table 2, 3 and 4). The effect of salinity on these parameters showed a significant genotypic variation. As expected, the highest reduction of shoot length was found in 12 dSm^{-1} treatment; considering the mean over all treatments, Conservalia and Shiraz cultivars had the highest shoot length while 'Dakal' showed the lowest one under each salinity stress conditions (Table 2). The highest reduction in number of branches was found in 8 and 12 dSm^{-1} treatments on average, the lowest branch number was found in 'Conservalia' whereas the 'Amigdalilolia', 'Dakal' and 'Tokhm-e-

Table 2 - Effect of interaction between salinity treatment and cultivar on total shoot length (in comparison to the beginning of the experiment

Cultivar	Total shoot lenght (cm)				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Conservalia'	158.4 ab*	158.7 a	131.2 a-e	106.3 def	138.6 A
'Shiraz'	149.1 ab	137.7 a-d	137.7 a-d	124.1 a-f	138.8 A
'Tokhm-e-Kabki'	149.1 a-d	119.6 b-f	114.7 def	112.7 def	121.0 BC
'Dezful'	159.9 abc	136.0 a-d	119.2 b-f	121.4 b-f	134.1 AB
'Zard'	156.3 ab	124.7 a-f	127.3 a-f	118.0 c-f	131.6 AB
'Dakal'	154.4 ab	102.7 F	104.8 ef	103.9 ef	116.4 C
'Amigdalilolia'	142.4 a-d	121.8 b-f	106.4 ef	115.7 c-f	120.2 BC
Mean	151.4 A	128.5 B	120.2 BC	141.6 C	

* Mean separation within columns and rows by DMRT, at 5% level.

Table 3 - Effect of interaction between salinity treatment and cultivar on branch number of olive cultivars

Cultivar	Branch number				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Conservalia'	21.0 h-k *	24.3 f-j	8.3 l	8.0 l	15.4 D
'Shiraz'	28.3 c-h	18.6 Ijk	18.4 ijk	17.6 ijk	20.7 C
'Tokhm-e-Kabki'	40.0 a	35.6 a-d	25.2 e-i	30.2 b-g	32.7 A
'Dezful'	29.8 b-h	28.5 c-h	22.9 g-k	20.9 h-k	25.3 B
'Zard'	27.6 d-h	17.6 Ijk	16.0 k	17.0 h-k	19.3 C
'Dakal'	29.3 c-g	31.0 b-f	32.0 b-f	37.6 ab	32.7 A
'Amigdalilolia'	36.0 abc	34.6 a-d	33.0 a-e	22.6 g-k	31.4 A
Mean	30.3 A	28.0 B	21.8 C	22 C	

* Mean separation within columns and rows by DMRT, at 5% level.

Table 4 - Effect of interaction between salinity treatment and cultivar on leaf number of olive cultivar (Calculated as: leaf number at the beginning of the stress-the leaf number at ending the stress)

Cultivar	Leaf number				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Conservalia'	151.0 a-f *	138.6 c-g	21.0 Jk	-56.0 l	66.3 D
'Shiraz'	114.6 b-f	113.6 e-i	74.3 g-j	-11.3 kl	80.3 C
'Tokhm-e-Kabki'	190.6 a-d	207.1 ab	100.0 f-i	73.6 g-j	142.8 B
'Dezful'	224.6 a	217.6 a	135.0 c-g	113.3 e-i	172.6 AB
'Zard'	160.0 a-f	96.0 f-i	65.6 g-j	62.6 hij	96.8 C
'Dakal'	218.3 a	218.6 a	200.6 abc	130 d-h	191.9 A
'Amigdalilolia'	185.3 a-d	177.3 a-e	174.0 a-e	98 f-i	158.6 B
Mean	177.8 A	167.0 A	110.1 B	60.0 C	

* Mean separation within columns and rows by DMRT, at 5% level.

Kabki' obtained the most one (Table 3).

Leaf number was also significantly reduced by salinity treatments. At 12 dSm^{-1}, the reduction in leaf number ranged from -56.0 for 'Conservalia' to 130 for 'Dakal' with respect to control plants. The greatest number of leaves was found in controls and 4 dSm^{-1} treatments (Table 4).

Effect of salinity on cell membrane injury (CMI)

Cell membrane injury (CMI) was significantly increased in all studied cultivars from the treatment of 12 dSm^{-1} except in 'Amigdalilolia', 'Zard' and 'Dakal'. The highest percentage of CMI was obtained in 'Conservalia' with respect to the control treatment (Table 5). Furthermore, the maximum and minimum CMI belonged to 4dSm^{-1} and control, respectively. Therefore, CMI was influenced by both salinity treatment and type of cultivar (Table 5).

Table 5 - Effect of interaction between salinity treatment and cultivar on CMI of olive leaf (%)

Cultivar	Cell membrane injury (%)				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Corservalia'	74.7 abc*	72.8 abc	67.9 b-f	49.1 g	66.1 C
'Shiraz'	66.6 c-f	60.4 f	52.7 g	52.9 g	58.2 D
'Tokhm-e-Kabki'	72.4 abc	71.6 a-d	63.6 def	63.1 ef	67.7 BC
'Dezful'	69.7 a-e	66.2 c-f	68.7 a-e	53.3 g	64.5 C
'Zard'	69.6 a-e	73.0 abc	68.3 b-f	69.4 a-e	70.1 B
'Dakal'	70.3 a-e	77.1 a	68.9 a-e	68.6 a-e	71.2 B
'Amigdalilolia'	76.7 ab	75.2 ab	76.3 ab	76.0 ab	75.6 A
Mean	71.4 A	70.9 A	66.6 B	61.6 C	

* Mean separation within columns and rows by DMRT, at 5% level.

Proline content

Leaf proline content of olive cultivars was significantly influenced by salinity treatment and type of cultivar (Table 6). Maximum proline content was obtained in the leaf tissue of 'Dezful' while the minimum proline content was determined in the leaves of 'Conservalia'. In general, the highest proline content was observed in 8 dSm^{-1} treatments and the lowest one was observed in 4 dSm^{-1} treatments (Table 6).

Soluble carbohydrate content

The concentration of soluble carbohydrates of leaves under salinity stress was significantly changed in all studied cultivars and the highest content of soluble carbohydrates was found in Shiraz and Zard cultivars and the lowest was observed in the leaves of 'Amigdalilolia' (Table 7). The maximum soluble carbohydrate content was found in 4 dSm^{-1} treatment and the minimum content was observed in control and 12 dSm^{-1} treatments, respectively.

Starch concentration content

The stored carbohydrate content of leaves was significantly affected by salinity treatments and type of cultivar. 'Conservalia' had the highest stored carbohydrate content whereas 'Shiraz' showed the lowest (Table 8). Plants grown at 12 dSm^{-1} had the highest stored leaf carbohydrate content and the lowest one was observed in 8 dSm^{-1} treatments (Table 8).

Table 6 - Effect of interaction between salinity treatment and cultivar on proline content of olive cultivar leaf (µM g DW^{-1})

Cultivar	Proline content (µm G DW^{-1})				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Conservalia'	29.9 b-e*	21.1 g	24.0 efg	22.1 fg	24.3 C
'Shiraz'	36.7 ab	22.7 fg	26.8 d-g	23.0 efg	27.3 BC
'Tokhm-e-Kabki'	28.9 b-f	28.9 b-f	33.2 a-d	26.6 d-g	29.4 B
'Dezful'	35.4 abc	32.7 a-d	40.7 a	37.8 ab	36.7 A
'Zard'	26.6 d-g	26.5 d-g	35.7 abc	28.6 b-f	29.3 B
'Dakal'	24.1 e-g	29.9 b-e	33.8 a-d	29.9 c-g	28.7 BC
'Amigdalilolia'	22.4 fg	25.9 d-g	26.5 d-g	34.0 a-d	27.2 BC
Mean	29.1 B	26.8 C	31.5 A	29.5 AB	

* Mean separation within columns and rows by DMRT, at 5% level.

Table 7 - Effect of interaction between salinity treatment and cultivar on soluble carbohydrate content of olive leaf (%)

Cultivar	Soluble carbohydrate content (%)				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Conservalia'	30.6 abc*	28.3 c-g	26.9 d-h	27.2 d-h	28.3 AB
'Shiraz'	31.5 a	28.7 a-f	29.9 a-e	28.7 b-f	29.7 A
'Tokhm-e-Kabki'	28.0 c-h	31.3 ab	29.0 a-f	26.8 e-h	29.0 AB
'Dezful'	26.7 d-h	29.1 a-f	26.8 e-h	28.3 c-g	27.6 BC
'Zard'	30.1 a-d	30.0 a-e	28.9 a-f	29.3 a-f	29.6 A
'Dakal'	23.5 h	31.2 ab	30.6 abc	28.6 b-f	28.7 AB
'Amigdalilolia'	24.5 gh	25.7 fgh	28.4 c-g	27.3 d-h	26.5 C
Mean	27.9 B	29.4 A	28.6 AB	28.1 B	

* Mean separation within columns and rows by DMRT, at 5% level.

Table 8 - Effect of interaction between salinity treatment and cultivar on stored carbohydrate content of olive cultivar leaf (mg g DW^{-1})

Cultivar	Stored carbohydrate content (mg g DW^{-1})				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Corservalia'	279.6 a-e*	280.6 a-d	251.3 b-g	339.1 a	290.7 A
'Shiraz'	221.5 d-g	164.0 h	191.0 gh	296.9 abc	220.2 D
'Tokhm-e-Kabki'	238.7 c-g	242.8 c-g	239.0 c-g	284.2 a-d	249.7 BC
'Dezful'	221.6 d-g	277.0 a-d	244.9 c-g	261.8 b-e	251.3 B
'Zard'	251.9 b-f	233.4 c-g	244.2 c-g	237.3 c-g	241.7 BCD
'Dakal'	254.6 b-f	229.4 c-g	211.5 e-h	198.6 fgh	221.6 CD
'Amigdalilolia'	288.9 a-d	319.7 ab	233.4 c-g	229.6 c-g	269.1 AB
Mean	249.4 AB	249.1 AB	230.0 B	264.5 A	

* Mean separation within columns and rows by DMRT, at 5% level.

Chlorophyll content

The highest chlorophyll content was observed in the 'Dakal', 'Dezful' and 'Amygdalolelia' while the lowest chlorophyll content was determined in the leaves of 'Shiraz'. In general, the highest chlorophyll content was measured in plants grown with the 4dSm^{-1} treatment and the lowest was observed for 12dSm^{-1} treatment (Table 9).

Table 9 - Effect of interaction between salinity treatment and cultivar on chlorophyll of olive cultivar

Cultivar	Chlorophyll				
	EC (dSm^{-1}) of irrigating water				
	0	4	8	12	Mean
'Corservalia'	1.84 efg*	2.56 a	2.31 a-e	0.74 i	1.86 B
'Shiraz'	2.10 a-e	1.61 fg	1.55 g	0.20 I	1.36 C
'Tokhm-e-Kabki'	2.23 a-e	2.30 a-e	2.12 a-e	1.02 h	1.97 B
'Dezful'	2.52 a	2.47 abc	1.95 d-g	2.05 a-f	2.25 A
'Zard'	1.92 d-g	2.24 a-e	1.98 c-g	1.59 fg	1.93 B
'Dakal'	2.25 a-e	2.48 abc	2.42 a-d	2.01 b-g	2.29 A
'Amigdalilolia'	2.51 ab	2.25 a-e	2.27 a-e	1.80 efg	2.21 A
Mean	2.19 AB	2.27 A	2.09 B	1.36 C	

* Mean separation within columns and rows by DMRT, at 5% level.

4. Discussion and Conclusions

The purpose of this study was to evaluate the salinity tolerance of seven olive cultivars ('Conservalia', 'Amigdalilolia', 'Dakal', 'Tokhm-e-Kabki', 'Shiraz', 'Dezful' and 'Zard) based on the effect of salinity on growth characteristics, and on physiological and biochemical response of different cultivars, grown in south of Iran. Salt stress has been reported to have genotypic-linked response (Chartozoulakis, 2005). Different parameters were used to indicate the response of seven cultivars to NaCl stress.

The main result concerns the reduced number of leaves in salt treated olive plant respect to control ones. Such decrease was not only connected with the growth inhibition effects of salt but also to plants defoliation (Karimi *et al.,* 2009).

The impact of salinity stress on reducing growth indices was clear and confirmed the response shown in previous research on different species and cultivars of olives (Perica *et al*., 2004). It was clear that the decline in growth rate of olive trees under salinity stress was dependent on the duration of salt exposure, the concentration of salt and the potential of tolerance of cultivars. In the current study, olive cultivars showed different reactions to salinity stress. In general, vigorous cultivars were more susceptible to salinity stress than low growth cultivars. Partial growth reduction of olive tree growth can be related to the osmotic stress resulting of elevated level of ions in soil solution and irrigation water (Weissbein *et al*., 2008). Because no visual signs of salt toxicity in the plants e.g. tip burn, necrosis and/or shoot die back (Chartozoulakis, 2005) under low salinity level; In addition, monitoring leaf number changes indicated no sign leaf abscission due to salt toxicity or oxidative damages under low salt stress level. Low osmotic potential of soil solution under salinity stress limits growth of plant by reducing water uptake, transpiration and stomatal conductance, which is associated with the reduced photosynthesis (Ben-Asher *et al*., 2006). Hence it can be concluded that, osmotic stress is the primary reason of olive growth limitation under salinity stress.

The CMI of leaf is the quantitative index which shows either health of plasma membrane or the rate of membrane disruption in plant leaf tissue under salinity stress. The reduction in CMI of olive cultivar was parallel to intensity of sodium chloride concentration in the irrigation water. Under the severe salinity stress, the highest CMI rate was observed in the 'Amigdalilolia', 'Dakal' and 'Zard' (101.8%, 99.4% and 97.6%), respectively and the lowest one belonged to 'Conservalia' (65.6%). Furthermore, leaf tip burning and leaf abscission were observed that might be due to accumulation of specific ions in olive leaf under salinity stress.

Though the leaf proline content in 'Shiraz' and 'Conservalia' declined with the increase of salinity stress, it increased in the other cultivars. During the salinity treatment, proline content was induced to accumulate in plant leaf tissue by the accumulation of the sodium and chloride ions and water stress as a result of enhancing of salt in soil solution (Delauney and Verma, 1993). In this study, despite salinity stress injuries, the increase in proline content may have played an important role in protection of olive cultivars in stress situations. The data presented here agreed with previous studies which show the direct relationship between tolerance of salinity stress and proline accumulation. It has been demonstrated that proline can protect protein from denaturation and maintain cytoplasmic membrane in salinity stress (Khedr *et al*., 2003; Karimi *et al*., 2009).

Data were shown that soluble sugar content of leaves of tolerant olive cultivars ('Amigdalilolia', 'Dakal') was enhanced during salinity period.

However in semi-tolerant cultivars ('Dezful', 'Zard' and 'Tokhm-e-Kabki') the sugar content was not significantly changed and in sensitive cultivars ('Shiraz' and 'Conservalia'), this factor in comparison to control treatment was reduced. Soluble sugars play an important role in maintaining turgor pressure in osmotic stress. Furthermore, they can protect the plasma membrane in the stress situation (Sanchez *et al*., 1998). This study showed that among tolerant olive cultivars, the accumulation of sugars could be due to the reduced consumption of stored carbohydrate contents (Chartzoulakis, 2002). Among the sensitive olive cultivars in which the starch and soluble sugar content were limited, it can be concluded that this phenomenon was related to photosynthesis disruption and the subsequent consumption of stored carbohydrate. Under severe salinity stress condition, starch accumulation in the tissue of sensitive cultivars, may be due to disruption of enzyme activity and plant metabolism which led to stored carbohydrate in the tissue.

Salinity stress decreased chlorophyll concentration in the olive cultivar leaf. Tolerant cultivars were shown more chlorophyll concentration than susceptible ones. Therefore, it can be concluded that leaf chlorophyll concentration might be the best index for evaluating of olive cultivars to tolerate salinity stress as reported by Noble and Rogers (1994). The same authors suggested that chloroplast dysfunctions and decrease in the number and volume of chloroplast were influenced by salinity stress, and as a result, a reduction in chlorophyll content was a main reaction of olive trees to salinity stress. Winicov and Seemann (1991) observed that a salt - tolerant alfalfa cell line exhibited an 11-fold increase in chlorophyll content compared to the unadapted cell line. The increase in chlorophyll content in alfalfa was associated with large increase in the activity of ribulose-1, 5- bisphosphate carboxylase (Winicove and Seemnann, 1991).

In conclusion, the relative tolerance of olive cultivars to salinity stress was in the following order: tolerant cultivars were 'Amigdalilolia' and 'Dakal', semi-tolerant cultivars included 'Dezful', 'Zard' and 'Tokhm-e-Kabki' and finally sensitive cultivars were 'Shiraz' and 'Conservalia'.

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The impact of fruit temperature dynamics on heat stress tolerance of selected oil pumpkin genotypes

A. Urbanek Krajnc *, J. Rakun, P. Berk, A. Ivančič
Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 10, Hoče, Slovenia.

Key words: breeding, *Cucurbita argyrosperma* var. *argyrosperma*, *Cucurbita moschata*, *Cucurbita okeechobeensis*, *Cucurbita pepo*, fruit temperatures.

Abstract: Fruit temperature is a key parameter for fruit growth and quality which is affected by climate, plant vigorousity, solar exposure and fruit thermal properties. In the present study, the variability in temperature dynamics of Styrian oil pumpkin fruits and selected interspecific hybrids involving *Cucurbita argyrosperma, C. moschata, C. pepo* was analysed in two different periods of hot weather. The temperatures were measured with thermistors on (a) attached fruits, (b) detached fruits exposed to the sun and (c) artificially black coloured fruits. The highest average temperatures were determined in the Styrian oil pumpkin, whereas the lowest temperatures were determined in genotypes with lighter fruit exteriors suggesting that those are less sensitive to heat stress conditions and may represent a good option for the improvements of adaptability to climatic changes. In order to combine lighter and harder pericarp, the most promising genotypes were crossed with wild *Cucurbita okeechobeensis*. The histological analysis showed that *C. okeechobeensis* was a good source of genes for obtaining a thicker sclerenchymatic layer within pericarp.

1. Introduction

Oil pumpkins play a significant role in human nutrition and health. The nutritional value of oil pumpkin seeds is based on high protein and antioxidant content, and high energy potential due to the high percentage of oil (Fruhwirth and Hermetter, 2007; Sari *et al*., 2008; Fokou *et al.,* 2009; Lelley *et al.,* 2009; Urbanek Krajnc *et al.,* 2016). During the last decade, oil pumpkin cultivation declined regarding productivity and quality due to the outbreaks of the *Zucchini yellow mosaic virus* (ZYMV), extremely high temperatures, radiation stress and prolonged periods of drought (Lelley *et al.,* 2009; Seebold *et al.,* 2009; Gong *et al.,* 2013). Years 2013 and 2015 have been excessively hot and we have seen serious problems in fruiting pumpkins related to weather conditions especially high day/night temperatures and drought stress (Yavuz *et al.,* 2015).

Heat stress depends on intensity, duration, and rate of increase in temperature. The extent to which it occurs, in specific climatic zones, depends on duration and level of high temperatures occurring during the day and/or the night. In general, a transient elevation in temperature, 10-15°C above ambient, is considered as heat stress (Wahid *et al*., 2007). It causes an array of morpho-anatomical, physiological and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in economic yield. On the morphological level, high temperature can cause considerable pre- and post-harvest damages such as scorching of leaves and stems, sunburns on leaves, stems and fruits, leaf senescence and wilting, shoot and root growth inhibition, fruit discoloration and damage, and reduced yield (Wahid *et al*., 2007; Ara *et al*., 2015; Johnson, 2015). The physiological changes caused by high temperatures include the negative effect on photosynthesis, respiration, water relations, and modulated levels of hormones and primary and secondary metabolites. On the biochemical and subcellular level the direct injuries due to high temperatures include protein denaturation and aggregation, disorganization of cytoskeleton and increased fluidity of membrane lipids. Indirect or slower heat injuries include inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity. These injuries eventually lead to starvation, inhibition of growth, reduced ion flux, production of toxic com-

(*) Corresponding author: andreja.urbanek@um.si
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pounds and reactive oxygen species (ROS), which coincide with increased synthesis of antioxidants and activity of antioxidant enzymes. Furthermore, enhanced expression of a variety of heat shock proteins and other stress-related proteins constitute major plant responses to heat stress (Iba, 2002; Howarth, 2005; Wahid *et al.,* 2007; Ara *et al*., 2013 a, b, 2015).

During the ontogenetic development of plants, flowering and fruit set are the most sensitive stages; fruit set of pumpkins is affected at day/night temperatures above 26/20°C and is severeliy affected above 35/26°C (Schrader *et al*., 2004, 2011; Saudreau *et al.,* 2011; Lei *et al*., 2014; Johnson, 2015). The decrease in yield is due to the rapid decrease of photosynthesis, which reduces the amounts of sugars and other storage products that can go into fruits and developing seeds. On the other hand, hot night temperatures lead to greater cell respiration. High temperatures can also cause increased developmental disorders in fruiting vegetables due to the reduced pollen production leading to a reduction in fruit and seed sets, smaller pods, and split sets. Another effect of heat stress in many plant species is induced sterility when heat is imposed immediately before or during anthesis (Siddique *et al*., 1999; Wahid *et al*., 2007).

Theoretical and experimental evidence shows that fruit temperature can be 10°C higher than the air temperature under sunny conditions (Schrader *et al*., 2004; Racskó *et al.,* 2005; Schrader, 2011; Lei *et al.*, 2014). Sunburn of fruits is a surface injury caused by solar radiation which, during the initial phase, results in a light corky layer, golden or bronze discolouration. The damage occurs mainly in the surface and subsurface layers. There are two types of sunburn damage which may have effects on fruits and fruiting vegetables. The first, sunburn necrosis, appears due to the thermal death of cells on the sun exposed side of the fruit; cell membrane integrity is lost and cells start leaking their contents (Schrader, 2011; Johnson, 2015). The critical fruit tissues' temperatures for sunburn necrosis vary with the type of fruit. The fruit surface temperature (FST) threshold for sunburn necrosis for cucumbers and pumpkins is between 37 and 42°C (Rabinowitch *et al.,* 1983, 1986; Ara *et al.,* 2013 a, b; 2015). The second type of sunburn injury is sunburn browning, which is caused by the combination of high FST and high solar radiation. It causes degradation of photosynthetic pigments resulting in yellow spots on the sun-exposed side of the fruit and occurs at a temperature about 5°C lower than sunburn necrosis (Schrader, 2011; Johnson, 2015).

Plants have three major ways in which they dissipate excess heat: (1) long-wave radiation, (2) heat convection into the air and (3) transpiration. If transpiration is interrupted by stomatal closure due to water stress, inadequate water uptake or other factors, a major cooling mechanism is not functioning. This will cause internal leaf/fruit temperatures to rise. Without transpiration, the only way that plants can reduce heat is by heat radiation back into the air or wind cooling. Under high temperatures, radiated heat builds up in the atmosphere around plants, limiting further heat dissipation (Wahid *et al*., 2007; Schrader, 2011; Johnson, 2015).

The adverse effects of high air and soil temperatures, and the high levels of solar radiation can be mitigated by developing plant genotypes with improved thermotolerance. Some attempts to develop heat-tolerant genotypes via conventional breeding protocols have been successful (Ehlers and Hall, 1998; Camejo *et al*., 2005). Breeding of cultivated cucurbits was mainly focused in combinig good attributes of *C. moschata* and *C. maxima.* (Balkaya and Karaagac, 2005; Balkaya *et al.,* 2009, 2010 a, b, 2011; Balkaya and Kandemir, 2015). It is well known that *C. moschata* is best adapted to hot climate and is successfully cultivated in tropical and subtropical regions (Balkaya *et al.,* 2010 a, b; Balkaya and Kandemir, 2015). Recently, a study was conducted to determine the extent of heat tolerance of newly developed interspecific squash hybrid named as 'Maxchata' compared to its parents *C. maxima* and *C. moschata* (Ara *et al*., 2013 a, b, 2015) under three different temperature regimes. Results showed that various gas exchange and photosynthetic attributes dropped significantly with increasing temperature, while intercellular CO_2 concentration increased showing the nonstomatal limitations. These trends were more abrupt in *C. maxima*, reflecting that *C. maxima* was the most susceptible, while 'Maxchata' showed intermediate response. *C. moschata* had the best photosynthetic attributes to sustain the heat regimes (Ara *et al*., 2013 a, b). The ultramorphological, biochemical, and transcriptional analyses gave similar results. The electron microscopy highlighted the maximum degradation of the leaf ultrastructure of *C. maxima*. *C. moschata* and '*Maxchata*' exhibited lower degree of subcellular injury upon heat exposure. The antioxidant enzyme activities and their expression were found to be highest in *C. moschata*, moderate in '*Maxchata*', and lowest in *C. maxima* (Ara *et al*., 2013 a, b; 2015). The authors concluded that the interspecific hybridization with *C. moschata* might significant-

ly contribute to heat tolerance (Ara *et al*., 2013 a, b, 2015).

The presented study is associated with creating heat tolerant pumpkins characterised by lighter exocarp. The work began in 1996 and is based on a modified recurrent selection approach. Its aim is to create cultivars characterised by bushy growth, resistance to all major pests and diseases, tolerance to drought and high temperatures, and large and thick seeds having thin seed coats and high concentrations of high quality oil.

The basic population (i.e., population of the cycle-0) was established by inter-crossing all available sources of genes (i.e., numerous local and commercial cultivars, populations and hybrids of *C. pepo*, following the semi-diallel scheme). In 1997, the most valuable progenies were planted in New Caledonia, at the CIRAD centre near Pouembout (South Pacific). Due to the favourable semi-tropical climate, it was possible to execute three cycles per year. The problems, however, were seed germination within fruits and rotting fruits due to overheating. As the genetic resources within *C. pepo* were found to be insufficient for overcoming these problems, it was decided to incorporate interspecific hybridisation and change the exterior fruit colour. The main sources of genes for lighter fruit exterior were *C. argyrosperma* var. *argyrosperma* and *C. moschata. Cucurbita argyrosperma,* which was the main source of genes for whitish exocarp and was also used as a genetic bridge between *C. pepo* and *C. moschata.* Another interesting trait obtained by interspecific crosses was dark yellow fruit exterior which was associated with the same colour of mesocarp. Some years later, a wild species *C. okeechobeensis* (Small) L. H. Bailey was added to the hybridisation programme, in order to improve the resistance to viruses and harder pericarp. The interspecific hybrids included in this study were indirect progenies developed within the recurrent selection programme which involved intra- and inter-population crosses, self-pollinations, and backcrosses.

The progress in breeding for heat stress tolerance strongly depends upon understanding the genetic and physiological mechanisms associated with stress tolerance of the whole plants well as at the molecular and cellular levels. Our study involved thin-coated seed pumpkins with lighter exocarp because they were considered to have the highest level of tolerance to high air temperatures and high levels of solar radiation during summer. In order to investigate their tolerance to heat stress, pericarp tissue temperature profiles were monitored on the exocarp surface, as well as in the meso- and endocarp of fully developed attached and detached fruits during two different periods of hot weather.

2. Materials and Methods

Plant material

Four different plant materials with thin coated seed were used in the study: (1) Styrian oil pumpkin *Cucurbita pepo* subsp. *pepo* var. *styriaca* (O), (2) progeny with whitish fruit derived from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* var. *argyrosperma* (O/A), (3) a progeny derived from crosses involving *C. pepo* (non-lignified seed type, oil type), *C. argyrosperma* and tropical *C. moschata* characterised by yellow fruits (A/Mo x O/A) and (4) a three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* var. *argyrosperma* and *C. okeechobeensis* (Oke × O/A) (Table 1, Fig. 1).

Table 1 - List of key plant materials with short explanations of abbreviations

Plant material	Abbreviations
Species	
Cucurbita argyrosperma. var. *argyrosperma*	A
Cucurbita moschata	Mo
Cucuribita pepo subsp. *pepo* var. *styriaca*	O
Cucurbita okeechobeensis	Oke
Interspecific hybrids	
Material obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma*. var. *argyrosperma*, followed by several seasons of intrapopulation recombinations and selection	O/A
Three-species hybrid involving *C. argyrosperma* (used as a genetic bridge), *C. moschata* and *C. pepo* (non-lignified seed coat, oil type), after several seasons of intrapopulation crosses and selection	A/Mo x O/A
A three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* var. *argyrosperma* and *C. okeechobeensis*	Oke x O/A

The temperatures were measured on (a) attached fruits (1st period, 27th July to 5th August 2012), (b) detached fruits exposed to the sun (2nd period, 1st to 11th September 2012) and (c) artificially black coloured fruits (on both periods). The fruits were exposed to sunlight due to loss of foliage caused by drought stress and diseases. For each experiment, three fruits of each studied material were used.

Fruit temperature measurements

The temperatures were measured during two different periods of hot weather, between the 27th July

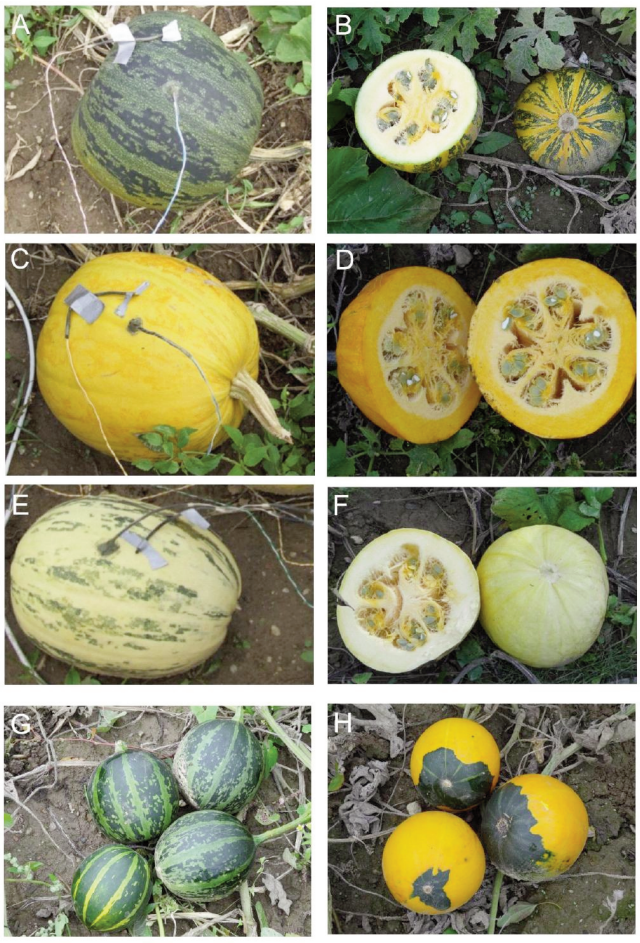

Fig. 1 - Fruits of selected genotypes with attached sensors, which were selected for lighter and harder fruit exterior and thin coated seeds: A, B) Styrian oil pumpkin (O). C, D) Three-species hybrid involving *C. argyrosperma* var. *argyrosperma* (used as a genetic bridge), *C. moschata* and *C. pepo* (non-lignified seed coat, oil type), after several seasons of intrapopulation crosses and selection (A/Mo × O/A). E, F) Genotype with lighter (i.e., whitish, white-green) fruits material obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma*, followed by several seasons of intrapopulation recombinations and selection (O/A). G) *C. okeechobeensis*. H) A three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* and *C. okeechobeensis* (Oke × O/A).

to 5th August 2012 and 1st to 11th September 2012. During both periods, three pumpkins of each progeny (O, O/A, A/Mo x O/A) were chosen and the thermistors were placed on exocarp the sun-exposed side, as well as inserted in meso- (2 cm deep) and endocarp (10 cm deep) (Fig. 1A, C, E). The temperatures were recorded every 15 seconds and stored as 10-min averages. The data represented in figures 3-6 show a diurnal and maximum day temperature as average of three fruits out of each progeny.

In order to measure temperatures, 32 BETATHERM 10K3A542I thermistors connected to a Campbell's CR1000 datalogger (Campbell Scientific Inc., Logan, UT, USA) were used. The data logger recorded the current times for each iteration supply voltage and analogue input voltage. The thermistors' readings were performed sequentially as they were all connected to the same ADC converter through a multiplexor; so only one of the thermistors was connected to the converter at a time.

The selected thermistors had accuracies of 0.2°C and were of negative-temperature-coefficient (NTK) type, which meant that their resistances decreased with the increases of temperature. As the data logger was unable to read the resistance of the thermistor directly, the thermistors were connected in a form of voltage divider, with an additional resistor with 1 KΩ±0.1% fixed resistance. As the resistive responses of the thermistors were nonlinear, the measured temperatures were calculated according to the temperature table from the datasheet (BETHATERM).

The results are represented by mean values (N=3), and were statistically evaluated by one-way analysis of variance (ANOVA), using the SPSS 21 software (SPSS 21 software, SPSS Inc., Chicago, IL, USA). Significant differences between mean values were determined using the post-hoc Duncan test. Significant differences (α<0.05) between means were indicated by different letters.

Air/soil measurements

Furthermore, air/soil temperatures and relative humidity were measured on the sun-exposed side and within the canopy (5 and 20 cm below soil surface, on soil surface as well as 10 and 20 cm above soil surface using 215 and 107 Temperature Probe sensors (Campbell Scientific Inc., Logan, UT, USA). The temperatures were recorded every 15 seconds and stored as 10-min averages.

Histochemical evaluation of pericarp

From each studied material, 3-6 fruits were taken for histological evaluation. Out of each fruit six pieces (diameter 8 mm) of pericarp were sampled on equator of the fruit positioned around the pumpkin in regular intervals. The pieces were cut with a cryotom in order to evaluate the size of the lignified cell layer. An Olympus microscope (Provis AX 70) with a 100 W mercury arc lamp was used to take analogue images with a 3-chip-colour video camera (Sony DXL 950 P, 3 CCD). Fluorescence images were obtained through an UplanFI 40x dry objective (n.a., 0.75), a PlanApo 60x oil immersion objective (n.a., 1.40), and an UplanApo 100 x oil immersion objective (n.a., 1.35). Lignified cells were visualised using an Olympus filter set (U-MWU) with 330-385 excitation and 420 nm emission.

3. Results and Discussion

Our research was based on the hypothesis that interspecific hybridization aimed in creating lighter exocarp, thicker hypodermal sklerenchyma layer and cuticle would reduce the heat load on the fruit.

Air/soil measurements

During the first period of measurements (Jul.-Aug.), on the sun-exposed sides, the highest temperatures were registered on the ground surface reaching 45°C, whereas 20 cm above ground the maximum daily air temperatures were about 38°C. On the sides shaded by a canopy, the ground temperatures remained cooler and more stable, varying between 18 during the nights and 32°C on the hottest days. Twenty cm above ground, the air temperatures reached 43°C. The underground temperatures (20 cm deep) remained stable with 20-22°C day/night variation (Fig. 2 A).

Additionally, the ground temperatures were also measured within the proximities of the studied pumpkins. In the proximity of Styrian oil pumpkins, from the 30th of July onwards, they increased from 27°C (daily maximum) to more than 47°C on the 1st August, and remained above 40°C for the next five days (Fig. 3 A, B). The maximum day ground temperature in the proximity of white pumpkins were similar, reaching 45°C during the first three days of August (Fig. 3 E, F).

At the beginning of September, the minimum night temperature was 10°C. One day, on the 7th September, it was particularly hot and the air temperature 20 cm above ground, on the sun-exposed side, reached 45°C, whereas the temperature of the ground surface reached 58°C. A similar situation was observed within the canopy. At 20 cm height, the temperature reached 45°C (Fig. 2 B).

On attached Styrian oil pumpkins (1st period), the maximum day temperatures measured on the exocarp varied between 38 and 50°C. In mesocarp the temperatures ranged between 32 and 51°C. Endocarp was more or less 2°C cooler than mesocarp (Fig. 3 A, B). On detached Styrian oil pumpkins (2nd period) the maximum day temperatures of exocarp varied between 20 and 54°C, whereas mesocarp heated up above the temperature of exocarp to 61°C on the hottest day (7th September), on later days, the maximum day temperatures were around 45°C (Fig. 4 A, B).

In the 'yellow fruit' pumpkin material (A/Mo × O/A), the temperatures of exocarp ranged between 42°C and 50°C on attached fruits during the first period and 17°C and 50°C on detached fruits during the second period of hot weather. The temperatures of endocarp varied between 33°C and 42°C during the first period, whereas during the second period the temperatures of mesocarp of detached pumpkins ranged between 16°C and 45°C (Fig. 3 C, D, Fig. 4 C, D).

'White fruit' pumpkin material (O/A) was characterised by the lowest exocarp temperatures, which ranged between 30°C and 41°C during the first period, whereas during the second period the temperatures ranged between 17°C and 44°C. The mesocarp temperatures varied between 35 and 44°C, the endocarp temperatures were more or less 2°C above the mesocarp temperatures. During the second period, where temperatures were measured on detached fruits, the average temperatures of mesocarp were

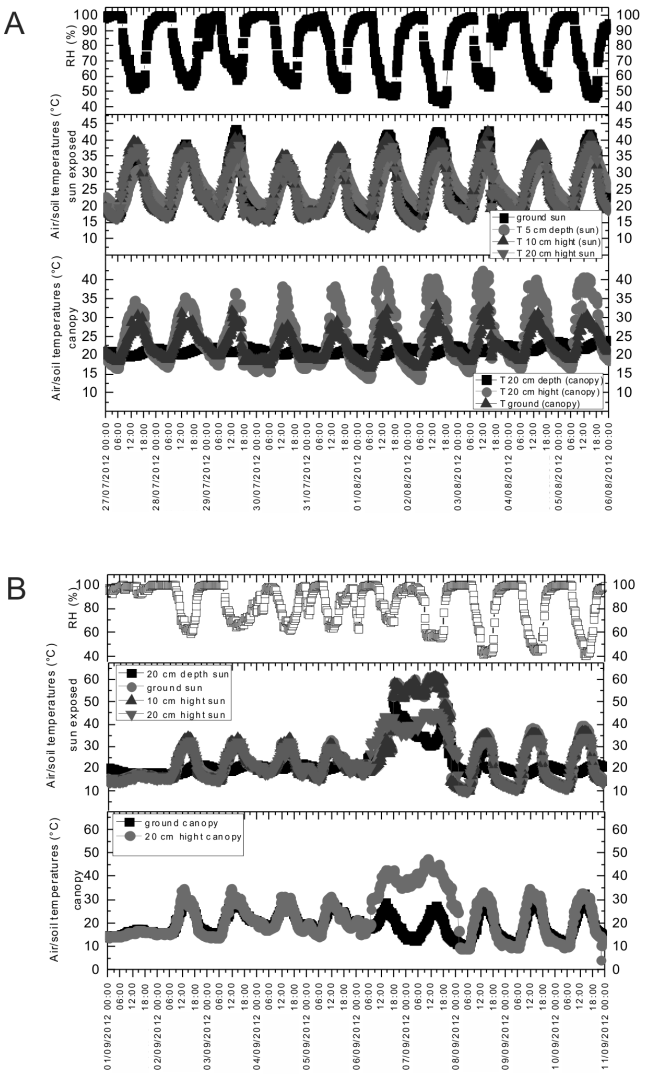

Fig. 2 - Relative humidity and the average air/soil temperatures measured within canopy and on sun exposed side during the period between 27 July-6 August 2012 (A) and between 1-11 September 2012 (B).

generally 2°C lower when compared to exocarp but the fruit flesh heated up to 56°C on the extremely hot days of the 6th and 7th September, similarly as was observed in the Styrian oil pumpkin (Fig. 4 A, B, E, F, Fig. S1 see supplementary material). Transpiration appeared to be vital for maintaining optimal growth temperatures in growing plants. Detached fruits, however, lacked the protective effects of transpiration, and direct sources of heat, such as sunlight, can rapidly elevate the internal fruit

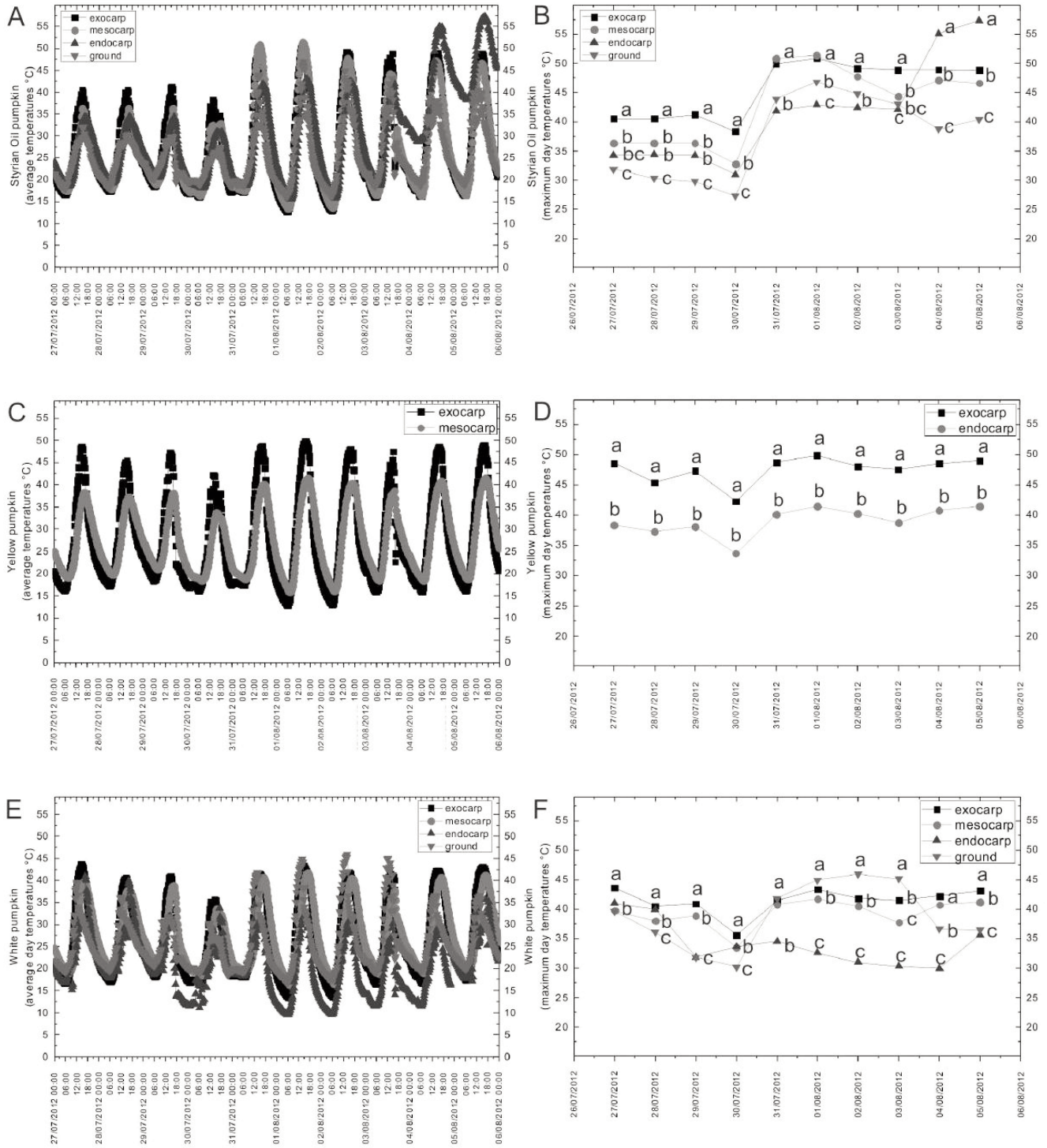

Fig. 3 - Average and maximum day temperatures measured in exocarp, mesocarp, endocarp of attached pumpkins and ground in the period between 27. July- 6. August 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A). E, F) Genotype with white fruits obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* (O/A).

temperatures to above that of exocarp and towards the thermal death points of their cells. In case of Styrian oil pumpkin these lead to localised bleaching and necrosis (sunburn or sunscald). Similarly, Rabinowitch *et al.* (1983, 1986) reported that detached cucumbers (*Cucumis sativus* L.) and peppers (*Capsicum annuum* L.) had significantly higher surface temperatures and more serious sunburn injuries than attached fruit.

Comparing the measured results of the different

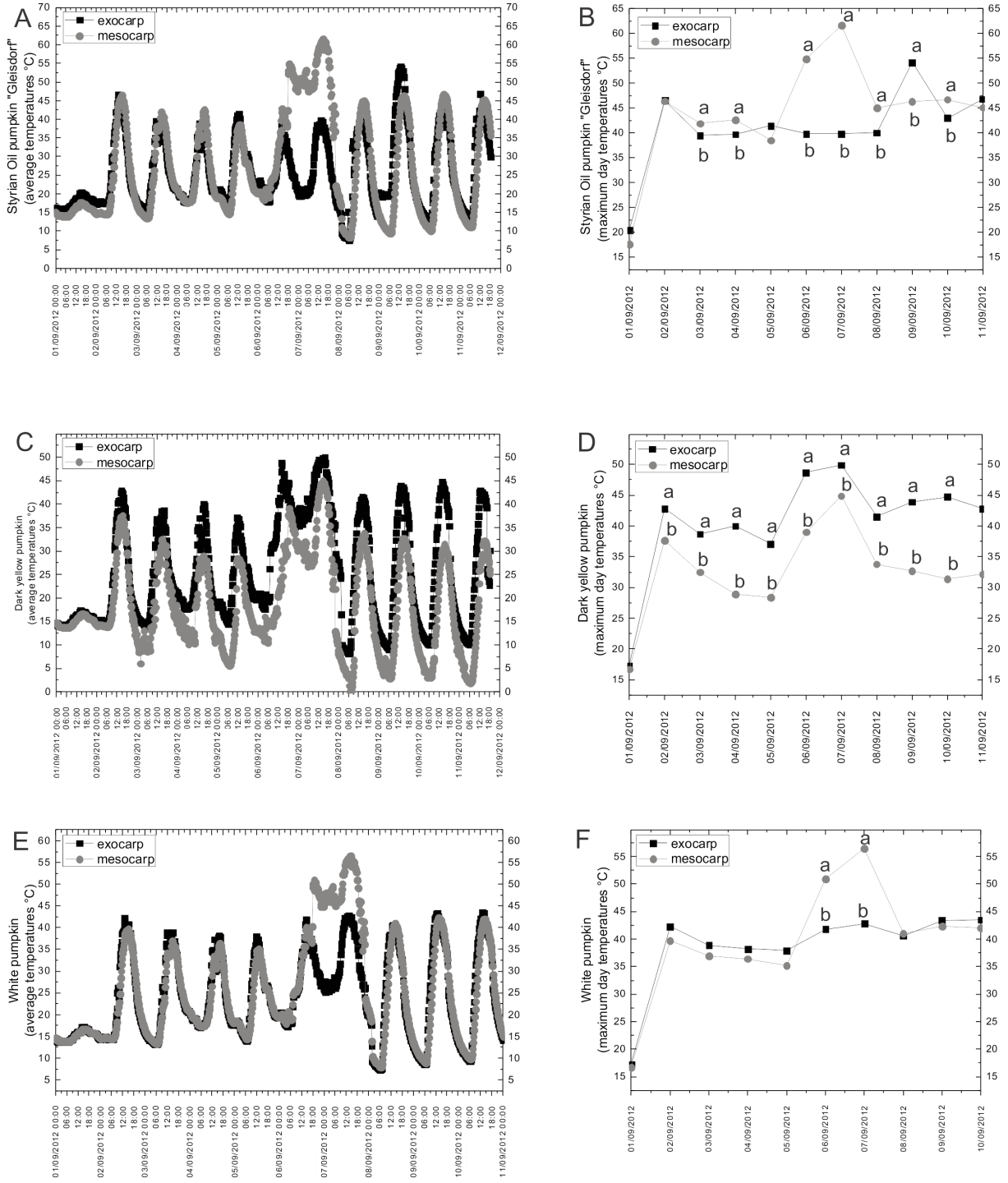

Fig. 4 - Average and maximum day temperatures measured in exocarp and mesocarp of detached pumpkins in the period between 1.-11.September 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A). E, F) Genotype with white fruits obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* (O/A).

varieties on attached fruits, we can conclude that Styrian oil pumpkin was characterised by the highest temperatures within all three tissues (Fig. S2), followed by yellow genotype, whereas white genotype had the lowest temperature, confirming the hypothesis that varieties with lighter fruit colour are less heated up. Our results of the experiment on detached fruits, however, suggest that the most tolerant is A/Mo × O/A which is a progeny derived from the interspecific cross involving the Styrian oil pumpkin, *C. argyrosperma* and *C. moschata*. It is well known that *C. moschata* is best adapted to hot climate and is successfully cultivated in the tropical and subtropical regions (Balkaya *et al*., 2010 a, b; Balkaya and Kandemir, 2015). Heat tolerance of *C. moschata* can be very useful in breeding involving interspecific hybridisation. One of the successful examples, mentioned earlier in the text, is interspecific hybrid of squash named as 'Maxchata'. The authors concluded that the interspecific hybridization involving *C. moschata* might significantly contribute to heat tolerance (Ara *et al*., 2013 a, b; 2015).

In order to test further the resistance of selected genotypes to heat stress, the selected pumpkins were sprayed with black colour and exposed to the sun in order to induce bleaching by the excess heat. The exocarp of the black coloured Styrian oil pumpkin fruit heated up to 52°C whereas the mesocarp temperature rose up to 43°C in the afternoon hours between 3 and 4 p.m. during both periods. When analysing the daily temperature curves of attached pumpkins during the July-August period, a depression in the curve was observed at around 12 a.m., reflecting a higher transpiration of fruits in response to heat stress; later, a rapid increase in temperature, may indicate stomata closure (Fig. 5A). It is well-

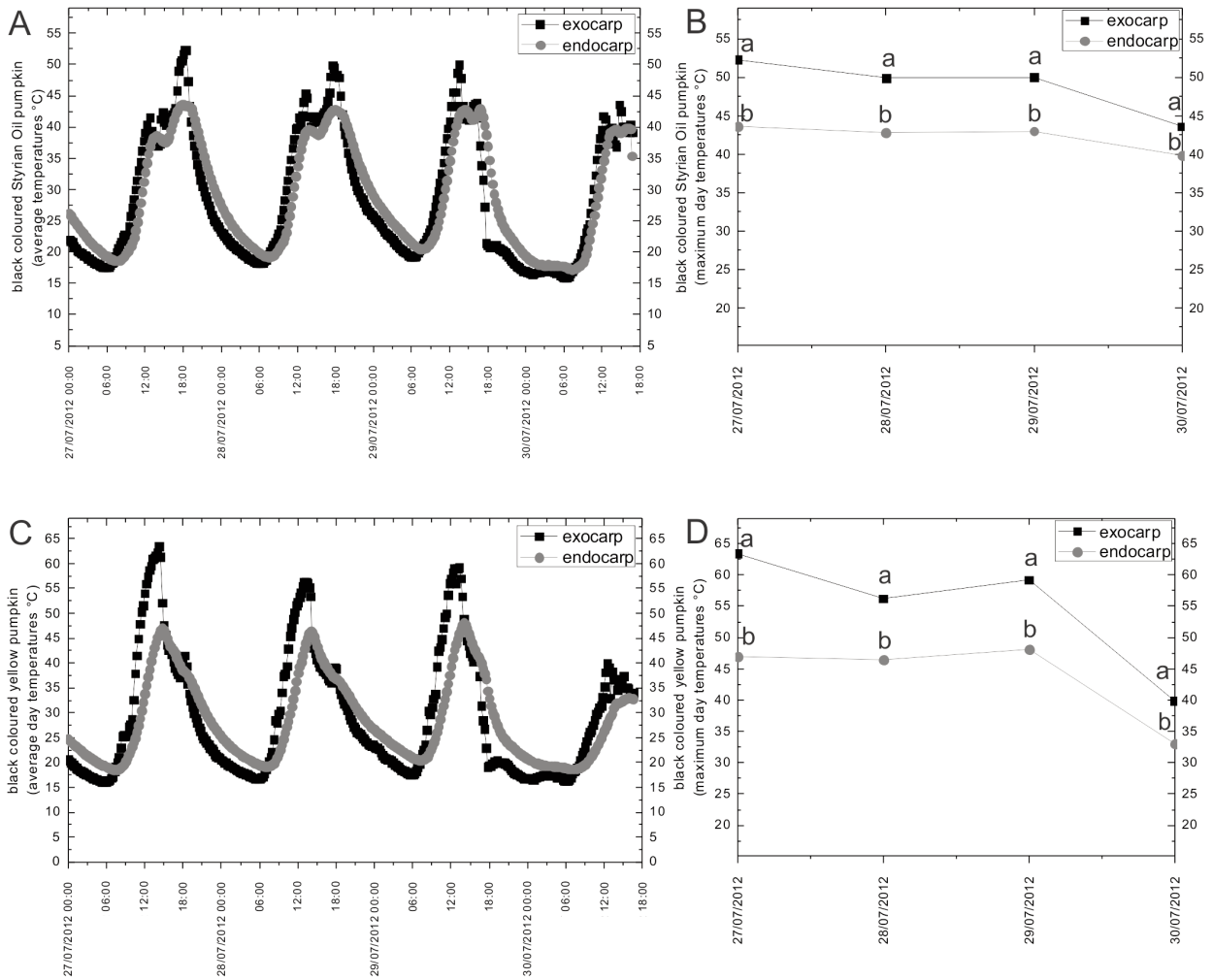

Fig. 5 - Average and maximum day temperatures measured in exocarp and endocarp of black coloured attached pumpkins measured in the period between 27 July- 6 August 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A).

known that if transpiration is interrupted by stomatal closure a major cooling mechanism is lost and the internal fruit temperatures raise (Wahid *et al*., 2007; Johnson *et al.,* 2015). The temperatures of coloured fruits were measured for three days since a general collapse and tissue death of the inner pericarp layers was observed later on. The fruits were too much damaged by sunburn and measurements of temperatures no longer made sense. In the September period, when the experiment was performed on detached fruits, the exocarp of Styrian oil pumpkin heated up to 51°C, similarly as on attached fruits. Mesocarp temperature raised to a higher level on detached fruits (61°C) in comparison to attached fruits (43°C) (Fig. 6A, Fig. 6B, Fig. S2).

Interestingly, during the 1st experiment on

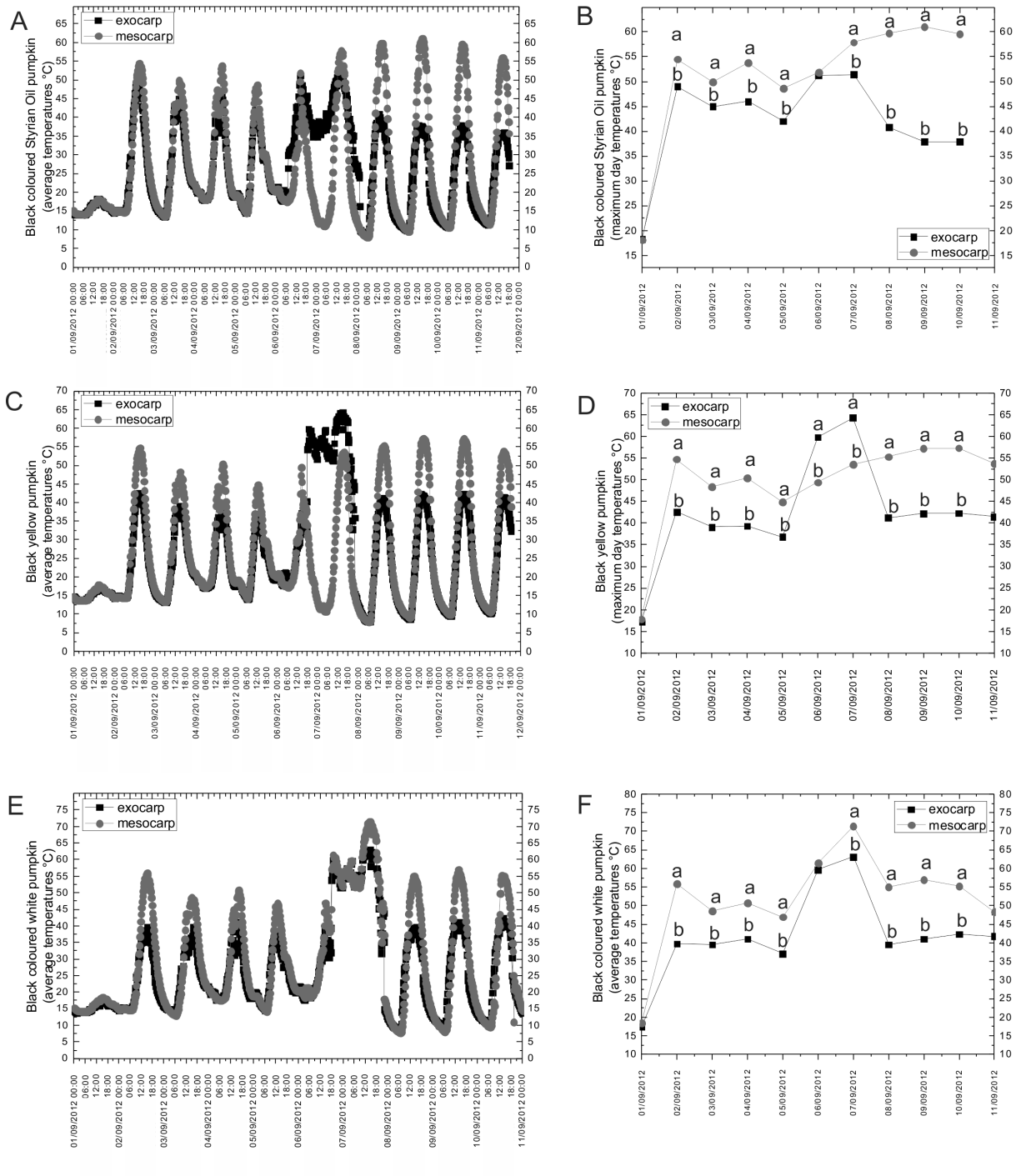

Fig. 6 - Average and maximum day temperatures measured in exocarp and mesocarp of black coloured detached pumpkins measured in the period between 1.-11.September 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A). E, F) Genotype with white fruits obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* (O/A).

attached pumpkins the exocarp of the black coloured 'yellow' pumpkin heated up to 64°C, whereas the endocarp up to 48°C during the July-August period (Fig. 5C, D). Compared to the Styrian oil pumpkin the temperatures were higher. A rapid increase of temperature was determined in the late morning hours until noon. Later, an abrupt decrease of temperature followed and maintained at around 38°C between 3 and 6 p.m. After that, during the night hours, the temperature dropped below 20°C (Fig. 5C). Similarly, in September, during the early afternoon hours of the hottest day (7th September), the maximum day temperatures on exocarp reached 64°C, whereas in mesocarp the temperatures reached 57°C. On later dates the daily maximum temperatures of exocarp constantly reached 42°C, whereas mesocarp was heated up to 57°C (Fig. 6 C, D).

The 'white fruit' genotype (O/A) was characterised by the highest mesocarp temperatures during the hottest days of the 2th period when compared to O and A/Mo × O/A progenies. During the first five days, the maximum exocarp temperatures were around 40°C, whereas the mesocarp reached maximum day temperatures between 48°C and 55°C. During the following two days, the exocarp temperatures reached 60°C and 63°C, respectively, whereas the mesocarp 62°C and 71°C. Later, the exocarp maximum temperature was comparable to that of the black coloured 'yellow' pumpkin, reaching 42°C in the early afternoon hours, whereas the mesocarp was heated up to approx. 55°C (Fig. 6 E, F).

Summarising the results of temperature measurements within the black coloured pumpkins, we can conclude that for the attached pumpkins, the 'yellow' genotype was heated up more than the Styrian oil pumpkin. By analysing the results of detached pumpkins during the first days of measurements, the highest temperatures of exo- and mesocarp were determined in the Styrian oil pumpkin, whereas the maximum day temperatures of both 'yellow' and 'white' genotypes did not differ significantly and were about 5°C lower than those of the Styrian oil pumpkin. However, on the hottest days (6^{th} and 7^{th} September), the temperatures of exocarp within 'yellow' and 'white' genotypes were 8°C higher than those of the Styrian oil pumpkin reaching 64°C. Consequently, when compared to the Styrian oil pumpkin, higher temperatures were also determined in fruit tissues of both studied interspecific hybrids.

During the last two centuries, pumpkins have been selected for their softer pericarp in order to ease the laborious and time-consuming hand-harvesting of seeds. However, the resulting pumpkins' genotypes became more susceptible not only to diseases but also to various types of abiotic stress such as heat stress and drought. In order to create a variety with harder pericarp and also better abscission of the fruit peduncle, those pumpkins with lighter exocarp were crossed with the wild species *C. okeechobeensis* (Fig. 1 G, H), which is characterised by hard perikarp, good abscission of the pedincle and resistance to viruses. The selected hybrids were histologically evaluated and compared to the Styrian oil pumpkin and O/A hybrids with lighter pericarps. The Styrian oil pumpkin (O) was characterised by its thick cuticle, a 15-cell layer of chlorenchyma cells and a 2-3 cell layer of more or less isodiametric lignified cells between the chlorenchyma and parenchyma of the mesocarp (Fig. 7 A, B). Both the 'yellow' (A/Mo × O/A) as well as the 'white' (O/A) genotypes were characterised by a similar 2-3 cell layer of thick lignified cells although the cell walls of this layer as well as the cuticle were thicker (Fig. 7 C, F). Crosses of O/A genotypes with *C. okeechobeensis* (Oke × O/A) became characterised by a thicker scklerenchymatic layer of 4-5 oblong cells in a radial direction, which were approx. 100 µm long (Fig. 7 G, H). This progeny represents a good material for the future breeding for increased heat tolerance. Furthermore, *C. okeechobeensis* has been recognised as promising for interspecific hybridization due to its central position in the genus *Cucurbita* (Gong *et al*., 2013).

We may conclude that the genetic breeding of oil pumpkins for heat tolerance is still in its infancy stage and warrants more attention than it has been given in the past. Considerable information is presently available regarding the physiological and metabolic aspects of plant heat-stress tolerance (Ara *et al.*, 2013 a, b, 2015). Furthermore, attempts have been made to include molecular marker technology for genetic characterization and/or development of plants with improved heat tolerance. Gong *et al.* (2013) analysed SSR polymorphisms on a large collection of *Cucurbita* materials in order to obtain an improved insight into the relationships amongst most of the species of the genus. Wild species *C. foetidissima* has been identified as resistant to numerous pathogens and pests but is most distant to other *Cucurbita* species. *Cucurbita pepo* and *C. ficifolia* were the most outlying of the mesophytic species. The clusters of the six remaining species form three pairs, *C. maxima* with *C. ecuadorensis*, *C. okeechobeensis* with *C. lundelliana*, and *C. moschata* with *C. argyrosperma*. Due to the genetic distances

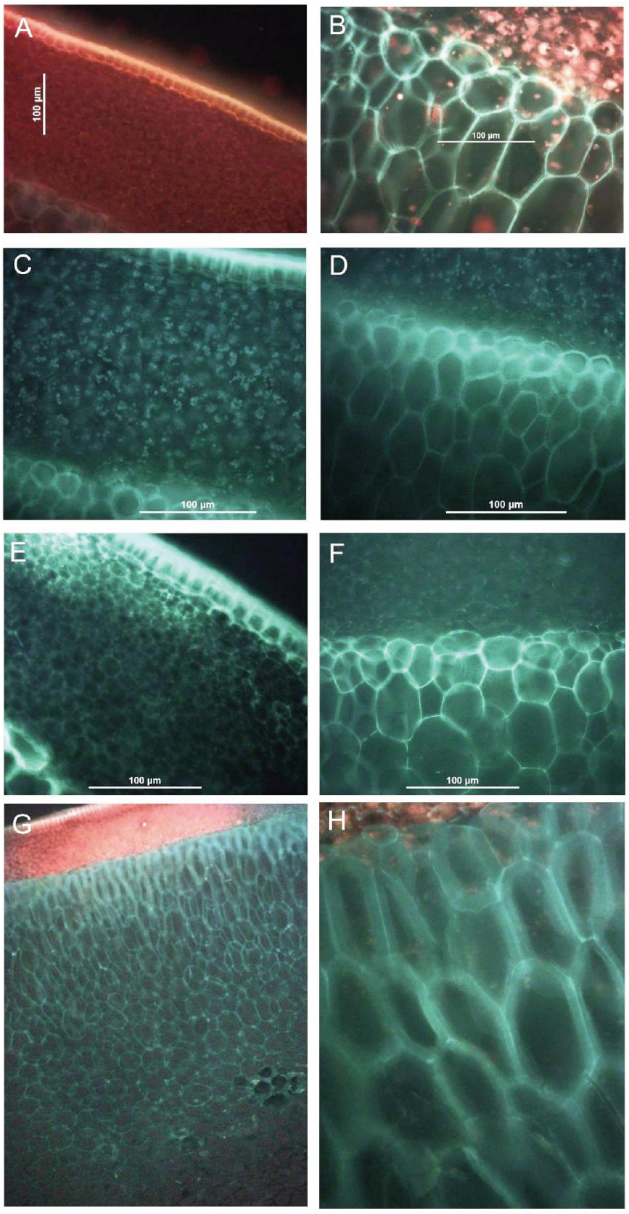

Fig. 7 - Histochemical analyses of perikarp. A, B) Styrian oil pumpkin (O) characterised by its thick cuticle, a 15-cell layer of chlorenchyma cells and a 1-2 cell layer of isodiametric cells and one layer of oblong cells with thick cell walls. C, D) Genotype with yellow fruits (A/Mo × O/A), with two layers of isodiametric cells with lignified cell walls. E, F) Genotype with white fruits (O/A) and three layer of lignified cells below the chlorenchyma. G, H) A three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* and *C. okeechobeensis* (Oke × O/A) exhibiting 4-5 cell layers of oblong sklerenchymatic cells below the chlorenchyma.

amongst the *Cucurbita* species, various breeding strategies and biotechnological approaches have been employed (Merrick, 1995; Lebeda *et al.*, 2007; Ortiz-Alamillo *et al.*, 2007; Lelley *et al.*, 2009; Karaağaç and Balkaya, 2013) but the success in introgressing desirable traits from one species to another have been limited. *Cucurbita pepo* excels in plant earliness and productivity but lacks genetic resources for disease resistance. *Cucurbita moschata*, on the other hand, carries resistance to various pathogens and is adapted to humid tropics but lacks earliness and productivity (Lebeda *et al.*, 2007; Lelley *et al.*, 2009; Karaağaç and Balkaya, 2013). Relatively high successes and fertilities have been observed for the cross-combination of *C. argyrosperma* and *C. moschata* (Montes-Hernandez and Eguiarte, 2002; Ortiz-Alamillo *et al.*, 2007) and *C. maxima* × *C. moschata* (Ara *et al.*, 2013 a, 2015).

However, despite all the complexity of heat tolerance and difficulties encountered during the genetic transfer of tolerance, few heat-tolerant inbred lines and hybrid cultivars with commercial acceptability have been developed and released (Montes-Hernandez and Eguiarte, 2002; Ortiz-Alamillo *et al.*, 2007; Ara *et al.*, 2013 a, b, 2015).

4. Conclusions

The presented study suggests that the colour of exocarp is probably one of the key parameters of tolerance to high temperatures. Darker colours are generally associated with higher fruit temperatures. In general, for attached pumpkin fruits higher temperatures were measured on exocarp, followed by meso- and endocarp. However, for the detached fruits on extremely hot days in September, the temperatures within mesocarp increased above those of exocarp. It is well known that if transpiration is interrupted by stomatal closure due to water stress and heat stress, a major cooling mechanism is not functioning. This was the reason of rising the internal fruit temperatures in case of detached fruits.

One could expect that the genotypes with whitish exocarp (i.e., O/A progenies derived from the cross Styrian oil pumpkin × *C. argyrosperma*) would be the most tolerant to heat stress. The experiment on detached fruits, however, suggest that the most tolerant is the A/Mo x O/A progeny derived from the cross involving the Styrian oil pumpkin, *C. argyrosperma* and *C. moschata*, which is characterized by yellow exocarp. One of the reasons for this could be the introgression of desirable traits of *C. moschata,* which is more adapted to high temperatures. This parental species was brought from the Island of Espiritu Santo, Vanuatu (tropical Pacific) and was obviously more tolerant to heat stress than the other two involved pumpkin species. The second important parameter appears to be the histological structure of

the pericarp. The genetic improvement of this complex trait should consider both parameters. Nevertheless, to accelerate such progresses, major areas of emphasis in the future should be: (1) development of accurate screening procedures at each stage of plant development; (2) identification and characterization of additional genetic resources associated with heat tolerance; (3) discerning the genetic inheritance of heat tolerance; (4) development and efficient screening of large breeding populations to facilitate transfer of genes for heat tolerance to commercial cultivars.

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The impact of fruit temperature dynamics on heat stress tolerance of selected oil pumpkin genotypes

A. Urbanek Krajnc *, J. Rakun, P. Berk, A. Ivančič
Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 10, Hoče, Slovenia.

SUPPLEMENTARY MATERIAL

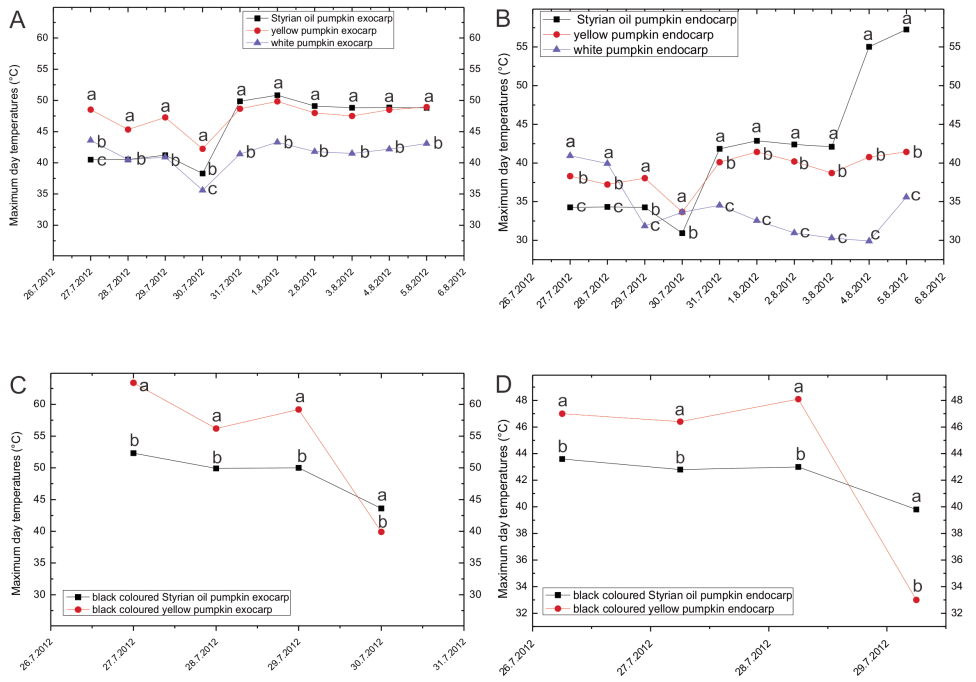

Fig. S1 - Comparison of the maximum day temperatures measured in the period between 27 July - 6 August 2012 in: A) Exocarp of Styrian oil pumpkin, yellow and white genotype; B) Endocarp of Styrian oil pumpkin, yellow and white genotype; C) Exocarp of black coloured Styrian oil pumpkin and yellow genotype; D) Endocarp of black coloured Styrian oil pumpkin and yellow genotype.

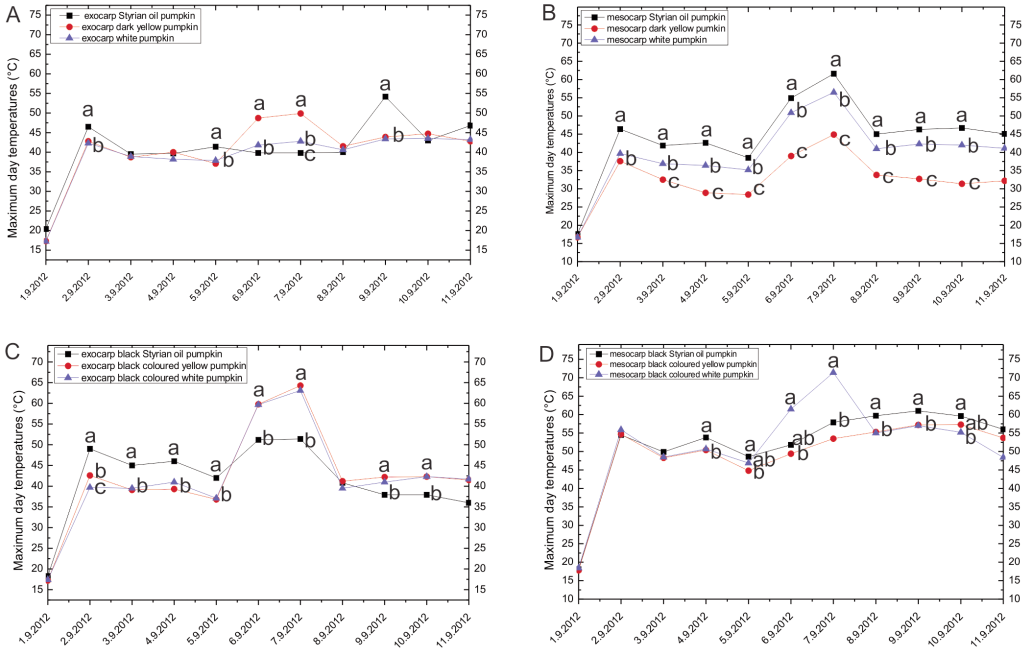

Fig. S2 - Comparison of the maximum day temperatures measured in the period between 1-11 September 2012 in: A) Exocarp of Styrian oil pumpkin, dark yellow and white genotype; B) Mesocarp of Styrian oil pumpkin, yellow and white genotype; C) Exocarp of black coloured Styrian oil pumpkin, yellow and yellow genotype; D) Mesocarp of black coloured Styrian oil pumpkin, yellow and yellow genotype.