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Advances in Horticultural Science

Postharvest melatonin treatment reduces chilling injury and enhances antioxidant capacity of tomato fruit during cold storage

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Key words: enzyme activity, lycopene, melatonin, proline, tomato fruit.

Abstract: In this study, tomato fruit was treated with 50, 100 or 200 µM melatonin and then stored at 5°C for 28 days to investigate the effect of melatonin treatment on chilling injury, nutritional quality and changes in the antioxidant system. Tomato fruit developed chilling injury, manifested as surface pitting and irregular red color development during storage. These chilling injury symptoms, ion leakage and malondialdehyde content were significantly reduced, and proline and carotenoids contents were significantly increased by melatonin treatment. Meanwhile, melatonin substantially reduced O_2^- production rate and H_2O_2 content, which result from significantly higher activities of superoxide dismutase, catalase, and peroxidase than control during the storage. These results suggest that melatonin treatment can effectively enhance chilling tolerance and reduce chilling injury. The reduction in chilling injury by melatonin may be associated with enhanced enzymatic and non-enzymatic antioxidants, in favor of membrane integrity and thus low cellular and tissue damage.

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The authors declare no competing interests.

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1. Introduction

Cold storage is one of the most effective postharvest technologies to preserve the quality of fresh produces from the time of harvest until final preparation for human consumption in food chain (Bourne, 2006). However, cold storage imposes great risk on postharvest commodities sensitive to chilling injury (CI). Tomato (*Lycopersicon esculentum*), as one of the most important tropical crops, is typically cold sensitive (Hong and Gross, 2006). The most common visual injury symptoms of CI depicted for tomato fruit include irregular ripening and red color development as well as surface pitting on the fruit. Furthermore, as the chilled tissues are weakened, they become prone to decay and microbial spoilage. This phenomenon limits postharvest life and leads to significant degradation of

produce quality (Wang, 1993).

Melatonin, first discovered in tomato in 1995, accumulates in the fruits as they mature (Dubbels *et al*., 1995; Hattori *et al*., 1995). Melatonin content, as an endogenous signaling molecule, increases in response to abiotic and biotic stress, such as drought, salinity, chilling, and pathogens to protect against damage caused by them (Zhang *et al*., 2014; Arnao and Hernández-Ruiz, 2015; Liu *et al*., 2016). Accumulation of higher levels of melatonin in horticultural crops is beneficial not only for human health, but also for prolonging storability (Tan *et al*., 2012). Melatonin, a naturally occurring indoleamine, acts as endogenous elicitor and signaling molecule for plants growth and development, decreasing of biotic and abiotic stress, as well as a potent hydroxyl radical scavenger and antioxidant (Zhang *et al*., 2014; Zhang and Zhang, 2014; Manchester *et al*., 2015; Zhang *et al*., 2015). Melatonin contribution has been evidenced in a semilunar rhythm in macroalgae guarding this plant against high temperature stress (Tal *et al*., 2011). Melatonin treatment decreased apoptosis chilling-induced in carrot suspension cells. Moreover, melatonin treatment alleviated chilling-induced shrinkage and disruption of carrot cell plasma membranes (Lei *et al*., 2004). It has been reported that melatonin treatment reduced chilling injury in peach fruits by enhancement of chilling tolerance and provoking of defense response during cold storage (Cao *et al*., 2016). Soleimani Aghdam and Rezapour Fard (2017) reported that melatonin treatment at 100 µM decreased strawberry fungal decay resulting from higher superoxide dismutase (SOD) activity, associated with lower catalase (CAT) and ascorbate peroxidase (APX) activities as well as higher phenylalanine ammonia lyase (PAL) enzyme activity leading to higher total phenols and anthocyanins accumulation along with higher DPPH scavenging capacity. Likewise, marssonina apple blotch caused by fungus *Diplocarpon mali* decreased by melatonin treatment at 0.1 mM which is caused by higher H_2O_2 accumulation leading to enhancing pathogenesis related (PR) proteins accumulation such as peroxidase, chitinase and b-1,3-glucanase, and triggering phenylpropanoid pathway by enhancing phenylalanine ammonia lyase (PAL) enzyme activity (Yin *et al*., 2013). It has been reported that the attenuating of postharvest physiological deterioration in cassava roots by melatonin treatment, obtained by lower H_2O_2 accumulation as a result of increasing antioxidant enzymes; SOD, CAT and GR activities causing higher membrane integrity indicated by lower malondialdehyde (MDA) accumulation (Ma *et al*., 2016). Gao *et al.* (2016) reported that lower O^-_2 and H_2O_2 accumulation in melatonin treated peach fruits resulted from higher antioxidant enzymes SOD, CAT, APX activities, concurrent with lower lipoxygenase (LOX) enzyme activity leading to higher membrane integrity indicated by lower MDA accumulation.

In addition to antifungal and antioxidant activities, melatonin is useful in increasing postharvest sensory and nutritional quality of fresh produces (Meng *et al*., 2015; Cao *et al*., 2016; Gao *et al*., 2016; Liu *et al*., 2016; Ma *et al*., 2016). It has been reported that preveraison melatonin-treated grape berries showed higher endogenous melatonin accumulation, which not only enhances berry size and weight, indicated by higher sugars accumulation and higher endogenous hormones GA/ABA ratio, but also enhances synchronicity of berry ripening (Meng *et al*., 2015). It has been observed that preharvest melatonin-treated tomato fruits showed higher fruits weight caused by higher sugars accumulation, as well as higher organic acids accumulation results in tomato fruits with favorable flavor. Moreover, higher lycopene and ascorbic acid contents were observed in preharvest melatonin-treated tomato fruits (Liu *et al*., 2016).

Since there is a lack of knowledge about the influence of melatonin on chilling tolerance of tomato fruit during low temperature storage, the present work was initiated to determine the efficacy of postharvest melatonin treatment on chilling demonstrations and enhanced fruits visual and nutritional qualities through augmenting antioxidant capacity of tomato fruits frequently encountered under cold storage.

2. Materials and Methods

Fruit and treatment

Tomato fruit (*Lycopersicon esculentum* Mill. cv Banemi) were harvested at the mature green stage (i.e., liquefying locular tissue, seeds not cut with a knife) (Saltveit, 1991) from a local producer in Mohammad Shahr, Karaj (Iran) and then immediately transported to Karaj Agricultural Engineering and Engineering Research Institute Laboratory. The green stage of maturity with homogeneous size and randomly allotted into three groups (100 fruits per grop) for treatment in triplicate by dipping of fruits at 0 (control), 50, 100 and 200 µM melatonin solution for

5 min at 20°C. The selected concentrations were based on published effects of these compounds on peach, strawberries and cherry tomatoes (Sun *et al*., 2015; Cao *et al*., 2016; Soleimani Aghdam and Rezapour Fard, 2017). Following immersion, the fruits were dried for 1 h at room temperature. The tomato fruits were then put in plastic baskets, covered with a perforated plastic bag to retard weight loss and stored at 5°C with 80-85% relative humidity for 4 weeks. The seven-day intervals during storage at 5°C followed by shelf life at 20°C for 1 and 3 days, the development of chilling injury and ripening characteristics as well as enhanced fruits nutritional quality through augmenting antioxidant capacity were measured, respectively (Ding *et al*., 2002).

Measurements of chilling injury and ripening characteristics

Chilling injury (CI) of fruits was evaluated at 20°C for 1 day after the 7-, 14- 21 or 28-day cold-storage periods. Symptoms of tomato fruit chilling injury were manifested as surface pitting and large green patches or blotchy yellow areas resulting from loss of full red color development ability (Wang, 1993). The severity of the symptoms was assessed visually according to the following four-stage scale: 0= no pitting; 1= pitting covering <25% of the fruit surface; 2= pitting covering <50%, but >25% of surface; 3= pitting covering <75%, but >50% of surface and 4= pitting covering >75% of surface. The average extent of chilling-injury damage was expressed as a chilling-injury (CI) index, which was calculated using the following formula:

$$\text{CI index (\%)} = \{[(\text{CI level}) \times (\text{number of fruit at the CI level})]/(\text{total number of fruits}) \times 4\} \times 100.$$

For determining the effect of different treatments on ripening, fruits following 28 days storage, were incubated in diffused light at 20°C for 3 days to full red color development (Ding *et al*., 2002). Measurement of full red color development in terms of carotenoids accumulation was conducted. Lycopene and β-carotene were determined by the method described by Nagata and Yamashita (1992). The amount of 0.1 g of fruit tissue was mixed with 20 mL of hexane:acetone solution (3:2). An aliquot was taken from the supernatant and measured at 453, 505, 645, and 663 nm in a spectrophotometer. The content of lycopene and β-carotene was estimated using the following equations:

$$\text{Lycopene} = -0.0458\ A_{663} + 0.204\ A_{645} + 0.372\ A_{505} - 0.0806\ A_{453}$$
$$\beta\text{-carotene} = -0.216\ A_{663} - 1.220\ A_{645} + 0.304\ A_{505} + 0.452\ A_{453}$$

The results were expressed in milligrams per 100 g fresh weight (mg 100 g^{-1} FW).

Measurements of ion leakage and malondialdehyde content

Ion leakage was measured at 20°C for 3 days after the 7-, 14-, 21- or 28- day cold-storage period according to the method of Zhao *et al.* (2009). 3 mm thick of mesocarp tissues were excised from equator part of 5 fruits. Disks were put into aqueous 0.1 M mannitol and shaken at 100 cycles/min for 2 h. The conductivity of the solution (L1) was measured with a conductivity meter. Solutions were boiled for 10 min and then cooled to 20°C. The conductivity of tissues (L2) was measured. Ion leakage was calculated as the ratio of L1 to L2. Malondialdehyde (MDA) content was measured at 20°C for 3 days after the 7-, 14-, 21- or 28- day cold-storage period using the thiobarbituric acid method described by Zhao *et al.* (2009) with modification. Absorbance at 532 nm was recorded and corrected for nonspecific absorbance at 600 nm. The amount of MDA expressed as µmol MDA per gram of pulp.

Measurement of proline content

Proline content was measured at 20°C for 3 days after the 7-, 14-, 21- or 28- day cold-storage period using the acid ninhydrin method described by Shan *et al*. (2007). Proline in tissues was extracted with 30 mL L^{-1} sulfosalicylic acid at 100°C for 10 min with shaking. The extract was mixed with an equal volume of glacial acetic acid and acid ninhydrin reagent and boiled for 30 min. After cooling, the reaction mix was partitioned against toluene and the absorbance of the organic phase was recorded at 520 nm. The resulting values were compared with a standard curve constructed using known amounts of proline and expressed as µg proline g^{-1} fresh weight (FW).

Measurements of O_2^- production rate and H_2O_2 content

The O_2^- production rate and H_2O_2 content were measured at 20°C for 3 days after the 7-, 14-, 21- or 28- day cold-storage period. O_2^- production was measured using the method of Elstner (1976) with modification. 4 g of fruit tissue was homogenized with 5 ml of 50 mM phosphate buffer (pH 7.8) and then centrifuged at 8000×g for 20 min at 4°C. The supernatant was used for the determination of O_2^- production and expressed as nmol g^{-1} FW min^{-1}.

For H_2O_2 measurement, 2 g of fruit tissue was homogenized with 5 ml of cold acetone and then centrifuged for 15 min at 8000×g at 4°C, the supernatant was collected immediately for H_2O_2 analysis according to the method of Patterson *et al.* (1984).

H_2O_2 content was expressed as nmol g^{-1} FW.

Enzyme extraction and analysis

Enzyme activities were measured at 20°C for 3 days after the 7-, 14-, 21- or 28- day cold-storage period. 5 g of fruits tissue were homogenized with 50 mmol/L phosphate buffer (pH 7) containing 0.2 mmol/L EDTA and 2% PVP. The homogenate was centrifuged at 12,000×g for 20 min at 4°C and the supernatant was used. SOD (EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977) with modification. One unit of SOD activity was defined as enzyme that caused 50% inhibition of nitro blue tetrazolium reduction by recording the absorbance at 560 nm. According to Zhang *et al*. (2013) with modification, 1 unit of CAT (EC 1.11.1.6) activity was defined as 0.01 decrease in absorbance at 240 nm per min. POD (EC 1.11.1.7) activity was determined according to Maehly and Chance (1954) with modification. One unit of POD was defined as 0.01 increase of absorbance at 470 nm as a result of guaiacol oxidation.

Statistical analysis

Experiments were performed using a completely randomized design. All statistical analyses were performed with SAS 9.2 software package. Data were analyzed by one-way analysis of variance (ANOVA). Mean comparisons were performed using HSD in Tukey's test for comparing treatment group at level of 1% (P<0.01) on three biological replicates.

3. Results

Chilling injury and ripening characteristics

The chilling injury (CI) symptoms were expressed on control group as surface pitting and large green patches or blotchy yellow areas resulting from loss of full red color development ability (Wang, 1993), only after 7-day of cold storage and following shelf life at 20°C for 1 and 3 days, respectively (Fig. 1). No significant difference in CI was observed between the control and 50 µM melatonin-treated fruit. Whereas, melatonin-treated groups with 100 or 200 µM underwent normal ripening at 20°C and only few visual chilling-injury symptoms were observed after 14 days storage at 5°C (Fig. 1). Fruits treated with 100 or 200 µM melatonin maintained the same quality as before chilling-temperature storage except for developing a slight yellow pigmentation, and the effect of the used formulas increased with increasing their concentrations (Fig. 2A). The results indicate that a 14-day storage was the maximum that could be tolerated by untreated mature green fruit. In this experiment, treatments with higher concentrations (100 or 200 µM) of melatonin were more effective in protecting against chilling injury than lower concentration (50 µM).

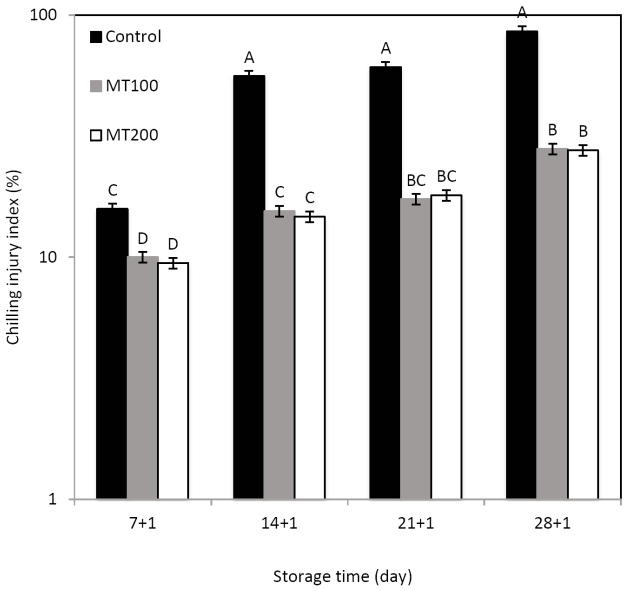

Fig. 1 - Chilling injury (CI) index (%) of tomato fruits treated with 100 and 200 µM during storage at 5°C and after 1 day of shelf life. All data are presented as a mean of three biological replicates, and vertical bars indicated the standard errors. Different letters indicate significant differences at P<0.01.

For examining the effect of melatonin treatment on color development of fruit after cold storage, mature green tomatoes were transferred to 20°C for 3 days for ripening. Treatment with 100 or 200 µM melatonin, prior to 5°C storage, was effective at alleviating chilling injury; this treatment category resulted in normally fruit ripening and uniform red color development caused by significantly (P<0.01) more lycopene and β-carotene accumulations, and the effect of the used formulas on fruit ripening and eventually visual quality increased with increasing their concentrations (Fig. 2). However, control and melatonin-treated group with 50 µM failed to develop the normal red color, with lower lycopene and β-carotene values, demonstrated by large green patches or blotchy yellow areas. Interaction effects and time of storage had no meaningful influence on these traits.

Ion leakage, malondialdehyde content

Ion leakage and MDA, as a consequence of membrane damage, are credible parameters for CI development and degree for postharvest tomato fruit

(Zhao *et al*., 2009). In this experiment, significantly the highest ion leakage was detected in control group (P<0.01) (Fig. 3A). However, no significant differences were statistically found in the ion leakage incidence between melatonin-treated groups with 100 or 200 µM. As shown in figure 2B, MDA content showed a similar pattern of change during storage.

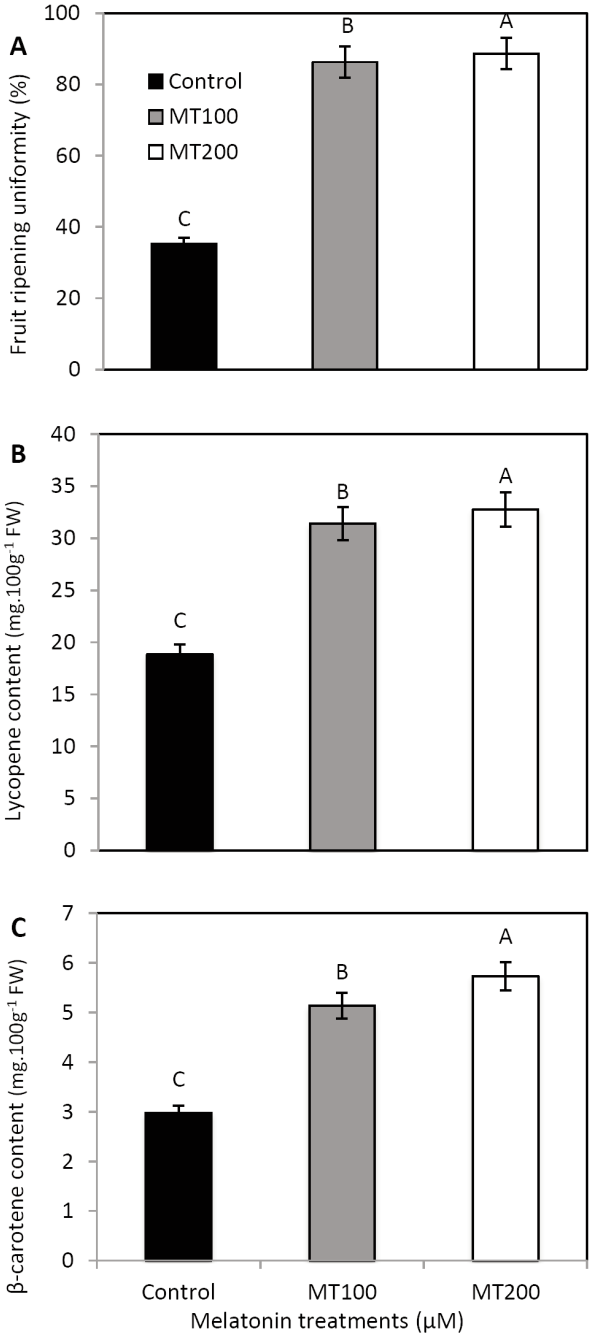

Fig. 2 - Fruit ripening uniformity (%), lycopene (A) and β-carotene (B) contents of tomato fruits treated with 100 and 200 µM during storage at 5°C and after 3 days of shelf life. All data are presented as a mean of three biological replicates, and vertical bars indicated the standard errors. Different letters indicate significant differences at P<0.01.

MDA content was significantly (P<0.01) lower in melatonin-treated groups with 100 or 200 µM compared with control at the same time of cold storage, and the highest level observed about 14 to 21 days (Fig. 3B).

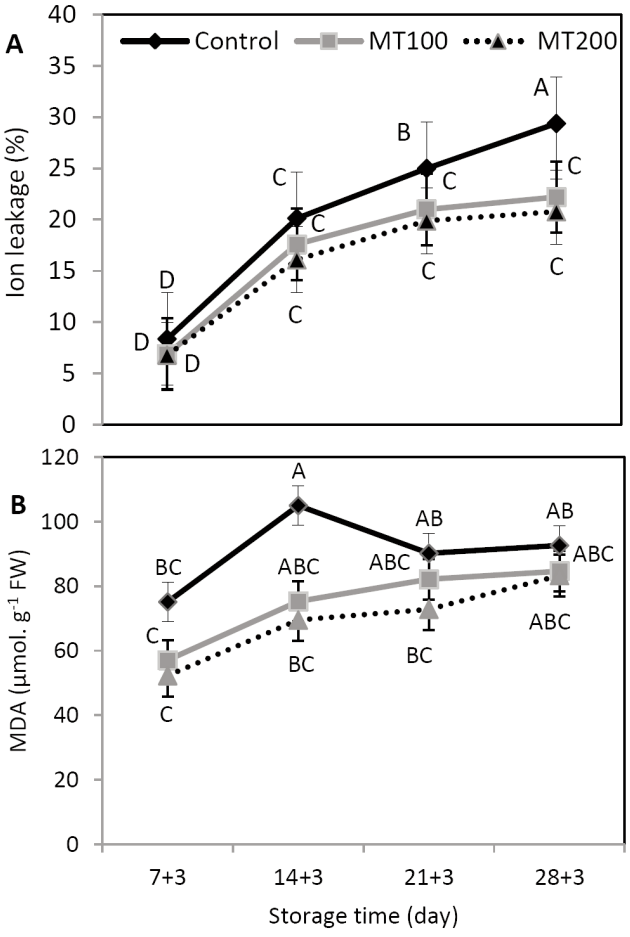

Fig. 3 - Ion leakage (%) (A) and MDA (B) content of tomato fruits treated with 100 and 200 µM melatonin during storage at 5°C and after 3 days of shelf life. All data are presented as a mean of three biological replicates, and vertical bars indicated the standard errors. Different letters indicate significant differences at P<0.01.

Proline content

There was a peak of proline content appearing in 14-day in all groups, which suggested that low temperature induced the proline synthesis mechanism in fruits (Zhao *et al*., 2009). However, proline accumulation was about 2 times high in the melatonin-treated groups with 100 and 200 µM compared to control from 14-day to the end of storage period, and the effect of the used formulas on proline content increased with increasing their concentrations (Fig. 4). No significant difference in proline content was observed between the control and 50 µM melatonin-treated fruit.

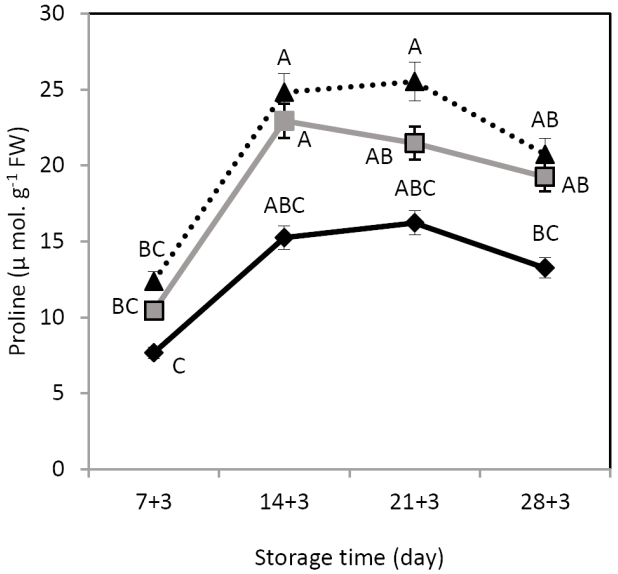

Fig. 4 - Prolin content of tomato fruits treated with 100 and 200 µM melatonin during storage at 5°C and after 3 days of shelf life. All data are presented as a mean of three biological replicates, and vertical bars indicated the standard errors. Different letters indicate significant differences at P<0.01.

O_2^- production and H_2O_2 content

In figure 5, the measured levels of O_2^- and H_2O_2 were shown as an influence of low temperature to ROS generation in fruits exposure to chilling stress. Contents of O_2^- and H_2O_2 remained relatively unchanged in control and melatonin-treated groups within the first 14 days of cold storage. Thereafter, both O_2^- and H_2O_2 contents increased rapidly, treatment with 100 and 200 µM melatonin significantly (P<0.01) restrained the enhancement of O_2^- and H_2O_2 contents, and again the effect of the used formulas on O_2^- production rate and H_2O_2 content decreased with increasing their concentrations (Fig. 4). No significant difference in O_2^- and H_2O_2 contents was observed between the control and 50 µM melatonin-treated fruit.

SOD, CAT, POD activities

As depicted in figure 6A, the SOD activity in both control and melatonin-treated groups steadily increased during storage, nonetheless significantly the highest SOD activity was observed in melatonin treated groups with 200 and 100 µM throughout the storage, respectively (P<0.01). The changes of CAT and POD activities in tomato fruit showed a similar pattern during the cold storage. The activities of both enzymes in control and melatonin-treated groups oscillatory increased with storage time. Melatonin treatment significantly promoted the increases in activities of CAT and POD, the activities of both enzymes were significantly higher (P<0.01) in these groups than those in control group during the whole storage (Fig. 6).

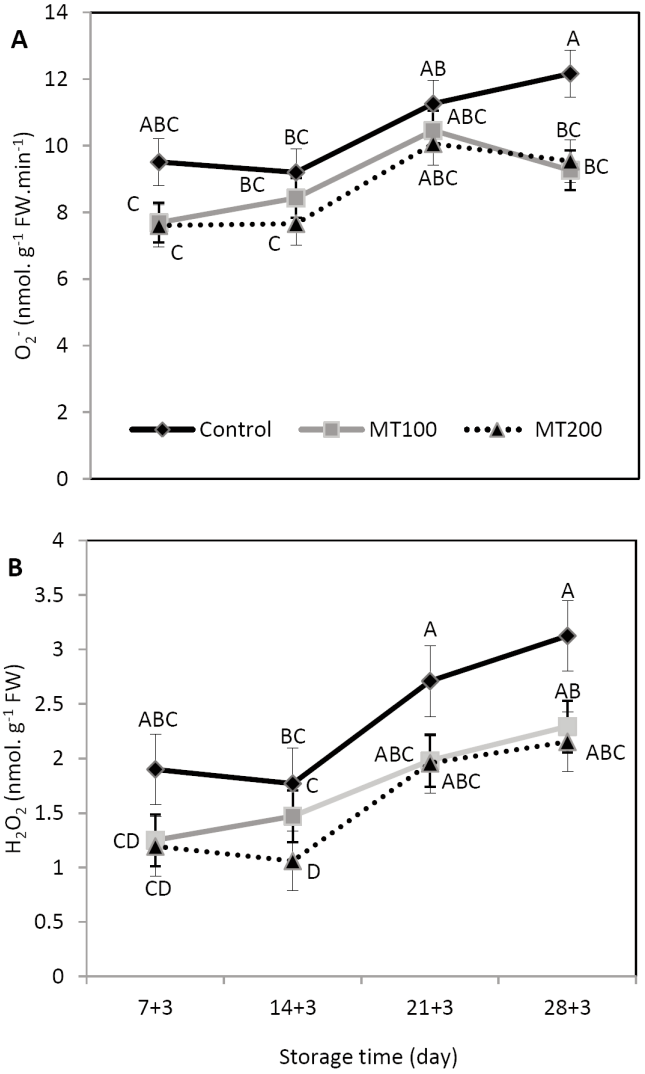

Fig. 5 - O_2^- Production (A) and H_2O_2 (B) content of tomato fruits treated with 100 and 200 µM during storage at 5°C and after 3 days of shelf life. All data are presented as a mean of three biological replicates, and vertical bars indicated the standard errors. Different letters indicate significant differences at P<0.01.

4. Discussion and Conclusions

Little information is available on melatonin treatment of horticultural commodities, even though there are many reports suggesting that melatonin is an endogenous signaling molecule for the activation of certain plant defense responses and the onset of the tolerance has often been correlated with the accumulation of defense-related enzymes and compounds (Zhang *et al.*, 2014; Arnao and Hernández-

Ruiz, 2015; Liu *et al.*, 2016). Exogenous melatonin application has been shown to result in an improved chilling tolerance and reduced incidence of chilling injury in peach and strawberry fruits (Cao *et al*., 2016; Soleimani Aghdam and Rezapour Fard, 2017). In this experiment, we found that melatonin treatment could effectively not only reduce development of surface pitting on the fruit and irregular ripening and full red color development (large green patches or blotchy yellow areas), the typical chilling injury

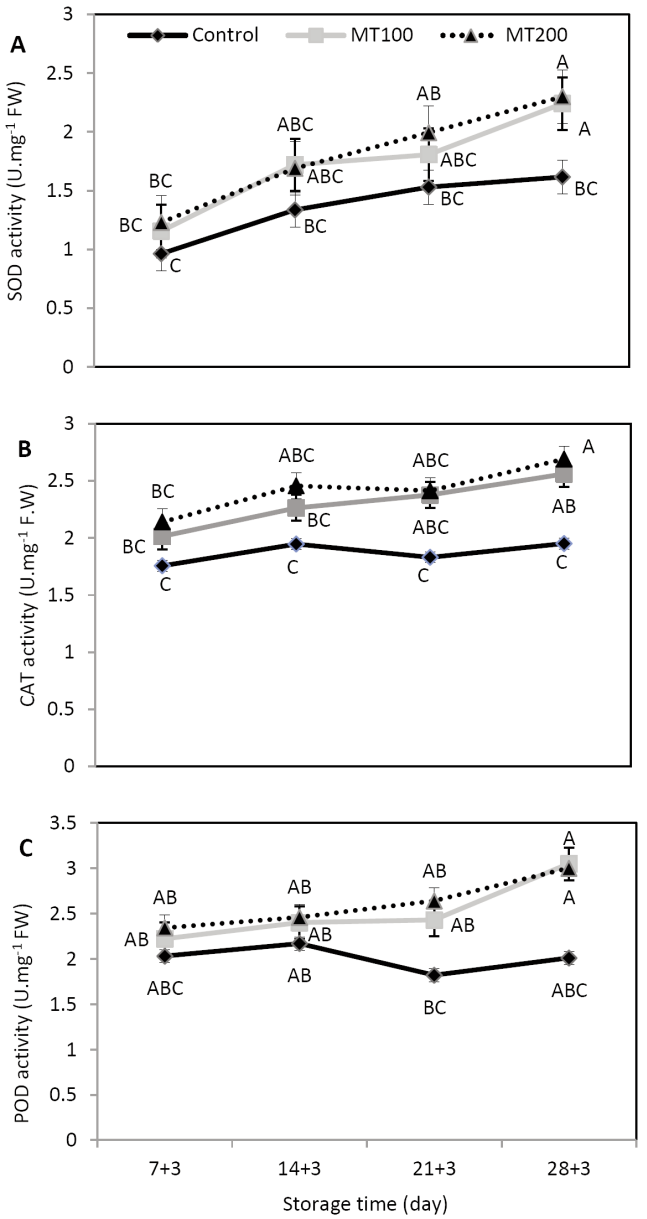

Fig. 6 - Superoxide dismutase (A), Catalase (B) and peroxidase activities (C) of tomato fruits treated with 100 and 200 µM during storage at 5°C and after 3 days of shelf life. All data are presented as a mean of three biological replicates, and vertical bars indicated the standard errors. Different letters indicate significant differences at P<0.01.

symptoms in tomato fruit, but also enhance fruits nutritional quality. This indicates that postharvest treatment with melatonin increased chilling tolerance in tomato fruit. Since melatonin treatment is easy to set up, inexpensive and safe, even if higher amounts are accumulated in the plant (Tan *et al*., 2012), it could be a functional method to decrease chilling injury, maintain quality and prolong shelf life of tomato fruit.

Carotenoids, highly characteristic phytochemicals, known to be potent ROS scavengers and antioxidants, act as a cell proliferation inhibitor and hindering of cancer cell growing (Tijskens and Evelo, 1994; Levi *et al*., 2001; Giovannucci *et al*., 2002; Stahl and Sies, 2005). During maturation/ripening, the green pigment chlorophyll degrades and carotenoids are synthesized. Carotenoids, particularly lycopene and β-carotene, represent the primary components of ripe fruit pigmentation in tomato pericarp and are responsible for the characteristic color of ripe tomatoes, conferring deep red and orange colors, respectively. These carotenoids largely influence flavor and nutritional qualities as well as commercial value and enhances consumer acceptance of fresh tomato fruit (Tijskens and Evelo, 1994). In this study, higher accumulation of lycopene and β-carotene in melatonin treated groups with 100 and 200 µM not only contributed to alleviate chilling injury to fruit, but also lead to normally fruit ripening with uniform red color development (panels B and C of Fig. 2). It has been reported that in tomatoes, the contents of lycopene and β-carotene increase from the green to the fully ripe stage (Fraser *et al*., 1994). Melatonin may affect directly or indirectly other carotenoid genes and/or enzymes in tomato fruit. This could be the case for example of lycopene cyclases, which is responsible for the formation of β-carotene from lycopene, which its accumulation is a ripening-related event in tomato (Giovannoni, 2001). The higher levels of these compounds in melatonin-treated red ripe fruits may be associated with a general acceleration in ripening and with some of the associated transcriptional events, leading to the color change of tomato fruit (Guo, 2015; Sun *et al*., 2015). Therefore, the improved capability of full red color development in chilling-faced melatonin-treated group is one of the most important outcome of this study for the quality of tomato.

Proline, an important amino acid, has been considered as a cellular osmotic regulator, protein stabilizer, free-radical scavenger, and lipid peroxidation inhibitor in plant (Sun *et al*., 2015). The elevated level

of proline found to be associated with improved cold tolerance in chilling-sensitive plants (Zhao *et al*., 2009; Shang *et al*., 2011; Zhang *et al*., 2013; Cao *et al*., 2016). Our findings are in agreement with the above reports (Zhang *et al*., 2010), because a significantly ($P<0.01$) higher content of proline was observed in melatonin-treated tomato fruits with 100 or 200 µM during the whole storage period along with the reduced CI incidence (Fig. 4). Cao *et al*. (2016) reported that higher transcripts of *PpP5CS* and *PpOA* were observed in melatonin-treated peach fruits which provokes proline accumulation. Zhao *et al*. (2009) claimed that proline levels in a tissue may be an effective indicator for CI analysis in postharvest tomato fruits.

The reduction of cell energy and/or induction of alterations in membrane integrity are occurred in chilling-sensitive horticultural commodities at low temperatures. Reducing scavenging potency through such factors as chilling-related inactivation of antioxidants and/or obstructed antioxidant turnover may result in the enhanced ROS generation. Chilling temperatures destroy the balance between ROS formation and defense mechanisms which cause oxidatively chilling injury and consequent cellular damage (Hodges *et al*., 2004). It is figured that antioxidant enzymes, SOD, CAT, and POD are the primary enzymatic scavenging mechanism of ROS that contribute to attenuate chilling injury to fruit (Sala and Lafuente, 2000; Mondal *et al*., 2004; Ding *et al*., 2007; Imahori *et al*., 2008). Thus, this balance between the generation and scavenging of ROS is crucial to cell survival during cold storage and is thought to be a major mechanism of resistance to chilling stress. It has been reported that in harvested commodities enhanced enzymatic antioxidant activities result in the improved chilling tolerance. A higher antioxidant enzyme activity was indicated in the chilling-tolerant mandarins compared with the chilling sensitive cultivars (Sala, 1998). In many other studies, enhancement of antioxidant enzyme activity through a number of postharvest treatments (e.g. heat shock, low temperature conditioning and superatmospheric oxygen treatment) provoked chilling tolerance and alleviated chilling injury to fruit (Wang, 1995; Sala and Lafuente, 2000; Zheng *et al*., 2008). Neutralizing of the O_2^- by SOD is the initial step of cell defense against free radicals (Bowler *et al*., 1992). CAT is one of the enzymes that protect cells against ROS because it catalyzes the decomposition of H_2O_2 to form O_2^- and H_2O_2 (Imahori *et al*., 2008). POD catalyzes H_2O_2 dependent oxidation of substrate (Fu and Huang, 2001). In the present work, the higher increases in activities of SOD, CAT, and POD concurrent with reduced O_2^- and H_2O_2 content in melatonin-treated groups than those in control group were indicated (Fig. 6). While, the levels of H_2O_2 and O_2^- significantly increased during the development of irreversible chilling injury symptoms surface pitting and irregular ripening and full color development in control group. Treatment with melatonin significantly alleviated these chilling-induced damages and increased the activities of SOD, CAT, and POD under cold stress. The increased SOD activity could enhance the ability of the fruit to dismutate superoxide radicals, while the increases in CAT and POD activities would contribute to the stronger omitting of hydrogen peroxide (Lukatkin, 2002), which may give an explanation for the lower levels of O_2^- and H_2O_2 observed in melatonin-treated groups. These results suggest that effect of melatonin in reducing the incidence of chilling injury was correlated to enhanced enzymatic scavenging mechanism of ROS. In melatonin-treated tomato fruits the continues functions of SOD, CAT, and POD may be associated with higher stress resistance and eventually extended shelf life.

Membrane lipid peroxidation may be one of the first events in the manifestation of CI, in which phase MDA as a final product of polyunsaturated fatty acid oxidation was produced and damaged to cell membrane, resulted in ion leakage (Lukatkin, 2002; Imahori *et al*., 2008). As depicted in panels A and B of figure 3, the increase in ion leakage and MDA from 14-day of storage period indicates that cold storage caused a distinct deterioration of membrane integrity and activation of lipid peroxidation in the non-treated control group, which could be attributed to the decreases in SOD and CAT activities as well as in antioxidant compounds including lycopene, β-carotene and proline. These reductions induced by chilling stress favor accumulation of O_2^- and H_2O_2, which can result in lipid peroxidation. Ion leakage and MDA content may be a reflection of CI development and fruit cold tolerance (Zhao *et al*., 2009). Furthermore, Posmyk *et al*. (2005) reported that ion leakage intensity and MDA content in a tissue can be a reliable indicators of the structural integrity of the membranes of plants exposed to low temperature. Given to these results, prevention of MDA accumulation and subsequent ion leakage by melatonin treatment could be related to a low degree of lipid peroxidation, which could result from the maintenance of high enzymatic and non-enzymatic antioxidants. It has been reported that melatonin efficiently con-

tributes to membrane integrity maintenance, and in turn, alleviates symptoms and severity of CI (Cao *et al*., 2016; Soleimani Aghdam and Rezapour Fard, 2017). Treatment with melatonin attenuated chilling induced shrinkage and disruption of carrot cell plasma (Lei *et al*., 2004).

As a whole, the results of this study show that melatonin treatment can effectively enhance chilling tolerance and reduce chilling injury of tomato fruit. The reduction in chilling injury by melatonin may be associated with enhanced enzymatic and non-enzymatic antioxidants, in favor of not only membrane integrity as well as low cellular and tissue damage, but also fruits visual and nutritional qualities. Practically, considering the economic aspect and nutritional risks of melatonin treatment, this compound may be used as an efficient bio-molecule for protecting tomato fruits encountered with chilling.

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Postharvest performance of cut rose cv. Lovely Red as affected by osmoprotectant and antitraspirant compounds

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Abstract: In cut flowers, the post-harvest turgor is a critical aspect in a system in which, in the absence of the root system, transpiration water losses must be compensated. Two experiments were conducted in order to elucidate the effect of osmoprotectants (L-Proline) as well as of molecules with antitranspirant behavior (ß-aminobutyric acid - BABA or Pinolene) on the water relations and vase life of rose cut stems. Applications of L-Proline enhanced water fluxes, water conductivity, relative water content and stomatal conductance of rose cut stems in comparison to untreated plants, thus increasing the vase-life of rose cut flowers. BABA treatment reduced the stomatal conductance in rose as well as the daily water consumption, on the other hand senescence phenomena occurred earlier. The water used by pinolene treated stems was lower compared to the control and this was associated with a medium increase of the vase life. Overall, enhanced osmoregulation prolonged the vase life of cut flowers since the improved water status allowed the cut stem to partially continue its metabolic functions. On the other hand, the control of transpirational flux was functional in maintaining cellular turgor in pinolene treated cut stems, whereas with BABA, senescence phenomena occurred probably due to the activation of biochemical pathway of senescence involving abscisic acid. Taking all together, osmoregulation or direct control of transpirational fluxes may provide a promising avenue for improving the post-harvest longevity of cut roses.

1. Introduction

Post-harvest efficiency is a crucial point of the cut flowers commercial value since it is related to growth and storage conditions interacting with the plant genetic background; those aspects will overall contribute to maximize the stems qualitative performance after cutting (Fanourakis *et al*., 2013). Cut flowers are subjected to a wide range of post-harvest losses as developmental senescence, leaf and petal abscission, leaf discoloration, premature wilting and disease from moulds and fungal pathogens (Scariot *et al*., 2014). However, among all, water balance is yet a major factor influencing the longevity of cut flowers, in a system in

which water losses must be compensated by water uptake and transport in the absence of the root system (Singh and Moore, 1992; Lu *et al*., 2010).

In particular, the post-harvest life of cut flowers is strongly dependent on their ability to maintain tissues hydration overtime, and water deficit or wilting mainly occur if the amount of transpiration exceeds the volume of water uptake (Halevy and Mayak, 1981; van Doorn, 2012).

One of the first plant responses to abiotic stress (as for cutting) is the stomatal closure, and this mechanism provides protection against tissues dehydration by reducing transpiration from the leaf surface (Hare *et al.*, 1998). However, in cut stems stomata are not completely closed after cutting, leading to a residual stomatal transpiration that together with cuticular transpiration, determine additional water losses from the stem (van Doorn, 2012). On the other hand, a phenomenon that can severely undermine the cut stems post-harvest performance, is the lowering of water uptake that is mainly due to occlusions located in the basal stem end (He *et al.*, 2006). In rose, one of the main causes of the cut flower wilting is the vascular occlusion determined by bacteria, air emboli and physiological responses to cutting (Fanourakis *et al*., 2013).

In rose cut flowers, the regulation of water balance has been in the past decades a key aspect in the improvement of the stems vase life (Alaey *et al*., 2011; Reid and Jiang, 2012). Among several mechanisms suggested that may improve water uptake in response to a stress, hydraulic conductivity variations and accumulation of compatible solutes are the most documented (Chen and Murata, 2002; Ehlert *et al.,* 2009). Compatible solutes or osmolytes are organic compounds which help in raising the osmotic pressure and thereby maintaining both the turgor pressure and the driving gradient for water uptake (Serraj and Sinclair, 2002). Common osmolytes found in plants mainly include proline, trehalose, fructan, mannitol and glycinebetaine and these compounds also help in maintaining the structural integrity of enzymes, membranes and other cellular components during the stress regime (Zhao *et al.,* 2007; Chen and Jiang, 2010). Compatible solutes may be constitutively overproduced in transgenic plants (Zhang *et al.,* 2004) or directly applied on plants to improve stress tolerance under both open-field and protected cultivation (Okuma *et al.,* 2004; Barbieri *et al.,* 2011; Cirillo *et al.,* 2016).

In addition to physiological mechanisms that can be exploited to improve water uptake, the reduction of transpirational flux has traditionally been one of the main objectives for controlling the cellular turgor after harvesting of fresh-cut vegetables and ornamentals (Prakash and Ramachandran, 2000). The reduction of transpiration can improve the water balance of cut flowers and extend their vase life, whereby artificial closure of the stomata might be an efficient strategy to reduce water losses (Lu *et al*., 2010; van Doorn, 2012).

One method of limiting water loss involves the use of antitranspirants, which reduce plant transpiration forming a vapour-impermeable film on the leaf surface and, among these, the natural terpene polymer di-1-*p*-menthene (pinolene) is widely applied on different crops (Francini *et al*., 2011; Abdel-Fattah, 2013). These polymers, also called "Film Forming antitranspirants", sprayed on crops in a form of water emulsion, are generally employed to reduce weathering and extend pesticide efficacy, improving distribution and adherence of agrochemicals and decrease water loss and wilting of young transplants (Gale and Hagan, 1996; Percival and Boyle, 2009).

Research conducted on cut roses revealed that treatments with a Film-forming antitranspirant are able to reduce the degree of fresh weight loss and water loss during transpiration, delay the process of flower opening and slow down the rate of stomatal conductance reduction (Song *et al*., 2011).

Alternatively to traditional antitranspirants, the use of compounds which may induce a series of stress adaptation mechanisms, such as stomatal closure, could also be considered. Beta-aminobutyric acid (BABA) is a non-proteinogenic amino acid known for its ability to increase plant resistance to biotic (Jakab *et al.,* 2001; Baider and Cohen, 2003) and abiotic stresses (Jakab *et al.,* 2005). Applications of BABA may improve the plant tolerance to stress by activation of defense mechanisms mediated by Abscissic Acid (ABA) and Salicilic Acid (SA) (Zimmerli *et al.,* 2000; Jakab *et al*., 2005; Baccelli and Mauch-Mani, 2016). Taking this background into consideration, it is clear that an efficient control of water balance is crucial to improve cut flowers vase-life and this can be achieved by using molecules that activate water transport in stem or inhibit transpiration. Therefore, the aim of this study was to assess the influence of exogenous applications of osmolytes such as proline or anti-transpiring solutions like β-aminobutyric acid and Pinolene on the water balance, vase-life and also to shed light on the potential

physiological mechanism(s) involved in cut stems of rose.

2. Materials and Methods

Plant material and growth conditions

Two experiments were carried out in order to assess the effects of exogenous applications of L-Proline (Experiment 1) or antitranspirants [specifically: an active compound film forming (pinolene) and a stomatal closure inducing active compound β-aminobutyric acid ; Experiment 2] on water control in cut stems of rose plants (*Rosa spp.* L.) cv. Lovely Red.

Cut flowers of rose were harvested from two years plants grown in closed soilless system in a heated greenhouse located in Naples, south Italy (40°51'55.5"N 14°20'30.1"E). Rose plants were grown in plastic channels containing pumice and lapillus. The basic nutrient solution was supplied through a drip-irrigation system at a flow rate of 2 L h^{-1}. Irrigation frequency was regularly adjusted during the growing cycle based on the crop water requirements.

At marketable harvest, cut stems were immediately transferred to the laboratory under room conditions, re-cut at the base (2-3 cm) and placed in graduated glass cylinders with 300 ml of deionized water and sodium hypochlorite (50 mg L^{-1}).

Application of compatible solutes and antitranspirants compounds

In the first experiment, two days before harvest, rose plants were treated with 10 mM L^{-1} of L-Proline (Sigma-Aldrich, Saint Louis, Missouri, USA) in 200 ml of deionized water per plant, applied in the growth substrate. Control plants were treated with deionized water only. The treatment was performed at the end of the last daily irrigation and repeated after 24 h.

In the second experiment, two days before harvest and at the end of the last daily irrigation, a substrate treatment was performed on rose plants, with of 0.5 mM L^{-1} of ß-aminobutyric acid (BABA - Sigma-Aldrich, Saint Louis, Missouri, USA) in 200 ml of deionized water per plant, whereas control consisted of plants treated with deionized water.

On a second plot of plants, the Pinolene treatment was performed in post-harvest once transferred to the laboratory. Stems were sprayed with a solution of 50 g L^{-1} of Pinolene (96% poly-1-p-menthene, NU-film, Intrachem bio, Italy) in deionized water. Control stems were sprayed with deionized water only.

Storage and physiological measurements

Part of the stems, weighted and sized based on length and diameters, were placed on ten precision balances (EK-410i, A&D Instruments Ltd, Abingdon, UK) connected via USB to a computer for automatic monitoring of weight through a specific software (RS-com®, Corby, U.K.). These cylinders were sealed with parafilm to avoid water losses through evaporation. RS-com® software was set to record three daily weights in order to determine stems water consumptions over storage. Cut stems were stored for 12 days under room conditions measuring daily mean temperature and relative humidity using a thermo-hygrometer (DO 9847K, Delta OHM Srl, Padova, Italy).

At storage days 2, 4 and 6, water flux measurements were recorded by using a Scholander pressure chamber (3005F01 Plant Water Status Console, Soil mosture Equipment Corp., Santa Barbara, California, USA). Twenty centimeter stem segments (5 cm below the calix after measuring stems diameter) were immersed into a cylinder containing distilled water, placed in the pressure chamber, while the other extremity, out of the chamber, was connected to falcon tubes to collect and weight the water efflux. The system was then subjected to increasing pressure (P= 0.05, 0.1, 0.2, 0.3 MPa) and maintained at each pressure value for 5 minutes up to a constant outflow from the stem.

Water flux (Jv) was expressed as Jv= kg H_2O m^{-2} s^{-1} m^{-1}. Water conductivity (Lp) of stems was then expressed by the slope of the regression function of Jv vs. P. Volumes of collected efflux per unit of time (Jv) were normalized to the cutting section surface.

Stomatal conductance (gs) was determined at storage days 2, 4 and 6 using a diffusion porometer (Delta P-4, Delta-T Devices, Cambridge, U.K.) in three daily measurements (h 9:00 AM; h 1:00 PM; h 7:00 PM). Osmotic Potential ($\Psi\pi$) was measured using a dew-point psychrometer (WP4, Decagon Devices, Washington) on frozen/thawed leaf samples. Relative Water Content (RWC) value was calculated as: RWC= (leaf fresh weight - leaf dry weight)/(leaf saturated weight - leaf dry weight) (Morgan, 1984). Leaf area was estimated by scanning cut stems leaves and using the *Image J®* software (Abramoff *et al.,* 2004) for image processing. The cut stems vase life was assessed visually using a "quality score" from 0 to 4.

Statistical analysis

All data were statistically analyzed by ANOVA using the SPSS software package (SPSS 10 for Windows, 2001). The RWC data were transformed in

arc-sin before ANOVA analysis.

3. Results

Experiment 1. Effect of L-Proline application on postharvest performance of cut rose

Exogenous applications of L-Proline enhanced significantly water fluxes of rose cut stems in comparison to the untreated control, for all the 3 days of measurements (Fig. 1A). During storage, water flux decreased from day 2 to day 6 in treated stems (Fig. 1A). Moreover, stems water conductivity (Lp) was 3.05 [(kg H_2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in control and 3.55 [(kg H_2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in L-Proline treatment (Table 1).

Significant Increase in stomatal conductance (gs) as well as in RWC were observed in L-Proline treatment compared to the control (Table 1). Similarly to the physiological measurements, water consumptions normalized per leaf area were higher in treated stems (Fig. 1B). As a result of cellular osmotic adjustment due to L-Proline application, leaf osmotic potential was lower for treated stems in comparison to untreated control (Table 1). The improved water status of L-Proline treated stems influenced positively cut stems longevity extending their vase life by 2 days compared to the untreated control.

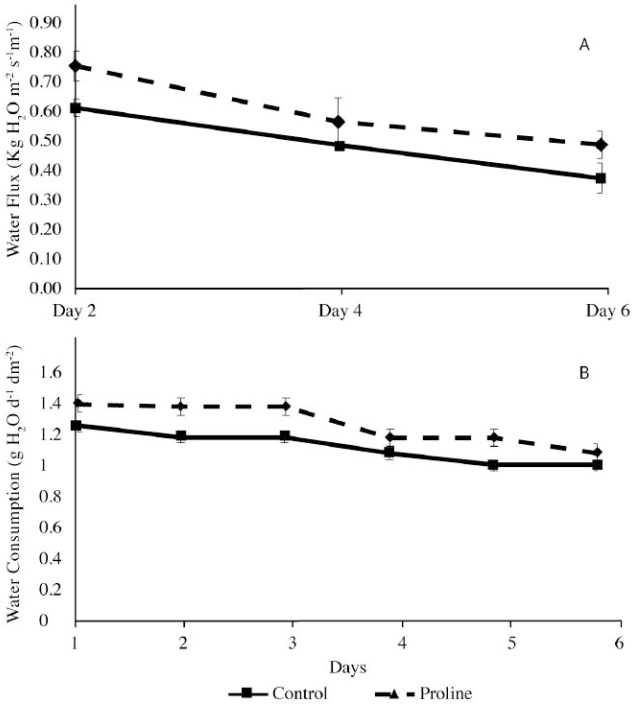

Fig. 1 - Effect of exogenous L-Proline on water flux (A) and water consumptions per leaf area (B) of rose cut stems during storage. Vertical bars indicate ± SE of means.

Table 1 - Effect of exogenous L-Proline on stomatal conductance (gs), relative water content (RWC), osmotic potential (Ψπ) and water conductivity (Lp) of rose cut stems

Treatment	gs (cm s^{-1})	RWC (%)	Ψπ (MPa)	Lp (Kg H_2O $m^{-2}s^{-1}$ m^{-1})
Control	0.38 b	84 b	-0.17 b	3.05 b
Proline	0.42 a	89 a	-0.34 a	3.55 a
Significance	*	*	*	*

NS,*, not significant or significant at P≤0.05 respectively. Within each column, different letters indicate significant differences.

Experiment 2. Effect of antitraspirants application on postharvest performance of cut rose

In our current study, stomatal conductance was reduced by BABA treatment respect to the control (Table 2). Consequently, daily water consumption, normalized per leaf area, was lower for BABA treated stems (Fig. 2B). The application of 0.5 mM of BABA significantly reduced water fluxes during storage (Fig. 2A) as well as the water conductivity (Lp) of rose cut stems (2.6 [(kg H_2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in control *vs.* 1.54 [(kg H_2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in BABA treatment). RWC was significantly higher in the control and it decreased during storage (Table 2). Furthermore, the water potential decreased over time and it was lower for rose stems treated with BABA (Table 2). The vase life, however, was not influenced by the treatment since in BABA treated stems the improvement of the water balance was accompanied by premature yellowing of the leaves. As a consequence of the mechanical stomatal closure, the water use of pinolene treated stems was always lower compared to control (Fig. 3). This was associated with a significant increase of the vase life by 1.5 days (Fig. 4).

Table 2 - Effect of exogenous β-aminobutyric acid (BABA) on stomatal conductance (gs), relative water content (RWC), osmotic potential (Ψπ) and water conductivity (Lp) of rose cut stems

Treatment	gs (cm s^{-1})	RWC (%)	Ψπ (MPa)	Lp (Kg H_2O m^{-2} s^{-1} m^{-1})
Control	0.41 a	79 a	-0,17	2.60
BABA	0.25 b	74 b	-0,27	1.54
Significance	*	*	*	*

NS,*, not significant or significant at P≤0.05 respectively. Within each column, different letters indicate significant differences.

4. Discussion and Conclusions

It is well established that osmotic adjustment contributes to maintain water uptake and cellular turgor (Maggio *et al.*, 2002; Heuer, 2003). Among all the osmolytes involved in this process, it has been sug-

gested that proline, exogenously applied via foliar spraying or through the irrigation water, could localize into the cytoplasm to reduce the cellular osmotic potential and to restore cellular hydration (Gadallah, 1999; Barbieri *et al.,* 2011). In our experiment, L-Proline treatment on rose plants has shown to substantially improve the hydration state of the cut stems, with an observed decrease of the leaf osmotic potential and increased stomatal conductance and leaf RWC. Consequently, the improved hydration state of tissues and stomatal conductance enhanced the water consumption in plants treated with L-Proline.

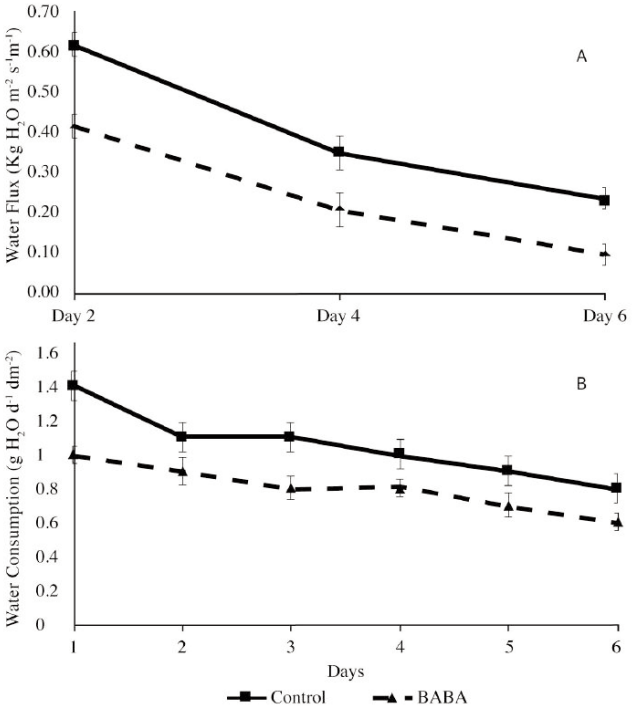

Fig. 2 - Effect of exogenous β-aminobutyric acid (BABA) on water flux (A) and water consumptions per leaf area (B) of rose cut stems during storage. Vertical bars indicate ± SE of means.

The decline in stem water conductivity, is one of the main reasons for impaired water balance, as well as water stress is the most common reason for reduced cut roses vase life (Halevy, 1976; Joyce and Jones, 1992). The increase in water fluxes and water conductivity for the L-Proline treated flowers was an evidence of improved water status of the plant tissues that probably was the main factor involved in the extended vase life of L-Proline treated stems. It has been demonstrated that, in cut flowers, osmolytes are fundamental compounds in maintaining water balance, a key factor to extend their longevity (Ichimura *et al*., 1997) as well as accumulation of these solutes, such as Proline, is one of the main mechanisms to alleviate the detrimental effects of dehydration (Morgan, 1984; Anjum *et al*., 2011). In fact, the role of osmolytes includes mainly protection against the deleterious effects of the low water activity, preserving appropriate cellular volume (Csonka and Hanson, 1991).

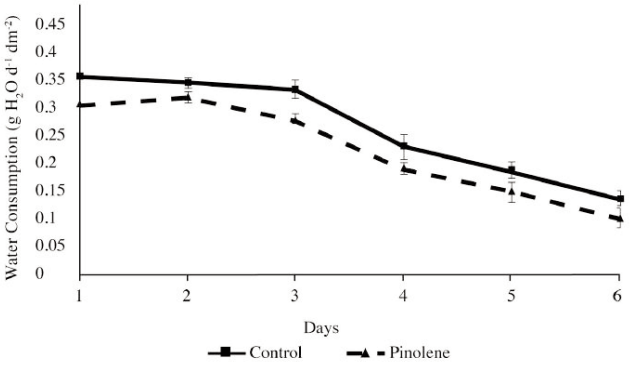

Fig. 3 - Effect of Pinolene on water consumptions per leaf area of rose cut stems during storage. Vertical bars indicate ± SE of means.

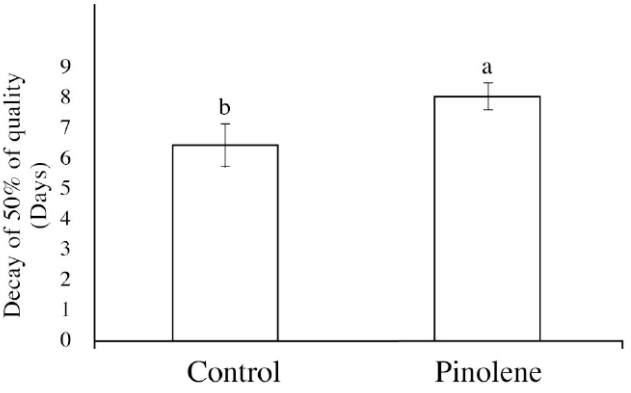

Fig. 4 - Effect of Pinolene on the vase life of rose cut stems expressed as decay of 50% of the stems quality. Different letters indicate significant differences at P≤0.05, vertical bars indicate ± SE of means.

However, even though it is not yet clear if an extension of the cut flowers vase life may be more related to the ability of stem to maintain sustained water uptake rates or to control water losses, the control of the stomatal conductance is a fundamental determinant of the tissue water balance (Fuchs and Livingston, 1996; Woodward *et al.*, 2002). In nature, it is well known that plants control water losses by regulating transpiration in response to environmental factors (Chaerle *et al*., 2005). In some respect, cut flowers respond to the same stimuli and the difference between the rate of water uptake and the tran-

spiration rate is one of the parameters that will define their hydration state.

As documented in different cases, BABA acts through potentiation of ABA-dependent signaling pathways (Ton and Mauch-Mani, 2004) and for this reason, we supposed that applications of ß-aminobutyric acid (BABA) may increase the tolerance to water shortage through the induction of functions associated to the synthesis of ABA, such as stomatal closure (Jakab *et al.,* 2001; Desikan *et al.,* 2004).

Applied on rose plants, BABA treatment has induced a decrease in stomatal conductance, with the consequent reduction of the stems water consumptions. Along with this decrease of the transpirational flux, water fluxes and RWC decreased over time and they were generally lower in BABA treated stems. In addition, Lp was lower in BABA-treated stems. The decreased RWC and water potential, together with a reduction of the cut stems hydration state, may be associated to senescence phenomena that occurred with the premature yellowing of the leaves, which is also mediated by ABA (Hunter *et al.,* 2004; Ferrante *et al.,* 2006). Accordingly, Mayak and Halevy (1972) reported that exogenous application of ABA to rose cut flowers accelerate senescence phenomena.

Another strategy to control the plants transpiration and sustain a favorable plant water status is the utilization of antitranspirants compounds (Del Amor and Rubio, 2009). Our results confirmed that film-forming antitranpirants are effective in reducing water losses providing a thin coating on the leaves surfaces leading to an improved tissues water status in cut roses (Moftah and Al-Humaid, 2005; Song *et al.*, 2011; Mikiciuk *et al.*, 2015).

Consistently, this mechanical effect on the transpirational flux regulation was observed on water consumptions normalized per leaf area rose cut stems, that were significantly reduced by pinolene application during the vase life. The reduced water use was correlated to an extended vase life compared to the water-treated control.

In conclusion our results demonstrated that both osmoregulation and direct transpirational control were effective strategies in maintaining an enhanced hydration state of rose cut stems, leading to a prolongation of the stems vase life. Treatment with 10 mM L-Proline has allowed the maintenance of higher stomatal aperture and improved cut stems RWC and Lp during storage. The positive effects on cut stems were measured as decrease of osmotic potential and increased stomatal aperture consequent to osmoregulation. These physiological conditions are crucial for prolonging the vase life of cut flowers because, despite the absence of the root system, allow the stem to partially continue its metabolic functions.

On the other hand, the reduction of transpiration that is considered a functional target for controlling cellular turgor after harvest thus prolonging cut flowers. Since ABA is involved in the induction of physiological mechanisms that facilitate adaptation to abiotic stress, it has been hypothesized that the administration of BABA, a mediator of ABA functions, may confer a stress protection that could result in enhanced turgor and vase life of cut stems. In fact, our results also demonstrated that applications of 0.5 mM BABA on rose has reduced water consumption by inducing stomatal closure. However, this was associated with a more rapid decay of the cut stems quality probably for earlier oncoming of senescence phenomena. Furthermore, pinolene treatment prolonged the vase life of cut stems, by reducing water losses through transpiration. This was likely due to the formation of a 'film' at the leaf surface that acts as a physical barrier to gas exchanges.

Taking all together, we can conclude that osmoregulation or direct control of traspirational fluxes may provide a promising avenue for improving the post-harvest longevity of cut roses. However, further investigations are required whenever other physio-chemical processes are involved such as the induction senescence phenomena.

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Shelf life of iceberg lettuce affected by hydro cooling and temperature of storage

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Key words: carbohydrates, chlorophyll, cooling curve, *Lactuca sativa* L., relative water content, weight loss.

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Abstract: Pre cooling is applied to remove the field heat of harvested horticultural produces. The goal of this work was to determine the cooling curve and the effects of hydro cooling on quality and shelf life of iceberg lettuce 'Lucy Brown' stored at 5°C and 22°C. Through shelf life, it was determined the changes on accumulated fresh weight loss, leaf relative water content and total chlorophyll, total soluble sugars, reducing and non-reducing sugars, and starch. The field heat from iceberg lettuce heads was removed within the first 10 min when submerged into cooled water at 4°C. Hydro cooled lettuce heads accumulated water over the leaf surfaces resulting in higher rate of fresh weight loss during storage when compared to control. Lettuce stored at 5°C kept higher relative water content in the leaves throughout the shelf life. Hydro cooling treatment delayed the wilting of the external leaves in three and two days when stored at 5 and 22°C, respectively. Hydro cooling did not influence the decrease on total soluble sugars, reducing sugars, non-reducing sugars and starch throughout shelf life, but affected the leaf chlorophyll content. Independent of the temperature in which the 'Lucy Brown' iceberg lettuce will be stored, hydro cooling is recommended to prolong quality and shelf life.

1. Introduction

Senescence is a natural process common to all fresh vegetables, which is intensified after harvest, by handling and storage conditions. In addition, the rate of deterioration is quickly intensified if a vegetable or a fruit is stored under stressed conditions. Storing fresh horticultural products under extremes of high temperature or under low relative humidity, results in intense water loss, triggers senescence and finally the death of the tissues.

The major important factor affecting the postharvest shelf life for most of fresh horticultural products is the temperature during storage or display. To preserve the quality and prolong the perishables commercial quality, it is necessary to rapidly remove the field heat to an optimum temperature for subsequent storage (Brosnan and Sun, 2001). Independent of the pre cooling method to be used, the premises as an useful postharvest practice, is based on the quick reduction of product temperature. In addition, it is recommendable to reduce the temperature as soon as possible after harvest, which will increase the beneficial effects of the rapid cooling. Among the many benefits of pre cooling, in keeping the quality of a produce, is the lower respiration rate, the reduction of water loss by the product, and less contamination by pathogenic microorganisms (Brosnan and Sun, 2001).

The rapid loss of quality and limited shelf life of leafy vegetables, like lettuce, parsley and jute leaves is mainly due to their fast postharvest dehydration (Tulio Jr. *et al*., 2002; Finger *et al*., 2008; Aguero *et al*., 2011). In these products wilting of leaves occur even faster when the storage is done under high storage temperatures or without any refrigeration and inadequate packaging.

There is a variety of pre cooling techniques available including cooling rooms, hydro cooling systems, air forced cooling, ice packaging, vacuum and cryogenic cooling (Brosnan and Sun, 2001). Hydro cooling is relatively inexpensive and very effective method recommended to remove the field heat of several leafy vegetables including kale, green onions and spinach (Sargent *et al*., 2007). Álvares *et al.* (2007) determined that hydro cooled parsley leaves had less water loss, resulting in longer shelf life without the appearance of wilting symptoms compared to control. Hydro cooling also proved to be a faster method for cooling peach pulp to 1°C compared to forced air and conventional cooling room methods (Brackmann *et al*., 2009).

The expansion of large cities in developing are pushing the vegetable farms farther away from the markets, making harder for them to deliver products with good quality to urban population. This situation demands the incorporation of appropriated postharvest handling, but most of the small farmers have no capital to purchase refrigeration systems. Iceberg lettuce is the most popular leaf vegetable used in burgers, sandwiches and salads by fast food stores in most of the countries, including Brazil. Thus, there is the need to evaluate the influence of pre cooling on the shelf life of this lettuce. Therefore, the objective of this work was to determine the cooling curve and the effects of hydro cooling on quality and shelf life of iceberg lettuce heads stored under cold and room temperature conditions.

2. Materials and Methods

Heads of iceberg lettuce 'Lucy Brown' were harvested from the field at Federal University of Viçosa (642 m asl, 20°45' lat. S and 42°51' long. W) in the morning between 7 and 7:30 hours. The heads of lettuce were taken to the laboratory quickly and the heads with external leaves with brown spots, wilted, or dirty leaves were discarded. The lettuce heads weighting between 300 to 400 g were subjected to the following treatments: 1) Hydro cooling followed by storage at 5°C; 2) Control without hydro cooling and storage at 5°C; 3) Hydro cooling followed by storage at 22°C; 4) Control without hydro cooling and storage at 22°C. Hydro cooling was performed by submerging the heads in a mixture of tap water and crushed ice at proportion of 3:1 (v:v) kept at 4°C. Temperature of lettuce heads was determined at every five minutes with the help of a digital infrared thermometer. The temperature of the heads before initiating the hydro cooling treatment was between 20 to 22°C. At every 5 minutes, two heads were quickly removed from the cold water to determine the changes in the temperature, repeating the procedure up to fifty minutes. At the end of hydro cooling, the heads were removed from the cold water and allow draining for 5 min in the air before storage in the plastic boxes. Hydro cooled and control lettuces were kept in plastic boxes at 5 and 22°C for the whole experiment. The boxes (18 cm height, 25 cm wide × 48 cm length) were covered with perforated (12 holes 1.1 cm in diameter) low density polyethylene plastic sheets to protect from excessive dehydration. The relative humidity inside the boxes was always between 85 and 90%.

Loss of fresh weight of heads, leaves relative water content, chlorophyll, total soluble sugar, reducing sugar, non-reducing sugar and starch leaf contents were determined at every 12 h up to the first 48 h and then at every 24 h until the end of the lettuce shelf life.

The end of shelf life was determined when the heads were wilted, yellowed or with signs of deterio-

ration, being unfit for commercialization. The wilting, yellowing or deterioration of 50% or more heads was used as the discard parameter.

The accumulated loss of fresh weight was obtained in relation to initial fresh mass of heads and during storage period. The leaf relative water content (RWC) was determined as described previously by Álvares *et al.* (2007) with modifications. Fifteen leaf discs with 1.1 cm in diameter were removed from the external, middle and internal position in the lettuce head, which were kept between two layers of wet sponge sheets until to obtain the leaf turgid fresh weight and then, they were oven dried at 70°C to obtain the total dry mass. The fresh weight of the disc, the turgid weight and the dry weight were used to estimate the RWC according to the formula established by Barr and Weatherley (1962).

Total chlorophyll content was determined in a combine sample of leaf discs removed from the external, middle and internal position in the head, following the method described by Inskeep and Bloom (1985) using 5,5-dimethylformamide as extractor. The absorbance of the filtrate was determined in a spectrophotometer at 647 and 664.5 nm and the results expressed in μg cm^{-2}.

Samples of five grams of leaves from the external, middle and internal position of the lettuce head were homogenized in 80% hot ethanol and centrifuged at 2000 rpm for 15 min. The pellet was then re-extracted twice with 80% ethanol, and the total soluble sugars were determined by the phenol-sulfuric acid reaction (Dubois *et al*., 1956). From the same extract was determined the reducing sugars content by the Somogyi-Nelson method (Nelson, 1944). For total soluble sugars analysis sucrose was used as standard and glucose for the reducing sugar analysis. Afterwards, the pellet from the ethanolic extraction was dried at 65°C and then the starch was hydrolyzed in 52% perchloric acid for 30 min with shaking (McCready *et al*., 1950). The procedure was repeated three more times. The quantification of starch was performed by the phenol-sulfuric acid reaction using sucrose as standard using the correction factor of 0.9. The total of non-reducing sugars was obtained by the difference between the total soluble sugars minus the content of reducing sugars.

The experiment was conducted in a split-plot scheme, with the treatments in the plots and shelf life in the subplots in a randomized block design with four treatments and four replicates per treatment. Each replicate was composed by one lettuce head. Individual analysis of variance was performed to evaluate the effect of the hydro cooling and temperature of storage by using the SAEG/UFV software, and the mean separation was done by Scott Knott test at 5% probability. The regression analysis was based on the regression coefficient using the t-test at 5% or 10% probability to establish the significance for the chosen regression model.

3. Results and Discussion

Initial temperature of the lettuce head showed a sharp drop within the first 10 min of hydro cooling time. The model that better explained the changes in temperature was exponential, with an estimated final temperature of 4.8°C after 10 min of hydro cooling (Fig. 1). Longer periods of cooling time did not remove additional field heat from the lettuce head. Based on the lettuce temperature record data, the total amount of heat removed from the lettuce, by the cold water under this experiment conditions, corresponded to 71% from the initial temperature (Fig. 1). The 87.5% or seven eighths cooling times recognized as the ideal theoretical reduction for the field heat presented by Brosnan and Sun (2001) was not achieved in this experiment, even after keeping the heads submerged in the cold water mixture for 50 min (Fig. 1). Using the same cooling technique of this experiment, Álvares *et al*. (2007) reported the removal of only 43% of the initial temperature in bunched parsley leaves. The reason why the hydro cooling of lettuce was much more efficient in removing the field heat than parsley remains to be the subject of further studies. Furthermore, the cooling time varied according to varieties of lettuce, as found by França *et al*. (2015) working with butter lettuce where the ideal hydro cooling time was 5 min instead

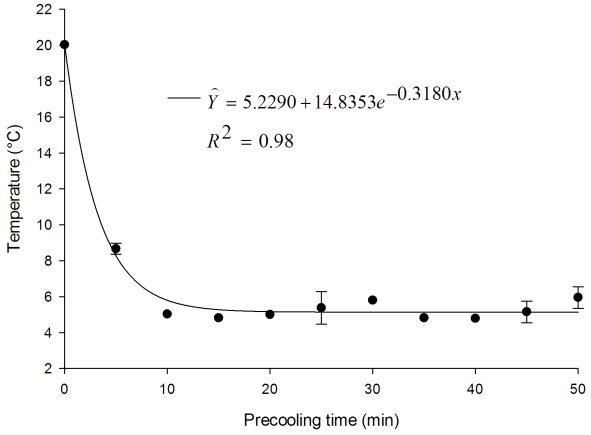

Fig. 1 - Influence of hydro cooling time period treatment on the temperature of iceberg lettuce 'Lucy Brown'.

of 10 min found for iceberg lettuce in this work (Fig. 1). The difference of hydro cooling time between the two cultivars of lettuce may relay on the thickness and compactness of the whole head. The leaves from iceberg lettuce are thicker and more compact head than butter lettuce, which can restrict the access of cold water into the more internal leaves of the head. In others leaf vegetables, including peppermint, coriander and basil, the ideal time of hydro cooling was 20, 10 and 16 min, respectively (Oliveira *et al*., 2015; Barbosa *et al*., 2016; Teixeira *et al*., 2016).

The end of shelf was established when symptoms of wilting and discoloration appeared in the external leaves (data not shown). Shelf life of hydro cooled lettuce and stored at 5°C was increased by 75% compared to not cooled lettuce, comprising a total of 168 h for the hydro cooled and 96 h for not cooled heads. For the lettuce that was hydro cooled and then stored at 22°C, the gain of shelf life was 50% or 72 h for hydro cooled and 48 h for not cooled lettuce. Álvares *et al*. (2007) also found beneficial effects to the shelf life of hydro cooled bunched parsley leaves followed by cold storage. The result of this study shows the importance of keeping the cold chain for fresh vegetables, but also shows the contribution of hydro cooling on extending the shelf life even without further cold storage. Because of the hydro cooling positive effects, the external leaves of the lettuce had a 72 h delay in showing wilt symptoms if stored at 5°C and 24 h delay for the lettuce stored at 22°C (data not shown). The increased shelf life of hydro cooled lettuce was determined by the higher leaf relative water content of hydro cooled lettuce (Table 1). The smaller effect of hydro cooling in the lettuce kept at 22°C compared to 5°C may be due to the greater gradient of water vapor between the leaf surface and the atmosphere of storage at 22°C. But, in a similar experiment with butter lettuce, the beneficial effect of hydro cooling on shelf life was greater for the lettuce stored at 22°C compared to the shelf life of hydro cooled heads followed by storage at 5°C (França *et al*., 2015).

Table 1 - Influence of the hydro cooling and temperature of storage on the leaf relative water content during storage period of iceberg lettuce heads 'Lucy Brown'

Treatments	RWC (%)
Hydrocooled + storage at 5°C	96.4 A
Storage at 5°C	92.8 B
Hydrocooled + storage at 22°C	94.0 B
Storage at 22°C	92.4 B
CV %	3.2

Means followed by the same letter do not differ by the Scott Knott test at 5% probability.

Regardless the treatment, the rate of fresh weight loss was constant, resulting in linear accumulation up to the end of the lettuce shelf life (Fig. 2). The lowest rate of weight loss was determined in the lettuce stored at 5°C without hydro cooling (0.109% h^{-1}) and the highest for the heads hydro cooled and stored at 22°C (0.26% h^{-1}), as previously observed in a similar experiment with butter lettuce by França *et al*. (2015). The higher weight loss rate of hydro cooled lettuce was due to the water accumulated at surface and in between the leaves after being removed from the cooled water. Regardless if the lettuce was hydro cooled or not, the lower rates of weight loss found for heads stored at 5°C was determined by the smaller gradient of water vapor compared to the storage room at 22°C (Wills *et al*., 2010). Like in this experiment, when coriander leaves were stored at 5°C also had lower rates of weight loss compared to leaves stored at 20°C, regardless if the leaves were submitted to hydro cooling treatment (Oliveira *et al*., 2015). During the whole period of storage, the leaves of hydro cooled lettuce had higher relative water content when stored at 5°C (Table 1). This higher content of water found in the hydro cooled lettuce leaves was clearly observed in the appearance of lettuces, which were more turgid than those not hydro cooled, which resulted in longer shelf life due to fresher appearance. Similar results were found for hydro cooled butter lettuce heads, peppermint and coriander leaves, which also had higher relative

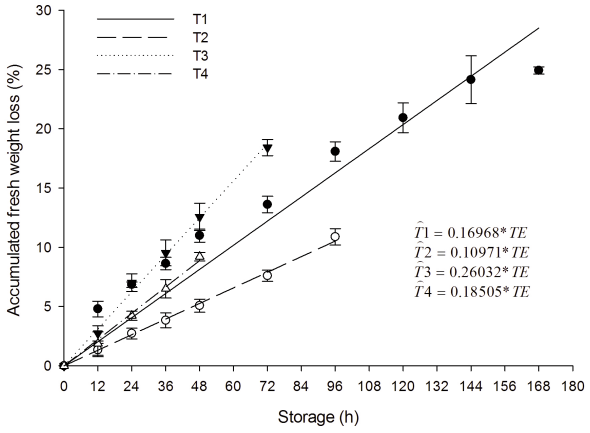

Fig. 2 - Accumulated fresh weight loss of iceberg lettuce 'Lucy Brown' submitted to the following treatments: T1) Hydro cooling for 10 min + storage at 5°C; T2) Control without hydro cooling + storage at 5°C; T3) Hydro cooling for 10 min + storage at 22°C; T4) Control without hydro cooling + storage at 22°C. TE= Time.

water content during their shelf life (França *et al.*, 2015; Oliveira *et al.*, 2015; Barbosa *et al.*, 2016). In conclusion, the hydro cooling treatment and storage 5°C resulted in higher level of water compared to not cooled heads, indicating the importance of field heat removal and followed by continuous cold chain.

Although hydro cooling reduced significantly the leaf chlorophyll content throughout storage for the lettuce stored at 5°C compared to the remaining treatments (Table 2), and the coefficient of variation was high (35.7%). This reflects the positions internal, middle and external which the leaf samples were taken. Because the relative water content present in the leaves of the hydro cooled lettuce stored at 5°C was higher, a much more favorable water status existed during storage. And at the same time, for the other treatments, the lower relative water content reflects a bigger dehydration rate of the cells, which resulted in higher chlorophyll concentration in the leaves (Table 1). But, the same effect on chlorophyll content induced by hydro cooling was not present on parsley and ora-pro-nobis leaves (Álvares *et al*., 2007; Barbosa *et al*., 2015). These differences may be related to the lower trend of parsley and ora-pro-nobis leaves in loosing water from the cell to the environment during storage.

Table 2 - Influence of the hydro cooling and temperature of storage on the total chlorophyll content during storage period of iceberg lettuce 'Lucy Brown'

Treatments	Total chlorophyll (μg cm^{-2})
Hydrocooled + storage at 5°C	6.00 B
Storage at 5°C	7.74 A
Hydrocooled + storage at 22°C	7.69 A
Storage at 22°C	7.81 A
CV (%)	35.7

Means followed by the same letter do not differ by the Scott Knott test at 5% probability.

Hydro cooling had no effect on leaf carbohydrate changes during storage. However, there was significant decrease in the total soluble, reducing, non-reducing sugars and starch contents in the first 12 h of storage, either at 5 or 22°C (Table 3). In the first few hours after harvest, a much greater amount of carbohydrate is required to maintain high respiratory demand, coinciding with the highest physiological activity (Wills *et al*., 2010). In the study, during the first 48 h of storage there was a drop of 37.2, 24.5, 52.1 and 23.7% in the total soluble sugars, reducing, non reducing sugars and starch, respectively (Table 3). In a similar work, França *et al.* (2015) found similar decreases on non-reducing sugars and starch content of butter lettuce on the first 12 hours, but not on reducing sugars. The reduction of all carbohydrates found in this work, reflects the high demand of glucose and fructose to keep the respiratory activity even at low temperature of 5°C. Since, leafy vegetables do not store large amounts of carbohydrates; their storage potential for longer shelf life is much smaller than tubers and fruits, which have large amount of stored carbohydrates. Thus, further work with the use of controlled and modified atmosphere should be applied to increase iceberg lettuce shelf life.

Table 3 - General mean values of total soluble sugars (TSS), reducing sugars (RS), non-reducing sugars (NRS) and starch contents in 'Lucy Brown' icerberg lettuce stored at 5 or 22°C during the first 48 h of shelf life storage

Time (h)	TSS*	RS *	NRS *	Starch **
0	2.72 A	1.73 A	1.25 A	3.36 A
12	1.84 B	1.12 B	0.71 B	2.65 B
24	1.82 B	1.21 B	0.60 B	2.55 B
36	1.62 B	1.06 B	0.55 B	2.32 B
48	1.71 B	1.11 B	0.59 B	2.57 B
CV (%)	24.79	25.71	57.92	19.33

Means followed by the same letter do not differ by the Scott Knott test at 5% probability.

4. Conclusions

Regardless the temperature of storage, application of hydro cooling treatment removed most of the field heat with beneficial effects on quality, prolonging the shelf life of 'Lucy Brown' iceberg lettuce, by keeping higher water status in the cells and reducing discolorations in the leaves. Hydro cooling had no influence on carbohydrate metabolism of the leaves throughout storage either 5 or 22°C.

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Evaluation of an alternative mean for controlling postharvest *Rhizopus* rot of strawberries

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Abstract: Crude extract of the brown seaweed *Laminaria digitata* was tested as an alternative mean to control postharvest *Rhizopus* rot of strawberries. The antifungal activity of four extracts (one un-fractionated and three soluble by hexane, ethanol, and water) was *in vitro* measured against one pathogenic isolate of *Rhizopus stolonifer* at a concentration range from 10 g L^{-1} to 30 g L^{-1}. The antifungal activity of the un-fractionated extract was *in vivo* measured into a climatic room at the same concentration range in comparison to fungicide Fenhexamid in preventive and curative treatments. The peroxidase activity in *L. digitata*-treated inoculated strawberries was performed. A significant inhibition of mycelia growth applying 30 g L^{-1} of un-fractionated extract (until 80% after 5 days) and suppression of sporangia germination (until 95% after 24 hours) were found by a dose-dependent manner of the treatment. Only the extracts fractionated by hexane and ethanol were likewise suppressive at the same concentrations against mycelia (until 71% and 66% respectively) and sporangia (until 82% and 69% respectively) such involving a direct toxicity induced by lipids and phenolic compounds to *R. stolonifer* suppression. Fruit decay inhibition of the *R. stolonifer*/strawberries pathosystem increased from 10 g L^{-1} to 30 g L^{-1} until 75% after 4 days only in preventive treatment. An increased peroxidase activity (4.84 Δ_{OD420} g^{-1} min^{-1}) seen in fruit tissue after one-day from the application of 30 g $^{-1}$ raw extract suggests that *in vivo* suppression could also be related to induced systemic resistance phenomena.

1. Introduction

Strawberry fruits are a perishable commodity due to industrial processing chain that occurs immediately after harvesting which causes mechanical injury, desiccation, physiological disorders, deterioration of quality and nutrient composition, decaying, abiotic stress, mycotoxin contamination, and reduction of their market value (De Cicco *et al*., 2008). The known fungi that are considered responsible of strawberry fruit

postharvest decay are *Botrytis cinerea* Pers. *ex* Fr. and *Colletotrichum* spp., the causal agents of gray mold and anthracnose respectively; *Penicillium* spp., the agent of green and bleu mold; *Rhizopus* spp., *Mucor* spp., and *Alternaria alternata* f. sp. *fragariae*, the main agents of rots (Husaini and Neri, 2016). Among the most important postharvest decay agents that occur in strawberries, the soft rot caused by the zygomycete fungus *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. induces economically severe losses both in the field and overall during long-distance transport, especially if storage temperature was more than 4-5°C and when strawberries are damaged during handling (Maas, 1998). Control of postharvest decay on strawberry (*Fragaria* × *ananassa* Duch.) fruit can be usually achieved by physical, chemical and biological methods (Ippolito *et al*., 1997; Droby *et al.,* 2009). Requirements from consumers for fruit free by chemical residues from synthetic fungicides have recently stimulated the researchers (Mari *et al.*, 2016) to find safer alternatives for environmental and human health based on the wide range of natural antifungal compounds, as phenolic substances given from wild edible herbs (Gatto *et al*., 2011, 2013), and terpenic compounds provided from aromatic plants (De Corato *et al*., 2010).

In this context, seaweed extract can stimulate growth in strawberries by protecting them from pathogens and physiological hazards under storage condition (Tuhy *et al*., 2013). Crude extract from brown seaweed contains a wide range of antifungal substances, mainly lipids (triglycerides), phenolic compounds (phlorotannins) and water-soluble polysaccharides (laminarans, fucoidans, and alginates), which were investigated for their antifungal properties in preventing postharvest fruit losses caused by plant pathogenic fungi (Washington *et al*., 1999). Recently, the use of crude extracts from brown and red seaweeds obtained by a supercritical carbon dioxide technique in preventing the severe fruit postharvest losses caused by several plant pathogenic fungi, has been investigated (De Corato *et al*., 2017). In addition to that, an excellent paper provides an overview of the most recent findings on the potential use of extracts from macroalgae for strawberry management, concerning both their biostimulant effects and antifungal properties against postharvest pathogens (Righini *et al.*, 2018). From this review, it is clear that very few papers about the practical use of algal extract as an effective alternative strategy to syntetic fungicides, such as Fenhexamid, for controlling postharvest strawberries rot by *R. stolonifer* are reported in literature, because this fungus is really most invasive and it shows a faster development of aerial mycelia in comparison to other fungi afore-mentioned, especially if storage temperature varies from 6°C to 10°C.

The purpose of this work was to assess a potential use of crude extract from the brown seaweed *Laminaria digitata* (Huds.) Lamouroux for effectively controlling strawberry fruit rot caused by *R. stolonifer* under postharvest condition. The first objective was to investigate the potential suppressive, under *in vitro* and *in vivo* conditions, of crude extracts of *L. digitata* in preventing postharvest losses of strawberries caused by infections of *R. stolonifer* which are of greater importance in the Italian markets. The second objective was to clarify the most probable hypothesis about the mechanisms explaining the suppressive effect observed. To reach these two objectives, the direct antifungal activity of four extracts (one un-fractionated and three fractionated by hexane, ethanol, and water) was *in vitro*, measured by a microbiological method within an increasing concentration range from 10 g L^{-1} to 30 g L^{-1}. The antifungal activity of the raw extract was *in vivo*, measured at the same concentration range by a phytopathological method into a climatic room in comparison to Fenhexamid during preventive and curative treatments. Finally, the peroxidase (POD) activity in *L. digitata*-treated inoculated strawberries was assessed by an enzymatic method to investigate an indirect antifungal activity of the raw extract by induced systemic resistance mechanisms.

2. Materials and Methods

Experimental trials

An amount of about 80 Kg of fresh algal biomass, collected from healthy and matured *L. digitata* cultures performed into a photo-bioreactor of 900 l capacity, was purchased from a marine biorefinery located near the coastal areas of Gibraltar and Morocco. Algal biomass was immediately refrigerated after harvesting thoroughly washed with seawater, and washed with tap water to remove all extraneous particles and epiphyte organisms. Fresh biomass was dried into an industrial drier located at ENEA - Trisaia Research Centre (Policoro, Matera, Italy) - chopped, finely pulverized, heat-treated for 24 hours with sodium hydroxide (1:10 w/w) for

triglycerides saponification, weighed, and stored at 4°C until extraction of biologically active substances. The extraction was carried out by using a mixture of un-polar and polar solvents in appropriate proportions (hexane:ethanol:water, 1:2:2 v/v) into a Clevenger apparatus. Extraction was repeated three times at room temperature, and total processing time was about 4-5 hours (Selvi *et al*., 2014). Stocks of raw crude extract were collected, suspended in methanol, collected into bottles, and stored at 4°C in the dark until tested. Crude seaweed extract yield was calculated on three independent replicated samples of 50 g each after drying at 55±5°C for 2 hours, and expressed as a percentage on the basis of fresh biomass weight. Stocks of crude seaweed extract were dried, weighed, and stored at -20°C in the dark till further uses. Aliquots of raw extract were fractionated into three fractions using three solvents with a different affinity towards fatty acids, phenolic compounds and water-soluble polysaccharides (Khanzada *et al*., 2007). Samples of 50 g of dry extract were suspended in n-hexane, or distilled water, or pure ethanol (1:5 w/v) in separating funnel for 20 days at room temperature to separate lipids, polysaccharides and phenolic substances, respectively from the crude extract. Suspensions were then filtered using Whatman filter paper and concentrated under reduced pressure at 35°C using a rotary evaporator (Strike 202, Steroglass, Perugia, Italy) till the extract become as a syrup. Three different fractions were finally separated from this residue. Each fraction was individually collected from the respective funnel, and each stock was air-dried, weighed, and stored at -20°C in the dark till *in vitro* and *in vivo* assayed by using a certified pathogenic strain of *R. stolonifer* (provided by the Collection of Microorganisms and Cell Cultures Institute, DSMZ, Braunschweig, Germany) causing severe rots on strawberries under postharvest condition.

Stocks of total extract, or fraction of it, suspended in sterile 0.1 M K-phosphate buffer were *in vitro* tested at the concentration range of 10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1} for determining a minimum bioactive concentration by poison food technique (Shahi *et al*., 1999). Mycelia inhibition was quantitatively assessed measuring radial growth in Petri plates (100 mm diameter) containing Potato Dextrose Agar (PDA, Sigma-Aldrich, Milan, Italy) adding 18 ml PDA per plate. In treated plates, aliquots of 2 ml of sterile stock suspension containing extract (both raw and fractionated) were added to PDA at 42±3°C before solidification. In untreated plates (control), 2 ml of sterile buffer was added to PDA in place of the extract. Three mycelia plugs measuring 5 mm diameter each were cut out by the margin of 3-day-old fungal cultures actively growing, and then aseptically placed on the upper PDA surface. Treated and control plates were incubated in the dark at 22±1°C for 5 days. Mycelia growth inhibition was measured at three time set points (after 1 d, 3 d and 5 d of incubation) with respect to the control plates by the index

$$MGI\% = [(Dco-Dse)/Dco] \times 100$$

where, Dco is the average of colony diameter (mm) in the control plates, and Dse is the average of colony diameter in the plates amended with seaweed extract at the three afore-mentioned concentrations. All experiments were carried out with three replications of 10 plates for each. Sporangia germination was evaluated on micro-cultures by a microassay on glass slides that allowed the quantitative analysis of sporangia suppression using an optical microscopy technique (Gatto *et al*., 2011; 2013). Assays on 96-microwell (100 µl volume) plates purchased from AES Laboratory (Milan, Italy) were performed. Each micro-well was set up with three replicates each containing 10 µl of Potato Dextrose Broth (PDB) provided from Sigma-Aldrich, 2 µl of sporangial suspension containing 10^8 CFU ml^{-1}, and 88 µl of extract (both raw and fractionated) suspended in sterile 0.1 M K-phosphate buffer to be tested at the concentrations of 10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1}. One micro-well row used as a control was filled with 10 µl PDB, 2 µl sporangial suspension, and 88 µl buffer. Each plate was incubated at 22±1°C for 24 hours. After incubation, aliquots of 5 µl sporangia cultures taken from each micro-well were sampled and mounted on the upper surface of glass slides sterilized with denatured ethanol. Number of the total, un-germinated, and collapsed sporangia were counted by a Burker's hemocytometer using a photomicroscope (40 × magnification) (BX60, Olympus, Milan, Italy). Sporangia germination suppression was measured at four time set points (after 2 h, 10 h, 18 h and 24 h of incubation) by the index

$$SG\% = Su/St \times 100$$

where, Su is the average of the sum of un-germinated + collapsed sporangia in un-supplemented cultures (control) or amended with the extracts at the three afore-mentioned concentrations, and St is the average of number of total sporangia in the same sample. All measurements were performed with three replications of 5 glass slides taken from each micro-well.

Preventive and curative treatments were *in vivo* evaluated on strawberries (cv. Camarosa) harvested from growers located in Basilicata (Policoro, Matera, Italy) under tunnel condition. Healthy fruit untreated with synthetic fungicides and selected for uniform size, same ripening stage, and absence of visible defects and injuries, were washed under running tap water, surface-disinfected by dipping for 1 min in 2% sodium hypochlorite solution, rinsed with tap water and allowed to dry. Each fruit was injured in the equatorial zone in two opposite points at the fixed dimensions (wide= 2 mm, deep= 2 mm), treated with the crude seaweed extract, and inoculated with a sporangial suspension of *R. stolonifer*. Trials treated with the stocks containing 10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1} of raw extract suspended in sterile 0.1 M K-phosphate buffer were set up. Two controls replacing the extracts, the first one with buffer alone, and the second one with one commercial fungicide containing as active substances 50% Fenhexamid (1.2 g l^{-1}) were both included for preventive and curative treatments. Aliquots of 30 µl of the stock suspension of extract were dispensed over each wound of the treated fruit allowing the droplet to be absorbed into the fruit. Aliquots of 30 µl of sterile buffer alone, or fungicide, were dispensed over the wound of the control fruit. Each wound was inoculated with 10 µl of a sporangial suspension containing 10^6 CFU ml^{-1}. In preventive treatments, the pathogen was inoculated over the injured area 2 days later from the application of the extract, or the fungicide, or the buffer alone, for enhancing plant defences before inoculation. Instead, in those curative, the extract, or the fungicide, or the buffer alone, were applied over the wound 8 hours later from inoculation of the pathogen allowing sporangia germination before treatment. Fruit were placed in trays, packaged in plastic bags and maintained into a climatic room for 4 days at the temperature of 20±2°C and relatively humidity (RH) of 96±2% in the dark. Trials were arranged in a completely randomized experimental design including six replicates per treatment, whenever thirty strawberry fruit with two wounds per fruit were considered per each replication. Disease incidence was assessed by counting the number of the infected wounds on each fruit. The disease incidence data were converted into the strawberries soft rot suppression data by the index

$$DI\% = [(Nco - Nse)/Nco] \times 100$$

where, Nco is the average of number of infected wounds in the control plots treated with buffer alone, and Nse is the average of number of infected wounds in the plots treated with extract at the three afore-mentioned concentrations, or with the fungicide (Arras *et al*., 1999).

Trials were also arranged to assess the POD activity including thirty strawberry fruit treated with 30 g l^{-1} un-fractionated extract in preventive treatment, and sampled after one-day and five-days of incubation at temperature of 20±2°C and RH of 96±2% in the dark. Inoculated and healthy fruit (not injured) treated with only sterile 0.1 M K-phosphate buffer were both included as positive and negative control, respectively. Small pieces of tissue (diameter= 2-3 mm, deep= 3-4 mm) randomly collected from six points of each fruit were powdered with liquid nitrogen. Samples of one-gram of tissue were extracted with 2 ml of 0.1 M sodium-phosphate buffer (pH 7.0) at 4°C and used for assessing POD activity. All experiments were performed with three replications of 10 fruit for each. POD activity was assayed according to Hammerschmidt *et al.* (1982). The reaction mixture consisted of one-gram of tissue, 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme (1.5 U ml^{-1}) and 0.5 ml of 1% H_2O_2 that it was incubated at 28°C. Absorbance measured at a wavelength of 420 nm were recorded every 30 s for 3 minutes and the boiled enzyme preparation served as blank. POD activity was expressed as change in absorbance of the reaction mixture on the fresh weight basis (Δ_{OD420} g^{-1} min^{-1}).

Statistical analyses

The suppressive effects of each *L. digitata* crude extract to mycelia growth and sporangia germination of *R. stolonifer* (using both raw extract and soluble extracts by hexane, ethanol and water), and *versus* strawberries soft rot and peroxidase activity (using only raw extract), were assessed by using variance analysis (ANOVA). In particular, two-way ANOVA was applied to test the interactions either among three extract concentrations (10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1}) and three incubation times (1 d, 3 d and 5 d) on mycelia growth inhibition index (MGI%), either between three extract concentrations (10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1}) and two treatment types (preventive and curative) on strawberries soft rot suppression index (DI%). For performing two-way ANOVA we have used the average data of MGI% and DI% assessed at the different extract concentrations, incubation times, and treatment types at *P*-value ≤0.05. Value percentage of the two indexed parameters was before transformed into arcsine for satisfying the assumption of normality, and then compared by applying the

Duncan Multiple Test Range (DMRT) whenever two-way ANOVA revealed a significant difference among the means at a $P \leq 0.05$ level. However, these data has been shown as un-transformed values. One-way ANOVA was instead used to test difference among four extract concentrations, including control (0 g l^{-1}), at the same incubation time (2 h, 10 h, 18 h and 24 h) on sporangia germination suppression index (SG%). SG% values were before transformed into arcsine and then compared by DMRT whenever one-way ANOVA revealed a significant difference among the means at $P \leq 0.05$. One-way ANOVA was also used to test difference among the three experimental trials at the same sampling time to assess peroxidase (POD) activity at the first and fifth day, and DMRT was used to compare data whenever one-way ANOVA revealed a significant difference among the means at $P \leq 0.05$. Regards to better clarify the effect of seaweed extract at the three considered concentrations including control on the un-germinated and collapsed sporangia vs. incubation time, the SG rates were submitted to regression analysis in order to obtain curves that were compared for slope and elevation for each set points. All statistics were managed by the 12.0 SPSS programme (Statistics Base™, Chicago, Illinois, USA).

3. Results

The seaweed extract yield obtained at the end of the extraction process was about 1.22±0.1 g on 100 g of fresh biomass. *R. stolonifer* mycelia growth was affected by extract concentration and incubation time showing a significant interaction between these two factors after supplementation to PDA with *L. digitata* crude extract (Table 1). The mycelia growth inhibition percentage by increasing doses of raw extract resulted of 10%, 31% and 48% after one-day of incubation; it increased until 22%, 45% and 58% after three-days; finally it reached 40%, 61% and 80% at the end of incubation time (after five-days) applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 1A). The mycelia growth inhibition percentage after supplementation to PDA with increasing doses of extract purified with hexane resulted of 4%, 15% and 22% after one-day of incubation; it increased until 16%, 31% and 53% after three-days, reaching 20%, 45% and 71% at the end of incubation time applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 1B). The mycelia growth inhibition percentage after amendment with increasing doses of extract soluble in ethanol resulted of 10%, 14% and 16% after one-day of incubation; it increased until 17%, 30% and 43% after three-days, it reached 19%, 43% and 66% after five-days applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 1C). No inhibition effect after amendment with extract purified by water was seen (data not shown). The un-germinated and collapsed sporangia percentage of *R. stolonifer* after supplementation to PDB with increasing doses of *L. digitata* un-fractionated extract resulted of 16%, 24% and 36% after 2 h of incubation, while it increased until 50%, 77% and 95% at the end of incubation time (after 24 h) applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 2A). The un-germinated and collapsed sporangia percentage following to supplementation with increasing doses of extract soluble in hexane resulted of 9%, 15% and 25% after 2 h of incubation, while it reached 40%, 65% and 82% after 24 h of incubation applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract (Fig. 2B). The un-germinated and collapsed sporangia percentage after adding of increasing doses of extract soluble in ethanol resulted of 5%, 13% and 14% after 2 h of incubation, while it reached 42%, 55% and 69% at the end of incubation applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract (Fig. 2C). No reduction of sporangia germination was found with respect to control after adding of extract soluble in water (data not shown).

Strawberry fruit soft rot suppression was affected by extract concentration and treatment type with a significant interaction between these two factors after adding of *L. digitata* raw extract over strawber-

Table 1 - Synthetic values to different two-way ANOVA analysis to *Rhizopus stolonifer* mycelia growth inhibition and strawberries *Rhizopus* soft rot suppression at P-value ≤ 0.05

Effect	df [c]	*F*	*P*-value
On mycelia growth inhibition [a]:			
1) - Extract concentration	2	44	0.02
2) - Incubation time	2	26.2	0.03
- Extract concentration × incubation time	4	2.8	0.02
On strawberries soft rot suppression [b]:			
3) - Extract concentration	2	31.6	<0.01
4) - Treatment type	1	6.6	0.01
- Extract concentration × treatment type	2	9.4	<0.01

[a] Extract concentration (10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1}) and incubation time (1 d, 3 d and 5 d) are the two factors considered on mycelia growth inhibition. [b] Extract concentration (10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1}) and treatment type (preventive and curative) are the two factors considered on strawberry fruit rot suppression. [c] Degree of freedom.

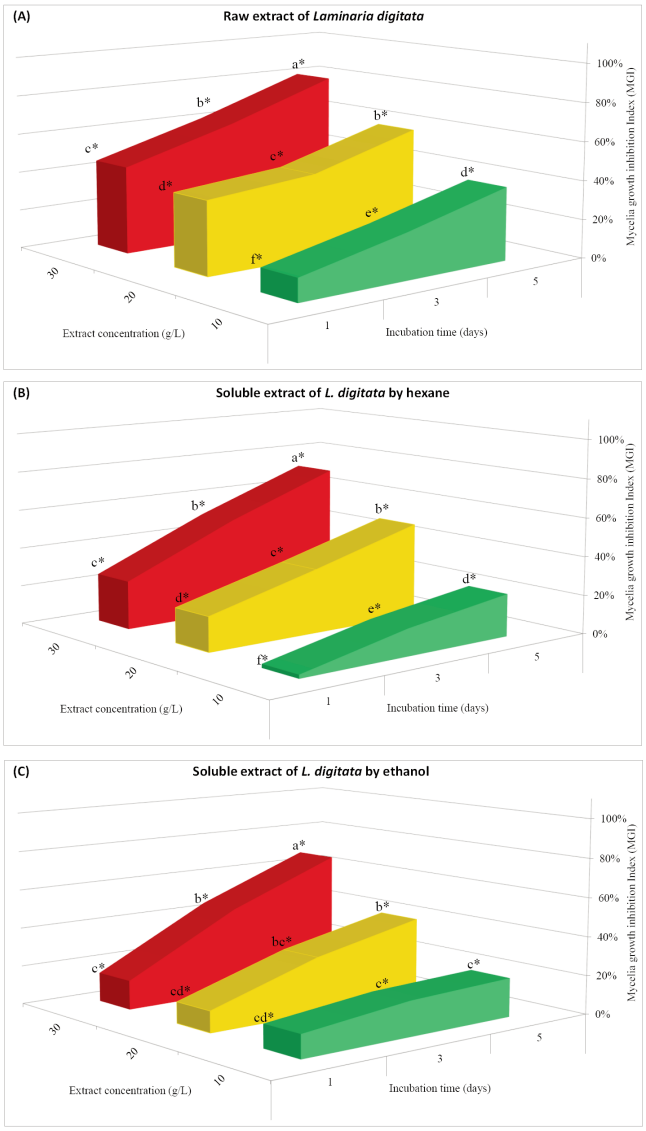

Fig. 1 - Suppressive effect of *Laminaria digitata* raw extract (A), and soluble extracts by hexane (B) and ethanol (C), on *Rhizopus stolonifer* mycelia growth inhibition index (MGI%). The suppressive activity of extracts applied at three different concentrations was tested after incubation at 22±1°C for 5 days into Petri plates on PDA medium. Each value, ranging from 0% (no inhibition) to 100% (total inhibition), represents the pooled mean of three replicates with 10 plates for each. MGI% values were collected after 1 d, 3 d and 5 d and analysed by two-way ANOVA. Asterisk indicates P-value ≤0.05 according to table 1. Histogram points with different letters are significantly different according to Duncan Multiple Test Range (DMRT) at a probability P≤0.05 level.

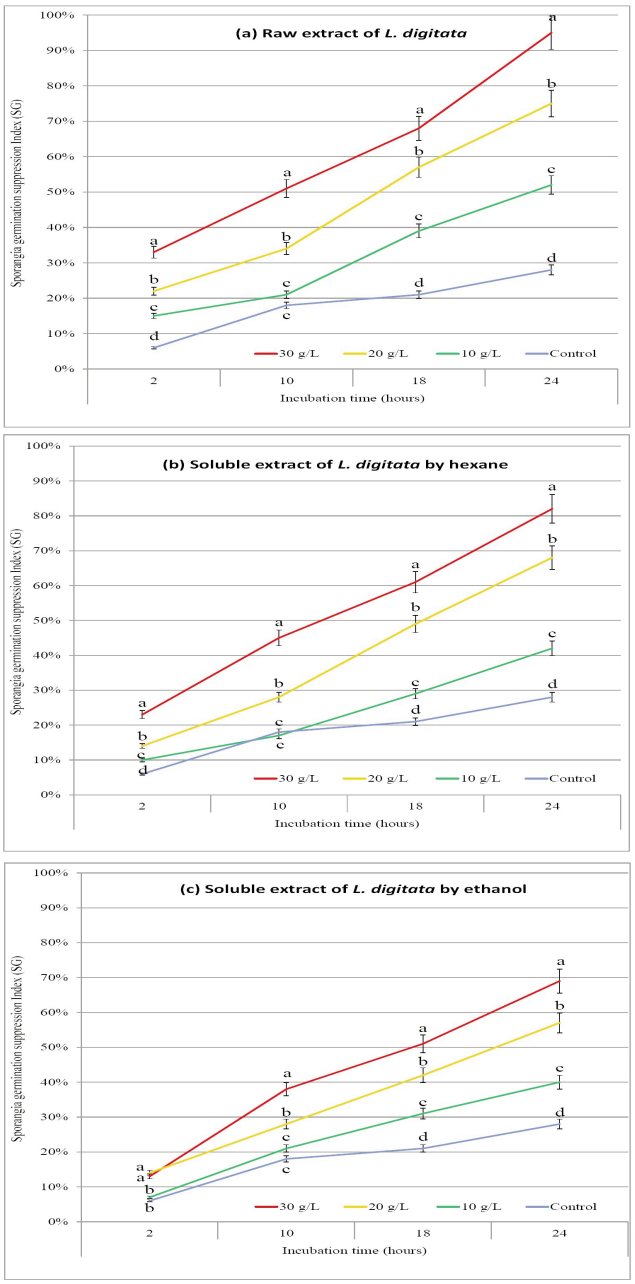

Fig. 2 - Suppressive effect of *Laminaria digitata* raw extract (A), and soluble extracts by hexane (B) and ethanol (C), on *Rhizopus stolonifer* sporangia germination suppression index (SG%). The suppressive activity of extracts applied at four different concentrations (including control) was tested after incubation at 22±1°C for 24 hours into micro-well plates filled with PDB medium. Each value, ranging from 0% (no suppression) to 100% (total suppression), is the pooled mean±SD (bars) of three replicates of 5 glass slides taken from each micro-well. SG% values were collected after 2 h, 10 h, 18 h and 24 h and submitted to regression analysis. For each incubation time, SG% values analysed by one-way ANOVA with different letters are significantly different according to DMRT (P≤0.05).

ries wound (Table 1). The fruit decay inhibition percentage of the *R. stolonifer*/strawberries system after preventive treatment with *L. digitata* was of 22%, 49% and 75%; but it remained of 2%, 11% and 21% in curative treatment applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} raw extract (Figs. 3, 4). The chemical treatment with Fenhexamid suppressed the strawberries rot until 100% in those preventive and 70% in those curative (Figs. 3, 4).

Finally, as regards to measurement of peroxidase activity in strawberries (Table 2), a significant increment of absorbance in plots treated with *L. digitata* raw extract was seen yet after one-day of incubation (6.84 Δ_{OD420} g^{-1} min^{-1}) if compared to those of the untreated-inoculated strawberries (5.01 Δ_{OD420} g^{-1} min^{-1}). Moreover, the untreated-healthy fruit shows a significant lower POD (1.12 Δ_{OD420} g^{-1} min^{-1}) with respect to untreated-inoculated strawberry fruit at the same sampling time.

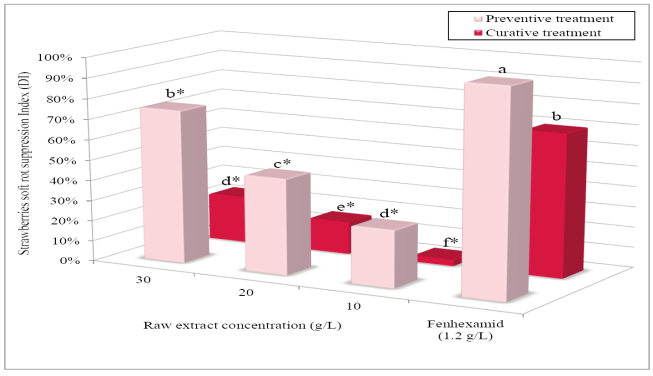

Fig. 3 - Strawberries *Rhizopus* soft rot suppression index (DI%) after 4 days of incubation at 20±2°C and 96±2% RH in the dark following to preventive and curative applications of 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} *Laminaria digitata* raw extract, with respect to trial treated with Fenhexamid. Values ranging from 0% (no suppression) to 100% (total suppression) are the pooled mean of six replicates per treatment, each one carried out with thirty strawberries with two wounds per fruit. DI% values were analysed by two-way ANOVA. Asterisk indicates P-value ≤0.05 according to table 1. Histograms with different letters are significantly different according to DMRT (P≤0.05).

4. Discussion and Conclusions

In vitro experiments performed with the *L. digitata* hexane-soluble extract fraction suggests that inhibition on mycelia growth and sporangial germination of *R. stolonifer* could be due to direct toxicity of the fatty acids found in extracts of *L. digitata* purified with chloroform (Løvstad Holdt and Kraan, 2011). By supporting this hypothesis, in ours finding the hexane-soluble extract fraction exerted a similar antifungal effect in comparison to those seen with the total extract. A very similar effect was also found testing the ethanol-soluble extract, suggesting that suppression could be due to direct toxicity exerted by the phenolic substances found in ethanolic extracts of *L. digitata* (Løvstad Holdt and Kraan, 2011). In fact, the ethanol-soluble extract fraction incited a similar antifungal effect in comparison to those seen either with the hexane-soluble fraction either with the un-fractionated extract. Instead, no suppressive effect on mycelia and sporangia was seen applying the water-soluble extract fraction, suggesting that the water-soluble polysaccharides (laminarans, fucoidans and

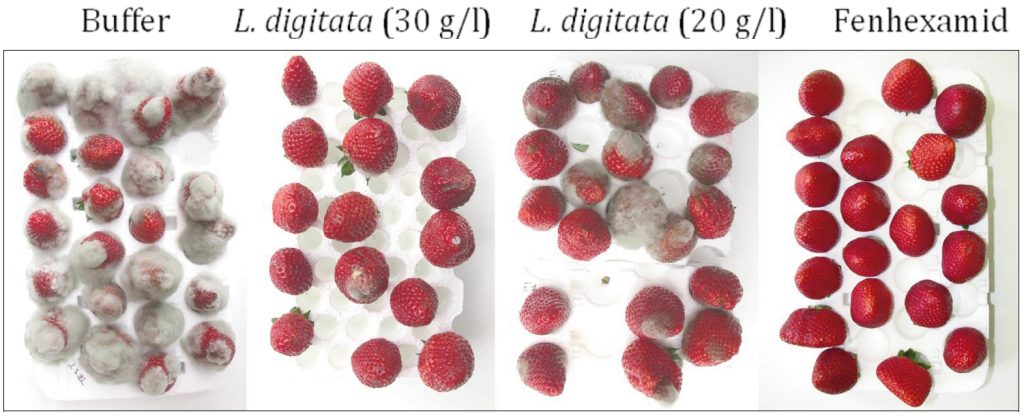

Fig. 4 - *Rhizopus* soft rot symptom on strawberries cv. Camarosa after 4 days of incubation at 20±2°C and 96±2% RH in the dark after a preventive application of 20 g L^{-1} and 30 g L^{-1} *Laminaria digitata* raw extract with respect to the control trial treated with buffer alone and Fenhexamid (1.2 g L^{-1}).

Table 2 - Peroxidase (POD) activity in strawberries inoculated with *R. stolonifer* and preventively treated with 30 g L^{-1} *Laminaria digitata* un-fractionated extract. POD assessment was carried out after one-day and five-days of incubation at 20±2°C and 98% RH in the dark

Experimental trial	One-day	Five-days
L. digitata-treated inoculated strawberries	6.84±0.3 a	53.15± 1.5 a
Untreated-inoculated strawberries	5.01±0.2 b	20.38±0.9 b
Untreated-healthy strawberries (not injured)	1.12±0.06 c	5.27±0.2 c

Values are the pooled mean±SD of three replicates of 10 fruit for each analyzed by one-way ANOVA. In each column, values followed by different letters are significantly different according to Duncan Multiple Test Range (DMRT) at a probability P≤0.05 level. Change in absorbance on the fresh weight basis (Δ_{OD420} g^{-1} min^{-1}).

alginates) present in aqueous extracts of *L. digitata* (Løvstad Holdt and Kraan, 2011) could not be involved in mycelia and sporangia suppression under *in vitro* condition. Moreover, mycelia inhibition was closely related to extracts concentration added into growing media, showing an increasing of antifungal activity as the dose of extract increased. A dose-dependent manner of the treatments with *L. digitata* extracts was therefore found at least in the concentration range considered here. Finally, our findings showed that mycelia inhibition increased from the first to the last day of incubation in a time-dependent manner, showing that the extracts (both un-fractionated and fractionated by hexane and ethanol) were really efficacy in suppressing mycelia growth during all incubation time. *In vivo* experiments performed with crude extract employed at 30 g l^{-1} dose showed a stronger efficacy of *L. digitata* in suppressing decay on infected strawberries by *R. stolonifer* in preventive treatments with respect to those curative. Findings coming from *in vivo* experiments highlighted an interesting and very competitive antifungal efficacy of *L. digitata* raw extracts against *R. stolonifer* on strawberries when compared to action of Fenhexamid in preventive treatments. Our findings show that strawberries soft rot suppression was closely related either to extract dose or treatment type, since a remarkable increasing of suppressivity was seen as the concentration of extract applied over the wounds increased, as well as passing from the curative treatment into those preventive. Therefore, an evident dose-effect of the preventive treatment was observed at least in the concentration range here considered. Finally, a significant POD activity in *L. digitata*-treated inoculated strawberries was early found with respect to untreated-inoculated fruit. Moreover, healthy strawberries treated with only sterile buffer showed a significant POD decreasing with respect to untreated-inoculated fruit. Increments of POD activity early found after one-day of incubation after treatment with 30 g L^{-1} crude extract, and confirmed after five-days of incubation, could be related to activation of induced systemic resistance mechanisms into the *R. stolonifer*/strawberries system. In fact, the artificial inoculation with the pathogen without extract application induced a lower absorbance change with respect to *L. digitata*-treated inoculated strawberries, while not injured fruit showed a lower absorbance change in comparison to untreated-inoculated strawberries.

Our findings show that a direct antifungal activity exerted by crude extract of *L. digitata* could be attributed to its content of fatty acids, accordingly with De Corato *et al.* (2017), which have investigated on the antifungal properties of five crude seaweed extracts obtained by a supercritical carbon dioxide technique, including those derived from *L. digitata*, on three fruit/pathogen pathosystems in preventing postharvest losses caused by *B. cinerea*, *Monilinia laxa* (Aderh. & Ruhland) Honey and *Penicillium digitatum* (Pers.) Sacc. on strawberries, peaches and lemons, respectively. In our findings, phenolic compounds could be considered as good candidates able to suppress mycelia growth and sporangia germination of the pathogen together to fatty acids accordingly with the *in vitro* tests. This finding is nevertheless divergent if compared to the observations of De Corato *et al.* (2017), whenever ethanolic extracts of *L. digitata* were weakly suppressive against *B. cinerea* and *M. laxa* when applied at 30 g L^{-1} dose with respect to extracts purified by hexane. The discordance between these two experimental evidences probably is due to the two different extractive techniques used, being employed a supercritical carbon dioxide technique to prevalently extract lipids rather than phenolic substances and water-soluble polysaccharides, while extraction by mixtures of polar and un-polar solvents in appropriate rates are generally less selective and more suitable to extract lipids, phenolic substances, and polysaccharides without preferences among them (Selvi *et al*., 2014). Several damaging mechanisms induced by exposure to vapour of essential oils derived from various aromatic plants, as well as after longer treatments with fatty acids and various phenolic substances were reported in literature, such as a partition of lipid layer of the cell membrane due to their hydrophobic nature, and affection of permeability of the cell membrane that cause leakage of cell components (Rasooli *et al*., 2006; Soylu *et al*., 2006; Laird and Phillips, 2011; da Cruz *et al*., 2013; Shao *et al*., 2013). In our findings, *in vitro* inhibition were not found by using aqueous extract revealing that the water-soluble polysaccharides fraction does not exert direct toxicity against mycelia development and sporangia germination. Therefore, is reasonable affirm that laminarans, fucoidans and alginates present in aqueous extracts of *L. digitata* (Løvstad Holdt and Kraan, 2011) could be involved in POD increasing by working most as resistance inducers (or elicitors) rather than as toxic chemicals. Peroxidases usually employ hydrogen peroxide as a substrate causing defence reactions which earlier occur in the fruit tissue after infection. Hydrogen peroxide has an antimicrobial properties due to its

strong oxidizing power and its capacity to generate other oxidizing species (hydroxyl radicals, singlet oxygen species and hydrogen peroxides), on the whole well known as ROS ('reactive oxygen species'), which are toxic to living cells. Inactivation of membrane respiratory chain enzymes and damage to DNA are the probable mechanisms of action for hydrogen peroxide and related ROS (Imlay and Linn, 1988; Tatsuzawa *et al*., 1998). The laminarin, a storage polysaccharide (β-1,3-glucan) isolated for the first time from cell walls of *L. digitata*, elicits host defence responses in grapevine against *B. cinerea* (Aziz *et al*., 2003), and the use of various chemical resistance inducers, including laminarin, for controlling postharvest gray mold and *Rhizopus* rot in strawberry fruit, was studied in the past years (De Miccolis *et al*., 2009; Santini *et al*., 2009).

We can conclude that the antifungal activity showed by crude extract from *L. digitata* could be mainly attributed to content of fatty acids and phenolic compounds extracted from this profitable algal biomass source by appropriate mixtures of polar and un-polar solvents; but, also an increased peroxidase activity probably elicited by the water-soluble polysaccharides content, as laminarin, could be related to activation of an induced systemic resistance mechanism able to suppress postharvest *Rhizopus* soft rot of strawberries under *in vivo* condition.

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Ascorbic acid content and senescence in blueberry (*Vaccinium corymbosum* L.) during storage

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Key words: controlled atmosphere (CA), malondialdehyde, oxidative stress, redox state and quality.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

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Abstract: Blueberry consumption increases because its health properties linked to antioxidants, easy cultivation and profitability. The ability to preserve fruits in controlled atmosphere (CA) allows extending the marketing calendar. The work evaluates parameters linked to the cellular redox state of blueberry fruits, cv. Brigitta, stored at 0°C at different atmosphere regimes (CA1= 10% CO_2, 4% O_2 and CA2= 9% CO_2, 2% O_2, compared to air as control). During storage, quality was assessed by the content of ascorbic acid (AA), antioxidant and index of fruit metabolic status, and of malondialdehyde (MDA), cell membranes oxidative stress and senescence marker; soluble solids content, titratable acidity and dry matter were also determined. Storage in CA increases the blueberries shelf life, particularly at the intermediate times; after 2 months there is a drastic lowering in AA levels and differences among treatments are no longer detectable. Ascorbate is confirmed to be an excellent index of oxidative stress in fruit senescence processes. In control, along with the AA decrease, there is a higher MDA content, in particular up to the intermediate dates. CA2 proves to be the most suitable atmosphere for delaying the senescence process. Titratable acidity and soluble solids remain constant in all samples throughout storage.

1. Introduction

The blueberries (*Vaccinium corymbosum* L.) industry is rapidly increasing because of its healthy properties linked to antioxidant content and its meeting the consumer expectation of healthy food (Gosch, 2003; Kähkönen *et al.*, 2003). Therefore the blueberry growing area is rapidly extending in many regions, taking advantage by the adaptability and ease of cultivation of this crop and its profitability. Blueberry cultivation is well adapted to mountain and hill soil and climate conditions and to oriented organic or environmental friendly agricultural methods with integrated pest management, endowing the growing areas with a benefit of local production and environmental respect. Moreover blueberry, as other small fruit, can be established as small-scale farms giving extra income to family businesses.

The quality of this fruit and the content of health-promoting compounds are influenced by many factors, such as environmental conditions, genetic diversity and degree of maturity at harvest (Ehlenfeld and Prior, 2001; Connor *et al.*, 2002). Blueberry has often been reported to be a highly perishable fruit, thus its commercial value could be strongly affected by storage conditions. Its profitability may take interesting advantage by a storage and shelf life extension that can be achieved by cold storage (0-1°C) and controlled atmosphere (CA) with low oxygen (1-4 kPa O_2) and high carbon dioxide (9-12 kPa CO_2) concentrations (Krupa and Tomala, 2007). After harvest, blueberry quality and product losses are mainly due to dehydration, weight loss, shrivel and fungal spoilage. To understand better the effects of long-term storage on the antioxidant components of blueberry the present study evaluates some parameters related to the cellular redox state of berries of the late cultivar Brigitta, stored at 0°C in different CA regimes.

2. Materials and Methods

Full ripe 'Brigitta' berries were harvested in the Valtellina area (northern Italy, lat: 46.1653333, long: 9.6461111) from 10-year-old plants. The same day of harvest, fruits were put in polyethylene punnets containing about 200 g of fruits each, labelled, weighed and randomly assigned to each of the different storage conditions. Berries were stored at 0°C, 95% relative humidity in the following controlled atmosphere modes: CA1: 4 kPa O_2 and 10 kPa CO_2; CA2: 2 kPa O_2 and 9 kPa CO_2. The control was kept in the air (20.1 kPa O_2 and 0.03 kPa CO_2).

Samples were taken for quality assessment at the following storage times: 0, 12, 33, 47, 61, 76, 94, 110, 132, 147 days.

At each sampling time, 3 punnets per storage condition were removed from the storage and held at -80°C until chemical analysis and quality measurements. Each parameter was determined on 3 replicates, obtaining one fruit sample from each of the 3 punnets per storage condition and time. Dry matter was determined on 50 g of homogenate placed in crucibles and left in an oven at 70°C for 24 hours.

Ascorbic acid was extracted in a 6% (w/v) metaphosphoric acid solution. The amount of 7.5 g of blueberries was homogenized in a mortar with 10 mL of cold extraction medium and centrifuged at 10,000 *g* at 4°C. The supernatant was transferred into a 25-mL volumetric flask at 4°C. The pellet obtained by centrifugation was washed with 7 mL of cold metaphosphoric acid solution and centrifuged. The supernatants were combined and brought to a final volume of 25 mL with cold 6% metaphosphoric acid. After filtration through 0.2-µm Nylon filter, a 10-µL sample aliquot was injected onto an Inertsil ODS-3 (5 µm; 4.6 mm × 250 mm) GL Science column at 20°C attached to a Series 200 LC pump (PerkinElmer, Norwalk, CT, USA). The column was eluted with 0.02 M orthophosphoric acid at a flow rate of 0.7 mL/min and ascorbic acid was monitored at 254 nm with a UV-975 intelligent UV-vis detector (Jasco model 7800, Tokyo, Japan). Ascorbic acid was identified by the retention time and quantification was achieved according to the concentration of a corresponding external standard (Sinelli *et al.*, 2008).

The determination of the thiobarbituric acid-reactive-substances (TBARS) content was carried out using a 5% trichloroacetic acid extract. Five grams of mesocarp were homogenized in 25 mL of 5% (w/v) trichloroacetic acid then centrifuged at 4°C at 10,000 *g* for 30 min. The extract was added to an aqueous solution of 15% (w/v) TCA and 0.5% (w/v) 2-thiobarbituric acid. Samples were mixed and heated at 95°C for 15 min in a water bath, cooled and centrifuged at 4,000 *g* for 15 min. Samples were then analyzed in a spectrophotometer (Jasco, model 7800, Tokyo, Japan) at 532, 600, and 440 nm. The value of absorbance at 532 nm was purged from the absorbance at 440 nm and at 600 nm due to sucrose and tonon-specific turbidity (Cocetta *et al.*, 2016). TBARS concentration was expressed in MDA equivalent (nmol/g fw) following the equation (Du and Bramlage, 1992):

$$\{[(A_{532} - A_{600}) - (A_{440} - A_{600})(8.4/147)/157\,000]\}10^6$$

For the determination of the titratable acidity (TA), a 5 g sample of homogenized blueberry puree was diluted with 30 mL of distilled water. The TA was measured after 15 sec stirring by titration with 0.1 N NaOH to an end-point of pH 8.3 by a Compact Titrator D (Crison Strumenti SpA, Carpi, Italy). The acidity was expressed as meq/100 g fw.

Total soluble solids (TSS), expressed as percent of soluble solids, were determined by a hand refractometer (Atago mod., N1, Tokyo, Japan) on juice obtained from squeezing the berries.

Analysis of variance was performed by SPSS software, IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL), using general linear model univariate analysis. Sources of variation were time of storage and atmosphere regimes. Significant differences between

means were calculated by Tukey's mean test. Differences at P≤0.05 were considered as significant.

3. Results

Blueberry fruit cv. Brigitta showed at harvest a percentage of dry matter of 12.52% (Fig. 1). Within the first 47 day storage period, berries maintained in air and in controlled atmospheres exhibited similar dry matter contents (13.45% in control; 12.55% and 12.14% in CA1 and CA2 stored berries, respectively). Thereafter, the level of dry matter increased in control samples until end of storage and was higher than in treated samples. In berries stored in both CA regimes dry matter did not change throughout the entire storage period. At the end of the trial DM accounted for 14.52% of the weight of control fruit and for 11.69% and 11.54% of the weight of CA1 and CA2 stored fruit, respectively.

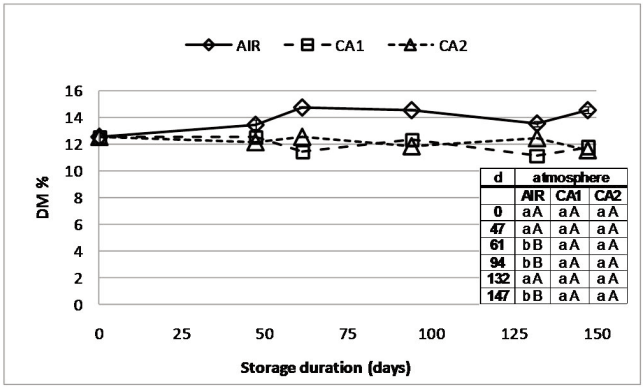

Fig. 1 - Changes in blueberry dry matter during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O_2 and 10 kPa CO_2; CA2= 2 kPa O_2 and 9 kPa CO_2). Values are mean ± SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time (P≤0.05).

The trend of total soluble solids fairly followed the parallel sample variation in dry matter content during storage (Fig. 2). Soluble solid content increased in control fruit during the first storage period, from 11.10% at harvest to 12.70% at 47 days storage, and during the trial it was always higher in fruit stored in air than that in berries stored in CA1 and CA2, except at 132 days of storage. During the trial, berries stored in CA showed no differences in this parameter until day 147, when a decrease was recorded. Comparing total soluble solids at the end of storage period with respect to the beginning, the amounts were 5% higher in berries maintained in air and 12% and 17% lower in berries under CA1 and CA2, respectively.

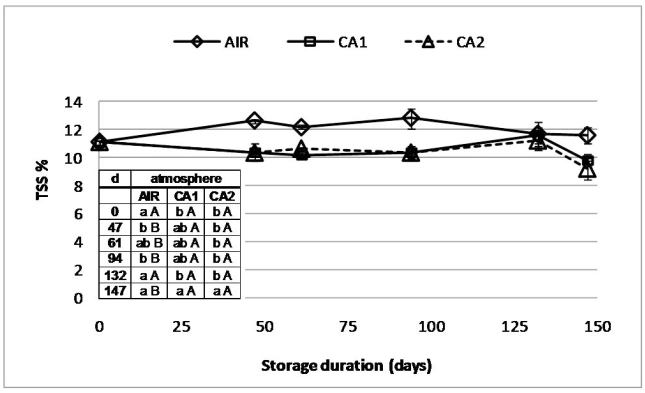

Fig. 2 - Changes in blueberry soluble solid content during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O_2 and 10 kPa CO_2; CA2= 2 kPa O_2 and 9 kPa CO_2). Values are mean ± SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time (P≤0.05).

The titratable acidity (11.91 meq/100 g fw at harvest) remained stable in the first 94 days of storage in all the samples and no differences were detectable among treatments (Fig. 3). After this sampling time, TA increased in all samples, reaching a maximum value at day 132 and either maintaining higher values at the last sampling point, in fruit under controlled atmosphere regimes (20.27 meq/100 g fw in CA1 and 19.69 meq/100 g fw in CA2 after 147 days storage), or decreasing, in control, at the end of storage to values not statistically different from those at the beginning of the trial (10.92 meq/100 g fw).

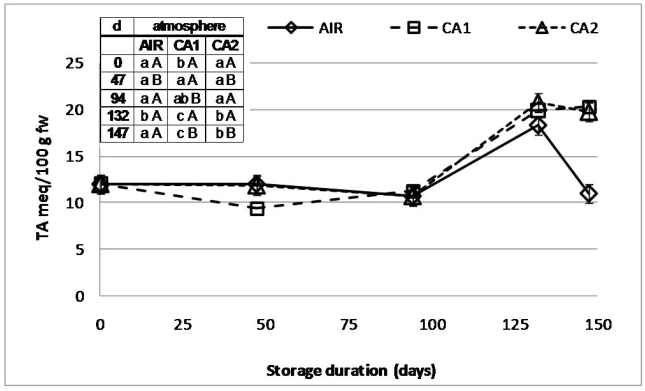

Fig. 3 - Changes in blueberry titratable acidity during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O_2 and 10 kPa CO_2; CA2= 2 kPa O_2 and 9 kPa CO_2). Values are mean ± SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time (P≤0.05).

Ascorbic acid content (0.35 mg/100 g fw at harvest) increases after the first 12 days of storage in control fruit by 377% and in CA1 and CA2 stored samples by 218% and 239%, respectively (Fig. 4). After this sampling time the levels in the control progressively declined reaching the minimum level of 0.18 mg/100 g fw, while ascorbate levels remained higher in the berries stored in both CA regimes until the day 61 (1.31 mg/100 g fw in CA1 and 1.39 mg/100 g fw in CA2). From that date the ascorbic acid content of treated fruit decreased drastically and after the sampling time at 94 days storage ascorbate content was no longer detectable in any sample.

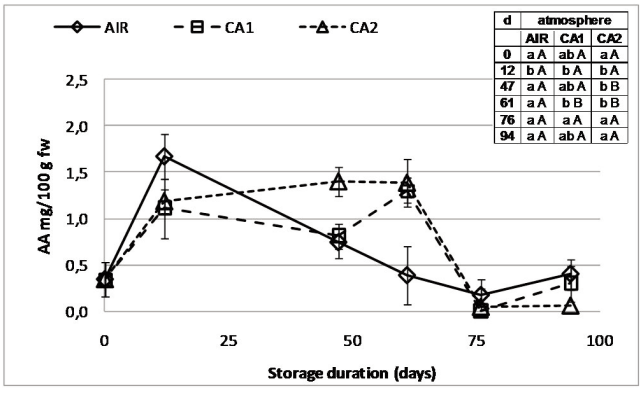

Fig. 4 - Changes in blueberry ascorbic acid content during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O_2 and 10 kPa CO_2; CA2= 2 kPa O_2 and 9 kPa CO_2). Values are mean ± SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time (P≤0.05).

In control fruit TBARS levels (197.95 nmol/g fw at harvest) decreased at the first sampling date and then increased markedly (+33%), reaching a maximum after 33 days of storage (Fig. 5). TBARS decreased over time, starting at 94 days of storage, from 222.10 nmol/g fw to 138.18 nmol/g fw at the end of storage. In CA stored berries, the trend of TBARS content progressively decreased, but no significant differences were observed compared to harvest date, except for fruit stored in CA1 for 61 days. At the end of the trial, TBARS levels reached values of 170.32 and 143.9 nmol/g fw in CA1 and CA2 stored berries, respectively.

4. Discussion and Conclusions

Dry matter content, linked to water and weight loss and to metabolic activity, is an important factor affecting blueberries storage life. As blueberries have a high surface-to-volume ratio, they are prone to water loss. The similar values showed by all the samples during the first 47 day of cold storage are in accordance with the research findings of Duarte *et al.* (2009). They reported a markedly low (0.9%) weight loss found in 'Brigitta' fruit at 48 d of cold storage, regardless of the gas mixture used, either air or CA.

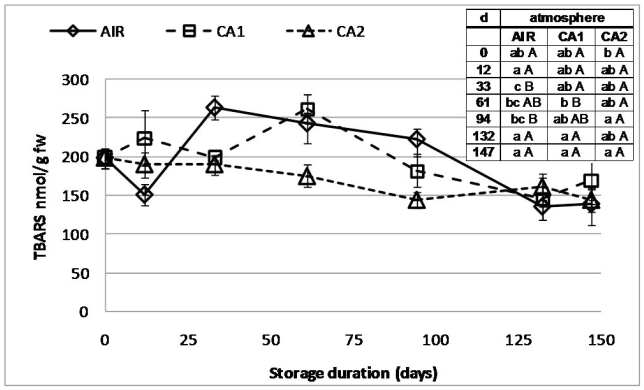

Fig. 5 - Changes in blueberry malondialdehyde level, expressed as TBARS content, during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O_2 and 10 kPa CO_2; CA2= 2 kPa O_2 and 9 kPa CO_2). Values are mean ± SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time (P≤0.05).

The higher percentages of dry matter determined in control starting from the sampling date after 61 days is due to the greater loss of water sustained by the berries kept in the air with respect to the samples stored in CA1 and CA2. In contrast to our data, Alsmairat *et al.* (2011) reported an effect of CA on moisture loss, yielding a 13-fold difference between the 0% CO_2/21% O_2 treatment (0.25% weight loss) and the 19% CO_2/2% O_2 treatment (3.3% weight loss). The authors reported that the greater weight loss for the highest CO_2/low O_2 treatment could be associated to physical causes, e.g. the greater flux of dry gas through the CA chambers, and secondly because high levels of CO_2 can stress blueberry fruit. On the other hand, in our trial a slight impact on moisture loss in control fruit may stem from similar physical causes, because the CA chambers are smaller than the storage room of control fruit and also because control storage condition could be more stressful to berries than the CA.

Blueberry fruit cv. Brigitta at harvest showed a total soluble solids content and titratable acidity according to other research data (Hancock *et al*.,

2008; Gündüz *et al.*, 2015).

Soluble solids content and titratable acidity level are important quality parameters that account for the flavor of fruit.

Total soluble solids of control samples increased by 13% during the first storage period of 47 days, according to Chiabrando *et al*. (2009) who recorded changes in soluble solid content in cv. Coville, with significantly higher values after 28 days of storage in air at 0°C. This increase in control samples cannot be completely explained on the basis of water loss by transpiration and could be more closely associated to the final events of the ripening process. Although blueberry is expected to stop sugar accumulation once the fruit is picked (nonclimacteric behavior) and does not have starch to support soluble sugar synthesis after harvest, an increase in carbohydrate levels may be a consequence of cell wall degradation. In fact, during this storage period of 47 days no significant increase in dry matter linked to a change in moisture content is observed in control fruit. Thereafter, soluble solids content did not change until end of the trial. Similar research findings were reported on cultivars Brigitta (Duarte *et al*., 2009), Bluecrop and Ivanhoe (Beaudry *et al.*, 1998), Burlington (Forney *et al.*, 2003). In CA stored fruit, soluble solids content shows similar values throughout the storage period until the last sampling time, when it decreased significantly both in CA1 andCA2 stored berries. The steady values of total soluble solids shown by the samples stored in CA starting from the beginning of cold storage can be due to a stronger effect of CA on fruit metabolism compared to air-stored fruit.

Tritatable acidity showed no changes in all samples stored up to 94 days. This is in accordance with previous studies carried out by Chiabrando *et al*. (2009) on 'Bluecrop' and 'Coville' (fruit stored for 35 days in air) and Smittle and Miller (1988) on rabbiteye blueberry (fruit stored for 42 days at different atmosphere regimes) which showed titratable acidity not to be affected by storage duration or atmosphere. On the other hand, after this storage period of 94 days, the parameter increased markedly at day 132 and then remained stable in CA stored fruit until end of the trial. Schotsmans *et al*. (2007) reported a significant increase in titratable acidity during CA storage (2.5% O_2, 15% CO_2) of rabbiteye blueberry 'Centurion' fruit from 35 days onwards.

In contrast to CA stored fruit, in control fruit titratable acidity decreased after the peak at day 132 to levels similar to that recorded at harvest. According to this trend, Harb *et al*. (2014) found no significant differences in titratable acidity between air-stored and CA stored blueberries cv. Duke after 4 weeks storage, but a 8 week storage period resulted in significant higher level of titratable acidity in CA stored fruit respect to control fruit.

Increased titratable acids contents of blueberries did not correspond proportionately and cannot be explained with declining moisture content of the berry, rather may be linked to the onset of senescence and to the release of additional acids associated with softening and cell wall breakdown (Proctor and Peng, 1989). The higher levels in titratable acidity at the end of storage in CA stored fruit compared to control could indicate that such fruit still had more reserves remaining whereas the reserves for the air-stored fruit were partially depleted.

Ascorbic acid is an antioxidant which concur to fruit quality and it is a marker of the metabolic status of fruit. Growing season, location, agricultural practices, cultivar and crop ripeness affected to varying degrees ascorbic acid levels at harvest. The levels of 1.7 mg/100 g recorded at full ripeness in control fruit of 'Brigitta' were low compared to some other research data (10.2 mg/100 g reported by Golding *et al*., 2014; 13.6 mg/100 g reported by Kozos *et al.*, 2014) but similar to other studies (4.8 mg/100 g reported by Spinardi *et al*., 2009; 2.6 mg/100 g reported by Sinelli *et al.*, 2008).

During the first 12 days of storage ascorbic acid levels increased markedly and reached a maximum in air-stored fruit, when the ripening process is completed. The significant increment in ascorbic acid content may rather be due to a partial disassembly of cell wall polysaccharides (Davey *et al*., 1999; Gilbert *et al.*, 2009; Cruz-Rus *et al.*, 2011) than to the activation of the ascorbic acid biosynthetic pathway. It could also be related to a more efficient ascorbic acid recycling pathway (e.g. enzymes of the ascorbate-glutathione cycle), which plays an important role in the response and adaptation to the stress (Stevens *et al.*, 2008). After this storage period, ascorbic acid content in control samples progressively diminished. This steady decrease could be due to less effective recycling of ascorbic acid or the presence of factors that promote oxidation of the ascorbic acid pool such as the enzymatic activity of ascorbate peroxidase (APX) or the direct interaction with reactive oxygen species (ROS). Zhou *et al*. (2014) found a progressive increment in APX activity in blueberries until 45 days of cold storage in air and a higher level after 60 days compared to the beginning of storage. Throughout

storage, they found also a steady increase in superoxide radical production rate and hydrogen peroxide content.

In contrast to the decrease recorded in control fruit after the first sampling time, ascorbic acid content did not change in CA stored fruit up to 61 days, when the levels were significantly higher than in control fruit. This evidence is partially in contrast to a previous report on cv. Bluecrop (Harb *et al.*, 2010) that indicate that a marked loss in ascorbic acid over the entire storage period of 6 weeks occurred under all storage conditions (ranging from 0% CO_2, 18% O_2 to 24% CO_2, 2% O_2) and stated that increasing CO_2 and/or decreasing O_2 partial pressures within the storage atmosphere did not decisively change this loss. On the other hand, Harb *et al*. (2010) found that storing fruit under low O_2 combined with high CO_2 level (up to 18%) resulted in better preservation of ascorbic acid and that the highest CO_2 level (24%) was injurious and resulted in lower ascorbic acid content.

The malondialdehyde (MDA) content (determined as TBARS level) of 198 nmol/g fw at harvest is similar to the levels reported on blueberries (Zhou *et al.*, 2014), on pears (Cocetta *et al.*, 2016) and grapes (Xu *et al.*, 2009). The levels of MDA were coherent in part with the ascorbic acid levels, and reflected more the differences between the various treatments than the trend of a single treatment during the storage period. In control fruit the decreasing trend of ascorbic acid levels during storage is accompanied with a significant higher malondialdehyde content than that recorded in fruit maintained in CA2, up to 94 days of storage. Malondialdehyde is considered a biochemical marker of lipid peroxidation of membranes. This data demonstrates a greater oxidative damage to the cellular components of fruit in a natural atmosphere. The drastic decrease in the ascorbic acid content in the second part of the storage period is not in fact accompanied by an increase in MDA levels. This could be due to the onset of senescence processes associated to a loss of cellular integrity and compartimentalisation, as a result of the drastic lipid peroxidation of cell membranes caused by ROS accumulation, and therefore not to a recovery of oxidative stress. The analysis of the TBARS content shows a positive effect especially of CA2 on the fruit, in which the senescence process is delayed and there is no increase in the levels of this parameter.

The present work confirms the ascorbic acid content as a parameter closely associated to the cellular metabolic state and therefore an excellent index of oxidative stress that intervenes in the processes of senescence of the fruits during storage. Moreover, the blueberry 'Brigitta' storage in Controlled Atmosphere, mainly at low O_2 level (CA2), shows a positive effect in delaying the fruit senescence as demonstrated by the reduced content of malondialdehyde.

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Water spectral pattern as a marker for studying apple sensory texture

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Key words: aquaphotomics, crispness, juiciness, *Malus x domestica* Borkh, mealiness, NIR.

Abstract: Aquaphotomics is a scientific discipline which investigates the water-light interactions in biological systems by using NIR spectroscopy and multivariate analysis to relate water absorption patterns to bio-functionalities. This work aimed at evaluating the feasibility of Aquaphotomics to study apple fruit sensory texture. 'Braeburn', 'Gala' and 'Kanzi®' apples were analyzed by a MicroNIR spectrometer and for mechanical, structural and texture sensory characteristics. Cluster analysis on sensory texture attributes showed four different profiles for each cultivar having different water spectral patterns (WASP). On average, the WASP of mealy apples showed the highest absorbance values at 1364, 1372 and 1382 nm and the lowest in the 1438-1492 nm range suggesting a preponderance of water structures with weak-hydrogen bonds; the opposite was found in crispy and juicy apples indicating the presence of more organized water structures with medium-strong hydrogen bonds. This WASP difference could be due to a different softening rate: apples clustered as firm/crispy/juicy had the highest firmness and the lowest intercellular spaces, while mealy apples had low firmness and high intercellular spaces indicating a more advanced softening. The chemical changes due to the pectin hydrolyzation could affect the water structures. The Aquaphotomics approach could be a useful tool for studying the sensory texture of fruits as water structures actually change in apples with different textural characteristics whatever the cultivars.

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1. Introduction

The preferences of apple consumers are generally based on a combination of texture and flavor (Harker *et al.*, 2003). Textural properties can be considered as the main factors responsible for fruit freshness, having crunchiness and juiciness a positive effect and mealiness a negative effect on consumer choice (Péneau *et al.*, 2006). Texture consists of a number of different properties perceived by human senses and depends on mechanical and structural characteristics of the fruit pulp. Texture changes with

fruit softening due to degradation and modifications of the cell wall and middle lamella structures (Gwanpua *et al.*, 2014). During softening, pectins undergo extensive structural modifications so that cell wall polymers are less bound together and become highly hydrated (Goulao and Oliveira, 2008). The hydrolysis of pectin requires water as substrate and occurs in the intercellular water (De Smedt *et al.*, 2002). All these changes influence the organization of water molecules (free water, dimers, trimers, solution shells) in the fruit tissue depending on the strength of hydrogen bonding, and produce changes in the water absorbance pattern. Thus, when an aqueous system, such as apple fruit, is measured by near infrared (NIR) spectroscopy, the light absorbance reflects the water vibrations and the contribution (concentration and structure) of the other molecules interacting with water. Tsenkova (2009) proposed the Aquaphotomics approach to relate water absorption patterns to bio-functionalities. In a series of experiments, it was found that Vis-NIR spectra acquired in living systems under various perturbations (temperature, ion concentrations, oxidative stress, illumination, disease, damage, etc) are characterized by twelve common water bands repeatedly occurring in different combinations in the spectral regression or classification models predicting the investigated perturbations. These water absorption bands (6-20 nm width, each) called Water Matrix Coordinates (WAMACs) corresponded to 1344 (C1), 1364 (C2), 1372 (C3), 1382 (C4), 1398 (C5), 1410 (C6), 1438 (C7), 1444 (C8), 1464 (C9), 1474 (C10), 1492 (C11) and 1518 (C12) nm (Tsenkova, 2009). The variation of WAMACs defines the water spectral pattern (WASP) which can be visualized by star-charts called 'aquagrams' (Tsenkova, 2010). Changes in water absorbance pattern have been used as biomarkers to monitor water quality (Kovacs *et al.*, 2016), to reveal the presence of bioactive compounds from propolis in smart packaging materials (Barzaghi *et al*., 2017), to detect honey adulteration with high fructose corn syrup (Bázár *et al*., 2016), to distinguish the effects of various coatings on the quality of different types of cheese and of winter melon (Cattaneo *et al*., 2016). Aquaphotomics has also been shown to be a useful tool for discriminating fresh and stored apples and for distinguishing apples stored under different atmospheres. Differences between fresh and stored apples involved C5, C7, C9 and C12 water matrix coordinates, while differences between apples stored in normal and in controlled atmosphere activate the C8 and C10 WAMACs (Barzaghi *et al*., 2014). Significant relationships were found between Vis/NIR wavelengths and some sensory attributes of apples, involving water absorption bands. Absorption measured at 720, 1440, and 2338 nm was positively correlated and at 1940 nm negatively related to mealiness, while the opposite was found for crunchiness and chewiness. The absorbance in the 630-700 nm range and at 1940 nm was positively correlated with juiciness, while negative correlations were found at 940 and 1450 nm (Mehinagic *et al*., 2004).

The aim of this work was to evaluate the feasibility of Aquaphotomics to study the texture sensory profiles of apple fruit belonging to three cultivars having different texture characteristics.

2. Materials and Methods

Fruit

'Braeburn' apples (808 fruits) picked in 2014, 'Gala' apples (270 fruits) picked in 2015 and 'Kanzi®' apples picked in 2014 (540 fruits) and in 2015 (270 fruits) at the experimental orchard of Laimburg (BZ) were stored for 4 ('Gala') or 6 months ('Braeburn' and 'Kanzi®') under controlled atmosphere ('Gala': 1% O_2, 1.5% CO_2; 'Braeburn': 1.8% O_2, 1.3% CO_2; 'Kanzi®': 1% O_2, 1.5% CO_2) and analyzed after 1 and 7 days of shelf life at 20°C ('Kanzi®'2014) or after 1, 7 and 14 days at 20°C ('Braeburn', 'Gala', and 'Kanzi®'2015).

All fruits of the experiment were individually measured by a MicroNIR spectrometer and individually analyzed for texture sensory properties (firmness, crispness, mealiness, juiciness), mechanical characteristics and Relative Internal Space Volume (RISV).

Sensory analysis

Sensory analysis was carried out separately per cultivar according to Eccher Zerbini *et al*. (1999) and Rizzolo *et al*. (2010). Each sensory session corresponded to a time of analysis, that was 9 sampling times for 'Braeburn', 'Gala' and 'Kanzi®'2015 apples and 6 sampling times for 'Kanzi®'2014 apples. As for 'Braeburn', 'Gala' and 'Kanzi®'2015 apples, in each session 9 fruits were presented to each panelist, while for 'Kanzi®'2014 apples 3 fruits per panelist were used. When 9 fruits were presented to each panelist, two sensory sessions were scheduled, the first with 5 fruits and the second with 4 fruits, at 10:30 and at 12:00 AM, respectively. A total of 810 ('Braeburn'), 540 ('Kanzi®' 2014) and 270 ('Gala', 'Kanzi®' 2015) apples were tested. The sensory tests

were performed by a panel of ten short-term-trained judges from Research Centre for Engineering and Agro-Food Processing of the Council for agricultural research and economics (CREA-IT, Milano), who had participated in prior studies on sensory evaluation of apples, in a sensory lab equipped with six computerized individual booths under white artificial lighting at room temperature (20°C). The FIZZ-Network 2.47B (Biosystemes, Couternon, France) software was used for test implementation and results collection.

Fruits (one peeled slice/apple) were presented to the judges at most 1 h after the cut to avoid the browning process, coded with three-digit random numbers and placed on a white flat-bottomed dish in randomized order for each assessor. At the beginning of each session, a peeled slice of a fruit not included in the experimental plan was tasted to eliminate the first tasting effect. Drinking water was provided as a palate cleaner between samples. As the quality of consumer perception is influenced by extrinsic attributes (e.g., price, dimension, size, origin, ripening stage), none of these attributes were mentioned to the judges (Taiti *et al*., 2017). Each sample was evaluated for the intensity of four sensory attributes related to fruit structure: firm, crispy, mealy and juicy. Evaluation was based on a form with a continuous open linear scale, consisting of 40 characters, each panelist rated the intensity of each attribute on the open linear scale anchored to 0 (no presence) and 120 (maximum intensity). Definitions of the sensory attributes are as follows: firm, the resistance to mastication perceived at the first and successive bites; crispy, the textural property perceived at the first bite when the fruit yields suddenly with a characteristic sound; mealy, the textural property consisting of the presence of lumps formed during mastication; juicy, the textural property giving the sensation of progressive increase in the free fluids in the oral cavity during mastication (Eccher Zerbini *et al.,* 1999).

Prior to statistical analyses, the rating scores of each attribute were standardized by panelist in order to remove the variability due to panelists using different parts of the scale according to Bianchi *et al.* (2009).

Mechanical properties and relative internal space volume

Individual fruit were analyzed for flesh firmness, compression and intercellular spaces (RISV). Flesh firmness and compression were measured on two opposite sides of each fruit (the blush side and the opposite one) in the equatorial region and data were averaged per fruit. Firmness was measured with an 11 mm diameter cylindrical plunger mounted on a TA-XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK) at the cross-head speed of 3.33 mm/s to a depth of 8 mm.

Mechanical properties were also nondestructively assessed by a uniaxial compression test. In this test, each apple was compressed between two steel parallel plates to a fixed deformation of 1 mm at a speed of 25 mm/min on an Instron Universal Testing Machine and the modulus of deformability (Ed) was computed according to Eccher Zerbini (1981):

$$\frac{Ed}{1-\mu^2} = \frac{F}{(d_L/2)^{3/2}\, D^{1/2}}$$

where F is the force at 1 mm of compression (N), d_L is the total deformation (mm), D is the fruit diameter (mm); μ is the Poisson's ratio. As Poisson's ratio was not measured in this work, it was fixed at a value equal to 0.3 (Ahmadi *et al.*, 2016).

Relative Internal Space Volume (RISV) was computed according to $RISV=100\times[1-(d_f-d_j)]$ where d_f is the density of the fruit (i.e. ratio fruit mass to fruit volume under water), and d_j is the density of the fruit juice (Baumann and Henze, 1983).

NIR analysis

NIR spectra were acquired on each intact fruit in reflectance mode using a diode array spectrometer (MicroNIR 1700 VIAVI, Diessechem Instrument, Italy) over the 900 to 1670 nm range (50 scans, 128 reading points) on two opposite sides of each fruit (the blush side and the opposite one) in the equatorial region and data were averaged per fruit. The spectra were pretreated using multiplicative scatter correction (MSC) to remove scatter effects, followed by Savitsky-Golay second derivative (15 points, second-order polynomial) after converting the spectra from reflectance to absorbance (The Unscrambler Software, ver. 10.0.1, CAMO Process AS, Norway). Aquagrams were built using the 12 characteristic water absorption wavelengths (1344, 1364, 1372, 1382, 1398, 1410, 1438, 1444, 1464, 1474, 1492 and 1518 nm) within each cluster sensory profile of each cultivar (see *Sensory analysis* paragraph). The values of the aquagrams (*Aq*) were obtained according to:

$$Aq_\lambda = \frac{A_\lambda - \mu_\lambda}{\sigma_\lambda}$$

where A_λ is absorbance, μ_λ is the mean value of all

spectra and σ_λ is the standard deviation of all spectra at wavelength λ (Cattaneo *et al*., 2016). Aquagrams were calculated using MS Excel 2010 (Microsoft, USA). The aquagram displays normalized absorbance values from different sample groups at several water bands on the axes originating from the center of the graph. Absorbance values at the WAMACs were placed on the respective radial axes.

Statistical analysis

RISV, mechanical and texture sensory data were submitted to analysis of variance (ANOVA) considering apple cultivar as factor and means were compared by Bonferroni's test at $P \leq 0.05$.

Data of texture sensory attributes were also analyzed by using an agglomerative hierarchical clustering of observations. Ward's clustering method and squared Euclidean distance were applied to create four data sets having distinctive sensory texture profiles. To form the clusters, the procedure begins with each observation in a separate group and then combines the two observations which are closer together to form a new group. After re-computing the distance between groups, the two groups then closest together are combined. This process is repeated until only the *n* fixed groups remained. As cultivars had different texture characteristics, Cluster Analysis on sensory texture attributes was carried out within each cultivar and year. Data of firmness, compression and RISV were submitted to ANOVA considering sensory cluster as factor and means were compared by Bonferroni's test at $P \leq 0.05$.

All the statistical analyses were performed using Statgraphics version 7 (Manugistic Inc., Rockville, MD, USA) software package.

3. Results

Mechanical and sensory analyses showed that the three cultivars had different texture characteristics (Tables 1 and 2). 'Braeburn' apples had very low firmness, intermediate RISV and the highest Ed, along with the lowest scores for firm, juicy and crispy and the highest scores for mealy. 'Gala' apples had the highest RISV, the lowest Ed and firmness, showing intermediate scores for juicy, crispy and mealy . 'Kanzi®' fruit had the highest firmness, the lowest RISV, intermediate Ed and were perceived as the most firm, juicy and crispy and the least mealy; Kanzi®'2015 showed lower firmness and Ed, higher RISV and higher scores for mealy than 'Kanzi®'2014.

Table 1 - Mechanical properties and intercellular spaces (RISV) of 'Braeburn', 'Gala' and ' Kanzi®' apples

Cultivar	Firmness (N)	Ed (N/mm^2)	RISV (%)
'Braeburn'	56.2 bc	10.5 a	17.6 b
'Gala'	55.2 c	6.5 d	19.6 a
'Kanzi®'2014	61.5 a	9.8 b	15.0 d
'Kanzi®'2015	57.6 b	7.5 c	15.9 c

Means in the same column followed by different letters are statistically different at P≤0.05 (Bonferroni's test).

Table 2 - Mean scores of texture sensory attributes of 'Braeburn', 'Gala' and 'Kanzi®' apples

Cultivar	Firm	Juicy	Mealy	Crispy
'Braeburn'	47.0 b	40.9 c	46.2 a	33.3 c
'Gala'	55.0 a	50.0 b	29.0 b	40.0 b
'Kanzi®'2014	57.2 a	62.0 a	18.1 d	53.4 a
'Kanzi®'2015	59.5 a	59.4 a	24.5 c	51.5 a

Means in the same column followed by different letters are statistically different at P≤0.05 (Bonferroni's test).

From an exploratory analysis carried out by dividing the standard score of each texture sensory attribute into five arbitrary classes according to: very low (<20), low (21-39), medium (40-59), high (60-80) and very high (>80) intensity of the attribute, and by pairing the classes of all attributes for every fruit, it was found that the minimum number of combinations of intensity classes between attributes was four. So clustering analysis was applied with the aim of creating the four data sets having distinctive texture sensory profiles, according to the descriptions and centroid values reported in Table 3. Profile W1 corresponded to a very firm, juicy and crispy texture for 'Kanzi®' apples, and to a very firm/juicy texture with a medium crispness for 'Braeburn' and 'Gala'. Profile W2 of 'Kanzi®' apples was very similar to profile W1 of 'Braeburn' and 'Gala' apples, while that of 'Gala' and 'Braeburn' was characterized by a medium juicy and crispy texture, with low scores for mealiness in 'Braeburn' fruit. Profile W3 of 'Kanzi®' apples showed a soft (2014) or a medium texture (2015) without mealiness, while that of 'Gala' and 'Braeburn' was quite firm with low ('Gala') or medium scores ('Braeburn') for mealiness. Profile W4 was characterized by a soft, dry and mealy texture with high scores for mealiness in 'Braeburn' and 'Kanzi®'2014. Considering the cluster distribution in relation to storage time and shelf life period, profile W1 was found in 65% of fruit at harvest and at stor-

Table 3 - Texture sensory profiles of the four clusters within each cultivar

Cluster number and texture sensory profile	firm	juicy	mealy	crispy	% obs
'Braeburn'					
W1 - very firm/juicy, medium crispy, not mealy	66.0	59.8	21.2	52.3	26.7
W2 - medium firm/juicy, quite crispy/mealy	52.4	40.7	35.4	37.3	23.9
W3 - quite firm/juicy/crispy, mealy	38.5	36.6	55.5	24.1	29.0
W4 - very soft, not juicy, not crispy, very mealy	28.0	22.4	78.2	16.9	20.4
'Gala'					
W1 - very firm/juicy, medium crispy, not mealy	75.5	67.9	19.2	58.6	31.9
W2 - very firm, medium juicy/crispy, not mealy	65.1	46.4	24.5	45.0	20.0
W3 - medium firm/juicy, quite crispy/mealy	43.4	44.4	32.5	30.6	30.4
W4 - very soft, not juicy, not crispy, mealy	26.7	31.6	45.7	17.1	17.8
'Kanzi®'2014					
W1 - very firm/juicy/crispy, not mealy	75.5	85.3	7.0	82.7	32.3
W2 - very firm/juicy, medium crispy, not mealy	61.6	64.5	17.0	54.7	34.8
W3 - low firm/juicy/crispy, not mealy	34.1	35.9	16.8	24.1	25.1
W4 - very soft, not juicy, not crispy, very mealy	35.5	37.8	73.8	20.8	7.8
'Kanzi®'2015					
W1 - very firm/juicy/crispy, not mealy	80.2	78.5	15.2	74.2	23.3
W2 - very firm/juicy, medium crispy, not mealy	67.2	61.7	20.0	55.6	36.7
W3 - medium firm/juicy/crispy, not mealy	51.9	48.9	25.7	41.7	20.0
W4 - very soft, not juicy, not crispy, mealy	28.8	43.4	42.3	27.5	20.0

For each cluster are reported: the description of the texture sensory profile, the values of centroids for each descriptor and the percentage of observations (% obs) grouped in the cluster.

age removal in 'Braeburn' and in 'Gala', and in 84% of 'Kanzi®' apples; profile W2 of the three cultivars and profile W3 of 'Kanzi®'2014 were equally distributed among the storage times and the days of shelf life; profile W3 was found in 75% of 'Kanzi®'2015, 'Braeburn' and 'Gala' apples kept at 20°C; profile W4 was characteristic of fruit held for 14 days at 20°C and was found in 75% of 'Kanzi®'2015, 80% of 'Gala' and 90% of 'Braeburn' apples. Each cluster showed different mechanical properties and intercellular spaces (Fig. 1). Firmness significantly decreased from W1 to W2, W3 and W4 profiles in 'Braeburn' and in 'Kanzi®' 2014 and 2015 apples, while in 'Gala' had the same values in W1 and W2 profiles and decreased in W3 and W4, where showed the lowest values. RISV significantly increased from W1 to W2, W3 and W4 profiles in Braeburn and in 'Kanzi®' 2015, while in 'Gala' had the same values in W1 and W2 and increased in W4; in 'Kanzi®' 2014, RISV showed the lowest values in W1 and the highest in W3 and W4. Ed distinguished only profile W1 from the other ones, except for 'Kanzi®'2014 (Fig. 1).

NIR spectra of the three cultivars (Fig. 2) show high variability in the 1344-1518 nm range corresponding to the first water overtone considered in the aquaphotomics approach.

Aquagrams showed a different water organization according to the different texture sensory profiles and to the cultivars (Fig. 3). 'Braeburn' apples

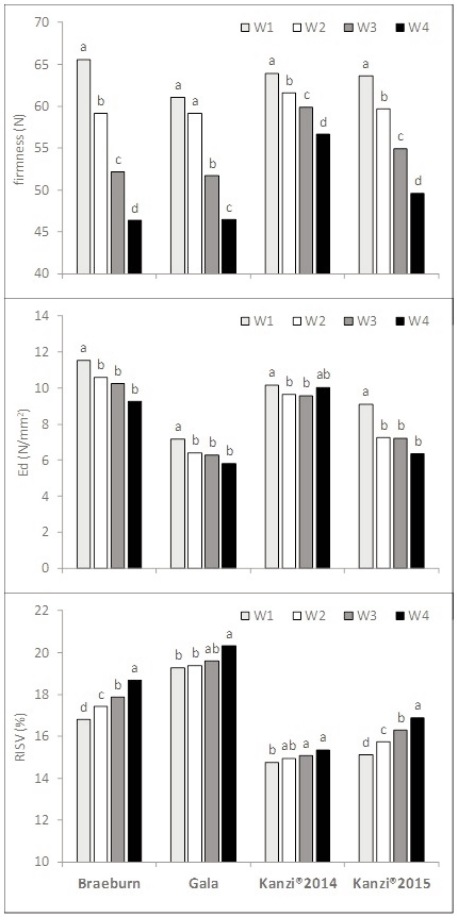

Fig. 1 - Mechanical properties and intercellular spaces of 'Braeburn', 'Gala' and 'Kanzi®' apples according to the texture sensory profiles described in Table 3.

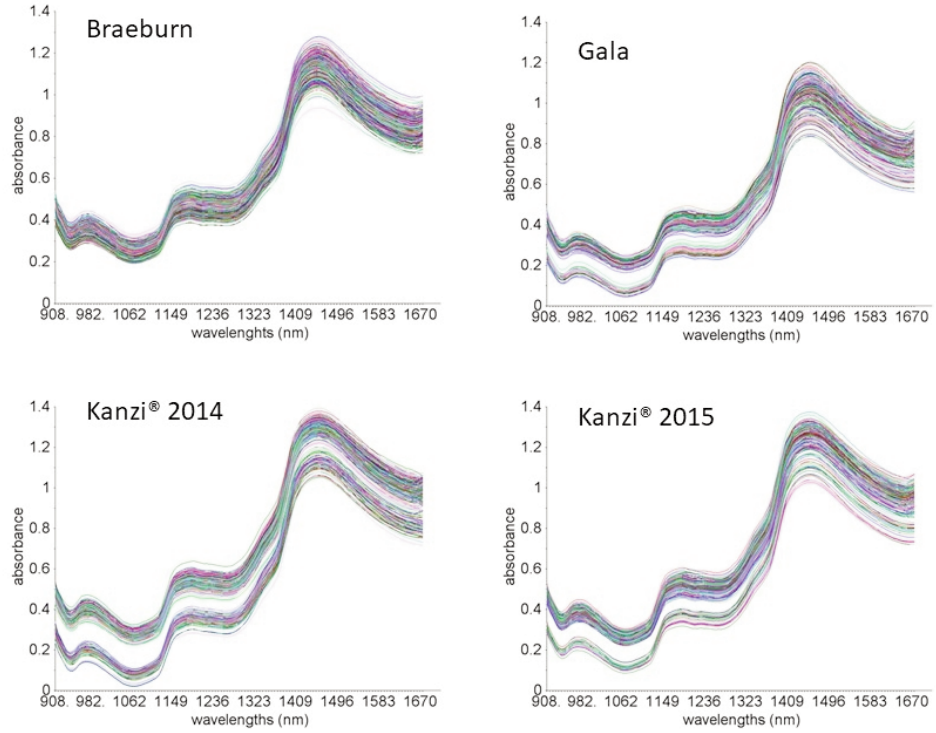

Fig. 2 - Raw NIR spectra of 'Braeburn', 'Gala' and 'Kanzi®' apples.

showed the greatest changes in the water absorbance pattern (WASP) according to the different texture profiles, while 'Kanzi®'2014 the lowest ones. Mealy apples belonging to 'Braeburn' W3 and W4, 'Gala' W4 and 'Kanzi®'2015 W4 profiles were associated to very high values in the 1364-1382 nm range and to very low values in the 1410-1518 nm range, while mealy apples belonging to 'Kanzi®'2014 W4 profile showed a balanced distribution of water structures with highest absorbance at 1344 nm and

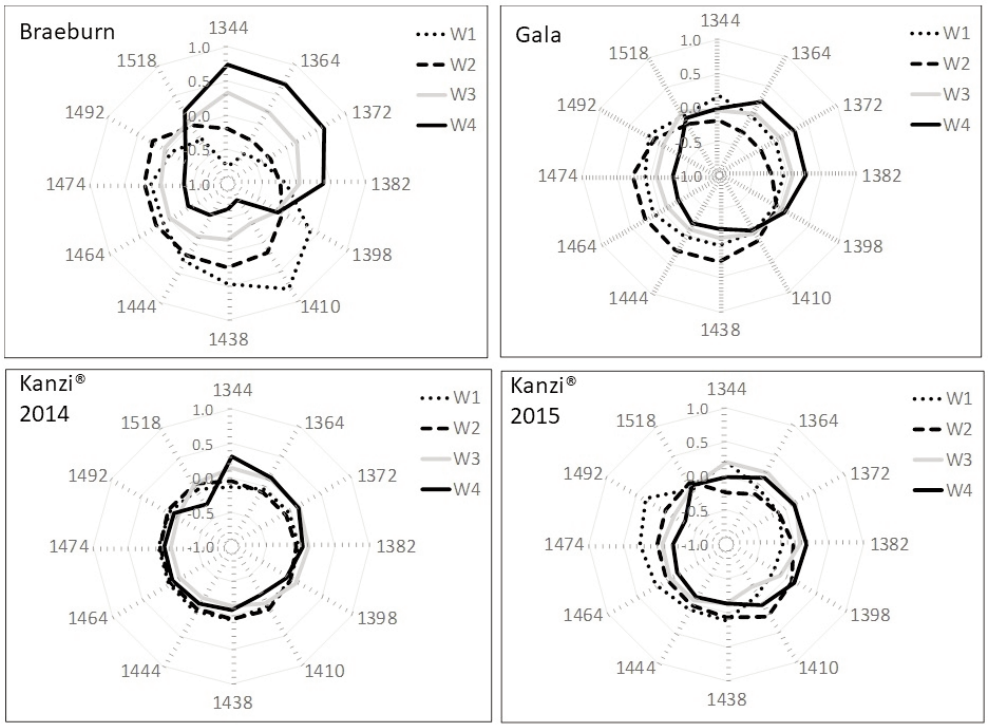

Fig. 3 - Aquagrams of 'Braeburn', 'Gala' and 'Kanzi®' apples according to the texture sensory profiles described in Table 3.

lowest absorbance values at 1518 nm. In low mealy apples ('Braeburn' W2, 'Gala' W3) the absorbance decreased at 1344-1382 nm while increased at 1410-1492 nm comparing to mealy apples of the same cultivars. In contrast, the WASP of firm, juicy and crispy sensory profiles ('Kanzi®'2014 and 2015 W1 and W2, 'Gala' W1) were characterized by a regular distribution of the different water structures with lower absorbance values at 1344-1382 nm and higher absorbance values at 1410-1492 nm comparing to the other textural profiles, while the WASP of 'Braeburn' W1 showed the lowest values at 1344 and at 1364 nm and the highest in 1398-1438 nm range.

4. Discussion and Conclusions

'Braeburn', 'Gala' and 'Kanzi®' apples showed different texture characteristics and were clustered according to the texture sensory attributes in four different profiles, ranging from a very firm/crispy/juicy texture to a mealy or a very mealy one. Each texture profile was characterized by specific mechanical and structural properties and showed different water spectral patterns. The differences in the textural profiles could be due to different softening rates occurring in apples. It is well known that fruit softening involves degradation and modifications of the cell wall and middle lamella structures, loss of turgor pressure, starch degradation and modification in the symplast/apoplast relations affecting the textural characteristics of the pulp: when cell-to-cell adhesion is weaker than the individual cell walls, cell separation occurs and the intact cells are responsible for the mealy texture, while when the individual cell walls are weaker than cell-to-cell adhesion, cell wall breakage occurs and the cellular content is released producing a juicy texture (Goulao and Oliveira, 2008; Harker *et al*., 2002; Vanoli *et al*., 2009). In this work it was found that apples clustered as very firm and firm had the highest firmness and the lowest RISV, whereas apples clustered as mealy showed low firmness and high RISV indicating a more advanced softening as reported by Ting *et al.* (2013), Vanoli *et al.* (2011) and Rizzolo *et al.* (2016). Moreover, mealy texture mainly belongs to apples held at 20°C for 7-14 days, that are fruits in which the softening process has already occurred, while the firm texture was typical of apples just picked or at storage removal, when softening was only at the beginning and apples had a rigid cellular structure with intact and adherent cell walls.

The chemical changes occurring with softening also affected the water structures with a similar pattern for the three cultivars and for the two seasons in 'Kanzi®' apples. The WASP of mealy apples were characterized by a preponderance of water structure with weak-hydrogen bonds (1364-1382 nm), whereas the WASP of crispy and juicy apples indicated the presence of more organized water structures (dimers, trimers) with medium-strong hydrogen bonds (1410-1492 nm). Peirs *et al*. (2005) found that when cell walls deteriorate, water molecules may relocate into the intercellular spaces, and this phenomenon changes the refractive indices at the cell walls. Møller *et al*. (2013) reported that apples treated with 1-MCP clearly differed in water state and dynamics compared to untreated fruit, as water in the cytoplasm and extra-cellular compartments and water in the vacuole were less restricted in treated apples, suggesting that a high firmness is associated with a low amount of vacuole water. The involvement of the first overtone of the OH vibration and pectin metabolism was observed by Boeriu *et al.* (1998) who found that the absorptions in the 1440-1445 nm range varied with the percentage of the degree of pectin esterification in green beans. Similarly, Sirisomboon *et al.* (2007) found a correlation between the absorption in the 1418-1464 nm range and the oxalate soluble pectin fractions in the AIS of intact pears and between the absorptions at 1368 and at 1452 nm and the total pectin content in pear juice. Barzaghi *et al.* (2014) observed that in stored apples the organization of water molecules involved more hydrogen-bonded water than in fresh fruit. This is contrary to our results; this difference could be due to the fact that in this work intact fruits were measured while Barzaghi *et al.* (2014) measured apple slices.

In conclusion, the aquaphotomics approach could be a useful tool and the water spectral pattern could be a marker for studying the texture sensory profiles in apple fruit as water structures actually change along with texture characteristics whatever the cultivar. Furthermore, being aquaphotomics based on NIR spectroscopy it could be possible to discriminate apples with different texture sensory properties in a nondestructive way. However, further studies are needed to better understand the relationships between the water spectral pattern and pectin metabolism and the different water structure organizations and the sensory profiles.

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Influence of soil and soilless agricultural growing system on postharvest quality of three ready-to-use multi-leaf lettuce cultivars

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Key words: ammonium, *Lactuca sativa* L., nitrate, microbial quality, postharvest storage, soilless cultivation.

Abstract: In this study the influence of soil and soilless growing systems (substrate 3:1 v/v perlite:peat) on quality and microbial traits of three multi-leaf lettuce cultivars (two green, 'Eztoril' and 'Ezabel', and one red, 'Ezra') was evaluated at harvest and after 7 and 13 days of storage at 8°C. At harvest, 'Ezra' showed a respiration activity and a total phenol content respectively 2-fold and 25% significantly higher than the green cultivars. Soil lettuces resulted more stressed than those grown in soilless, as indicated by their initial content in antioxidants. As for nitrate content, soilless grown lettuces at harvest showed an average concentration higher than soil-grown ones, although values are generally lower than limits imposed by the EU Regulation (No. 1258/2011). During storage, soilless lettuces showed no ammonium accumulation, differently from those cultivated in soil. In addition, lettuce cultivars grown in soilless condition showed unchanged content in the antioxidant activity and total phenols, and lower microbial counts than soil lettuces. Results of the present study showed that soilless growing system can positively affect qualitative and microbiological parameter of lettuces studied, and it can be considered a good soilless growing technique in order to obtain high quality multi-leaf lettuces for ready-to-use industry.

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1. Introduction

The consumer's demand of ready-to-use fruits and vegetables, and in particular that for minimally processed leafy vegetables, is continuously growing. Although iceberg lettuce is still the main lettuce used in the ready-to-use industry, consumers are requesting other types of lettuce with attractive colours and shapes combining the best quality characteristics from all varieties (Rijk Zwaan, 2009). The new baby-sized leaves,

baby- and multi-leaf have been developed recently as high quality lettuce varieties for the ready-to-use market. Some benefits of baby-sized lettuce, when compared with whole-head lettuce, include: i) greater efficiency with higher percentage of usable product; ii) easier and faster processing; iii) more attractive colour and shapes, and iv) minimal oxidation due to smaller stem diameter (Martínez-Sánchez *et al.,* 2012). Moreover, for both multi- and baby-leaf lettuces, no physical wounding was undertaken, except that of the harvesting, avoiding the physical damage that occurs during preparation of fresh-cut lettuce that causes an increase in respiration activity, biochemical changes and microbial spoilage, which may result in degradation of colour, texture and flavour of the ready-to-use produce (Cantwell, 2004). Likewise, cultivar selection is of great importance in the ready-to-use industry since quality characteristics (such as leaf colour, shape, freshness, texture and browning potential) can change largely depending on the genotype (Nicola *et al.,* 2009). The quality and shelf-life of ready-to-use leaves depend on genotypic traits of raw material and on several aspects from preharvest to postharvest processing (Clarkson *et al.,* 2003; Cantwell, 2004). Some physical and chemical indicators can be used for objective assessment of visual quality (Barrett *et al.*, 2010; Salinas-Hernández *et al.*, 2015). Among these, ammonium (NH_4^+), produced during storage as a consequence of senescence in various vegetables (Cefola *et al.*, 2010; Pace *et al.*, 2014), might be used as predictors of shelf-life (Cefola *et al.*, 2017). In general, preharvest factors should be aimed to optimize their impact on postharvest quality (Crisosto and Mitchel, 2002). From this point of view, soilless system is becoming of high interest since it can improve both, preharvest and postharvest quality of vegetables (Rodríguez-Hidalgo *et al.,* 2010). In particular, soilless agricultural growing system allows to set optimal conditions and nutrient concentration for plant growth (Silberbush and Ben-Asher, 2001; Selma *et al.,* 2012) with the following advantages: higher yields (Lopez-Medina *et al.,* 2004; Recamales *et al.,* 2007), better quality vegetables (Recamales *et al.,* 2007; Cefola *et al.,* 2011) and higher earliness (Recamales *et al*., 2007; Valenzano *et al*., 2008), compared to soil cultivation. The success of lettuce production depends to a great extent on the maintenance of a continuous growth rate by the optimal management of nutrients (Luna *et al.,* 2013). In addition, especially for leafy vegetables, the use of soilless system can avoid soil contaminants and improve the sanitary quality respect to traditional soil cultivation, leading benefits on raw materials for postharvest industry (Selma *et al.,* 2012). Starting from these findings, the aim of this work was to evaluate the influence of two growing system (soil and soilless) on postharvest quality of three multi-leaf lettuce genotypes, including two green and one red, stored under refrigeration for 13 days.

2. Materials and Methods

Reagents

Extraction solvents (MeOH, EtOH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and all standards used in the experiments were obtained from Sigma-Aldrich (St. Louis, Mo., USA). Folin-Ciocalteu's phenol reagent was purchased from Merck (Germany).

Plant material and growing system

Three types of Dutch multi-leaf lettuces (*Lactuca sativa* L.), two green (cv. Eztoril and Ezabel) and one red (cv. Ezra) (Enza Zaden, Enkhuizen, Netherland) were cultivated in an unheated plastic tunnel under soil (S) or soilless (SL) agricultural growing system in the same greenhouse at the experimental farm "La Noria" of the Institute of Sciences of Food Production (CNR-ISPA) located in the South of Italy (Mola di Bari). A split-plot design with three replications was applied, randomizing the growing systems (GS) in the main plots and cultivars in the subplots. Main plots were of 3.6 m^2 (0.9 m wide and 4 m long). The SL system consisted of three single benches (4 m long x 0.3 m wide x 0.1 m high, with a slope of 2%) each plot containing a 3:1 (v:v) perlite:peat mixture as substrate. The nutrient solution was supplied to the SL system without recirculation and had the elemental composition given in Table 1, where the soil characteristics are reported too. The irrigation water had the following composition (expressed in mmol L^{-1}): 0.3 $N\text{-}NO_3$, 0.23 K, 0 P, 1.73 Mg, 1.82 Ca, 7.39 Cl, 4.05 Na. Nutrient solution and water were supplied to SL and S units based on a timer controlled schedule, using minimum substrate water content values, monitored by tensiometers. Furthermore, as additional reference control taking into account two different threshold levels of -5 and -25 kPa to start the irrigation supply in SL and S, respectively. For SL system a nutrient supply level criterion was additionally

Table 1 - Soil and soilless nutrient solution composition. Soil classified as clay soil (USDA textural soil classification, 1987). Values of nutrient solution are expressed in mmol L^{-1}. Micronutrients were supplied according to Johnson *et al*. (1957)

Mineral composition	Soil composition	Soilless nutrient solution
Sand	24.30%	-
Silt	31.90%	-
Clay	43.80%	-
pH	7.6	6.5
EC (dS m^{-1})	2.5	2.3
Cl^-	-	7.39
Mg^{2+}	-	1.73
Na^+	-	4.05
K^+	-	5.12
Ca^{2+}	-	4.74
NH_4+	-	0.5
NO_3^-	-	9.43
P-H2PO^{4-}	-	1.61
S-SO4^{2-}	-	0.81
CEC (cmol kg^{-1} dw)	31.8	-
Organic matter (g kg^{-1} dw)	14	-
Total N (g kg^{-1} dw)	0.95	-
Available P (g kg^{-1} dw)	110	-
Available K (g kg^{-1} dw)	244	-
$CaCO_3$ (g kg^{-1} dw)	0.11	-

applied. At transplant soil plots were fertilized with ammonium nitrate and monopotassium phosphate giving the equivalent of 50, 80, 50 kg ha^{-1} of N, P_2O_5 and K_2O, respectively, and after a month a 30 kg ha^{-1} integration of N from ammonium nitrate was applied. Seedlings were produced in greenhouse in polystyrene trays on peat and were transplanted 25 days after sowing on February 22. Harvest was performed 55 days after transplanting (on April 18) for the SL and after 73 days (on May 6) for the S system. Greenhouse ventilation temperature was 20°C. In figure 1 the climatic parameters measured in the greenhouse are reported. Daily air temperature was on average 21°C, and minimum and maximum air temperature ranged from - 0.2 to 17.5 and from 17.5 to 46.0°C, respectively (Fig. 1A). Air relative humidity was on average 50.5%; daily minimum and maximum relative humidity ranged from 6 to 56% and from 45 to 85% (Fig. 1B). The average photosynthetically active radiation was 282 µmol m^{-2} s^{-1}; its mean and maximum values changed from 80 to 420 and from 400 to 2,080 µmol m^{-2} s^{-1}, respectively (Fig. 1C). After harvest, lettuces were immediately transported under refrigerated condition in polystyrene boxes to the CNR ISPA- postharvest laboratory.

Processing and storage

After harvest, for each multi-leaf lettuce cultivar ('Ezra', 'Eztoril' and 'Ezabel'), and for each agricultural growing system (S or SL), leafs were selected in order to avoid damaged samples, and no washing or pre-treatment were applied. For each cultivar and GS about 600 g of leaves were used for quality evaluation at harvest, whereas about 1.2 Kg were used for the quality evaluations during storage. Thus, leaves were put in open polyethylene bags (about 200 g each bag), and stored at 8°C in dark conditions. For each cultivar, 12 bags (3 replicates × 2 GS, S or SL, × 2 storage periods, 7 and 13 days) were prepared.

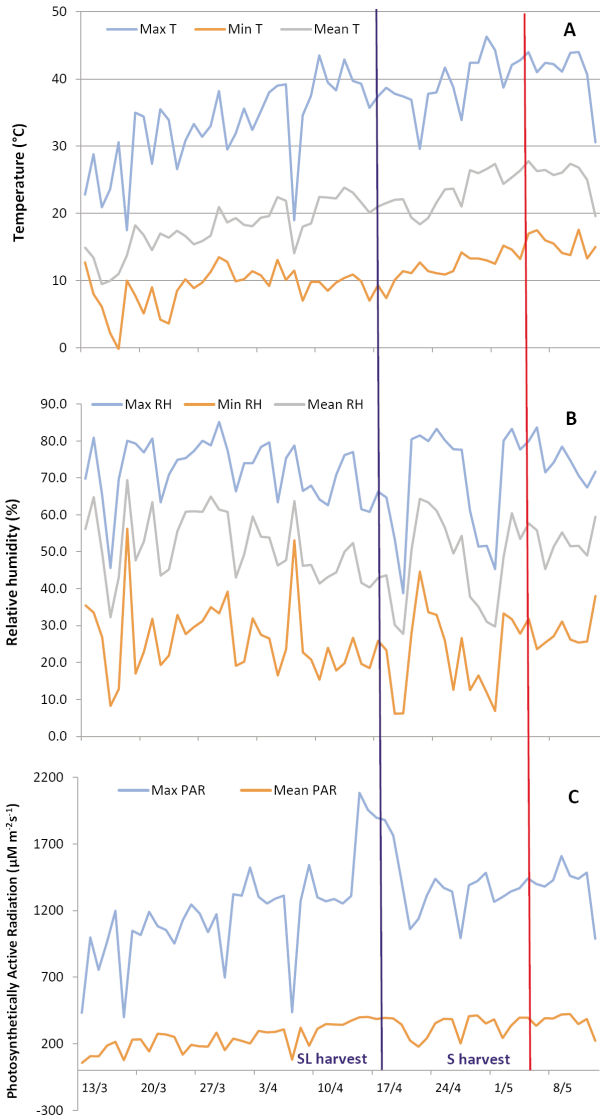

Fig. 1 - Climatic parameters (A= Temperature; B= air Relative humidity; C= Photosynthetically Active Radiation) measured in the greenhouse during the experiment, from seedling transplantation to harvest days. The blue and red perpendicular lines indicate the harvest day for lettuce cultivated in soilless and soil condition, respectively.

Respiration activity and the analysis of nitrate content were performed at harvest. In addition, antioxidant activity, total phenols, ammonium content and microbiological analysis were evaluated at harvest and after 7 and 13 days of storage.

Respiration activity

The respiration activity of each cultivar was measured using a closed system as reported by Kader (2002). About 100 g of leaves for each replicate were put into 6 L sealed plastic jars (one jar for replicate) where CO_2 was allowed to accumulate until the value of 0.1%. The time needed to reach this value was calculated, making CO_2 measurement at regular time intervals. For the CO_2 analysis, 1 mL gas sample was taken from the head space of the plastic jars through a rubber septum and injected into the gas chromatograph (p200 micro GC, Agilent, Santa Clara, CA) equipped with dual columns and thermal conductivity detector. CO_2 was analyzed with a retention time of 16 s and total run time of 120 s on a 10 m porous polymer (PPU) column at a constant temperature of 70°C. Respiration activity was expressed as mL $CO_2 \cdot kg^{-1} \cdot h^{-1}$.

Antioxidant activity, total phenol, ammonium and nitrate content

To determine both antioxidant activity and total phenols content, the extraction procedure reported by Cefola *et al.* (2012), was followed. In detail, 5 g samples were homogenized (Ultraturrax T-25, IKA Staufen, Germany) in a MeOH: water (80:20) solution for 1 min, and then centrifuged at 5°C at 6440 x *g* for 5 min. The supernatant was therefore used for the assays. The antioxidant activity assay was performed following the procedure described by Brand-Williams *et al.* (1995) with minor modifications. Briefly, the supernatant, proper diluted, was pipetted into 0.95 mL of DPPH solution to start the reaction. The absorbance was read after about 30 min at 515 nm. Trolox was used as a standard and the antioxidant activity was expressed in g of Trolox equivalents per kg of fresh weight (g TEAC kg^{-1} fw). The total phenol content was determined according to the method of Singleton and Rossi (1965). Each extract (100 µL), proper diluted, was mixed with 1.58 mL water, 100 µL of Folin-Ciocalteu reagent and 300 µL of sodium carbonate solution (200 g L^{-1}). The absorbance was read after 2 h at 765 nm. Total phenol content was calculated on the basis of the calibration curve of gallic acid and expressed as g of gallic acid per kg of fresh weight (g GA kg^{-1} fw).

For ammonium content the method reported by Weatherburn (1967) was used. In detail, 5 grams of chopped sample were homogenized (Ultraturrax T-25, IKA Staufen, Germany) with 20 mL distilled water for 2 min, centrifuged at 6440 x *g* for 5 min, and 0.5 mL extract was used for the analysis. Color development, caused by the reaction with a phenol nitroprusside reagent and alkaline hypochlorite solution, was determined after an incubation of 20 min at 37°C, by reading the absorbance at 635 nm (UV-1800, Shimadzu, Kyoto, Japan). Ammonium content was expressed as µmole NH_4^+ per kg of fresh weight (µmole NH_4^+ kg^{-1} fw).

As for nitrate content, samples (about 100 g for replicates) were dried in the oven (65°C until constant weight) and were ground to fine powder. The powder (0.5 g for each replicate) were extracted on orbital shaker for 20 minutes with 50 mL of a solution containing 3.5 mmol L^{-1} of sodium carbonate and 1 mmol L^{-1} sodium bicarbonate. Analysis were carried out using a ion exchange chromatography (Dionex DX 200, Dionex Corp, Sunyvale, CA, USA) with a conductivity detector, using an IonPac AG14 precolumn and an IonPac AS4A separation column (Dionex Corporation). Results were expressed in mg of nitrate per kg of fresh weight (mg NO_3^- kg^{-1} fw).

Microbiological analysis

Samples (30 g for replicates) were homogenized for 1 min in 0.1% sterile buffered peptone water (Difco Laboratories, Detroit, MI, USA) (1:5 dilution) using a stomacher (Seward, London, UK). Total aerobic mesophilic bacteria count was evaluated using plate count agar (Difco) incubated at 30°C for 48 h. Yeasts and moulds were counted on Sabouraud Dextrose Agar (Difco) supplemented with chloramphenicol and chlortetracycline (both 0.05 g L^{-1}) and incubated at 25°C for 5-7 days. Total counts of *Enterobacteriaceae* were obtained by pour-plating dilutions (1 mL) in Violet Red Bile Glucose agar (Difco) and plates were incubated at 37°C for 24 h. Microbiological counts were expressed as log CFU g^{-1} of fresh weight (log CFU g^{-1} fw).

Statistical analysis

In order to study the effect of GS (S or SL), cultivars, CV ('Ezra', 'Eztoril' and 'Ezabel') and their interaction (GS x CV) on quality parameters at harvest, and the effect of GS, CV, storage (0-7-13 days) and their interaction (GS x CV x storage) on quality parameters, two multifactor ANOVA were performed (Statistica Software). When significant effect of factors were detected, the Student Newman Keuls (SNK) test was applied to separate means. For a visual

analysis of the data, principal component analysis (PCA) (PRINCOMP procedure, SAS software, Cary, NC, USA; biplot by XLStat, Addinsoft, Paris, France) was performed on mean centered and standardized (unit variance scaled) data prior to analysis. The data matrix submitted to PCA was made up of 18 observations - 3 cultivars ('Ezra', 'Eztoril' and 'Ezabel') x 2 growing system (S and SL) x 3 storage times (0-7-13 days) and 6 quality parameters (antioxidant activity, total phenols, ammonium, mesophilic bacteria, yeasts and moulds, *Enterobacteriaceae*).

3. Results

Effect of growing systems and cultivars on lettuces quality traits at harvest

Yield response was influenced by genotypes more than GS (S or SL), since a lower fresh weight was produced by the red lettuce compared to the other two cultivars (2.7 *vs* 3.6 kg m^{-2}) and only in cv. *Eztoril* there was a higher yield in S compared to SL system (4.3 *vs* 3.3 kg m^{-2}).

The effect of GS, multi-leaf lettuce CV ('Ezra', 'Eztoril' and 'Ezabel') and their interaction on the quality parameters measured at harvest was investigated (Table 2). Ammonium content, antioxidant activity, total phenols and nitrate content were significantly affected by GS and CV, while respiration activity was affected only by CV. The interaction GS x CV was statistically significant only for nitrate content (Table 2). The respiration activity of cv. Ezra was two-fold higher than the green cultivars ('Eztoril' and 'Ezabel'). Regarding ammonium content, the values found for lettuces cultivated in SL were statistically higher respect to S and, between cultivars, red multi-leaf lettuce had mean values statistically higher than green cultivars (Table 2). Growing system affects significantly the antioxidant activity and total phenols: plants cultivated in S showed significantly higher mean content than SL samples (Table 2). Regarding CV, there were no differences between green multi-leaf lettuces in antioxidant activity and total phenols, while the red cultivar Ezra had lower values of antioxidants and higher values of total phenols respect to the green cultivars (Table 2). As for nitrate content was almost double in SL lettuces than S grown ones (838 *vs* 432 mg NO_3^- kg^{-1} fw). The red multi-leaf lettuce (cv. Ezra) had mean values of nitrate statistically higher than cv. Eztoril but not different from cv. Ezabel (Table 2).

Effect of growing systems and cultivars on lettuces' quality traits and microbial parameters during cold storage

The results of Multifactor Anova on antioxidant activity, total phenols and ammonium content as affected by GS, CV, storage time (0, 7 and 13 days) and their interaction were reported in Table 3. Growing system affected antioxidant activity and total phenols, CV affected total phenols and ammonium, while storage time affected antioxidant activity

Table 2 - Effect of growing system (soil and soilless) and cultivar (Ezra, Eztoril and Ezabel) on quality parameters measured at harvest

	Respiration activity (mL CO_2 kg^{-1} h^{-1})	Ammonium content (µmole NH_4^+ kg^{-1}fw)	Antioxidant activity (g TEAC kg^{-1} fw)	Total phenols (g GA kg^{-1} fw)	Nitrate content (mg NO_3^- kg^{-1} fw)
Growing system (GS)					
Soilless	47.44	66.7	2.33	1.32	838.12
Soil	45.73	49.6	4.82	1.84	431.96
Cultivar					
Ezra	72.67 a	72.80 a	3.07 b	1.89 a	765.71 a
Eztoril	35.82 b	48.10 b	3.82 a	1.41 b	524.32 b
Ezabel	31.26 b	53.60 b	3.84 a	1.44 b	615.09 ab
GS	NS	**	***	**	***
Cultivar	***	**	**	*	*
GS x cultivar	NS	NS	NS	NS	*

When interaction among factors was not significant, the results of the mean separation test (SNK test) are reported. Different letters indicate statistical difference within cultivars, respectively, for P≤0.05. NS, not significant; * P≤0.05; ** P≤0.01; *** P≤0.001.

and ammonium (Table 3). Considering the interaction among factors, antioxidant activity was affected by GS x Storage and CV x Storage; total phenols were influenced by GS x CV and by GS x CV x Storage, and ammonium was affected only by GS x Storage (Table 3).

Table 3 - Multifactor Anova of antioxidant activity total phenols and ammonium content as affected by growing system (soil or soilless), cultivar ('Ezra', 'Eztoril' and 'Ezabel') and storage time (0, 7 and 13 days)

	Antioxidant activity (g TEAC kg^{-1} fw)	Total phenols (g GA kg^{-1} fw)	Ammonium content (µmole $NH_4^+ kg^{-1}$fw)
Growing system (GS)	***	**	NS
Cultivar (CV)	NS	**	**
Storage time	***	NS	*
GS x CV	NS	**	NS
GS x Storage	*	NS	*
CV x Storage	***	NS	NS
GS x CV x Storage	NS	*	NS

NS= not significant; * P≤0.05; ** P≤0.01; *** P≤0.001.

In figure 2, changes in antioxidant activity (A), total phenols (B) and ammonium content (C) during storage of the three multi-leaf lettuce cultivars, cultivated in S and SL conditions, are reported. At harvest, lettuces cultivated in S showed values of antioxidant activity significantly higher than SL lettuce. However, during storage, antioxidant activity of lettuces cultivated in S decreased rapidly, reaching approximately the same values of samples cultivated in SL after 7 day of storage at 8°C; after it remained unchanged for cv. Ezabel (about 2 g TEAC kg^{-1} fw) and slightly increased for cv. Eztoril (about 3 g TEAC kg^{-1} fw). The cultivar Ezra cultivated in S showed a content in antioxidant activity almost constant during storage, with a 30% reduction at the end of storage (Fig. 2A). Whereas, lettuce cultivated in SL showed unchanged values of antioxidant activity during time, with a slight reduction at the end of storage for cv. Ezabel (about 1.3 g TEAC kg^{-1} fw) (Fig. 2A). Regarding the content of total phenols (Fig. 2B), green multi-leaf lettuces cultivated in S showed a slight decrease during the first week of storage, after then values rise again until the end of storage, reaching values of about 1.6 and 1.7 g GA kg^{-1} fw for cv. Eztoril and Ezabel, respectively (Fig. 2B). A specular trend for green multi-leaf lettuce cultivated in SL was observed (Fig. 2B). The cv. Ezra cultivated in SL showed the same behavior of green lettuces; whereas 'Ezra' cultivated in S showed an initial total phenol content of about 2.3 g GA kg^{-1} fw, which increased during the first week of storage, reducing to initial values until the end of the storage (Fig. 2B).

As regard data of ammonium content (Fig. 2C) lettuces cultivated in SL showed unchanged values during postharvest storage, starting from the initial mean values of about 79.9±7.5, 57.0±9.3 and 63.2±9.6 µmole NH_4^+ kg^{-1} fw in cv. Ezra, Eztoril and Ezabel, respectively. Whereas, lettuces cultivated in S showed an increase in ammonium content during storage, which doubled for all cultivars, starting from initial mean values of 65.5±15.7, 39.2±3.5 and 44.0±7.0 µmole NH_4^+ kg^{-1} in cv. Ezra, Eztoril and Ezabel, respectively.

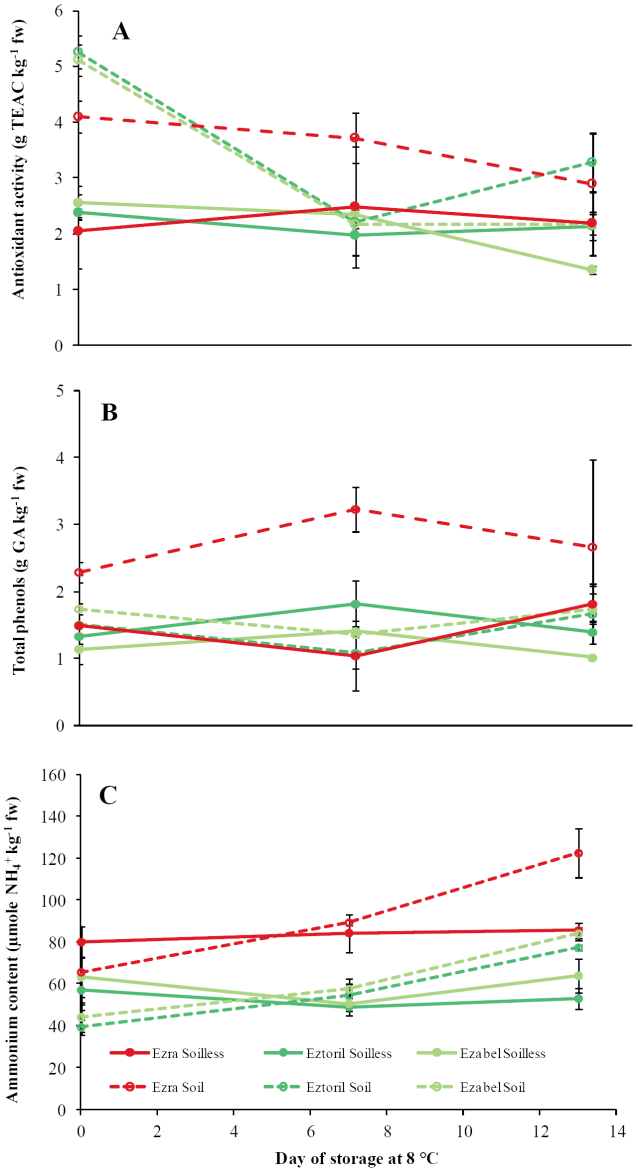

Fig. 2 - Changes in antioxidant activity (A), total phenols (B) and ammonium content (C) of three multi-leaf lettuce cultivars ('Ezra', 'Eztoril' and 'Ezabel'), cultivated in soil or soilless condition, during storage at 8°C. Mean ± SD.

As concerns microbial populations evaluated during the trial, results of multifactor Anova statistical analysis of mesophilic bacteria, yeasts and moulds and *Enterobacteriaceae* as affected by growing system (GS), cultivars (CV) and storage time are reported in Table 4. All factors and their interactions affected significantly the microbial populations evaluated, with the exception of GS x CV interaction for yeasts and moulds. As concerns total mesophilic bacteria, lettuces cultivated in SL showed an increase of about 1.5 log unit during storage, starting from initial mean values of 3.7±0.05 log CFU g^{-1} (cv. Eztoril), 4.9±0.22 log CFU g^{-1} (cv. Ezabel) and 6.1±0.29 log CFU g^{-1} (cv. Ezra), whereas lettuces cultivated in S growing condition showed an higher significant increase in mesophilic population during storage, of about 3 log unit, starting from a mean initial count of about 4.67±0.68 log CFU g^{-1} (Fig. 3A). Yeast and mould loads from S and SL were not found to be significantly different. However, during 13 days of cold storage their amount increased significanly for all cultivars, resulting statistically higher in multi-leaf lettuces cultivated in S respect to the SL ones (Fig. 3B). Also in the case of *Enterobacteriaceae*, lettuces cultivated in S showed a significant increase of about 4 log unit at the end of the storage, starting from initial values of 3.4±0.9, 2.4±0.26 and 4.10±0.10 log CFU g^{-1} for cv. Ezra, Eztoril and Ezabel, respectively. In lettuces cultivated in SL conditions, the increase of about one magnitude order was found for this microbial population (Fig. 3C).

Table 4 - Multifactor Anova of mesophilic bacteria, yeasts and moulds and *Enterobacteriaceae* as affected by growing system (soil or soilless), cultivar ('Ezra', 'Eztoril' and 'Ezabel') and storage time (0, 7 and 13 days)

	Mesophilic bacteria	Yeasts and moulds (log CFU g^{-1} fw)	*Entero-bacteriaceae*
Growing system (GS)	**	*	*
Cultivar (CV)	***	*	***
Storage time	***	***	***
GS x CV	***	NS	***
GS x Storage	***	***	***
CV x Storage	***	**	***
GS x CV x Storage	***	*	***

NS= not significant; * P≤0.05; ** P≤0.01; *** P≤0.001.

Principal component analysis

Principal component analysis revealed that almost 84% of the total variability of data was explained by the first two principal components. PC1 resulted mainly and positively correlated to ammonium and the counts of the three microorganisms groups. Each of them contributed to PC1 for 20-25% of the total variability (Fig. 4). On the other hand, antioxidant activity and total phenols contributed to PC2 for more than 45% each. Among observations, all S growing system samples after 13d storage showed a stronger and positive correlations with PC1, ammonium and the microbiological counts. At a proximate position collocated samples of the red cultivar Ezra collected from SL and stored for 7 and 13 days. On the contrary, all cultivars grown on the S system, at

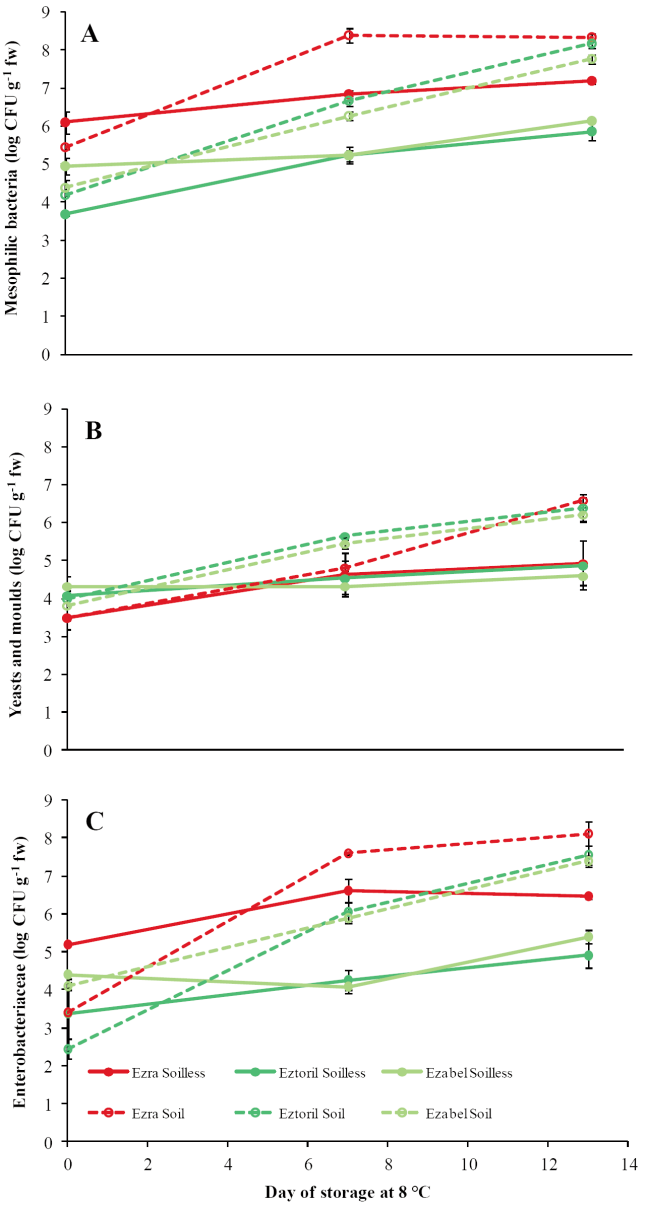

Fig. 3 - Changes in total mesophilic bacteria (A), yeasts and moulds (B) and *Enterobacteriaceae* (C) of three multi-leaf lettuce cultivars ('Ezra', 'Eztoril' and 'Ezabel'), cultivated in soil or soilless condition, during storage at 8°C. Mean ± SD.

harvest, were negatively correlated to PC1, on the opposite site of ammonium and microbial parameters. It seems that no observations were strongly correlated to antioxidant activity and total phenols, with the exception of soil-grown Ezra leaves sampled at harvest and at 7 days storage, followed by the other two cultivars coming from S at harvest and soil-grown Ezra at 13 days storage (Fig. 4). Among observations negatively correlated to PC2, one result noteworthy, the SL-grown 'Ezabel' sampled at 13 days after storage, since it showed a sharp decrease in the antioxidant activity at the end of the storage, as described in figure 2A.

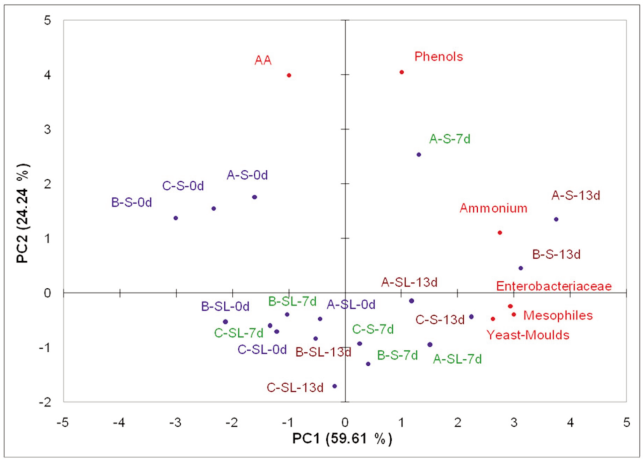

Fig. 4 - PCA biplot (PC1 *vs* PC2) describing the spatial distribution of quality and microbiological parameters of three multi-leaf lettuce cultivars ('Ezra', 'Eztoril' and 'Ezabel') grown in soil (S) and soilless (SL) system during storage; A= Ezra; B= Eztoril; C= Ezabel. AA= antioxidant activity; Phenols= total phenols; 0d – 7d – 13d: 0-7-13 days of storage.

4. Discussion and Conclusions

The three multi-leaves lettuces studied in this research showed different qualities at harvest. In particular, the green cultivars resulted very similar and suitable for postharvest processing, on the basis of respiration activity and ammonium content. At contrary, the red cultivar was considered more perishable than the green ones, due to the high respiration activity (Kader, 2002) even though it showed an higher content in polyphenols than the green cultivars, as previously reported by other authors (Martínez-Sánchez *et al.,* 2012; Selma *et al.,* 2012). As regards antioxidant activity and total phenols, the higher contents measured in S cultivated lettuces could be a plant response to applied stress treatments (Oh *et al*., 2009). Compared to the SL growing system, S irrigation management implies necessarily different water conditions, keeping S plants in not optimal and constant water availability all-day. As a consequence, S plants may have occasionally experienced a water stress combined to heat stress during the highest temperature hours under greenhouse, in the few days preceding harvest (Fig. 1). Even a time-limited stress can activate the antioxidant synthesis in the plant metabolism (Oh *et al*., 2009). On the other hand, the more constant availability of water and nutrients in SL grown plants allowed a higher nitrogen uptake, partially accumulated as nitrate in the vacuoles at higher rate than in S cultivated lettuces. However, the nitrate content measured at harvest was generally low in both GS compared to the limits imposed by the EU Regulation No. 1258/2011. In compliance with the current regulation, nitrate accumulation in lettuce grown in the spring-summer period under greenhouse should not exceed 4,000 mg kg^{-1} fw. This limit is in agreement with the potentially high nitrate accumulating capacity of lettuce. However, at our latitude, the optimal light conditions found by plants during the spring months allow an efficient and fast assimilation of the up-taken nitrate. During cold storage, the SL growing system resulted able to preserve the quality of lettuces since no increase in ammonium content (senescence indicator) was registered whereas multi-leaf lettuces cultivated in S that resulted more senescence-prone. Ammonium accumulates in leafy vegetables during storage, as consequence of protein catabolism. Thus, ammonium was used as indicator of quality and shelf-life of green vegetable. (Chandra *et al*., 2006; Pace *et al.*, 2014; Cefola *et al.*, 2015; Cefola and Pace, 2015; Cefola *et al.*, 2017). Data from ammonium confirms that SL could be considered a suitable growing system to preserve postharvest quality of the cultivars analysed, although genotyping characteristics of each cultivar need also to be taken into account (Selma *et al*., 2012). During storage nitrate measurements were not carried out, since in preliminary trials performed on the same lettuce genotype (cv. Ezra) no nitrate changes after storage at 8°C for 10 days were detected. In particular nitrate remained unchanged at 1550 and 1800 mg kg^{-1} fw, in soilless and soil lettuce, respectively (data not shown). This was supported by several contributes in literature, referring about no modification of nitrate concentration in lettuce and other species during storage at temperature in a range from 1 to 10°C (Siomos *et al*., 2002; Chung *et al*., 2004; Konstantopoulou *et al*., 2010). The SL cultivation showed a positive effect

also on microbiological quality of the green cultivars during storage. Similarly, results were reported by other authors on table grape (Cefola *et al.,* 2011) and on soilless growing systems (Scuderi *et al*., 2011; Selma *et al*., 2012).

In conclusion, the three multi-leaves lettuces studied in this research showed different qualities at harvest. In particular, green cultivars resulted very similar and suitable to postharvest processing, whereas, the red one was considered more perishable. At harvest, lettuces grown in soil showed the higher content in antioxidant activity and total phenols and the lower in nitrate than soilless samples. However, the nitrate content measured at harvest was generally low in both growing systems compared to the limits imposed by the EU Regulation No. 1258/2011. Regarding the postharvest storage, ready-to-use lettuces cultivated in soilless showed microbiological and qualitative performance better than those grown in soil. In particular, soilless growing system improved the storability of lettuces and allowed to the production of clean raw material, particularly suited for ready to use industry. It is interesting to note, as soilless system resulted able to limit ammonium accumulation (senescence indicator), also in red cultivar, which for genotipic traits resulted more senescence prone than green lettuces.

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Advances in Horticultural Science

Assessment of vase life and postharvest quality of cut rose (*Rosa hybrida* cv. Angelina) flowers by application of cumin (*Cuminum cyminum* L.) essential oil and 8-hydroxyquinoline sulfate

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

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The authors declare no competing interests.

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Abstract: Natural preservatives such as herbal essential oils have potential ability for extending postharvest vase life of cut flowers. In this study, application effect of cumin (*Cuminum cyminum* L.) essential oil and 8-hydroxyquinoline sulfate on vase life and postharvest quality of cut rose (*Rosa hybrida* cv. Angelina) flowers were investigated. A factorial experiment with three levels of each in different time after harvesting was conducted. Results showed that usage of different level of cumin essential oil and hydroxyquinoline sulfate had significant effects on rose attributes at the level of 0.05. The results showed that the interaction effect of cumin essential oil and hydroxyquinoline sulfate in measuring time was significant ($P<0.05$) on all of parameters except for anthocyanin content in rose petals in a way that the highest amount for measured traits was obtained with treatment of 150 mg L^{-1} cumin essential oil and 400 mg L^{-1} 8-hydroxyquinoline sulfate.

1. Introduction

Roses have a critical role in the manufacturing of various medicinal and nutritional products. Rosa, known as the symbol of affection and elegance in Iran, is one of the leading cut flower in global floriculture trade including our country (Butt, 2005; Zamani *et al*., 2011). The genus *Rosa* belongs to the family Rosaceae and includes 200 species and more than 18,000 cultivars (Ahmad *et al*., 2013). Cut flower trading is the prime purpose of rose cultivation, but short vase life is the most crucial problem. Commercially, post-harvest longevity of cut flowers is of importance. Many studies have therefore focused on its quality both in pre and postharvest periods (Mirjalili, 2015). The most common physiological and morphological responses after harvesting are wilting or bent neck caused by pathogens

especially bacteria, resulted in decreasing the vase life of cut rose flowers (Leiv and Hans, 2005; Thwala *et al*., 2013). The development of such symptoms is resulted from vascular occlusion, mainly located in the basal stem end (Lü *et al*., 2010; Farahi *et al*., 2013).

Study on effects of natural plant products, including essential oils as preservatives hasted during last decades (Elgimabi and Ahmed, 2009). In nature, essential oils play an important role in the protection of the plants as antibacterial, antiviral, antifungal and insecticides (Bakkali *et al*., 2008). Cumin (*Cuminum cyminum* L.) is an aromatic plant in the family Apiaceae. Cumin seeds are rich of essential oil especially cumin aldehyde, used as a stimulant as well as carminative and therapeutics (Iacobellis *et al*., 2005; Asghari Marjanloo *et al*., 2009).

There are reports on preservative effects of plant essential oils on other plants pathogens, such as tea essential oil on the Botrytis in grape (Jobling, 2000) and antifungal effect of Persian thyme essential oil on strawberry (Nabigol and Morshedi, 2011). Positive effects of plant essential oils have been reported on longevity of cut flowers' vase life (Deans and Ritchie, 1987; Dudai *et al*., 1999). Thwala *et al*. (2013) used cumin essential oil for decreasing degradation and vessel boring in orchids resulted in delay of senescence.

8-hydroxyquinoline sulphate (8-HQS) as a very important germicide in preservatives is used in floral industry. HQS acts as an anti-microbial agent and increases water uptake (Ali and Hassan, 2014). The positive effect of 8-HQS and calcium chloride alone or in combination with 4% sucrose as chemical preservative solutions to improve postharvest quality of cut gerbera flowers has been shown (Soad *et al*., 2011). It reported that HQS extended the vase life of rose cut flowers, whereas sucrose can promote the effect of HQS (Ichimura *et al*., 1999). It documented that vase life and postharvest quality of different cut flowers were enhanced by 8-HQS treatment through improving water uptake, fresh weight and carbohydrate content (Kim and Lee, 2002; Hassan *et al*., 2003; 2004; Ali and Hassan, 2014). Despite the valuable reports on successful use of various phytochemicals for improving longevity of fresh cut flowers, screening for introducing and developing an exact, cheap and easy-to-use preservative is of importance for floriculture (Wu *et al*., 2016).

The objective of this study was to investigate the effect of different cumin essential oil concentrations and 8-hydroxyquinoline sulphate (8-HQS) on vase life and postharvest quality of cut rose flowers in different measuring time.

2. Materials and Methods

Cut rose (*Rosa hybrida* cv. Angelina) flowers were obtained from the commercial greenhouse around Shiraz. Cut rose flowers were harvested when florets were not opened but sepals were turned back and separated from petals during September 2014 and immediately transported to the laboratory. Prior to insert in solutions, flowering stems of plants were cut under water to prevent air entrance into the xylem conduits that were opened by cutting.

This factorial experiment was conducted in randomized complete blocks design with three replications. Treatments were 8-hydroxyquinoline sulfate (8-HQS) at four level (0, 200, 400 and 600 $mg \cdot L^{-1}$) and cumin essential oil at three level (0, 100, 150 $mg \cdot L^{-1}$) in three measuring time (1st day, 8th day and 16th day) after treatment. After the duration of treatments, the flowers were placed in beakers containing 400 ml distilled water during the vase life evaluation period. The control flowers were kept in distilled water. Replications included five flowers per treatment.

Vase life room conditions was 12 hours day length, 18±2°C, 60±5% RH and 12 µmol s^{-1} m^{-2} light intensity and measured traits were vase life determination (days), petal fresh weight/dry weight rate (%), flower diameter (mm), anthocyanin content (mg 100 g^{-1} F.W.), relative water content (RWC) (%), leakage of ions (%), catalase enzyme activity (CAT) ($Ua \cdot mg^{-1}$ pro), peroxidase enzyme activity (POD) ($Ua \cdot mg^{-1}$ pro), membrane stability index (MSI) (%).

Vase life determination

In this study, vase life was considered as the time during which cut-flower can keep its marketability quality and before senescence symptoms including bending of petal margins and wilting are appeared (Singh, 1994). Cut-flower durability was evaluated from cut flower treatment till their ornamental value has disappeared.

Leakage of ions

Floret samples from each treatment were taken on first day and were repeated on day 7 for determining ions leakage by using the method of Sairam *et al*. (1997). Two florets samples (0.2 g) were taken and placed in 20 ml of double distilled water in two different 50 ml flasks. The first one was kept at 40°C for 30 min while the second one was kept at 100°C in boiling water bath for 15 min. The electric conductivity of the first (C1) and second (C2) samples were measured with a conductivity meter. The leakage of ions was expressed as the membrane stability index according

to the following formula (Ezhilmathi *et al*., 2007):

$$\text{Membrane stability index (MSI)} = [1-(C1/C2)] \times 100 \quad \text{(Eq. 1)}$$

Petal anthocyanin

The amount of 200 mg petal samples was pulverized in 3 ml 99:1 (v/v) methanol and hydrochloric acid and obtained extracts were centrifuged at 12000 rpm for 20 min at 4°C. Supernatants were kept in 4°C and under darkness condition for 24 h. After that, light absorption was estimated by spectrophotometer in 550 nm wavelength and using silence coefficient (ε =33000 mol^2 cm^{-1}) (Krizek *et al*., 1993).

Petal membrane stability index

For determining petal membrane stability, two samples of petals each including 200 mg of each replication were weighted and dipped in 10 ml double distilled water. One of them was placed in 40°C Benmary for 30 min and second one at 100°C Benmary for 15 min. After reaching to the room temperature, electrical conductivity of the solutions was measured with a EC meter and the stability percent of the membrane was determined according Ezhilmathi *et al*. (2007), as equation 1.

Enzymes assays

Peroxidase (POD) enzyme was extracted from 200 mg homogenized samples in 25 mM Na-phosphate buffer (pH 6.8) followed by centrifugation at 12000 rpm for 30 min at 4°C. For assay, a mixture consisting of 25 mM Na-phosphate buffer (pH 6.1), 28 mM Guaiacol, 5 mM hydrogen peroxide and crude extract was prepared and its absorbance at 470 nm was detected during 1 min, using spectrophotometer (BIO-RAD). Enzyme activity was expressed as absorption delta of 470 nm per mg protein (Chance and Maehly, 1995).

Catalase (CAT) enzyme was extracted from 200 mg samples homogenized in 25 mM Na-phosphate buffer (pH 6.8) followed by centrifugation at 12000 rpm for 30 min at 4°C. The supernatant was transferred to 15 ml tubes and referred to enzyme extract. For assay, a mixture consisting of 25 mM Na-phosphate buffer (pH 6.1), 10 mM hydrogen peroxide and crude extract was prepared and its absorbance at 240 nm was detected using a spectrophotometer (BIO-RAD). Enzyme activity was described by measuring the conversion rate of hydrogen peroxide to water and oxygen molecules, as the decrease of absorbance per time per mg of protein (8). Enzyme activity was expressed as absorption delta of 240 nm per mg protein. All steps of enzyme extraction were performed on ice. Cumin essential oil and 8-hydroxyquinoline sulphate (8-HQS) were purchased from Zardband Pharmaceuticals - Medicinal Plants Production Co., Yasuj, Iran and were used.

Statistical analysis

All data were analyzed for significant differences using analysis of variance (ANOVA) using the SAS (Statistical Analysis System) statistical package (SAS Institute, Cary, NC, USA). Data were then subjected to mean separation by the least significant difference test (LSD) at P<0.05.

3. Results

According to results of variance analysis, interaction effects of cumin essential oil (CEO), 8-hydroxyquinoline sulfate (HQS) application and measuring times was significant (P<0.05) on measured traits of vase life, petal fresh/dry weight rate, flower diameter, relative water content (RWC), leakage of ions, catalase enzyme activity (CAT), peroxidase enzyme activity (POD), membrane stability index (MSI) except for anthocyanin content. Interaction effect of HQS and measuring times was insignificant on anthocyanin content too, while main effects of each factor and interaction effects of CEO × HQS and HQS × T were significant (P<0.05) (Table 1).

Table 1 - Analysis of variance for measured traits in cut rose (*Rosa hybrida* cv. Angelina) flowers treated by cumin (*Cuminum cyminum* L.) essential oil and 8-hydroxyquinoline sulfate in different measuring times

S.O.V	DF	Mean squares								
		Vase life	Petal dry weight	Flower diameter	Anthocyanin content	Relative water content	Leakage of ions	CAT	POD	MSI
CEO	2	16.02 *	0.52 *	2.686 *	0.0020 *	17.33 *	37.31*	26.45 *	23.30 *	69.35 *
HQS	3	45.99 *	0.30 *	1.542 *	0.0016 *	48.76 *	44.97*	51.20 *	54.21 *	47.07 *
Time	2	11.33 *	0.78 *	1.033 *	0.0011 *	93.32 *	52.47 NS	21.30 *	23.22 NS	99.33 *
CEO×HQS	6	24.35 *	0.89 *	2.037 *	0.0034 *	58.25 *	41.25 *	35.15 *	35.81 *	49.99 *
CEO×T	4	20.46 *	0.55 *	2.432 *	0.0037 *	48.39 *	39.56 *	28.14 *	38.92 *	58.23 *
HQS×T	6	21.32 *	0.53 *	3.321 *	0.0061 NS	59.41 *	45.81 *	32.18 *	40.25 *	63.28 *
CEO×HQS×T	12	25.41 *	0.24 *	1.421 *	0.0061 NS	39.99 *	21.34 *	45.23 *	39.48 *	48.49 *

*,**, shows significant differences at 5%, 1%, respectively. NS= not significant.

CEO = Cumin essential oil. HQS= 8-hydroxyquinoline sulfate

Concerning the mean comparison, the maximum vase life was obtained by application of 100 mg·L^{-1} cumin essential oil and 600 mg·L^{-1} 8-hydroxyquinoline sulfate. However, the minimum vase life was observed in control treatments (Fig. 1). The greatest petal fresh/dry weight rate was evident in the treatment of 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate and the least with control treatments (Fig. 2). The results indicated that the highest flower diameter was found in 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate and the lowest diameter in control treatments (Fig. 3). Relative water content showed the maximum and minimum value in 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate and control treatment, respectively (Fig. 4). The greatest amount of ions leakage in control treatments and the lowest amount in 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate were found (Fig. 5). According to results of mean comparison, the highest catalase enzyme activity was attained in 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate, while the lowest of that was reported in control treatments (Fig. 6). The greatest peroxidase enzyme activity was observed in 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate and the least activity in control treatments (Fig. 7). According to the obtained results, the highest membrane stability index was obtained in 150 mg·L^{-1} cumin essential oil and 400 mg L^{-1} 8-hydroxyquinoline sulfate (Fig. 8).

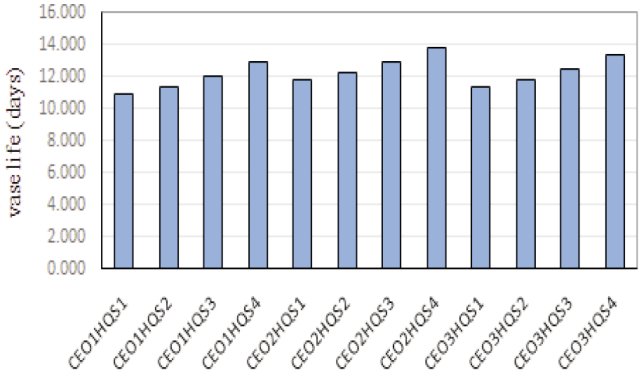

Fig. 1 - Changes of vase life under different levels of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS).

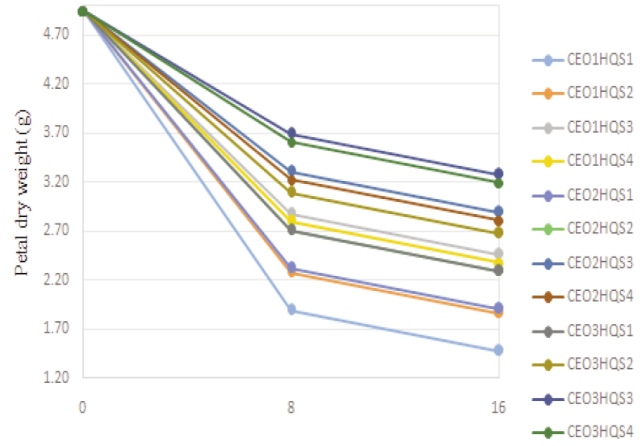

Fig. 2 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on petal dry weight (g) in different measuring times.

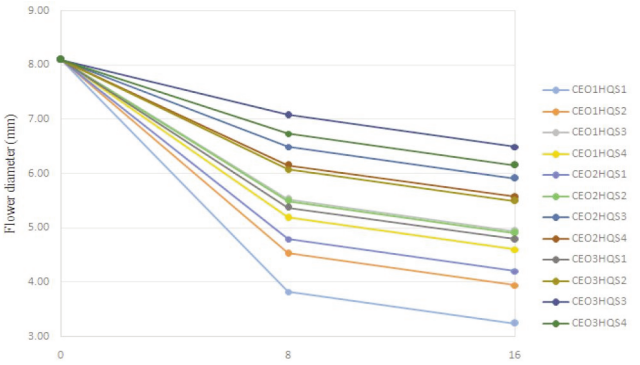

Fig. 3 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on flower diameter (mm) in different measuring times (days).

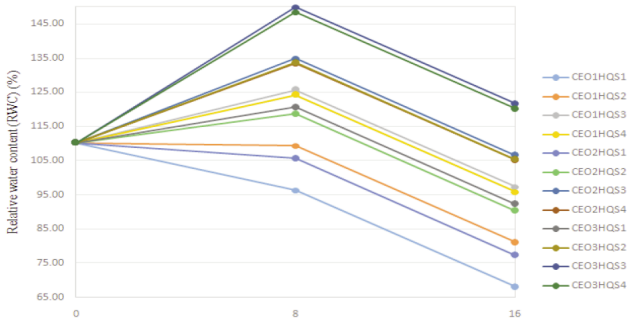

Fig. 4 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on relative water content (RWC) (%) in different measuring times (days).

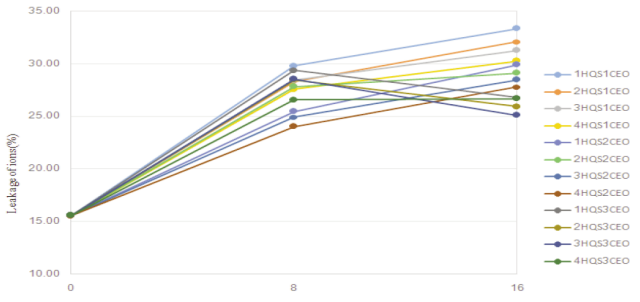

Fig. 5 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on leakage of ions (%) in different measuring times (days).

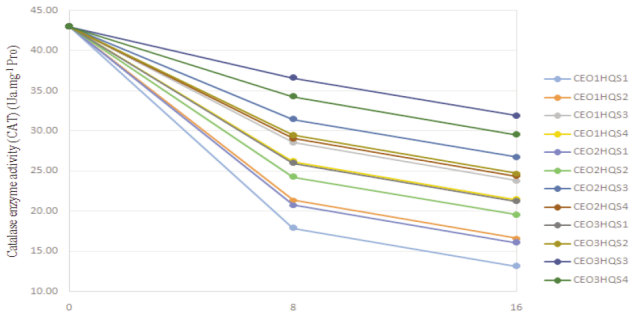

Fig. 6 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on catalase enzyme activity (CAT) (Ua mg^{-1} Pro) in different measuring times (days).

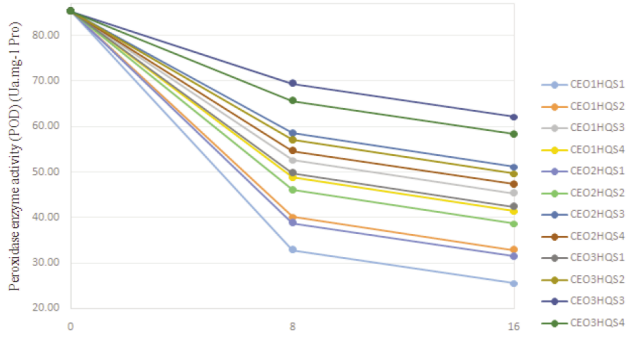

Fig. 7 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on peroxidase enzyme activity (POD) (Ua mg^{-1} Pro) in different measuring times (days).

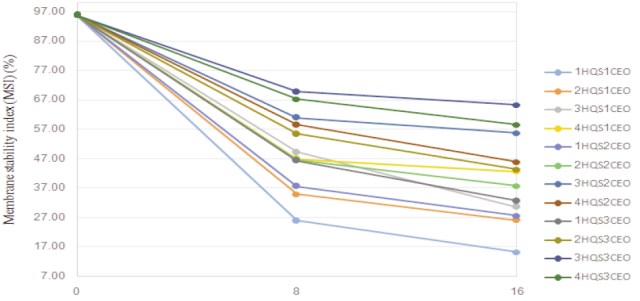

Fig. 8 - Mean comparison for interaction effects of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) different levels on membrane stability index (MSI) (5) in different measuring times (days).

4. Discussion and Conclusions

The results showed that the application of cumin essential oil (CEO) and 8-hydroxyquinoline sulfate (HQS) had positive effect on vase life and postharvest quality of cut rose (*Rosa hybrid* cv. Angelina) flowers in different measuring time. Application of 100 mg·L^{-1} cumin essential oil and 600 mg·L^{-1} 8-hydroxyquinoline sulfate increased vase life of cut rose flowers. This result was in accordance with results of Hussein (1994) and Knee (2000). The application of 8-HQS may prevent the accumulation of microorganisms in xylem vessels and suppressed the xylem occlusion due to its role as anti-microbial agent and hence, it might reduce stem plugging. Essential oils like CEO play an important role in the protection of the plants as antibacterial, antiviral, antifungal, insecticides and also against herbivores by reducing their appétit for such plants (Bakkali *et al.*, 2008). Petal fresh/dry weight rate was improved significantly by application of 100 mg·L^{-1} cumin essential oil and 600 mg.L^{-1} 8-hydroxyquinoline sulfate. These results are in line with results of Ali and Hassan (2014) on *strelitzia* cut flowers with application of 8-hydroxyquinoline sulfate and gibberlic acid treatments.

The application of 8-HQS may reduce the plasmolysis of cells which occurred when the rate of cellular water loss is too rapid. The cut rose flowers reached to the highest diameter with application of 100 mg·L^{-1} cumin essential oil and 600 mg·L^{-1} 8-hydroxyquinoline sulfate. These findings are in according to reports of Kim and Lee (2002). HQS not only prevents the vascular obstruction caused by the microorganisms, but also prevents the blockage stimulated by the plant itself. The highest relative water content in cut rose flowers was related to treatment of 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate. These results are similar to Knee (2000) findings on cut carnation flowers. Leakage of ions was occurred in control treatments in the highest amount. Essential oil of cumin mainly conjugated to compounds that have known as phenolic compounds, are responsible for pathogen control in plants (Plotto *et al.*, 2003). These compounds prevent senescence and wilting by their antibacterial property and reducing the pH of the environment (Elgimabi and Ahmed, 2009). Catalase and peroxidase enzymes activities increased significantly by treatments of 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxy quinoline sulfate. These results are consistent with results of Ranjbar *et al.*, 2015. Catalase is an important biological factor with major function in superoxide metabolism and plays an important role in releasing oxygen and hydrogen peroxide free radicals and prevents creation of hydroxyl radicals (Spanou *et al.*, 2012). Peroxidase has different biolog-

ical functions such as detoxification of hydrogen peroxide, lignin biosynthesis, hormonal signaling and response to stress (Gao *et al*., 2010). Maybe the treatment of 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate decreases oxidative stresses in cut rose flowers (Hassan and Ali, 2014). Membrane stability index showed the highest percent in treatment of 150 mg·L^{-1} cumin essential oil and 400 mg·L^{-1} 8-hydroxyquinoline sulfate. These findings are compatible to results of Kazemi and Ameri (2012). They showed the positive effect of herbal essential oils of thyme and lavender on the stability of the membrane and reduction of MDA. The senescence of cut flowers with hormonal regulatory mechanism is involved in changing the physical and biochemical features of cellular membrane (Buchanan Wollaston, 1997). Oxidative membrane injury allows the mixing of the normally separated enzyme (PPO) and oxidizable substrates (polyphenols), which lead to browning (Hodges, 2003). According to Palma *et al*. (2002), the herbal essential oils by preventing the activity of oxygen species reduce the lipid peroxidation in cell membrane and the concentration of MDA. Plant essential oils are bioactive in the vapor phase, and this makes them fumigants for postharvest rotting fungi control in fruits and grains (Paster *et al*., 1995; Hammer *et al*., 1999; Feng and Zheng, 2007). Different studies showed postharvest disease control in different fruit species by using biological agents including essential oils (Bishop and Thompdon, 1997; Feng and Zheng, 2007; Amiri *et al*., 2008).

A limiting factor in cut flower marketing is postharvest senescence. There are many reports used different materials for extending rose cut flower vase life. We studied application of cumin essential oil and 8-hydroxyquinoline sulfate. They had positive effects ($P<0.05$) on vase life and postharvest quality of cut rose (*Rosa hybrida* cv. Angelina) flowers. Results showed they affect some growth and development parameters such as relative fresh weight, flower and stem diameters, anthocyanin and chlorophyll contents as well catalase and peroxidase activities that cause improving vase life of rose cut flowers.

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Postharvest application of hydrogen peroxide and salicylic acid differently affects the quality and vase life of cut rose (*Rosa hybrida* L.) petals and leaves

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Key words: chlorophyll fluorescence, cut flowers, elicitors, oxidative stress, phenolic compounds, senescence.

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Abstract: Rose represents an important product in the market of cut flowers. Its quality is related to visual appearance and is affected by oxidative stress and senescence. To maintain the quality and extend the product vase life, innovative technical solutions to be applied in postharvest are needed. In this work the effectiveness of hydrogen peroxide (H_2O_2) and salicylic acid (SA), was assessed on cut rose leaves and petals. Several physiological indicators of quality were studied, including chlorophyll content, chlorophyll *a* fluorescence, color, lipid peroxidation, phenolic compounds and anthocyanins. After 7 days of vase life 73.33% of untreated roses were not marketable anymore, while the percentage was lower in response to treatments (47.06% in SA and 25.53% in H_2O_2). The application of H_2O_2 induced a reduction in leaf chlorophyll and in the performance index which, at the end of the vase life decrease by 76% and 49% respectively. Consistently, the lipid peroxidation in leaves treated with H_2O_2 increased by a 53%. After 4 days of vase life, SA allowed maintaining higher levels of anthocyanins in petals compared to H_2O_2 and to controls. The results obtained allowed individuating different responses, depending on the treatment applied as well as on the plant organ.

1. Introduction

The market of cut flowers is characterized by the high perishability of the commercialized products. The pipeline from growers to the final consumers, is often long and at the same time, the item must have very high-quality standards. Flowers showing discoloration, shriveling, bending or any other visible sign of damage can no longer be commercialized, and product losses represents a crucial problem for this sector.

Rose (*Rosa hybrida* L.) is one of the most important species in the flower market and it is particularly appreciated by the consumers as cut flower. The quality of cut roses is strictly related to the integrity and quality of petals, but also to the color of leaves, which contributes to their visual appearance. Chlorophyll degradation and leaf yellowing reduce the

ornamental value of cut flowers and compromise their commercialization. During transportation and handling, leaves and petals can undergo mechanical damage, which can lead to tissue browning with negative effect consequences on quality.

On the physiological point of view, the loss of quality is a direct consequence of oxidative stress which in turn can lead to senescence (Kumar *et al.*, 2008). These phenomena are characterized by the activation of several physiological responses at cellular level and involve signaling molecules, enzymes, and transcriptional regulators such as genes and transcription factors (Gregersen *et al.*, 2013; Thomas, 2013). In the recent years, several efforts have been made to maintain the quality of cut roses and extend their vase life. These efforts include breeding programs the improvement of growing conditions, and the optimization of transportation. Moreover, the effectiveness of several treatments applied during the vase life has been assessed (Reid and Jiang, 2012).

Salicylic acid (SA) is a plant hormone involved in several plants functions, including stress responses, development, and plant signaling (Hayat *et al*., 2010; An and Mou, 2011; Rivas-San Vicente and Plasencia, 2011). Spray application of SA has been reported to prolong vase-life in cut rose flowers by increasing the activity of the ROS-scavenging enzyme catalase and improving the water balance. Interestingly, the lowest dosage applied (50 µM) was the most effective (Alaey *et al.*, 2007). Also, in other studies, low concentrations of SA (0.01 mM) have been reported as the most effective in inducing plants responses to stress (Horváth *et al*., 2007).

Hydrogen peroxide (H_2O_2) is an important molecule produced and accumulated in plant cells because of the aerobic metabolism. It belongs to the reactive oxygen species (ROS) and can play a key role in stress metabolism and senescence. The increment in ROS levels is, in fact, a typical feature of senescence and can lead to cellular damage and finally to cell death (Panieri *et al*., 2013). However, slight alterations of the ROS balance, including H_2O_2 accumulation, can be considered as important signals in plant cells and can trigger a series of defense responses, which in some case can also involve SA (Kalachova *et al*., 2013; Herrera-Vásquez *et al*., 2015). While high concentrations of H_2O_2, can directly induce oxidative stress in plant tissue, low doses can lead to the activation of positive physiological responses. It has been suggested that H_2O_2 priming can help in modulating ROS-mediated stress tolerance in plants (Hossain *et al*., 2005). Non-toxic levels of this molecule must be maintained to have beneficial effects and to improve the quality and vase life of cut flowers, as reported for lily (Liao *et al*., 2012).

The aim of this work was to evaluate the effect of low concentrations of H_2O_2 and SA when applied to cut roses during the vase life. Attention has been given to the individuation of specific and common responses induced by these two molecules. To achieve this goal, several indices in both petals and leaves were considered, including chlorophyll content, chlorophyll *a* fluorescence, color, lipid peroxidation, phenolic compounds and total anthocyanins.

2. Materials and Methods

Plant material

Cut rose stems (*Rosa hybrida* L. 'Tacazzi') at the commercial developmental stage were purchased on July 20th, 2017, from the Milan flowers market and brought to the laboratory within one hour. Each stem was around 60 cm-long. Once in the laboratory, 20 stems were randomly selected from a pool of 60 and placed in a beaker filled with 2 L of distilled water (control). Similarly, two other groups of 20 stems each, were placed in a SA (0.01 mM) and H_2O_2 (0.1 mM) solution respectively. The concentrations of the treatments were chosen among the lowest effective dosage found in the literature to reduce the risk of toxicity. After 24 hours, all the solutions were substituted with 2 L of fresh distilled water and the volume was maintained until the end of the trial. The vase life conditions were: 20±2°C, 55% relative humidity (RH), 18 µmol m^{-2} s^{-1} light intensity, with a photoperiod of 12 hours. Samples of petals and leaves were collected at the beginning of the trial as well as after 4 and 7 days of vase life. After 7 days the remaining stems were maintained until all of them were considered unmarketable.

Vase life and damaged flowers (%)

At each time point, the number of damaged flowers were estimated based on the visual appearance. Those flowers showing wilting, shriveling or desiccation were considered as not marketable anymore and thus counted as damaged. This information was used to calculate the percentage of damaged flowers and the average vase life (reported as days).

Water content (%)

At harvest and after 7 days three roses for each treatment (the whole stem) were weighed and

immediately placed in an oven at 115°C until the complete drying. The water content was calculated as percentage of water respect to fresh weight.

Chlorophyll and chlorophyll a fluorescence-related parameters

During vase life *in vivo* determination of leaves chlorophyll content was performed using a CL-01 chlorophyll mater (Hansatech, United Kingdom). Similarly, chlorophyll *a* fluorescence was measured on dark adapted leaves using a portable fluorimeter (Handy PEA, Hansatech, United Kingdom). The parameters measured were: the quantum maximum efficiency of PSII (Fv/Fm), the performance index (PI), the number of reactive centers per cross section (RC/CSm), and the amount of energy dissipated per reaction center (DIo/RC).

Color measurement

Changes in petal coloration in response to treatments and to vase life were evaluated at each time point by using a Minolta CR-400 colorimeter (Konica Minolta, Inc., Japan). The color parameters were described using the Lab* and the Munsell (L*C*h) color spaces. Briefly, in the Lab*color space the lightness is represented by L* (with 0 indicating black and 100 white). The colors in the red green region are represented by a* (red with positive values and green with negative values) while b* describes the interval from yellow (positive values) to blue (negative values). In the Munsell color space h represents the hue while C* is the chroma (or color purity).

Phenolic index and total anthocyanins

For the extraction of phenolic compounds and anthocyanins, around 1 g of petals and leaves, collected at each time point, were homogenized in 8 mL of acidified methanol (1% HCl V/V) and extracted overnight in the dark. The phenolic index was calculated as the absorbance at 320 nm of the diluted extracts, normalized to fresh weight (Ke and Saltveit, 1989). Total anthocyanins were determined spectrophotometrically at 535 nm using an extinction coefficient (ε) of 29,600 mM^{-1} cm^{-1} and expressed as cyanidin-3-glucoside equivalents and (Klein and Hagen, 1961).

Lipid peroxidation

The level of lipid peroxidation was measured with the thiobarbituric acid reactive substances (TBARS) assay (Heath and Packer, 1968). Briefly, around 1 g of petals and leaves was homogenized in 5 mL 0.1% trichloroacetic acid (TCA) solution. The extract was mixed with 4 mL of 20% (W/V) TCA, 25 µL of 0.5% thiobarbituric acid (TBA) and distilled water. After vortexing, the mixture was heated at 95°C for 30 minutes in a water bath and then cooled on ice. Absorbance at 600 nm was subtracted from the absorbance at 532 nm (as an index of non-specific turbidity) and the concentration of TBARS was expressed as malondialdehyde (MDA) equivalents (nmol g^{-1} FW), calculated using an extinction coefficient (ε) of 155 mM^{-1} cm^{-1}.

Statistical analysis

All data were subjected to two-way ANOVA followed by Bonferroni's multiple comparisons test, except for vase life data which were subjected to a one-way ANOVA. Statistics were performed using GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla, California, USA (www.graphpad.com).

3. Results

Vase life and water content

After 4 days of vase life all the flowers treated with H_2O_2 and SA were considered marketable, while 10% of untreated flowers showed a marked loss of quality. After 7 days, the higher rate of damage was recorded in the controls (73.33%), followed by SA (47.06%) and H_2O_2 (25.53%) (Fig. 1 A). After 8 days all

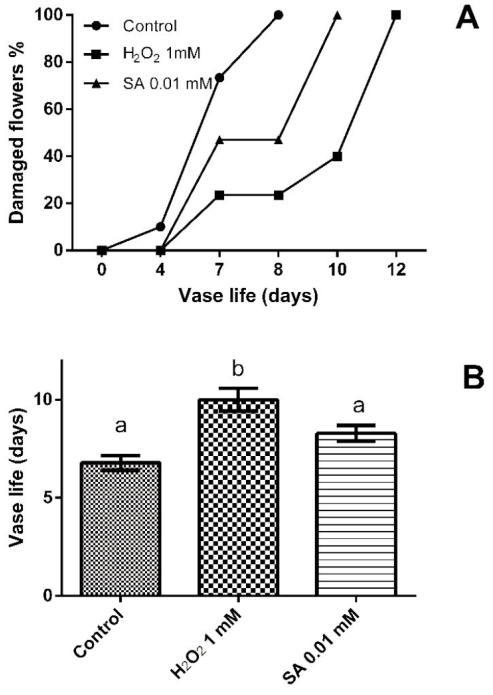

Fig. 1 - Percentage of damaged flowers (A) and average vase life (B) measured in cut 'Tacazzi' rose. Data are means ± SE (B: n=14; C: n=3). Different letters indicate significant differences ($p<0.05$).

the control flowers were already damaged, while 6 flowers treated with SA were still marketable until 10 days, and 6 among those treated with H_2O_2 lasted until 12 days.

The treatment with H_2O_2 determined a significant increment in the average vase life compared to SA and control (Fig. 1 B).

The initial water content was 77.86% and showed a slight progressive decline without any significant difference among the treatments. After 7 days the values were: 70.01%, 70.61%, 70.64%, in control, SA and H_2O_2 respectively. The flowers treated with H_2O_2 showed a reduced opening compared to those treated with SA or to controls (Fig. 2 A, C).

Color

After 7 days of vase life, all the petal samples looked paler compared with to the beginning of the trial and an increment in the L* value was recorded despite the treatments applied (Table 1). H_2O_2-treated flowers did not show any change in the color-related parameters compared to controls. On the other hand, after 7 days, SA induced a decrement in all the color attributes compared to the beginning of the trial, including a*, b*, hue (h) and chroma (C*). This variation was consistent with the visual observation of the petals which appeared darker and turned from red to a more violet coloration (Fig. 2 C).

Chlorophyll content and chlorophyll a fluorescence-related indexes

Treatment and vase life duration significantly affected the chlorophyll content of cut rose leaves. After 4 days of vase life, the chlorophyll content was

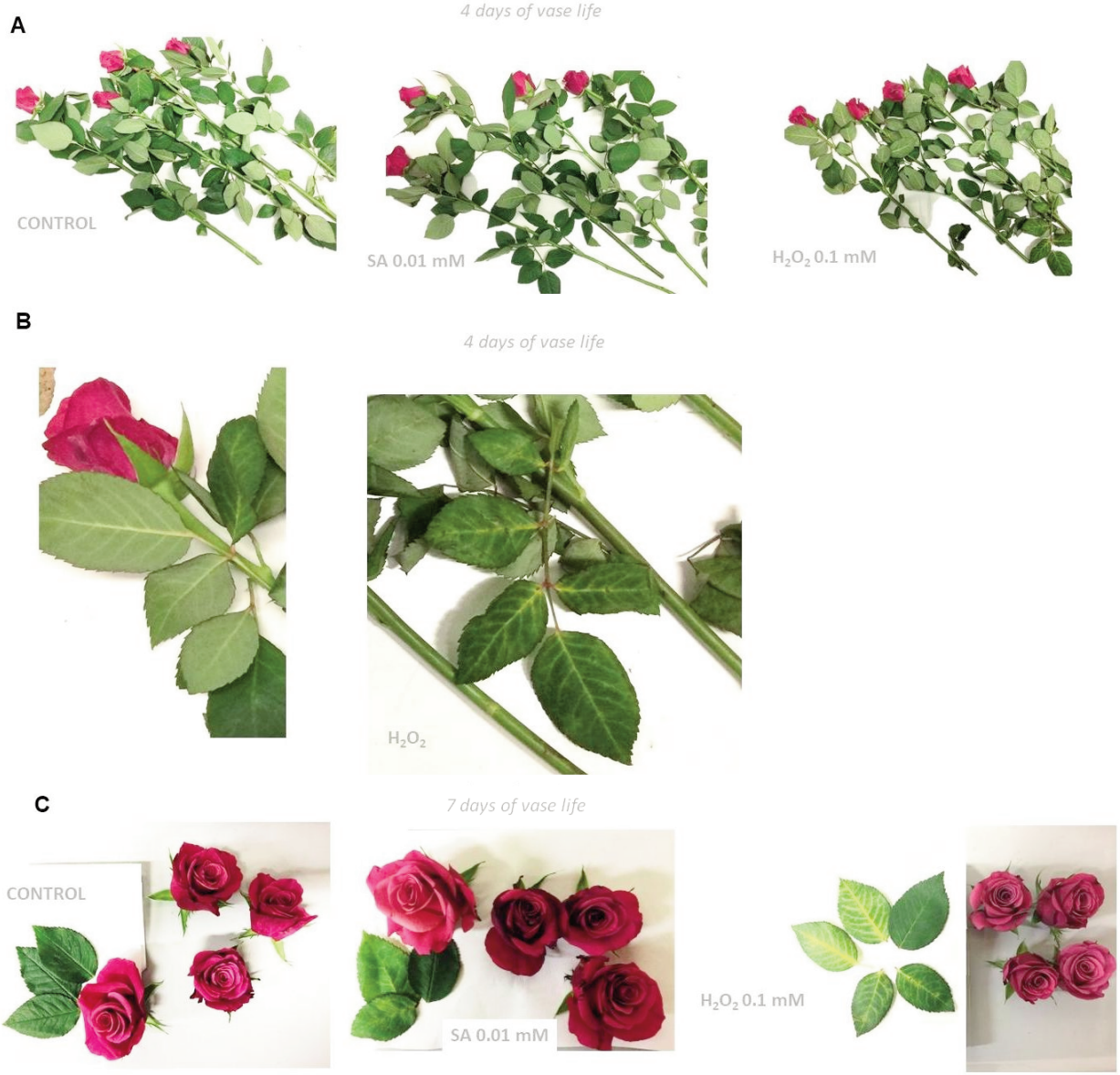

Fig. 2 - Visual appearance of cut roses (cv. Tacazzi) as affected by H_2O_2 or SA application after 4 (A, B) and 7 (C) days of vase life.

Table 1 - Changes in petal color parameters of cut 'Tacazzi' rose during vase life and in response to treatments

Treatment	Days of vase life	L*	a*	b*	h	C*
-	0	41.17±0.64 a	55.93±0.31 a	14.10±.01 a	14.14±0.98 a	57.70±0.37 a
Control	4	34.13±1.93 a	48.57±1.89 ab	9.75±1.12 ab	11.29±1.05 ab	49.56±2.00 ab
H_2O_2 0.1 mM	4	39.00±2.16 a	49.08±1.69 ab	10.46±2.13 ab	11.81±2.09 ab	50.77±2.30 ab
SA 0.01 mM	4	36.09±1.18 a	50.09±1.78 ab	10.12±1.94 ab	11.21±1.77 ab	51.17±2.11 ab
Control	7	47.73±3.02 b	48.12±2.83 ab	9.45±0.56 ab	11.16±0.63 ab	49.05±2.84 ab
H_2O_2 0.1 mM	7	51.12±1.22 b	50.13±1.67 ab	9.80±0.99 ab	11.00±0.81 ab	50.97±1.78 ab
SA 0.01 mM	7	50.19±2.67 b	44.57±3.14 b	6.49±1.02 b	8.52±1.64 b	45.10±3.04 b

Data are means ± SE (n=6). Different letters indicate significant differences ($p<0.05$).

significantly lower in the controls and in SA-treated leaves compared to H_2O_2-treated ones. However, H_2O_2 induced a pronounced discoloration of leaves, particularly at the end of the trial (Fig. 2 B, C). This observation is confirmed by the significant reduction in chlorophyll content which has been recorded after 7 days of vase life (Fig. 3 A).

All the parameters related to chlorophyll *a* fluorescence showed a trend consistent with the one observed in chlorophyll content. In particular, stems treated with H_2O_2 showed a loss in the leaf PSII functionality after 7 days of vase life, evidenced by a significant decrement in the PI value (Fig. 3 C). No significant changes were observed in Fv/Fm, RC/CSm and in DIo/RC (Fig. 3 B, D, E), even though these indexes showed a trend similar to the one observed in the PI.

Lipid peroxidation

The level of lipid peroxidation in leaves was significantly affected by treatment and by vase life duration. In leaves treatments induced higher oxidative damage compared to control, already after 4 days

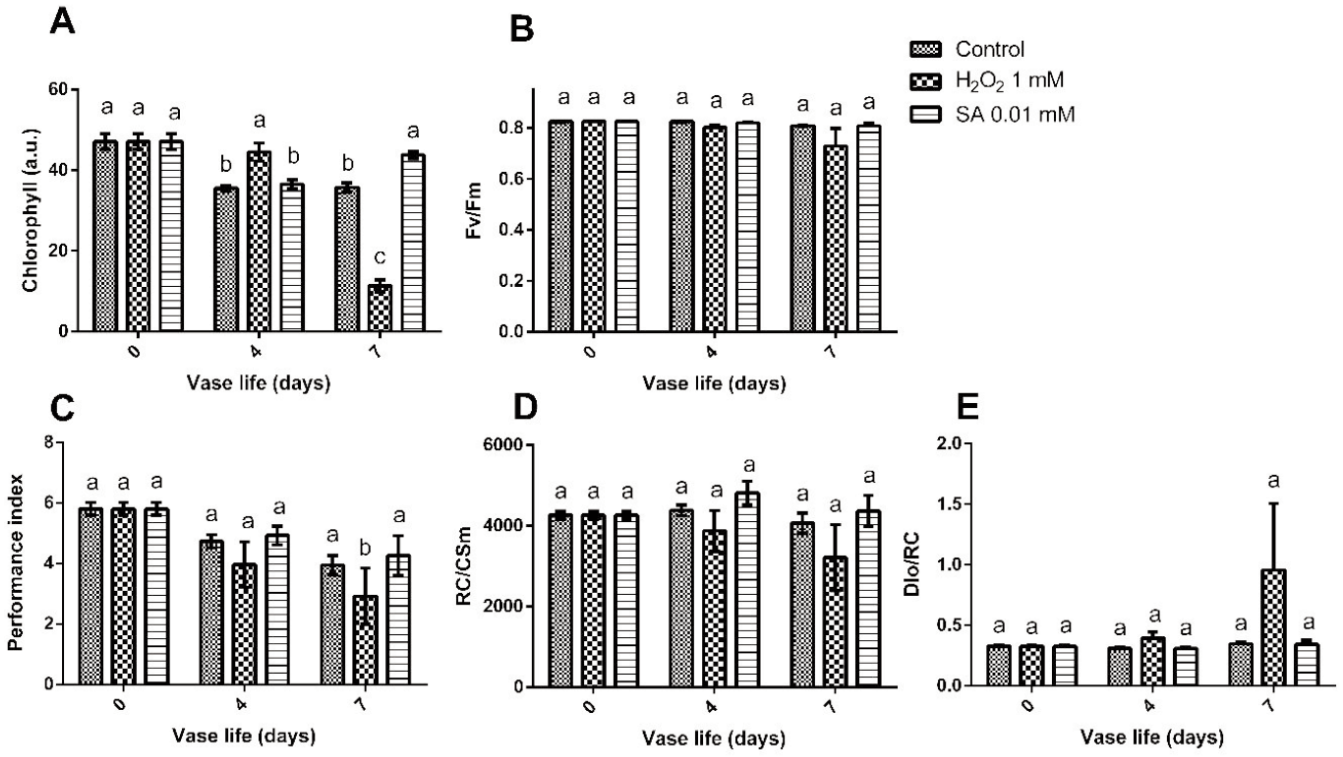

Fig. 3 - Chlorophyll content (A) and chlorophyll a fluorescence-related indexes: maximum quantum efficiency of PSII (Fv/Fm) (B), performance index (PI) (C), number of reaction centers per cross section (RC/CSm) (D), energy dissipated per reaction center (DIo/RC) (E). Measurements were conducted on leaves of cut 'Tacazzi' rose. Data are means ± SE (n=6). Different letters indicate significant differences ($p<0.05$).

and lipid peroxidation reached the maximum level in H_2O_2-treated leaves after 7 days of vase life (Fig. 4 A). The amount of MDA equivalents was markedly higher in flowers (Fig. 4 B) compared to leaves, but no significant changes were observed in petals in response to vase life or to treatments.

Phenolic index and total anthocyanins

The levels of total phenolic compounds, estimated with the phenolic index, were stable, with similar values between leaves and flowers and without showing any significant change in response to treatment or to vase life (Fig. 5 A and B).

On the other hand, total anthocyanins, which were highly accumulated in petals, progressively declined during the vase life (Fig. 5 C and D), with a significant effect of vase life time in both leaves and petals. This trend was more pronounced in petals, which showed a significant decrement already after 4 days in controls and in response to H_2O_2 treatment.

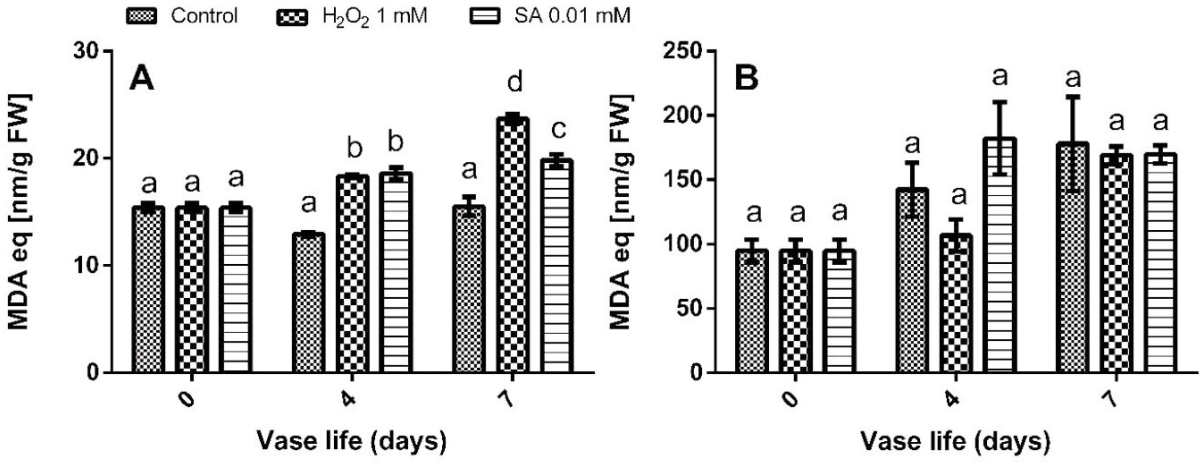

Fig. 4 - Changes in lipid peroxidation in leaves (A) or petals (B) of cut 'Tacazzi' rose during vase life and in response to treatments. Data are means ± SE (n=3). Different letters indicate significant differences ($p<0.05$).

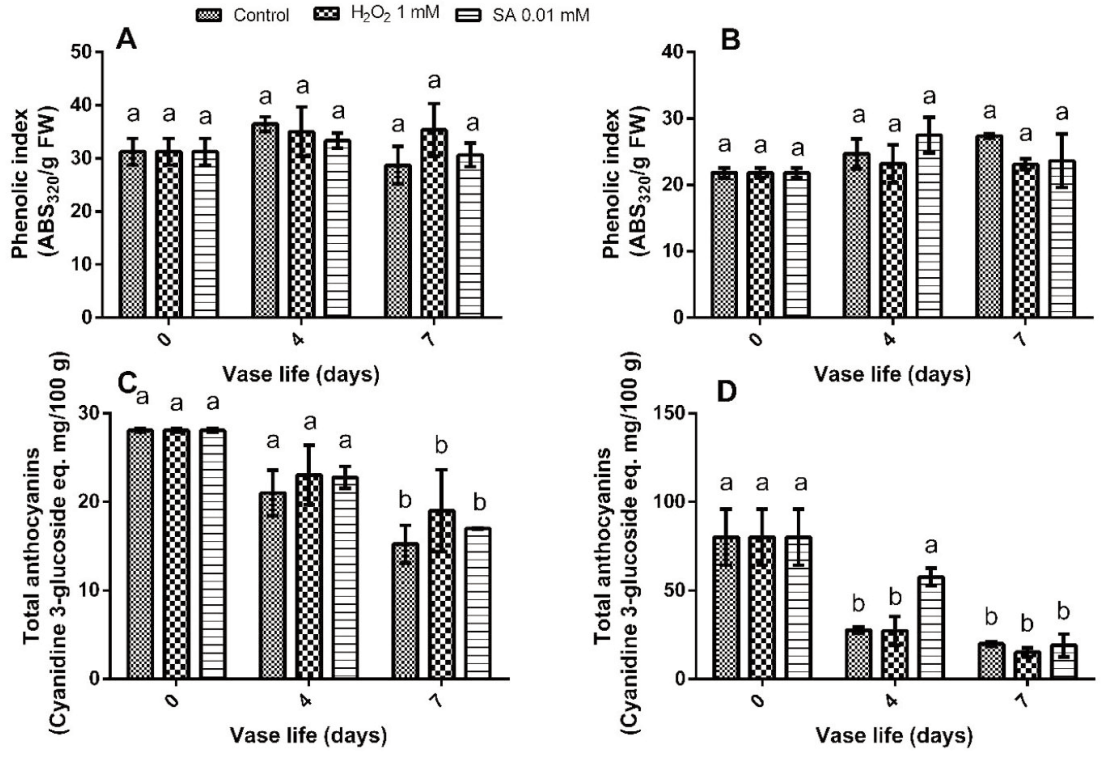

Fig. 5 - Changes in the phenolic index (A: leaves; B: petals) and in total anthocyanins levels (C: leaves; D: petals) of cut 'Tacazzi' rose during vase life and in response to treatments. Data are means ± SE (n=3). Different letters indicate significant differences ($p<0.05$).

Then again, after 4 days SA-treated petals showed significantly higher levels of anthocyanins compared to H_2O_2 and to control.

4. Discussion and Conclusions

The results obtained in this work allowed individuating independent ways of action for the two treatments and evidenced positive and negative aspects of both molecules.

As a first consideration is important to observe that both treatments determined a reduction in the product losses compared to control and in case of H_2O_2, a significant extension of the vase life. This positive result is consistent with other previously published works (Gerailoo and Ghasemnezhad, 2011; Liao *et al.*, 2012). It is also interesting to point out that the two treatments applied, induced different responses in rose leaves and petals.

The application of H_2O_2 determined a delay in the flower opening compared to control and to SA, this is consistent with what already observed in cut lily (Liao *et al.*, 2012) and in tree peony (Shi *et al*., 2015), and can have positive implications in the postharvest management of cut roses. In fact, the possibility of modulating or delaying the flower opening could be successfully be used to extent the commercial life of cut flowers and to better manage the storage and transportation. Moreover, H_2O_2 helped in maintaining low levels of MDA in petals during the first days of vase life. As a drawback, H_2O_2 induced leaves yellowing which in turn can affect the visual appearance of the stem. The detrimental effect of H_2O_2 on leaves has been confirmed by the marked loss of chlorophyll and by the higher level of peroxidation recorded at the end of the vase life. The parallel decrement in the PI, suggests a possible positive association between chlorophyll *a* fluorescence and plant stress, as previously reported (Kalaji *et al.*, 2006). Considering the results obtained in this trial, it appears that H_2O_2 is not affecting the accumulation of phenolic compounds and pigments in cut 'Tacazzi' rose.

It is known that the effect of H_2O_2 depends on the dosage (Liao *et al*., 2012), thus it is possible that the concentration of H_2O_2 applied in this trial was enough to induce oxidative stress and damage in the leaves while having a positive effect on flowers.

There are not many reports about the effect of SA in ornamental leaves, however recently, a role for SA in extending the vase life of *Acacia holosericea* foliage has been suggested (Chen and Joyce, 2017). The positive effect of SA on the leaves of cut roses has been confirmed in the present work, since at the end of the trial, SA-treated leaves showed a better maintenance of chlorophyll and leaf functionality indexes compared to controls and to H_2O_2-treated stems. The positive effect of SA could be due to the activation of plants defenses against ROS, including the increment in antioxidant compounds and the activation of antioxidant enzymes. Treatments with SA have been reported to reduce lipid peroxidation in gladiolus cut flower spikes (Rahmani *et al.*, 2015). Also, SA alone or in combination with sucrose, was shown to affect the rate of lipid peroxidation (MDA) and chlorophyll content in carnation petals (Kazemi *et al.*, 2011), but different results were observed depending on the dosage applied. Unexpectedly, in the present work, the application of 0.01 mM of SA was not effective in preventing lipid peroxidation of cut roses, perhaps because of the too low concentration applied.

The degradation of anthocyanins is a typical phenomenon which negatively affects the quality of ornamental flowers, including rose (Luo *et al.*, 2017). The role of exogenous SA in stimulating the biosynthesis of anthocyanin pigments has been documented (Ram *et al*., 2013), thus it can be hypothesized that SA treatment could delay the degradation of pigments in petals. The increase of phenolic compounds as well as the anthocyanins content could help in counteracting the senescence and extend the flower life as observed in model plants (McNish *et al.*, 2010). Despite the low dose applied, SA helped in maintaining higher levels of anthocyanins in cut rose petals after 4 days of vase life. Also, the petal coloration was affected by SA after 7 days.

In conclusion, the negative effect of H_2O_2 in leaves compared to the beneficial effect observed in flowers is controversial and it would surely worth to be investigated further. Perhaps the negative effects could be circumvented by testing other forms of application, such as direct spraying on the petals avoiding contact with leaves. The application of SA did not induce any damage, but at the same time, the positive effects were not as evident as expected compared to untreated control. This suggests that the conditions of treatment with SA can be further perfectioned. In future experiments the application of higher doses of SA could intensify the positive effects observed in this trial. Given the results obtained, the assessment of the PI, based on the chlorophyll fluorescence mea-

surement, could represent an innovative approach in the evaluation of the vase life of cut flowers, as it followed a trend consistent with the decline observed in chlorophyll content and with the increment in MDA. In fact, the non-destructive estimation of leaf functionality through this index could be further studied, implemented and successfully used for the rapid quality control of cut flowers and ornamentals.

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Yield, quality and antioxidants of peeled tomato as affected by genotype and industrial processing in Southern Italy

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

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Abstract: Research was carried out on processing tomato in San Severo (Tavoliere delle Puglie, Foggia, Italy) in order to compare four long-type fruit hybrids oriented to peeled produce (Abbundo, Umex, Superpeel, Taylor), using a randomized complete block design with three replicates. The hybrid Superpeel reached the highest marketable yield due to the highest fruit number and mean weight; along the peeling chain, Umex and Taylor showed the highest processing efficiency. Titratable acidity and sodium were highest in Taylor fruits, whereas the highest fiber content was detected in Abbundo fruits. Compared to pre-processing fruits, peeled tomatoes showed increased values of total and soluble solids as well as reducing sugars, but decreased sugar ratio and colour. The highest concentrations of antioxidants in processed fruits were recorded in Umex for lycopene and in Superpeel for β-carotene. Compared to pre-processing fruits, in peeled tomatoes lycopene and β-carotene concentrations remained stable and polyphenols increased referring to fresh weight. The hybrids examined did not show univocal trends in terms of sensorial features.

1. Introduction

Tomato is the most cultivated vegetable species worldwide with 5,023,810 ha (FAO, 2014); Italy is a major European producer of processing tomato with a surface area as much as 79,761 ha devoted to this crop (ISTAT, 2017 - dati.istat.it), of which 32% are located in Emilia-Romagna and 25% in Apulia. The Italian industry of tomato and its derivatives (peeled, diced and sauce) attained an export gross marketable yield as

much as about 1.5 billion of euros in 2017 (ISTAT, 2017). These products are traditionally addressed to United Kingdom, USA, France, Australia, Japan, Germany, Belgium, but also to growing demanding markets, i.e. eastern Europe, South America (Brazil and Argentina), South Africa, South Corea, United Arab Emirates.

The interest of processing factories in southern Italy to valorize peeled-oriented tomato type matches the seed company goal to provide farmers with hybrids showing high yield, improved taste and flavour features, and better industrial requirements compared to the current genotypes. With these prospects, the new hybrids are evaluated with regard to the main traits, such as: plant architecture and parasite resistance; morphology, number, weight, ripeness uniformity, technological and quality performances of fruits.

In order to carry out tomato genotype selection, some authors suggested to perform a comprehensive evaluation using synthetic agronomic and quality indexes (Carli *et al.*, 2011), upon assessing an appreciable number of related variables such as dry matter, soluble solids, sugars, acidity and antioxidants. Notably, high dry matter and soluble solids are desirable characteristics for the canned tomatoes industry since they improve the quality of the processed product (De Pascale *et al.*, 2001). Indeed, soluble solid content and titratable acidity are the main components responsible for tomato flavor (Kader, 1986; Flores *et al.*, 2008) and they are most likely to match the consumer perception of the internal quality (Baldwin *et al.*, 1998; Arazuri *et al.*, 2007). In this respect, the balanced ratio between sugars and organic acids is important to sweetness, sourness and overall flavor intensity in tomatoes (De Bruyn *et al.*, 1971; Stevens *et al.*, 1977). In fact, high acids and low sugars will produce a tart tomato while high sugars and low acids will result in a tasteless tomato (Kader, 1986).

Due to their antioxidant attributes in addition to sensorial appealing (Raiola *et al.*, 2016), tomato-based products are associated with a low risk of both cancer and incidence of coronary heart disease (Pernice *et al.*, 2010; Ilahy *et al.*, 2011).

The present research was carried out since new hybrids for peeled tomato with improved features are needed for the cultivation in Southern Italy and, in this respect, they were compared with a widespread cultivar in southern Italy, in terms of yield, technological, quality and sensorial characteristics.

2. Materials and Methods

Field conditions

Research was carried out on processing tomato at De Maio farm, located in San Severo (Tavoliere delle Puglie, Foggia, southern Italy) in 2017 on silty-sandy soil.

The experimental protocol was based on the comparison between 4 long-type hybrids oriented to peeled produce: Abbundo (HM Clause Italia SpA., Italy); Umex (Syngenta Italia SpA., Italy); Superpeel (United Genetics Italia SpA., Italy); Taylor (Nunhems Italy Srl, Italy) as a control. A randomized complete block design was used for the treatment distribution in the field, with three replicates, and the elementary plot had a 67 m^2 surface area.

The transplant was performed on 28 April, arranging a double-row layout, with 35 cm between the plants spacing along the rows, 40 cm between the two rows in each couple and 150 cm between the outer rows of two adjacent couples, thus achieving a density of 3 plants per m^2.

The ordinary farming technique related to processing tomato in Tavoliere delle Puglie was followed and harvests were practiced between 3 and 7 August.

Yield, quality and technological determinations

When the 90% fruits were ripe, the following agronomic determinations were made in each plot: weight of marketable fruits (red + colour turning point) and waste berries (green + rotten); mean fruit weight on a random 100 fruit sample; middle length and width on a random 20 fruit sample; percentage of fruit coverage exerted by plant canopy, referred to the ranges of 0-25%, 25-50%, 50-75%, 75-100%.

Quality analyses of pre-processing fruits sampled in each field plot, as well as technological and quality determinations of processed fruit samples of each hybrid were performed at the laboratories of Stazione Sperimentale per l'Industria delle Conserve Alimentari in Angri (Salerno).

As for technological determinations, the processing yield was assessed, representing the ratio between the canned tomato fruit amount, after selection and technological process, and the marketable yield obtained in the field. The fruit processing was performed using the pilot plant available at SSICA. In this respect, tomato peeled production was carried out on a semi-industrial scale, with juice addition, packaged in painted tinplates of 1 kg; notably, the juice obtained by the same cultivar was added

after partial concentration at 7.5°Brix. In order to assess the processing yield, weights were recorded at each different step, the product to be peeled underwent selections and weighing of each fruit fraction such as yellow and necrotized, rotten, broken, undersized; moreover, skins were weighed after peeling. As concerns the product submitted to the juice chain, yellow, necrotized and rotten fruits, as well as skins and refining seeds were weighed. Next, the drained fruit percentage was assessed, calculated as a mean of five cans; all the determinations were performed in triplicate and averaged.

The fruit quality features assessed on both fresh and peeled fruits, referred to fresh weight, were: total solids (TS), soluble solids (SS), reducing sugars (glucose and fructose) and sucrose, colour (a/b ratio), antioxidants (lycopene, b-carotene, total polyphenols). In addition, the components included in the current European nutritional label, according to EU Regulation 1169/2011, were determined: proteins, titratable acidity, lipids, fatty acids, fiber, salt and sodium. The analytical procedures were performed according to Caruso *et al*. (2012) for total and soluble solids, sugars, proteins, fiber, ash and sodium; MiPAF (1973) for titratable acidity and fats; Golubkina *et al*. (2015) for fatty acids; Conti *et al.* (2015) for colour; De Sio *et al.* (2001) for carotenoids; Golubkina *et al.* (2017) for polyphenols. Briefly, total solids were assessed in oven at 70°C under vacuum until steady weight, whereas soluble solids by means of a digital refractometer. Sugars were determined by HPLC, using the 600E Waters chromatographic system and a column Sugar-pak Waters at 85°C, EDTA-Ca in water solution as eluent (50 mg L^{-1}). Proteins were assessed with the Kjeldahl method, by a Foss Tecator digestor with a Kjeltec 2300 distiller. Fibre was determined after the samples were weighed, dried (105°C), gelatinized in the presence of heat-resistant α-amylase and enzymatically digested by proteases and amydoglucosydase, to remove proteins and starch, whereas soluble fibre was precipitated by ethanol; the residue was filtered, washed with ethanol and acetone, dried, weighed and split into two fractions to determine proteins and ash, and fibre content as the difference to the residue weight. Sodium was assessed by atomic adsorption spectrophotometry, after sulpho-nitric mineralization, with a model 1100 Perkin-Elmer spectrophotometer. Fatty acids were detected by gas chromatography via appropriate methyl ethers chromatography on capillary glass column, using an Agilent 6890 Gas Cromatograph, equipped with a flame ionization detector; the peaks of fatty acids methyl ethers were identified by comparison to the retention times of reference standards. Colour was assessed by a Hunter Associate Laboratories D25-A model colourmeter, using a suitable measurement cell with the standard BCR n. 1266 reference whose values are L= 25.7, a= 23.7, b= 14.8; chromatic parameter values are expressed in the Hunter scale as a/b. Carotenoids were assessed through HPLC, using a Waters Alliance chromatograph equipped with photodiode array detector mod. 996, performing the determinations at 450 nm on a reversed phase column YMC-Pack C30 (250 x 4.6 mm i.d.) filled with 5 µm average particle size. Polyphenols were determined in water extract through a spectrophotometer (Unico 2804 UV, USA); the concentration was calculated according to the absorbance at 730 nm, using 0.02% gallic acid as an external standard.

Sensorial determinations

Sensorial or organoleptic determinations were performed on processed tomato samples of each hybrid, which were coded and anonymously analyzed by a panel test team composed of ten specialists in tomato derivatives and five fellows at the first panel experience. Each expert evaluated the samples under neutral light (4000 K) and his opinion was reported in a form including 11 sensorial variables. Among the latter, five were considered of primary importance and the remaining as their detailing. With regard to primary variables, the score ranged from zero (extremely unpleasant) to ten (extremely pleasant) and, in particular: colour score zero was matched to brick red and ten to bright red; firmness score zero was associated to chewiness resistance absence and ten to extremely tough product. As for secondary variables, the form delivered to the panel test team was elaborated in order to minimize the fluctuations caused by the first-experience fellows. Moreover, the following perceptions and the related scores in brackets were taking into account: absence (2), mild presence (4), medium presence (6), strong presence (8); the scores were used to calculate the average value per each sample.

Statistical processing

The data relevant to agronomic, technological, quality and sensorial determinations were statistically processed by analysis of variance, with the ensuing Duncan's multiple range test for mean separation at 0.05 probability level. The percentage values were subjected to angular transformation before processing.

3. Results and Discussion

The hybrids did not significantly differ in terms of crop duration, presumably due to the high temperatures and lack of rainfall during the crop cycles (Fig. 1), which led to fruit ripeness and harvest anticipation. From yield and biometrical data reported in Table 1, it arises that hybrid Superpeel attained the highest marketable yield (175.2 t·ha^{-1}), as much as 95.2% of the total yield, due to the very low waste production. The productive result derived from the combination of the fruit number per plant (90.7) and mean weight (77.4 g), with the berries showing higher values of diameter and length compared to Abbundo and Umex respectively, and higher thickness than Abbundo and Taylor.

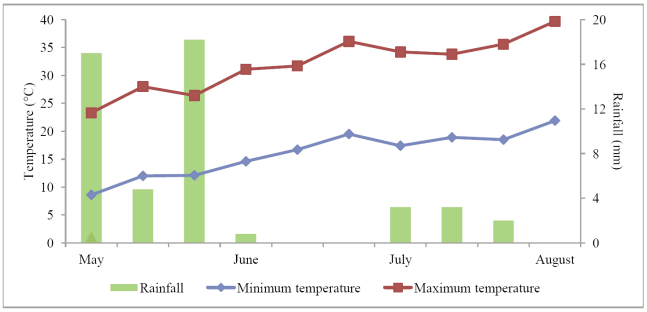

Fig. 1 - Ten-day means of temperatures and rainfall in San Severo (Foggia, Italy) in 2017.

The hybrids Abbundo and Umex gave 32% lower marketable yield than Superpeel due to lower values of the fruit number, size and weight as well as a higher waste production.

The hybrid Taylor ranked in the middle between the highest yielding Superpeel and the least productive Abbundo and Umex. Indeed, it provided with a 19.6% lower yield than Superpeel but 15.4% higher production than the other two genotypes; this result was the consequence of the lowest fruit number (71) per plant but the highest berry dimensions and mean weight (79.3 g) as well as also the lowest fraction of waste produce.

No statistically significant differences were recorded between the hybrids in terms of fruit covering by vegetation, which exceeded 75%.

Consistently with our findings, in previous investigation (Portugal *et al.*, 2015) hybrid productive performances ranged between 110 and 160 t·ha^{-1}, whereas in other research (Caruso *et al.*, 2016; Peixoto *et al.*, 2017) tomato genotypes showed a wide range of yields under the 70 t·ha^{-1} threshold.

With regard to processing efficiency (Table 2), Umex showed the highest value (87.0%) though not statistically different from the control. Similarly, along the peeling chain Umex and Taylor showed the best performances (81.5% as an average), whereas

Table 1 - Yield and biometrical parameters of long-type processing tomato hybrids

Hybrid	Total fruits		Marketable fruits								Waste fruits weight (%/total)
	Weight (t·ha^{-1})	Number per plant	Weight (t·ha^{-1})	Weight (%/total)	Number per plant	Mean weight (g)	Diameter (cm)	Length (cm)	Length/ width	Flesh thickness (mm)	
Abbundo	128.7 c	93.0 b	119.3 c	92.7	82.7 b	57.8 b	4.1 c	7.8 ab	1.89	7.0 b	7.3 a
Umex	126.0 c	91.0 b	118.9 c	94.3	78.5 b	60.4 b	4.3 bc	7.7 b	1.79	7.5 ab	5.7 b
Superpeel	184.1 a	97.7 a	175.2 a	95.2	90.7 a	77.4 a	4.6 ab	8.1 ab	1.76	7.8 a	4.8 c
Control	146.8 b	79.0 c	140.8 b	95.9	71.0 c	79.3 a	4.7 a	8.4 a	1.77	7.0 b	4.1 d
				NS					NS		

NS= not significant; within each column, the values followed by different letters are statistically different according to Duncan's multiple range test at P≤0.05.

Table 2 - Processing yield of four hybrids for peeled tomato

Hybrid	Processing yield (%)			Waste fruits along peeled chain (%)					Waste fruits along juice chain (%)		
	Total	Peeled	Juice	Yellow and necrotized	Rotten	Broken	Undersize	Skins	Yellow and necrotized	Rotten	Skins and seeds
Abbundo	81.5 b	72.4 b	90.1	13.3 a	0.6 a	6.3 a	1.3	6.3 c	5.0 a	3.0 b	2.0 c
Umex	87.0 a	82.0 a	92.2	4.0 d	0.0 b	5.8 b	0.8	7.4 b	2.8 c	2.2 c	2.9 a
Superpeel	82.7 ab	75.1 b	90.5	9.3 b	0.7 a	6.1 ab	0.6	8.2 a	4.3 b	2.9 b	2.3 b
Control	86.3 ab	80.9 a	91.8	7.9 c	0.2 b	3.8 c	0.7	6.5 c	2.9 c	3.4 a	1.9 c
			NS				NS				

NS= not significant; within each column, the values followed by different letters are statistically different according to Duncan's multiple range test at P≤0.05.

no significant differences arose as concerns the juice yield (91.2% as an average).

In terms of waste along the peeling chain, Abbundo attained the highest occurrence of yellow and necrotized fruits, Umex the lowest and Taylor the lowest broken berry percentage. The undersized and rotten fruits were very few and Superpeel had the highest skin fraction.

As for waste along the juice chain, Abbundo showed the highest percentage of yellow and necrotized fruits, which was 79% higher than the lowest one corresponding to Umex; the latter hybrid also had the lowest value of rotten berries and the highest of skin and seeds.

The results relevant to the quality indicators of processed tomato (Tables 3 and 4) show that significant effects of the hybrid were recorded on the following parameters: titratable acidity and sodium attained the highest value in Taylor fruits (0.37% and 7.4 mg·100 g^{-1} respectively) and lowest in Abbundo; the fiber content recorded in Abbundo fruits was 17% higher than Taylor one; ash and salt concentrations showed the highest levels in Umex (0.54% and 20.4 mg·100 g^{-1} respectively) though the latter variable was not significantly different from Taylor.

No statistically significant differences were recorded between the hybrids referring to: total solids (7% on average), soluble solids (6.1%), reducing sugars (3.8%), sugars ratio (54.3%), proteins (1.6%), lipids (0.3 %), energetic value (24.5 kcal·100 g^{-1} or 104,5 kJ·100 g^{-1}), colour (1.82), glucose (1.5%), fructose (1.6%), sucrose (0.04%), fat acids expressed as saturated (0.07%), monounsaturated (0.06%) and polyunsaturated (0.14%).

Compared to pre-processing fruits, peeled tomatoes showed increased values of total and soluble solids as well as reducing sugars, but decreased levels of sugar ratio and colour; moreover, pH of fresh fruits was not significantly affected by the hybrid, ranging between 4.4 to 4.5. In previous research (Raiola *et al.*, 2018) the comparison between the values recorded before and after processing was genotype-dependent, except for titratable acidity and sugar ratio which always decreased and increased respectively, upon processing.

High total solids content in fruits is an industrial target, as it reduces the processing costs; this parameter in tomato varies around the 5-6% average and it is affected by some factors such as cultivar, soil type and climate conditions during the growing and

Table 3 - Quality features (referred to fresh weight) of peeled tomato fruits obtained from four hybrids

Hybrid	Total solids (g·100 g^{-1})	Soluble solids (°Brix)	Reducing sugars (g·100 g^{-1})	Titratable acidity (g anhydrous citric acid 100 g^{-1})	Sugar ratio (%)	Proteins (g·100 g^{-1})	Fats (g·100 g^{-1})	Fiber (g·100 g^{-1})	Energetic value (Kcal·100 g^{-1})	Colour (a/b)
Abbundo	7.0	6.1	4.0	0.27 c	56.7	1.56	0.27	1.32 a	24	1.82
Umex	7.0	6.1	3.8	0.29 c	53.5	1.52	0.31	1.23 b	25	1.81
Superpeel	7.0	6.1	3.7	0.33 b	53.5	1.59	0.26	1.19 bc	24	1.80
Control	7.1	6.2	3.8	0.37 a	53.6	1.64	0.32	1.13 c	25	1.85
	NS	NS	NS		NS	NS	NS		NS	NS
Average	7.0	6.1	3.8		54.3					1.82
Peeled/Fresh (%)	+ 24.7	+ 21.8	+ 18.2		- 5.2					- 30.6

NS= not significant; within each column, the values followed by different letters are statistically different according to Duncan's multiple range test at P≤0.05.

Table 4 - Sugars, fatty acids and mineral components (refereed to fresh weight) in peeled tomato fruits produced by four hybrids

Hybrid	Sugars (g·100 g^{-1})			Fatty acids (g·100 g^{-1})			Ash (g·100 g^{-1})	Sodium (g·100 g^{-1})	Salt (g·100 g^{-1})
	glucose	fructose	sucrose	saturated	monounsaturated	polyunsaturated			
Abbundo	1.48	1.57	0.04	0.06	0.06	0.13	0.47 c	5.6 c	10.3 b
Umex	1.54	1.58	0.04	0.07	0.07	0.15	0.54 a	6.4 b	20.4 a
Superpeel	1.46	1.58	0.04	0.06	0.05	0.13	0.51 ab	5.9 c	9.8 b
Control	1.49	1.60	0.04	0.08	0.07	0.15	0.50 bc	7.4 a	19.5 a
	NS	NS	NS	NS	NS	NS			

NS= not significant; within each column, the values followed by different letters are statistically different according to Duncan's multiple range test at P≤0.05.

harvesting season (Siddiqui *et al.*, 2015). Other authors (Majkowska-Godomska *et al.*, 2008) recorded a total solids content of tomato fruits ranging from 3.83 to 7.00%.

Campos *et al.* (2006) and Kader *et al.* (1987) reported that values of soluble solids below 4.5% are considered low for industrial tomatoes; in this respect, Turhan and Seniz (2009) found this quality indicator ranging between 5.0 to 5.5 % in processing tomato fruits and in other studies (Cramer *et al*., 2001; De Pascale *et al.*, 2001) soluble solids values of tomato fruits ranged from 4 to 6%.

Sugar content is positively and highly correlated with total soluble solids in tomato fruit and, notably, fructose has a large impact on the sweetness perception (Tieman *et al.*, 2012). In our research the sum of glucose and fructose accounted for the 80% of the total sugars, whereas it attained about 65% in previous investigations (Stevens *et al.*, 1977; Jones and Scott, 1984; Malundo *et al.*, 1995). Other authors found the total sugar content of ripe tomato ranging from 1.7 to 4.7% (Petro-Turza, 1987; Turhan and Seniz, 2009) or from 0.54 to 3.44% (Melkamu *et al.*, 2008) of fresh weight.

In previous research, titratable acidity (TA) in tomato fruits ranged from 0.22 to 0.40% (Turhan and Seniz, 2009) or even from 0.25 to 0.70 (George *et al.*, 2004). According to Beckles (2012), values of total soluble solids and titratable acidity as much as 5.0 and 0.4% respectively are considered desirable to produce a good-tasting tomato. Titratable acidity is mainly affected by citric and malic acids which reportedly attain about 15% of the dry content in ripe fresh tomatoes (Petro-Turza, 1987). Kamis *et al.* (2004) states that taste and flavour of tomato fruits are positively correlated to sugars and organic acids content. Moreover, in addition to flavour the organic acids influence pH, the latter being an important factor in canned tomato products to control the growth of thermophilic microorganisms (Yousef and Juvik, 2001). The influence of pH on the thermal conditions applied along the tomato processing chain is mainly associated to producing safe products and, in this respect, values below 4.5 prevent microorganism proliferation in the final product (Campos *et al.*, 2006; Garcia and Barrett, 2006). Notably, some authors did not detect varietal dependent pH differences in tomato berries (Kerkhofs *et al.*, 2005), whereas in other research pH showed differences among cultivars (Benal *et al.*, 2005; Frusciante *et al.*, 2007) even in the wide range of 3.78 to 5.25 (Turhan and Seniz, 2009).

Mineral element concentration in tomato fruits may reach 8% of dry matter and influences tomato fruit taste through the effect on pH and titratable acidity (Siddiqui *et al.*, 2015).

With regard to antioxidants (Table 5), lycopene attained a 16% higher concentration in Umex compared to Abbundo; Superpeel showed a 43% higher β-carotene content than Abbundo; polyphenols concentration was the lowest in the control fruits, but did not significantly differs between the three hybrids examined.

Compared to fresh fruits, in peeled tomatoes lycopene and β-carotene concentrations remained stable and polyphenols increased referring to fresh weight, whereas in relation to total solids lycopene had a 19.1% decrease whereas polyphenols just a slight reduction (5.2%).

In previous research (Binoy *et al.*, 2004) significant differences were found in lycopene and phenolic contents between the different genotypes, with lycopene showing 1 to 4 fold and 1 to 2 fold variation on fresh and dry weight basis respectively. Moreover, unlike our findings where lycopene remained stable and polyphenols increased upon industrial processing

Table 5 - Antioxidants concentration in peeled tomato fruits obtained from four hybrids

Hybrid	Lycopene		β-carotene	Total polyphenols	
	mg·kg^{-1}	mg·100 g^{-1} TS	mg·kg^{-1}	mg eq. gallic acid 100 g^{-1}	mg eq gallic acid g^{-1} total solids
Abbundo	127.0 c	181.9 c	2.1 c	36.7 a	5.3 a
Umex	146.6 a	208.3 a	2.3 bc	36.0 a	5.1 a
Superpeel	136.2 b	195.3 b	3.0 a	35.7 a	5.1 a
Control	131.0 bc	184.7 bc	2.4 b	31.7 b	4.5 b
Average	135.2	192.6	2.5	35.0	5.0
Peeled/fresh (%)	0.9	-19.1	- 1.0	17.6	-5.2

Within each column, the values followed by different letters are statistically different according to Duncan's multiple range test at P≤0.05.

and referred to fresh weight, Dewanto *et al.* (2002) reported the increase of lycopene concentration with no changes in polyphenols content in processed tomato fruits compared to raw berries. However, in other research (Pavlović *et al*., 2017) the antioxidants content in tomato fruits decreased upon processing thermal treatment, though the significance and amplitude of the differences are genotype dependent, and it is also affected by temperature settings (Jabbari *et al.*, 2018).

The scores resulted from the panel test performed by an expert team in tomato organoleptic evaluation and their subsequent statistical processing allowed to obtain the sensorial profiles which convey the immediate and clear quantitative and descriptive perception of the analyzed products.

The graphic representation of QDA (Quantitative Descriptive Analysis) obtained by processing the evaluation forms filled in by the experts is shown in figure 2 a. Taking into account the high number of data and in order to make it easier to interpret the profiles, the sensorial variable data considered negative for the relevant hybrids were extrapolated. In particular, the data related to strange taste and flavour and to acidity were clustered (Fig. 2 b): the profiles and the statistical processing performed show that there are no significant differences between the hybrids. The sensorial profiles of the positive variables are shown in Figure 2 c. Moreover, from data statistical processing reported in Table 6, it can be observed that the peeled product obtained from the hybrids Abbundo and Umex is statistically different in terms of colour, aspect and flavor; in addition, Abbundo is statistically different from Taylor for the colour and from Superpeel for the sensation of fresh flavor.

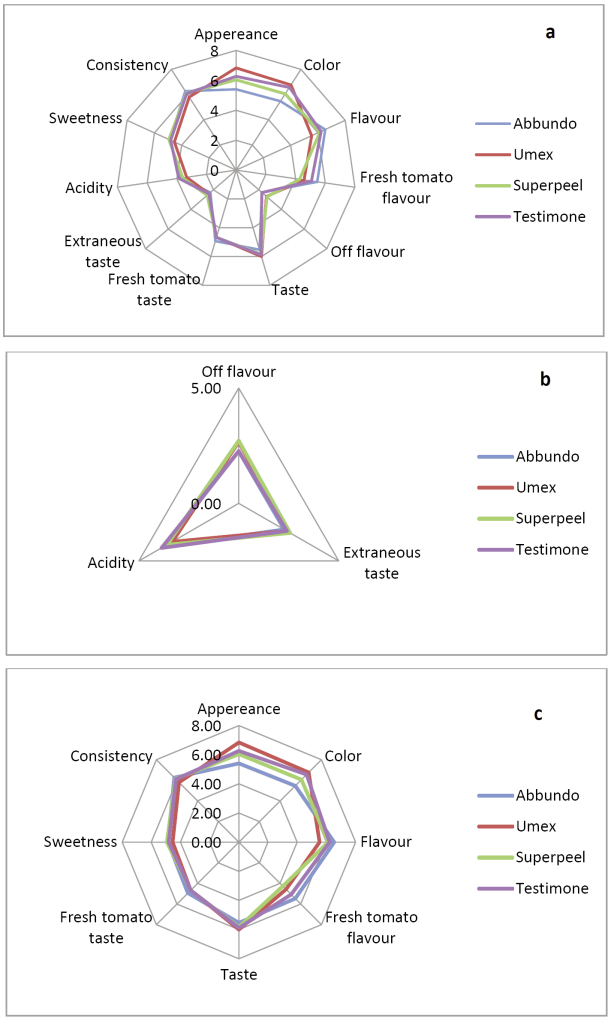

Fig. 2 - Organoleptic evaluations of peeled tomato fruits: comparison of the sensorial profiles of the four hybrids (a), sensorial profiles of the undesired features, named negative (b); sensorial profiles of the desired features, named positive (c).

Table 6 - Organoleptic features of peeled tomato fruits produced by four hybrids: analysis of variance and F significance

Variable	Cultivar		Δ	σ
Aspect	Abbundo	Umex	-1.43	0.007
Colour	Abbundo	Umex	-1.30	0.013
Colour	Abbundo	Control	-1.10	0.035
Flavour	Abbundo	Umex	1.00	0.050
Flavour as fresh	Abbundo	Superpeel	1.20	0.023

Δ= differences between the means; σ= F significance.

4. Conclusions

From research carried out on the comparison between long-type hybrids oriented to peeled produce in Tavoliere delle Puglie (Foggia, southern Italy), Superpeel showed the best yield performances (175.2 t·ha^{-1}) with about 21% higher production than the average value attained by Abbundo, Umex and Taylor, as a consequence of the combined effects of fruit number and mean weight. However, the highest processing efficiency was recorded for the hybrid Umex and Taylor, whereas no hybrid displayed an overall superiority in terms of quality features and sensorial profile.

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Postharvest control of *Aspergillus niger* in mangos by means of essential oil

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Key words: *Aspergillus niger*, essential oil, mango, postharvest.

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

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The authors declare no competing interests.

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Abstract: The use of essential oil as an alternative mean to synthetic fungicides has been considered in the past years for management of the postharvest decay of fruits in order to ensure more safe and long storage life of these perishable commodities. *Aspergillus niger* is one of the most dangerous fungal pathogen which can cause postharvest diseases in fresh mangos. The aim of this study was to assess the effectiveness of essential oil from four aromatic plants (*Thymus vulgaris*, *Salvia mirzayanii*, *Artemisa persica*, and *Rosmarinus officinalis*) in comparison to fungicide 'Mancozeb' against *A. niger* under *in vitro* and *in vivo* conditions. After inoculation of mango fruits with an isolate of *A. niger* followed by curative treatments with essential oil, the main physical and chemical attributes of mangoes were determined under postharvest condition. The *in vitro* results showed that colonies of *A. niger* were totally inhibited by application of essential oil of *T. vulgaris* (at all the tested concentrations) and *A. persica* (1500 µl/l). While, *S. mirzayanii* showed the lowest effect at 1000 µl/l if compared with the other essential oils. The results of the *in vivo* experiments showed that treatments with *T. vulgaris* and *S. mirzayanii* essential oil had significant (P<0.05) effects in preventing fruit decay at 1000 µl/l after 10 days of storage, while, *R. officinalis* essential oil significantly (P<0.05) reduced deterioration of mango fruits at 500 µl/l, followed by *A. persica*. Rosemary also showed the highest fruit firmness in comparison with other treatments. Also, the essential oils maintained higher chlorophyll content. The results of this work showed that application of essential oil on mangos assurance both a significant preservation on their quality attributes by controlling, at the same time, decaying caused by *A. niger* during the postharvest phase.

1. Introduction

Postharvest diseases are among the major causes of losses of mangos (*Mangifera indica* L.) fresh produce throughout the supply chain. The incidence of the postharvest diseases can also affect the quality of mangos limiting their shelf life up to 3-4 days. In literature is reported that about 17-37% of fresh mangos is wasted after harvesting and marketing (Madan

and Ullosa, 1993). Sharma *et al*. (1994) have reported that about 17.7% of this fresh produce is lost during the storage and marketing. Mango decay caused by the plant pathogenic fungus *Aspergillus niger* is one of the most dangerous postharvest diseases, leading to the losses of fruit quality during storage (Duamkhanmanee, 2008).

It well known from published reports as more negative effects associated to use of chemical fungicides for controlling postharvest diseases have been reported on the human's health and environment (Wightwick *et al.*, 2010). Furthermore, consumers believe that fruits not treated (or minimally-treated) with fungicides are safer for fresh consumption (Du Plooy *et al*., 2009). In the past 20 years there has been a great interest in using essential oils (EOs) to control postharvest diseases, such increasing shelf life of stored fruits (Tripathi and Dubey, 2004). Several studies have also reported on the antifungal activity of *Thymus vulgaris* against different strains of *Colletotrichum gloeosporioides*, *Rhizopus stolonifer*, *Penicillium digitatum* (Abdolahi *et al*., 2010; Sellamuthu *et al.*, 2013), *Aspergillus flavus*, *Aspergillus niger, Aspergillus fumigatus, and Alternaria alternata* (Kumar *et al.*, 2008). Different species of *Salvia* are yet used as antimicrobial agents (Fiore *et al.*, 2006; Kelen and Tepe, 2008), however *Salvia mirzayanii* is an endemic plant which grows only in some parts of Iran, and thus there is no an exhaustive information regarding its effect on *A. niger* (Rechinger, 1986*).* Centeno *et al.* (2010) have reported that extracts from *Rosmarinus officinalis* and *T. vulgaris* could have a significant effect on the control of fungal decaying. Previous studies have also confirmed the interesting antimicrobial activity of *R. officinalis* EO against spoilage and pathogenic food-related fungi (Abdolahi *et al.*, 2010). De Sousa *et al.* (2013), for instance, detected the strong effect of the *Origanum vulgare* and *R. officinalis* EOs in controlling *A. flavus*.

On the other hand, fewer papers report issues regarding to postharvest control of *A. niger* on mango fruits using EOs as an alternative mean to synthetic fungicides. This study was focused to evaluate the effectiveness of EOs derived from four aromatic plants (*T. vulgaris*, *S. mirzayanii, Artemisa persica* and *R. officinalis*) to *in vitro* and *in vivo* suppress the growth of one pathogenic strain of *A. niger* by preserving the mango fruit quality attributes under postharvest condition.

2. Materials and Methods

Plant material and extraction of essential oil

S. mirzayanii and *A. persica* samples were collected from Lar region of Fars Province, Iran (Lat. 27°41' 3'' N and Long. 54°2' 10''E). A *T. vulgaris* sample was collected from Geno region of Hormozgan Province, Iran (Lat. 25° 38' 37.9'' N and Long. 57° 46' 28'' E) and *R. officinalis* samples from Kerman Province, Iran (Lat. 30° 17' 2.1'' N and Long. 57° 5' 0.1'' E). Samples were harvested in vegetative stage (before flowering). The leaves of samples were cut into small pieces and shade-dried at room temperature. The material was then ground to fine powder. The 80 g of plant material were subjected to extraction of EOs by hydro-distillation method for 6 h using a Clevenger's apparatus (Moghaddam *et al*., 2011). The EOs were separately collected, dehydrated using sodium sulphate (Na_2SO_4), and finally stored in a dark bottle at 4°C until tested.

In vitro experiments

Fungi were isolated from mango and their identity was confirmed

The *Aspergillus niger* (PTCC 5010) was supplied by Iranian research organization for science and technology (IROST). Culture of the pathogen organism was maintained on potato dextrose agar (PDA) medium. Stock cultures were grown at 25°C for 7 days to allow for sufficient sporulation. Antifungal effects of EOs were carried out by the Solution Method (SM) according to Pitarokili *et al.* (1999). Inhibitory effects of EO extracted from *S. mirzayanii, A. persica, R. officinalis* and *T. vulgaris* were determined by *in vitro* antifungal assays. To measure the direct fungal inhibition of each EO on mycelial growth of *A. niger*, three different concentrations of them (1000, 1200 and 1500 µl/l) were added to potato dextrose agar (PDA; provided by Scharlau) media before solidification into Petri dishes (8 cm diameter) at 45-50°C. Fungal disks with 5 mm diameter were placed on the middle of Petri dishes and incubated at 25°C for 10 days. Three replicates per each treatment (4 EO × 3 concentrations) including control plate without EO were prepared. Inhibition percentage was determined at the end of incubation time by the index:

$$IP = (dc-dt)/dc \times 100$$

IP = inhibition percentage, dc = mycelium diameter in the control plate, and dt = in the EOs-treated plate.

In vivo experiments

Mangos cv. Halily were harvested from Minab (Lat. 27°07'51'' N and Long. 57°05'13'' E) at the maturity stage of development. Fruit surface was before disinfected with 2% sodium hypochlorite for 3 min, and then artificial inoculations were done by puncturing fruit surface (4 mm deep and 2 mm wide for each inoculation point) with a sterile needle on two sides of each fruit with 40 µl of a conidial suspension containing 10^6 UFC/ml of *A. niger* that it has been sprayed above each wound. After one-day of incubation at 25°C to allow conidia germination into fruit tissue, fruits were treated with 500 and 1000 µl/l EO of each plant in comparison to 0.5 and 1.0 mg/l mancozed. After the treatment (curative), all fruit trials, including the control (fruits were inoculated with conidial suspension without the treatment with essential oils), were placed into boxes and kept at 25°C for 1, 2, and 3 weeks. Decay percentage of fruit was calculated as the number of decayed fruit/ total number of fruit at each replication* 100 (El-Anany *et al.*, 2009).

Physical-chemical analysis

Firmness of each fruit was measured at two points of the equatorial region by using a texture analyzer with a 5 mm probe (Lurton, Taiwan) with units expressed in kg/cm^2. Surface color was measured on each fruit at two opposite sides using a chromameter (CR 400, Minolta) which provided CIE L*, a*, and b* values. L* is color lightness (0= black and 100= white). The a* scale shows in the maximum the red (+a*) and in the minimum the green color (-a*) while the b* ranged from yellow (+b*) to blue (-b*).

The content of ascorbic acid (AA) expressed as mg/100 g fruit weight was determined as described by Molla *et al*. (2011). Aliquots of 10 ml of each sample was homogenized in 100 ml of extraction buffer containing 3% metaphosphoric acid. Aliquots of 10 mL of homogenate was titrated against standard dye 2,6-diclorophenol indophenols to a faint pink color. The method proposed by Lichtenthaler (1987) was used to determine the total chlorophyll and carotenoids content of fruit. The 'Total Soluble Solids' (TSS) content was determined at 20°C using a digital refractometer, and expressed as °Brix. The pH of fruit juice was measured using a Jenway 3320 pH meter calibrated by pH 4 and 7 buffer solutions. The 'Titratable Acidity' (TA) was determined by titration of 5 mL extract with 0.1 mol L^{-1} sodium hydroxide at pH 8.1 and expressed as percent citric acid (Molla *et al.*, 2011). Weight loss, fruit firmness, surface color change, content of AA, total chlorophyll, and carotenoids, and TSS, TA and pH were determined after 1, 2, and 3 weeks of storage at 25°C. These characteristics was done with 3 replicates (3 large fruits for 1 replicate).

Statistical analysis

The experiment was conducted in a randomized factorial designed whit essential oils treatment and storage time as the two factors. Data were submitted to one-way analysis of variance (ANOVA) using SAS version 16.0 and means were separated by the Duncan test at $P<0.05$ (n=3).

3. Results and Discussion

Aspergillus niger mycelia inhibition

In vitro experiments showed that mycelia growth of *A. niger* was significantly suppressed (P<0.05) when treated with the different concentrations of each EO (Fig. 1). The fungal growth was totally inhibited (IP= 100%) by all the concentrations of *T. vulgaris* EO and with 1500 µl/l *A. persica* EO. On the other hand, *S. mirzayanii* EO showed lower effect (IP= 72%) than other EOs when tested at 1000 µl/l concentration.

Our findings are in agreement to the ones described by Kohiyama *et al.* (2015) who reported that *T. vulgaris* EO was able to control the growth of *A. flavus*. Similar observations on the prevention of

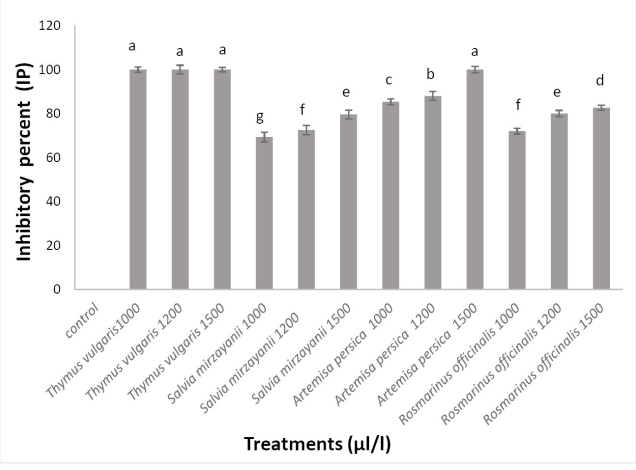

Fig. 1 - Inhibitory effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 1000, 1200 and 1500 µl/l on mycelia growth of *Aspergillus niger* cultures incubated at 25°C for 10 days. Bars indicate the SD of the mean. Different letters indicate significant differences in mean values (p<0.05).

different pathogenic fungi by using EOs have been reported in the previous studies, such as those conducted by Tripathi and Dubey (2004) and Pawar and Thaker (2006). Boubaker *et al.* (2016) reported the antifungal activity of four *Thymus* species EOs against *Penicillium digitatum*, *Penicillium italicum* and *Geotrichum citriaurantii*. Pawar and Thaker (2006) showed that *Cinnamomum zeylanicum, Cinnamomum zeylanicum, Cinnamomum cassia, Cymbopogon citratus* and *Syzygium aromaticum* were the best plant sources for EOs extraction showing a noticeable inhibitory effect against *A. niger.* It was also reported that the mycelial growth of *A. niger* was inhibited by application of 2.5 and 3.0 µg/ml of *Citrus sinensis* oil (sweet orange) in Potato Dextrose Broth and Agar Water medium, respectively (Sharma and Tripathi, 2008).

Modifications on fungal structures induced by the EOs afore-quoted might be due to interactions of their components (Carvacrol, thymol, eugenol, vanillin and etc.) with cell wall synthesis, which could affect fungal growth and its morphology (Rasooli *et al.*, 2006; Rao *et al.*, 2010). Some researchers have stated that some phenolic compounds present in the EOs could affect the plasma membrane and the cellular organelles, such as mitochondria of the fungi by decreasing the lipid and saturated fatty acid levels and increasing the unsaturated fatty acids, resulting in the leakage of Ca^{2+}, Mg^{2+} and K^{+} (Sharma and Tripathi, 2008). In addition, the existence of the hydroxyl groups and the aromatic nucleus could be a other important factor for the EOs antimicrobial activity (Numpaque *et al.*, 2011).

Fruit decay suppression

As shown in figure 2, the decay percentage of fruits increased with the storage time, variable from 1 to 3 weeks of incubation. The percentage of decay significantly decreased ($P<0.05$) with increasing of the concentration of *T. vulgaris* and *A. persica* EOs after 3 weeks of storage. After one week, no significant differences were observed between control and treated fruits (data not shown). After two weeks, significant differences were found among treatments with *R. officinalis* EO at 500 µl/l and *T. vulgaris* at 1000 µl/l. At the end of experiment, after two weeks, the maximum level of decay was related to the control fruits (70%), and the minimum one was attributed to *R. officinalis* (500 µl/l) and *A. persica* (1000 µl/l) EOs, reaching 12% and 13.3% decay, respectively.

These data agree with those obtained by Ramezanian *et al.* (2016) who showed the possibility of using *Z. multiflora* and *T. vulgaris* EOs to control postharvest citrus *Alternaria* decay (black rot). In addition, Elshafie *et al.* (2015) reported that *O. vul-*

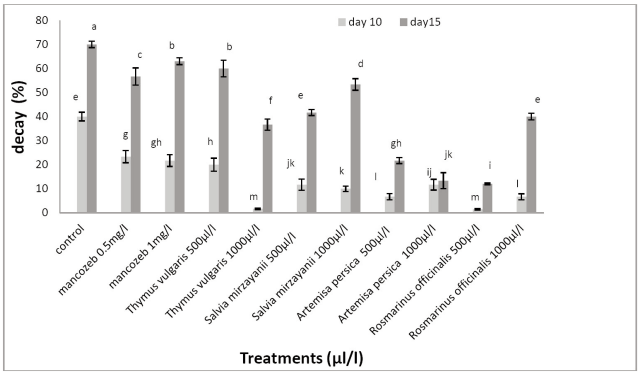

Fig. 2 - Suppressive effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 500 and 1000 µl/l on mangos decay after 10 and 15 days of storage compared to 'mancozed' (0.5 and 1.0 mg/l). Bars indicate the SD of the mean. Different letters indicate significant differences in mean values ($p<0.05$).

gare EO can control the brown rot of peach. Jhalegar *et al.* (2015) addressed their study on the influence of lemon grass, eucalyptus, clove and neem EOs against *P. digitatum* and *P. italicum* in 'Kinnow' mandarin. These authors showed that the decay rot during storage was less in the treated fruits than in the control ones. Duamkhanmanee (2008) reported that 4000 ppm lemon grass EO could control anthracnose by *C. gloeosporioides* decay of mangos. Phenolic compounds such as Carvacrol and thymol (Rao *et al.*, 2010) contained in EOs have a lipophilic molecular structure, therefore it interfer with membrane-catalyzed enzymes and cell wall, causing the cell death of microbes (Shirzad *et al.*, 2011). Many researchers believe that the type and the amount of phenolic compounds present in the oil can determine the antifungal activity of the EOs (Tripathi and Dukey, 2004).

Weight loss

The weight loss of fruit was increased strongly during the early weeks, but this increase was gradual throughout the storage period (Fig. 3). After two weeks of storage, the highest and lowest weight loss was observed in the control samples and those treated with 1000 µl/l *S. mirzayanii* (12.7% and 9.8% respectively). During the storage, the main mango weight loss (13.2%) was found in the control fruits, while the lowest one (10.8%) was observed in *R. officinalis* treated fruits at 500 µl/l concentrations.

The mechanism of EOs for reducing physiological

loss in weight might be related to the reduction of ethylene production and the respiration rate. Also, EOs cover the peel of fruit, creating the water barrier between the fruit and the environment, thereby reducing water exchange (Morillon *et al.*, 2002). This agrees with previous studies showing the efficacy of EOs in reducing the weight loss of cherries and grapes (Serrano *et al.*, 2005). Similarly, Du Plooy *et al.* (2009) reported that the use of *Mentha spicata* and *Lippia scaberrima* EOs reduced weight loss in 'Valencia' oranges.

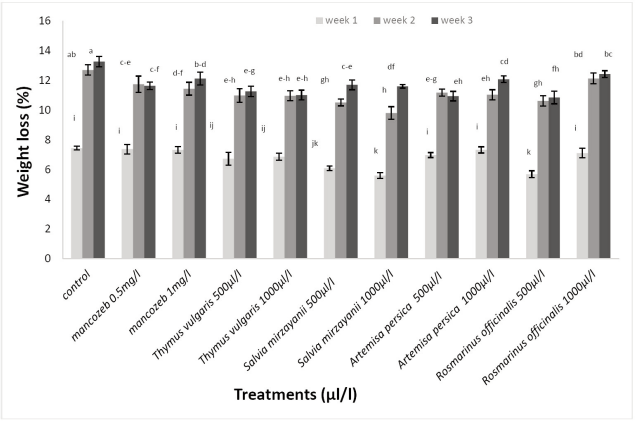

Fig. 3 - Effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 500 and 1000 µl/l on mangos weight loss after 1, 2, and 3 weeks of storage compared to 'mancozed' (0.5 and 1.0 mg/l). Bars indicate the SD of the mean. Different letters indicate significant differences in mean values ($p < 0.05$).

Fruit firmness

A continuous decline in mangos firmness was observed throughout storage (Fig. 4). However, the fruits treated with EOs showed higher firmness than the control ones. In each stage of storage, no significant difference was identified in the firmness of fruits, at different concentrations of EOs. After 3 weeks of storage, the firmness of control fruits was around 3.06 kg/cm^2, while the treated fruits were significantly firmer ($P<0.05$). In this stage, fruits treated with mancozeb showed no significant difference, as compared with those treated with 500 µl/l *T. vulgaris* EO. However, *R. officinalis* in both concentrations showed the highest firmness, as compared with other treatments.

Firmness, as one of the fruits properties, is a complex sensory attribute that also includes crispiness and juiciness; it is important in determining the acceptability of horticultural crops. It has been accepted that the loss of fruit firmness throughout the storage is mainly due to the depolymerization of cell wall components. Breakdown and the enzymatic degradation of insoluble protopectins into more simple soluble pectin can be associated with softening (Willats *et al.*, 2001). Ramezanian *et al.* (2016) found that the EOs reduced the activity of polygalacturonase and galactosidase, which are softening enzymes in the cell wall components, and maintained orange fruit firmness through the storage. The results obtained in the present study agree with those of Maqbool and Alderson (2010), who showed that by the application of lemongrass oil (0.05%) and Cinnamon oil (0.4%), the firmness of banana and papaya fruits was maintained during storage. However, Tzortzakis (2007) reported that eucalyptus and cinnamon EOs had no effect on the tomato and strawberry firmness.

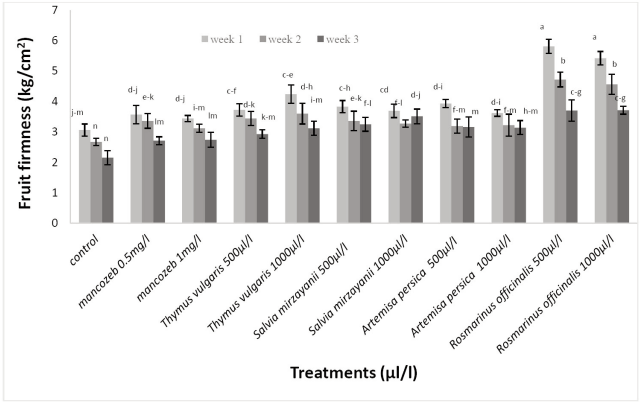

Fig. 4 - Effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 500 and 1000 µl/l on mangos firmness after 1, 2, and 3 weeks of incubation compared to 'mancozed' (0.5 and 1.0 mg/l). Bars indicate the SD of the mean. Different letters indicate significant differences in mean values ($p < 0.05$).

Surface color change

The results related to the changes in the fruit color (in terms of L*, a* and b*) of the treated mango showed that the lightness of the fruits peel was decreased throughout the storage time (Table 1). The fruits treated with 1000 µl/l *R. officinalis* EO retained higher L* over other treatments and control samples, after three weeks of storage. The highest and lowest L* was found in *R. officinalis* and *A. persica* at 1000 µl/l concentration, respectively. The results showed that a* was significantly decreased during the storage. However, the fruits treated with EOs maintained a higher a* than did the control ones. At the end of the storage, the lowest a* (7.46) and the highest a*(11.73) were found in the control and *R. officinalis* treated fruits at 1000 µl/l concentra-

tion, respectively. The results also showed that b* was increased during the storage time, but the trend in fruits treated by EOs was slower than that in the control. At the end of storage, *S. mirzayanii* and *R. officinalis,* at 500 µl/l concentrations, showed the minimum b* value (40.7 and 40.6), respectively. However, control and mancozeb samples showed the maximum b* value (56.6 and 55.9), respectively. The results obtained in the present study showed that EOs treatment could have better litheness with lower a* and b*, as compared to mancozed and control groups.

Ramezanian *et al*. (2016) showed the effect of the *Zataria multiflora* and *T. vulgaris* EOs on the black rot of 'Washington Navel' orange fruit. They found that the best color was related to zataria at 300 µl/l and thyme EOs at 400 µl/l concentrations. In agreement with our findings, Marjanlo *et al.* (2009) showed the effect of Cumin EO on the postharvest quality of strawberries, finding that the essential oil treated fruits maintained a higher L* during storage in comparison with the controls.

AA content

Ascorbic acid (vitamin C) content was gradually decreased during storage; however, its strength was lower in the treated samples (Table 2). Different concentrations of the EOs significantly maintained ascorbic acid content, as compared to the control. Overall, the most (14 mg/100 g/1) and the least (9 mg/100

Table 1 - Effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 500 and 1000 µl/l on mangos color change ± SD after 1, 2, and 3 weeks of storage compared to 'mancozed' (0.5 and 1.0 mg/l)

Testing index	Treatment (concentration)	Storage time		
		Week 1	Week 2	Week 3
L*	Control	63.3±0.43 a	47.5±0.56 n	46.2±0.56 n
	Mancozeb 0.5 mg/l	64.9±0.5 a	59.8±0.48 c-g	54.1±0.59 j-m
	Mancozeb 1 mg/l	55.3±.23 f-l	55.9±0.75 f-l	56.7±0.76 f-l
	Thymus vulgaris 500 µl/l	62.1±0.33 a-d	54.8±0.46 i-m	51.4±0.36 mn
	Thymus vulgaris 1000 µl/l	60.6±0.54 b-f	57.8±0.49 e-j	51.8±0.66 mn
	Salvia mirzayanii 500 µl/l	54.6±0.32 i-m	51.9±0.58 l-n	56.1±0.54 f-k
	Salvia mirzayanii 1000 µl/l	59.9±0.33 c-g	58.4±0.44 d-g	49.7±0.38 mn
	Artemisa persica 500 µl/l	59.7±0.43 c-g	56.9±0.58 f-l	48.7±0.65 n
	Artemisa persica 1000 µl/l	57.9±0.23 e-j	58.8±0.75 d-g	41.4±0.65 o
	Rosmarinus officinalis 500 µl/l	59.8±0.19 c-g	60.7±0.66 a-d	54.1±0.39 j-m
	Rosmarinus officinalis 1000 µl/l	62.2±0.25 a-d	65.3±0.76 a	59.5±0.53 c-g
a*	Control	16.4±0.87	12.6±0.87 g-i	7.4±0.87 l
	Mancozeb 0.5 mg/l	17.2±0.87	15.8±0.88 a-e	8.1±0.98 l
	Mancozeb 1 mg/l	16.8±0.99	16.1±0.98 a-d	8.6±0.99 kl
	Thymus vulgaris 500 µl/l	16.4±0.67	16.1±0.76 b-f	8.6±0.76 kl
	Thymus vulgaris 1000 µl/l	17.2±0.89	15.3±0.87 a-c	11.2±0.85 ij
	Salvia mirzayanii 500 µl/l	17.1±0.78	16.7±0.88 c-f	11.1±0.76 ij
	Salvia mirzayanii 1000 µl/l	17.6±0.77	15±0.68 e-g	8.4±0.87 kl
	Artemisa persica 500 µl/l	17.1±0.77	14.2±0.87 f-g	10.8±0.97 ij
	Artemisa persica 1000 µl/l	17.4±0.69	13.5±0.88 f-h	11.6±0.97 h-j
	Rosmarinus officinalis 500 µl/l	16.8±0.90	15.8±0.98 a-e	11.7±0.98 ij
	Rosmarinus officinalis 1000 µl/l	14.6±1.02	16.3±0.96 a-d	10.1±1.03 jk
b*	Cntrol	25.5±0.78 ij	34.7±1.02 e	56.6±2.2 a
	Mancozeb 0.5 mg/l	20.7±1.03 l-n	30±1.03 fg	55.9±3.2 a
	Mancozeb 1 mg/l	21.5±0.85 l-n	32.9±1.07 ef	46.7±2.9 bc
	Thymus vulgaris 500 µl/l	20.8±0.87 mn	25.1±0.84 h-j	46.7±3.8 bc
	Thymus vulgaris 1000 µl/l	20.6±0.78 mn	30.6±1.06 fg	49.4±3.5 b
	Salvia mirzayanii 500 µl/l	17.0±0.78 ab	30.7±0.94 fg	40.6±2.6 d
	Salvia mirzayanii 1000 µl/l	23.8±1.03 i-l	27.3±0.99 gh	48.3±3.5 bc
	Artemisa persica 500 µl/l	19.4±0.86 n	26.6±0.95 hi	47.8±3.6 bc
	Artemisa persica 1000 µl/l	20.8±0.98 l-n	30.8±1.04 fg	47.6±3.2 bc
	Rosmarinus officinalis 500 µl/l	20.7±1.03 l-n	24.4±0.90 i-k	40.7±2.8 d
	Rosmarinus officinalis 1000 µl/l	22.8±0.99 j-m	23.4±1.05 i-l	45.5±3.5 c

In each character, different letters indicate significant differences in mean values ($p<0.05$).

g/1) amount of ascorbic acid content was detected in the fruits treated with *S. mirzayanii* at the concentration of 500 µl/l and control after three weeks of storage, respectively. In general, fruits treated with mancozeb showed lower ascorbic acid content in comparison with those treated with EOs throughout the storage.

In agreement with our findings, Geransayeh *et al.* (2012) showed that the vitamin C content of grapes was decreased significantly during the storage; however, a higher vitamin C amount was observed in the samples treated with *T. vulgaris* EO. Our results were nevertheless in contrast with those of Marjanlo (2009) who did not detect any significant difference in the amount of ascorbic acid in strawberry fruits treated by the Essential Oils.

Carotenoids and total chlorophyll content

Analysis of the variance of carotenoids content revealed a significant difference ($p< 0.05$) between treatments (Table 2). The concentration of carotenoids content was low at the initial time of storage and then significantly increased during storage. At the end of the process, control fruits showed the highest content of carotenoids (1.94 mg 100 g^{-1}). *R. officinalis* and *T. vulgaris*, tested at 500 µl/l showing 1.09 and 1.15 (mg 100 g^{-1}) as carotenoids content, respectively, were the lowest one, as compared with other treatments. The total chlorophyll content was gradually decreased to a lower concentration in all

Table 2 - Effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 500 and 1000 µl/l on the ascorbic acid, carotenoids and total chlorophyll content ± SD in mangos after 1, 2, and 3 weeks of storage compared to 'mancozed' (0.5 and 1.0 mg/l)

Active metabolite	Treatment (concentration)	Storage time (week)		
		Week 1	Week 2	Week 3
Ascorbic acid	Control	12.3±3 cd	11±2 cd	9.5±4 d
	Mancozeb 0.5 mg/l	13.3±4 c	12.6±3 cd	11±3 cd
	Mancozeb 1 mg/l	13.5±4 c	12.3±3 cd	11±2 cd
	Thymus vulgaris 500 µl/l	14.8±7 bc	13.6±4 c	12±4 cd
	Thymus vulgaris 1000 µl/l	14.5±4 bc	13.7±4 c	12±5 cd
	Salvia mirzayanii 500 µl/l	17.4±4 a	16.4±4 b	14±5 bc
	Salvia mirzayanii 1000 µl/l	13.3±6 c	12.9±3 cd	12±4 cd
	Artemisa persica 500 µl/l	15.9±7 bc	14.8±2 bc	12±5 cd
	Artemisa persica 1000 µl/l	13.4±4 c	12.6±5 cd	11±5 cd
	Rosmarinus officinalis 500µl/l	17.5±4 a	17±5 b	13±3 c
	Rosmarinus officinalis 1000 µl/l	15.8±3 bc	14.5±4 bc	11±c 2 d
Carotenoids	Control	0.85±0.03kn	1.64±0.06 b	1.94±0.03 a
	Mancozeb 0.5 mg/l	0.69±0.04 m-o	1.57±0.03 b	1.43±0.03 b-e
	Mancozeb 1 mg/l	0.74±0.03 m-o	1.52±0.04 bc	1.48±0.08 b-d
	Thymus vulgaris 500 µl/l	0.74±0.07 m-o	1.12±0.05 g-j	1.15±0.03 f-i
	Thymus vulgaris 1000 µl/l	0.55±0.03 o	1.28±0.08 c-g	1.91±0.06 a
	Salvia mirzayanii 500 µl/l	0.69±0.08 m-o	1.26±0.05 d-h	1.39±0.07 b-f
	Salvia mirzayanii 1000 µl/l	0.7±0.06 m-o	1.1±0.06 g-k	1.53±0.03 bc
	Artemisa persica 500 µl/l	0.56±0.05 o	0.85±0.04 l-n	1.31±0.04 c-g
	Artemisa persica 1000 µl/l	0.6±0.06 o	0.87±0.08 j-m	1.22±0.03 e-h
	Rosmarinus officinalis 500 µl/l	0.52±0.04 o	0.93±0.05 i-m	1.09±0.04 g-l
	Rosmarinus officinalis 1000 µl/l	0.5±0.07 o	1.01±0.03 h-l	1.16±0.05 f-i
Total chlorophyll	Control	2.72±0.2 f	1.19±0.02 gh	0.03±0.002 i
	Mancozeb 0.5 mg/l	3.98±0.4 e	1.31±0.02 g	0.53±0.03 h
	Mancozeb 1 mg/l	3.44±0.2 f	1.72±0.02 g	0.31±0.02 h
	Thymus vulgaris 500 µl/l	4.86±0.5 de	2.5±0. 6 f	0.11±0.01 h
	Thymus vulgaris 1000 µl/l	4.33±0.2 de	2.66±0. 5 f	0.11±0.02 h
	Salvia mirzayanii 500 µl/l	5.43±0.3 d	3.55±0.2 f	0.16±0.01 h
	Salvia mirzayanii 1000 µl/l	7.62±0.2 bc	3.47±0.2 f	0.15±0.02 h
	Artemisa persica 500 µl/l	9.61±0.2 a	2.58±0.7 f	0.16±0.03 h
	Artemisa persica 1000 µl/l	8.38±0.3 b	2.35±0.5 f	0.22±0.02 h
	Rosmarinus officinalis 500 µl/l	8.69±0.2 ab	3.27±0.8 f	0.16±0.04 h
	Rosmarinus officinalis 1000 µl/l	8.19±0.4 b	3.3±0.6 f	0.32±0.5 h

In each character, different letters indicate significant differences in mean values ($p<0.05$).

treatments throughout the storage (Table 2); however, the highest reduction was observed in the control fruits. At the end of storage, the minimum chlorophyll content (0.03) was recorded in the control, while the highest was found in mancozeb with 0.5 mg/l concentration.

TSS, TA and pH

A gradual increase in TSS percentages was determined in all treatments (Table 3). Generally, the fruits treated with EOs had lower TSS percentages than the control fruits throughout the storage. However, the treated fruits did not show any significant difference in TSS, as compared with the controls. These results were in agreement with those in other studies (Marjanlo *et al.*, 2009). Nevertheless, they are in discordance with Rabiei *et al.* (2011), who reported that thyme EOs treatment had a significant effect on the pH of apples. As shown in Table 3, TA values were also gradually decreased during storage. At the end of storage, the maximum TA values (0.27%) were observed in *A. persica* (1000 µl/l), while the minimum (0.10%) was in the control fruits. Among the EOs treatments, *R. officinalis* (1000 µl/l) resulted in the lowest acidity (0.17%), this was followed by *T. vulgaris* (0.16%) with 1000 µl/l concentration during storage; however, no significant difference was observed between the treatments. These results are in agreement with those reported by Maqbool and Alderson (2010), who showed that the

Table 3 - Effect of the *Thymus vulgaris*, *Salvia mirzayanii*, *Artemisia persica* and *Rosmarinus officinalis* EOs tested at 500 and 1000 µl/l on Total Soluble Solids (TSS), pH, and Titratable Acidity (TA) ± SD in mangos after 1, 2, and 3 weeks of incubation compared to 'mancozed' (0.5 and 1.0 mg/l)

Quality parameter	Treatment	Storage time (week)		
		Week 1	Week 2	Week 3
TSS	Control	8.9±3 c	9.6±3 b	9.9±2 a
	Mancozeb 0.5 mg/l	8.06±3 cd	9.23±5 b	9.5±3 b
	Mancozeb 1 mg/l	8.33±4 c	9.13±5 bc	9.5±2 b
	Thymus vulgaris 500 µl/l	8.96±5 c	9.63±4 b	9.7±2 ab
	Thymus vulgaris 1000 µl/l	8.83±4 c	9.76±4 ab	9.8±4 ab
	Salvia mirzayanii 500 µl/l	8.7±4 c	9.26±2 b	9.3±3 b
	Salvia mirzayanii 1000 µl/l	8.33±3 c	9.26±5 b	9.6±2 b
	Artemisa persica 500 µl/l	8.66±3 c	9.3±3 b	9.6±3 b
	Artemisa persica 1000 µl/l	8.1±5 cd	9.53±5 b	9.7±4 b
	Rosmarinus officinalis 500 µl/l	7.5±3 d	9±4 bc	9.2±2 b
	Rosmarinus officinalis 1000 µl/l	8.66±4 c	9.53±5 b	9.5±3 b
pH	Control	1.15±0.03 cd	2.89±0.2 c	4.75±1.4 a
	Mancozeb 0.5 mg/l	1.06±0.02 cd	2.38±0.4 c	4.3± 2 ab
	Mancozeb 1 mg/l	1.09±0.02 cd	2.59±0.3 c	4.3± 2 ab
	Thymus vulgaris 500 µl/l	1.06±0.02 cd	2.51±0.2 c	4.18± 2. 2 ab
	Thymus vulgaris 1000 µl/l	1.26±0.02 cd	2.54±0.3 c	4.31±1.8 ab
	Salvia mirzayanii 500 µl/l	0.99±0.02 d	2.74±0.2 c	4.24±1.9 ab
	Salvia mirzayanii 1000 µl/l	1.12±0.02 cd	2.26±0.2 c	4.42±2 ab
	Artemisa persica 500 µl/l	1.03±0.02 cd	2.45±0.3 c	4.48±3 ab
	Artemisa persica 1000 µl/l	1.08±0.02 cd	2.17±0.2 c	4.47±1.4 ab
	Rosmarinus officinalis 500 µl/l	1.02±0.02 cd	1.76±0.1 cd	4.36±1.2 ab
	Rosmarinus officinalis 1000 µl/l	1.07±0.02 cd	2.52±0. 5 c	4.56±2.1 ab
TA	Control	0.208±0.03 c	0.196±0.01 c	0.10±0.009 d
	Mancozeb 0.5 mg/l	0.254±0.02 bc	0.255±0.01 bc	0.23±0.02 bc
	Mancozeb 1 mg/l	0.27±0.02 bc	0.234±0.02 bc	0.23±0.02 bc
	Thymus vulgaris 500 µl/l	0.328±0.02 b	0.26±0.03 bc	0.23±0.02 bc
	Thymus vulgaris 1000 µl/l	0.308±0.01 b	0.262±0.02 bc	0.16±0.01 cd
	Salvia mirzayanii 500 µl/l	0.384±0.02 b	0.267±0.03 bc	0.25±0.02 bc
	Salvia mirzayanii 1000 µl/l	0.352±0.03 b	0.299±0.03 bc	0.24±0.02 bc
	Artemisa persica 500 µl/l	0.288±0.02 bc	0.277±0.02 bc	0.23±0.02 bc
	Artemisa persica 1000 µl/l	0.405±0.02 b	0.307±0.01 b	0.27±0.02 bc
	Rosmarinus officinalis 500 µl/l	0.471±0.02 a	0.302±0.02b	0.26±0.02 bc
	Rosmarinus officinalis 1000 µl/l	0.328±0.01 b	0.261±0.01 bc	0.17±0.01 cd

In each character, different letters indicate significant differences in mean values ($p<0.05$).

maximum reduction of the TA values was observed in the control fruits of bananas and papayas. Data related to the changes in the pH of fruits during storage revealed a significant increase in all treatments (Table 3). In each time, a higher pH value was found in the control groups. These results were in line with those reported by Du Plooy *et al*. (2009) and Tzortzakis (2007), showing no significant differences between the pH of control and treated fruits

4. Conclusions

The present study proves that the *T. vulgaris*, *A. persica*, *R. officinalis* and *S. mirzayanii* EOs could be employed under postharvest condition to control a pathogenic isolate of *A. niger* on mango fruits. The effectiveness of these EOs was more than mancozeb. So that, the EOs here tested could be used as a natural fungicide to control an isolate of *A. niger* during postharvest mangos. However, further studies are needed to fully understand the antimicrobial mechanisms incited by these EOs on a wide range of *A. niger* isolates, and evaluate their commercial implementation in order to increase the storage lifetime and quality of this marketable commodity.

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Ready-to-eat raspberries: qualitative and nutraceutical characteristics during shelf-life

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Abstract: Raspberry (*Rubus idaeus* L.) fruits are characterised by a high content of nutraceuticals, such as vitamin C, polyphenols and anthocyanins, which are considered antioxidant compounds. The ready-to-eat raspberry product could increase the market opportunities and the consumption of this high-value-added fruit. The aim of this research was to evaluate the evolution of qualitative and nutraceutical characteristics during the shelf-life of ready-to-eat raspberries. Samples from three raspberry cultivars ('Glen Magna', 'Tulameen' and 'Heritage') were sanitized and then packed in polypropylene bowls. The analyses were carried out at harvest (raw material) and after 3, 6 and 8 days of storage at 3°C. The study indicated the loss of fruit firmness as the most problematic aspect, followed by a less important change in hue values from light red to dark red. The modifications of chemical-physical parameters (soluble solids content, pH and titratable acidity) during shelf-life did not compromise the product quality. Processing and cold storage affected only slightly the nutraceutical profile (scavenging activity, phenols and anthocyanin content), except for ascorbic acid, therefore, the ready-to-eat raspberries could be considered a good source of compounds with potential health benefits. Some handling difficulties were highlighted during processing due to the high fragility of fruit which caused a high percentage of waste.

1. Introduction

Increasing epidemiological data suggested the correlation between the consumption of fresh fruits and vegetables with the prevention, delay or onset of chronic degenerative diseases, including cancer (Kaur and Kapoor, 2001). Fruit and vegetables are, in fact, rich of the so-called

nutraceuticals, which are compounds with a significant biological action, especially as antioxidants (Szeto *et al.*, 2002; Fu *et al*., 2011). Phenolics compounds, as an example, have a significant antioxidant activity (Szajdek and Borowska, 2008). They can donate electrons to the reactive oxygen species (ROS), converting them into innocuous molecules (Haminiuk *et al.*, 2012) and they can also exert beneficial modulatory action in cells (Williams *et al.*, 2004). Ascorbic acid (AA) is an essential vitamin that is found in fruits and vegetables and it has several positive functions: in plant and animal systems, AA interacts enzymatically and non-enzymatically with the reactive oxygen species and it is able to terminate the radical chain reactions by converting the ROS in non-toxic products (Davey *et al.*, 2000).

Red raspberry fruit (*Rubus idaeus* L.) are highly appreciated by consumers for their aromatic taste, especially when they are fully ripe. They are an important dietary source of bioactive compounds (Kähkönen *et al.*, 1999) such as anthocyanins, hydrosoluble pigments belonging to the class of polyphenols (Benvenuti *et al.*, 2004; Pantelidis *et al.*, 2007; Szajdek and Borowska, 2008) and vitamin C (Mazur *et al*., 2014 a, b). Unfortunately the postharvest life of this small fruit is short, because of its high respiration rate, loss of firmness and freshness, susceptibility to fruit rot and darkening (Krüger *et al.*, 2011); consequently it loses very early its market viability.

Ready-to-eat fruits and vegetables are one of the most important novel products introduced into the Italian agro-food system over the last 30 years and they represent a dynamic and innovative sector. In the last few years, the consumption of these products has increased, because the doubt and mistrust that characterised them in the 90's have now disappeared. Ready-to-eat products are very convenient because they do not need any preparatory operation (washing, cutting) before consumption. There are obviously some disadvantages in using this kind of products: they are usually costly and the processing operation can shorten the postharvest life of the fruit/vegetable. However, since fruit are poorly eaten by kids and teens (OECD, 2012) the diffusion of ready-to eat products is often encouraged in order to increase fruit consumption among young people. Raspberries are generally eaten fresh and a ready-to-eat product could increase the consumption of this bioactively rich fruit. The aim of this research was to evaluate the qualitative and nutraceutical characteristics during the shelf-life of ready-to-eat raspberries.

2. Materials and Methods

Raw material

Investigations were carried out on raspberry fruits of three different cultivars picked at commercial maturity: 'Heritage' and 'Glen Magna', grown at the fruit experimental centre of Milan University (Italy) and 'Tulameen', grown at the experimental comparative field for berry fruits of the Catholic University of Piacenza (Italy). On arrival at the laboratory the fruits were stored at 3±1°C till the processing time, always within 24 h.

Processing

Raspberries were manually selected in order to remove damaged fruits. Fruits were dipped in a disinfectant solution (0.022% of active chlorine) for 10 min and successively rinsed in tap water for 1 minute. Raspberries, after being softly dried with adsorbent paper, were packed and hermetically sealed in polypropylene (Transpiration rates (TR): O_2TR: 0.117 $mm^3\ m^{-2}\ d^{-1}\ Pa^{-1}$; CO_2TR: 1.847 $mm^3\ m^{-2}\ d^{-1}\ Pa^{-1}$) bowls (100 g per bowl), previously sanitized by UV radiation, and hermetically sealed with a film (O_2TR: 0.476 $mm^3\ m^{-2}\ d^{-1}\ Pa^{-1}$; CO_2TR: 2.17 $mm^3\ m^{-2}\ d^{-1}\ Pa^{-1}$) using a packaging machine Mod. TSM 95 (MINIPACK-TORRE, Dalmine, Bergamo, Italy). A rack, made of the same material was inserted inside the bowl in order to drain any dripping liquid. The packed fruits were then stored at 3±1°C for 8 days.

Chemical and physical analyses

Chemical and physical analyses were carried out on six samples (100 g each) of raspberry fruits per treatment at harvest (raw material) and after 3, 6 and 8 days of storage at 3°C. Fruit of each bowl were weighed and the amount of weight loss was measured by the percentage alteration between the initial (packaging) and final weight of the fruit of each bowl. Tititratable acidity [g citric acid/100 g fresh weight (FW)] and pH were assessed according to the Official Methods of Analysis (AOAC, 1985) and the soluble solids content (SSC, %) was measured by a multiscale automatic refractometer (RFM91 model, BS, UK). Colour parameters L*, a* and b* were measured by using the spectrophotometer CM-2600-D Minolta and hue was calculated as arctangent (b*/a*). Texture was measured on 15 fruit per treatment and sampling date, using an Instron Universal Testing Machine (model 1140, Instron, High Wycombe, UK), as maximum force (g) used to compress by the fruit (crosshead speed 10 cm/min).

Analysis of the nutritional compounds

Nutritional compounds were analyzed on 3 samples (100 g each) per treatment and sampling date. The fruit extracts (2 per sample) were prepared as follows: 2 g of homogenized flesh were extracted with 18 mL of solution made of EtOH 95% and HCl 0.02N (1:1 v/v), vortexed for 30 sec, centrifuged (15 min, 4°C, 10000 g) and filtered through a cheesecloth.

Since the most important nutraceuticals in *Rubus* fruits belong to the phenols, this class of compounds was analysed by two different assays.

i) Total phenols content (TPC) was measured by Folin-Ciocalteu method, as described by Singleton and Rossi (1965). An aliquot of 150 µL of fruit extract, 5 mL of distilled water and 1 mL Folin-Ciocalteu reagent were incubated for 8 min, followed by the addition of 2 mL of 20% Na_2CO_3. After 2h in the dark, the absorbance was measured at 730 nm against blank with a UV-UVIDEC 320 spectrophotometer (Jasco, Japan). Results were expressed as mg of gallic acid equivalent (GAE) per 100 g of fresh weight (FW).

ii) Total monomeric anthocyanin pigment was estimated by the pH differential method described by Giusti and Wrolstad (2001) using a UV-UVIDEC 320 spectrophotometer (Jasco, Japan). Extracts were diluted 1:10 with two buffers at pH 1.0 and 4.5; readings of each sample were made at 510 nm and 700 nm and the absorbance (A) of the diluted sample was calculated using the formula:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

The anthocyanin concentration was calculated using the cyanidin 3-glucoside (C3G) molar extinction coefficient 26,900 and it was expressed as mg of C3G equivalents in 100 g of FW.

Ascorbic acid (AA) was determined according to the method described by Davey *et al.* (2003) with some modifications. Briefly, 300 mL of the extract were added to 600 mL of 6% meta-phosphoric acid solution, diluted 1:20 in ortophosphoric acid solution (0.02M) and analyzed by an HPLC system (Jasco, Japan). AA was separated on an Inertsil ODS-3 (GL Science) column kept at 37°C and connected to a variable wavelength detector (UV- 1575, Jasco) set at 254 nm, using as mobile phase 0.02M orthophosphoric acid at the flow rate of 0.7 mL/min. Ascorbic acid concentration was expressed as mg in 100 g of FW.

Samples were also subjected to one of the most used free radical scavenging assays, which is relatively easy and cheap in execution and is strongly correlated with the sample's nutraceutical content: the 2,2-diphenyl-1-pycrilidrazyl ($DPPH^{\cdot}$) assay (Brand-Williams *et al.*, 1995). The amount of 500 µL of a solution 0.5 mM DPPH (dissolved in EtOH) 2.0 mL EtOH and 100 µL of sample was mixed in a 1-cm path cuvette and the absorbance at 517 nm against blank (2.5 mL EtOH and 100 µL of sample) was recorded at time 0 s and 180 s. The percentage of DPPH decrease was computed as:

$$\text{DPPH } \% = [(A_{t0} - A_{t180})/A_{t0}] \times 100$$

and the results were expressed as mg Trolox equivalents in 100 g of FW.

Statistical analysis

Statistical analyses were carried out with the Statgraphics software v.5.1 package (Manugistics, Rockwell MD). Data were submitted to multifactor ANOVA evaluating the main effects of the factors "cultivar" and "shelf-life". Differences among the treatments were determined by Tukey multiple range test or by Least significant distance (LSD) test ($P \leq 0.05$). Data were also examined using multivariate analysis by Principal Component Analysis (PCA).

3. Results

At the end of the shelf-life of the ready-to-eat raspberries, a fairly limited weight loss (0.1-0.3%), was noticed in all samples, mostly due to a slight juice dripping (data not shown).

Samples from different cultivars were characterized by different soluble solids content (Table 1): the cultivar Tulameen showed higher SSC values, followed by 'Heritage' and 'Glen Magna'. During shelf-life, this parameter decreased slightly in the cultivars Tulameen and Glen Magna while it did not change in the 'Heritage' fruit.

Total acidity decreased during shelf-life in 'Heritage' and 'Glen Magna' and slightly in 'Tulameen' (Table 1). No important changes in pH values were shown by 'Heritage' and 'Tulameen', while a significant decrease of pH was found in 'Glen Magna' fruit during the storage at 3°C (Table 1).

The compression test showed similar firmness for all the cultivars (Table 1). After only 3 days of shelf-life at 3°C, firmness decreased markedly in 'Heritage' (-32%) but only slightly in 'Tulameen' and 'Glen Magna' (Table 1). At the day 6 a significant decrease in firmness was observed in all the cultivar and, at end of the shelf-life (8 days) all the fruits had a firmness loss between -43% and -48%.

Table 1 - Maturity indices of ready-to-eat fruit from different raspberry cultivars, at harvest (raw) and after 3-6-8 days shelf-life at 1°C

Cultivar	Day of shelf-life	pH	SSC (%)	Acidity (g citric acid/100 g FW)	Firmness (g)
Heritage	raw	2.66 a	10.78 a	2.13 b	14.38 b
	3	2.62 a	10.68 a	2.03 a	9.82 a
	6	2.61 a	10.47 a	2.01 a	8.75 a
	8	2.67 a	10.49 a	1.97 a	7.46 a
Tulameen	raw	2.77 a	11.52c	1.55 b	14.50 b
	3	2.71 a	11.13 b	1.55 b	12.29 b
	6	2.75 a	10.83 ab	1.52 b	9.30 a
	8	2.76 a	10.74 a	1.38 a	8.23 a
Glen Magna	raw	2.82 a	9.54 b	1.72 c	15.05 b
	3	2.82 a	9.31 ab	1.56 b	11.89 ab
	6	2.90 ab	9.18 ab	1.49 ab	10.13 a
	8	2.97 b	9.01 a	1.42 a	8.63 a
Main Factors					
Cultivar	Heritage	2.64 a	10.60 b	2.03 b	10.13 a
	Tulameen	2.75 b	11.05 c	1.50 a	11.07 a
	Glen Magna	2.87 c	9.26 a	1.54 a	11.42 a
Day of shelf-life	raw	2.74 a	10.61 b	1.80 c	14.64 c
	3	2.71 a	10.37 ab	1.71 b	10.97 b
	6	2.75 a	10.10 a	1.67 b	9.74 ab
	8	2.80 a	10.13 a	1.58 a	8.10 a

Different letters indicate significant differences among the days of shelf-life of the same cultivar or among the main factors (Tukey test).

Visual appearance is a key issue for commercial shelf-life as consumer acceptance is often based on fruit colour which is used to make conclusions on the freshness of the product. The colour of 'Heritage' and 'Tulameen' fruits was characterized, at harvest, by lower L*, a* and b* values (Table 2) than those reported in literature (Çekic and Özgen, 2010; Krüger *et al.*, 2011). Fruit of 'Glen Magna' showed higher lightness (L*) and lower a* and hue values than the other two cultivars (Table 2). During shelf-life, the trend of L*, a* and b* values was not well defined, while hue decreased significantly in 'Glen Magna' and 'Heritage' indicating a darker red colour of the fruit. 'Tulameen' sample was, instead, more stable from the chromatic point of view.

At harvest the amounts of total anthocyanin of 'Heritage' (Granelli *et al.*, 2010), 'Tulameen' (Kruger *et al*., 2011) and 'Glen Magna' (Mazur *et al.*, 2014 b) were within the range of the values reported in literature (Fig. 1A) and the cv. Heritage showed the highest content. The cold storage induced a slight but significant loss of anthocyanin in all samples: after only 3 days (-20%) for 'Tulameen' and after 8 days for 'Heritage' (-12%) and 'Glen Magna' (-13%).

The average total polyphenols content (Fig. 1B) was higher in 'Glen Magna', followed by 'Tulameen' and 'Heritage'. TPC resulted quite stable over the shelf-life and no significant decrease was recorded. In our study the 'Tulameen' fruits showed the highest ascorbic acid content (Fig. 1C) at the harvest. AA content decreased significantly after only 3 days in in 'Tulameen' fruit which showed a loss of 26%. The final losses were about 26% for 'Heritage' and 'Tulameen' and even higher (-42%) for 'Glen Magna'.

The cultivar Heritage showed a significantly lower scavenging activity value (Fig. 1D) than the other cultivar at harvest and this difference was confirmed over the shelf-life. During storage the free radical scavenging activity had an up and down trend, and the end of the shelf-life it resulted slightly, even though significantly, decreased only in the 'Tulameen' samples.

Principal components analysis (PCA) can provide an overview of the shelf-life behaviour of ready-to-eat raspberries from different cultivars (Fig. 2). From the PCA analysis of the raspberry samples three functions were extracted, explaining the 91.2% of total variance. Considering the first two principal components PC1 (54.3% of the total variance) grouped the

Table 2 - Color parameters of ready-to-eat fruit from different raspberry cultivars, at harvest (raw) and after 3-6-8 days shelf-life at 1°C

	Day of shelf-life	a*	b*	L*	Hue (h°)
Heritage	raw	21.75 ab	9.75 a	27.44 ab	24.03 b
	3	22.84 ab	9.10 a	27.60 ab	21.58 a
	6	21.65 a	8.23 a	26.20 a	20.73 a
	8	24.38 b	9.67 a	29.77 b	21.54 a
Tulameen	raw	18.90 a	8.36 a	29.51 a	23.64 a
	3	20.53 a	8.99 a	29.83 a	23.58 a
	6	19.23 a	8.20 a	29.33 a	23.07 a
	8	20.48 a	8.90 a	30.31 a	23.44 a
Glen Magna	raw	18.17 a	7.25 a	30.83 b	21.89 b
	3	19.76 a	7.12 a	28.18 a	19.45 a
	6	19.13 a	6.83 a	28.96 a	19.53 a
	8	19.34 a	6.43 a	31.30 b	18.32 a
Main Factors					
Cultivar	Heritage	22.65 b	9.18 b	27.75 a	21.96 b
	Tulameen	19.78 a	8.61 b	29.74 b	23.43 c
	Glen Magna	19.09 a	6.90 a	29.81 b	19.79 a
Day of shelf-life	raw	19.60 a	8,45 a	29,25 ab	23,18 b
	3	21.04 ab	8.39 a	28.53 a	21.53 a
	6	20.00 ab	7.75 a	28.16 a	21.11 a
	8	21.40 b	8.33 a	30.45 b	21.09 a

Different letters indicate significant differences among the days of shelf-life of the same cultivar or among the main factors (Tukey test).

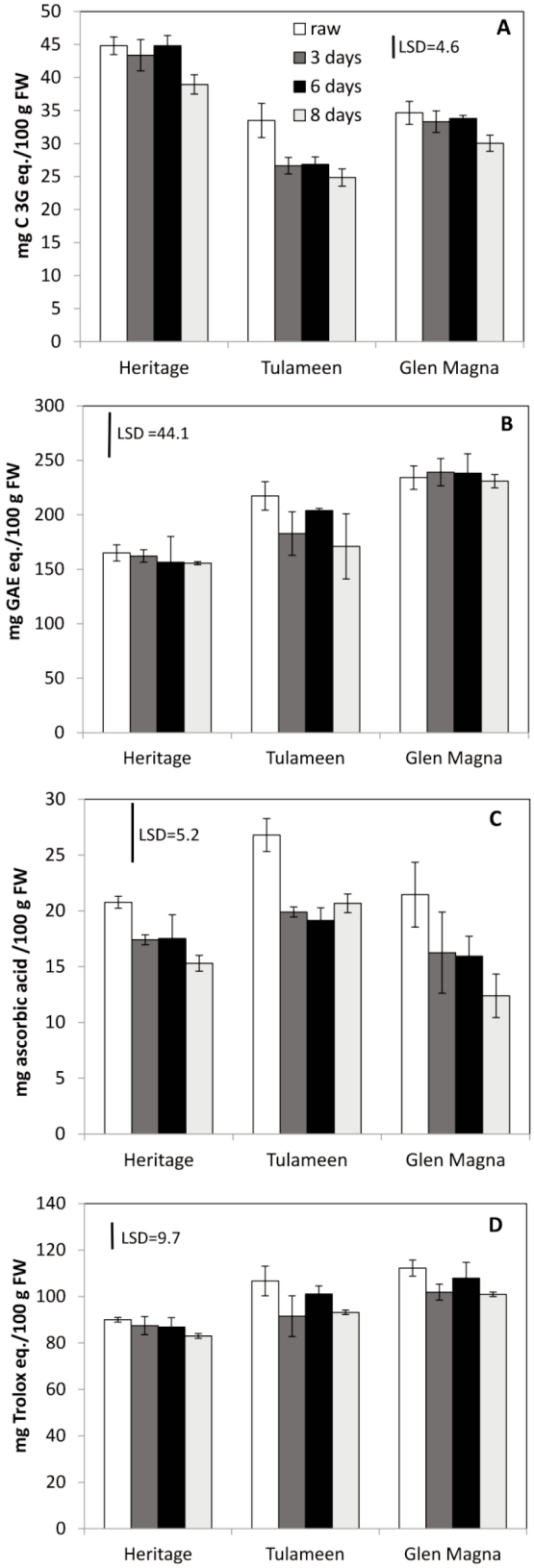

Fig. 1 - Total content (mean±standard error) of anthocyanins (A), phenols (B), Ascorbic acid (C) and total scavenging activity (D) measured in ready-to-eat samples from three raspberry cultivars, at harvest (raw) and after 3-6-8 days of shel-life at 3°C. The least significant distance (LSD) for P≤0.05 is indicated on every single graph.

colour parameters a* and b*, anthocyanins, acidity and SSC, opposite to scavenging activity, polyphenols content, L* and pH. PC2 (23.7%) was positively related to ascorbic acid, hue and SSC. In the biplot PC1 versus PC2 the three cultivars are very well distinguished. Heritage samples showed positive PC1 scores, linked to their high values of anthocyanins, acidity, a* and b* values. On the other side of the plot, 'Glen Magna' samples were characterized by low values on PC1 , due to their high DPPH scavenging activity and polyphenols content, L* parameter and pH values. All the 'Tulameen' samples had positive scores on PC2, linked to high ascorbic acid, hue and SSC. All the raw samples showed positive PC2 scores, being characterized by higher ascorbic acid content and more brilliant red colour (higher hue value). These values decreased markedly during shelf-life and, after only 3 days, all the samples showed lower PC2 scores.

4. Discussion and Conclusions

The weight loss can be an important problem during the postharvest of raspberry fruit (Haffner *et al*., 2002) however, in this experiment it always remained far below the limit of marketability (6%) (Haffner *et al*., 2002) and all fruit showed a good hydration state at the end of the shelf-life.

As it is visually shown in the PCA analysis, the raspberry cultivars were characterized by different quality parameters: in particular 'Heritage' by a higher anthocyanin content, acidity, a* and b* value, while 'Glen Magna' by a higher phenolic content and scavenging activity and 'Tulameen' by a higher ascorbic acid content.

At harvest SSC of raw fruit of 'Heritage' and 'Tulameen' was lower than those reported by the literature (Çekic and Özgen, 2010; Granelli *et al.*, 2010; Krüger *et al.*, 2011;) while the values of 'Glen Magna' were similar to those described by Mazur *et al.* (2014 a). The decrease in soluble solids content showed by all the cultivars during storage, as well as the reduction in acidity, was probably due to the normal respiration activity of the fruit (Giuggioli *et al.*, 2015). In any case, SSC was still at a good level at the end of shelf-life, showing a fairly good potential for these raspberry cultivars as a ready-to-eat product. It is well established, in fact, that SSC value is generally correlated with desirable flavor quality (Kader, 1997). The decrease of total acidity and the drop of the pH over time were also observed in other fresh-cut

products (Wright and Kader, 1997).

Tissue softening is a very serious problem and it often represents a limiting factor for many ready-to-eat products. Since consumers tend to want firm fruit, it is essential to evaluate fruit firmness in order to better assess the global quality of the product during shelf-life. Firmness of fresh-cut fruits has been widely reviewed but no data about this kind of ready-to-eat whole product are available. Besides, raspberry fruit is very fragile and the firmness is difficult to measure. The decrease in the firmness showed after only 3 days, even though the fruit tissues weren't injured by cutting, could be due to the necessary washing and sanitizing step. This operation consisted of a first dip in chlorinated water, to reduce the microbial loads on the fruit surface, and a second rapid dip in tap water, to eliminate residual chlorine and keep the sensorial properties of the untreated fruit. Moreover, the gentle drying of wet surfaces by adsorbent paper, a crucial step to remove the excess water, could cause mechanical damage to such delicate raspberries structures even though it is carried out carefully.

The stress caused from the washing step might also have affected the fruit color. By the way, the darkening of the fruit, which could affect the consumer choice, is reported by other authors (Haffner *et al.*, 2002; Giovanelli *et al.*, 2014) for fresh or packed raspberries stored in normal atmosphere at low temperature. In addition, Haffner *et al.* (2002) showed that storage in controlled atmosphere kept the berries more attractive than when stored in normal atmospheres.

Raspberry red colour is usually related to their anthocyanin composition (García-Viguera *et al.*, 1998). The higher initial anthocyanin content (Fig. 1A) and the higher value of the red parameter a* (Table 2) observed in 'Heritage', if compared to the other two cultivar, would confirm this positive correlation. The slight decrease of anthocyanin showed by the packed raspberry fruit during storage is in accordance with the results of Nunes *et al.* (2005) on strawberry stored at 1°C for 8 days. The author asserted that pigment degradation may be caused by the increased polyphenols oxydase activity as a result of physiological stress due to water loss during storage. Conversely, other authors reported an increase of the total anthocyanin content both in raspberry packed and stored at 4°C for 7 days (Giovanelli *et al.*, 2014) and in fresh fruit stored at 20°C for 1 day (Krüger *et al.,* 2011).

The total phenolics content (TPC) (Fig. 1B) of raw

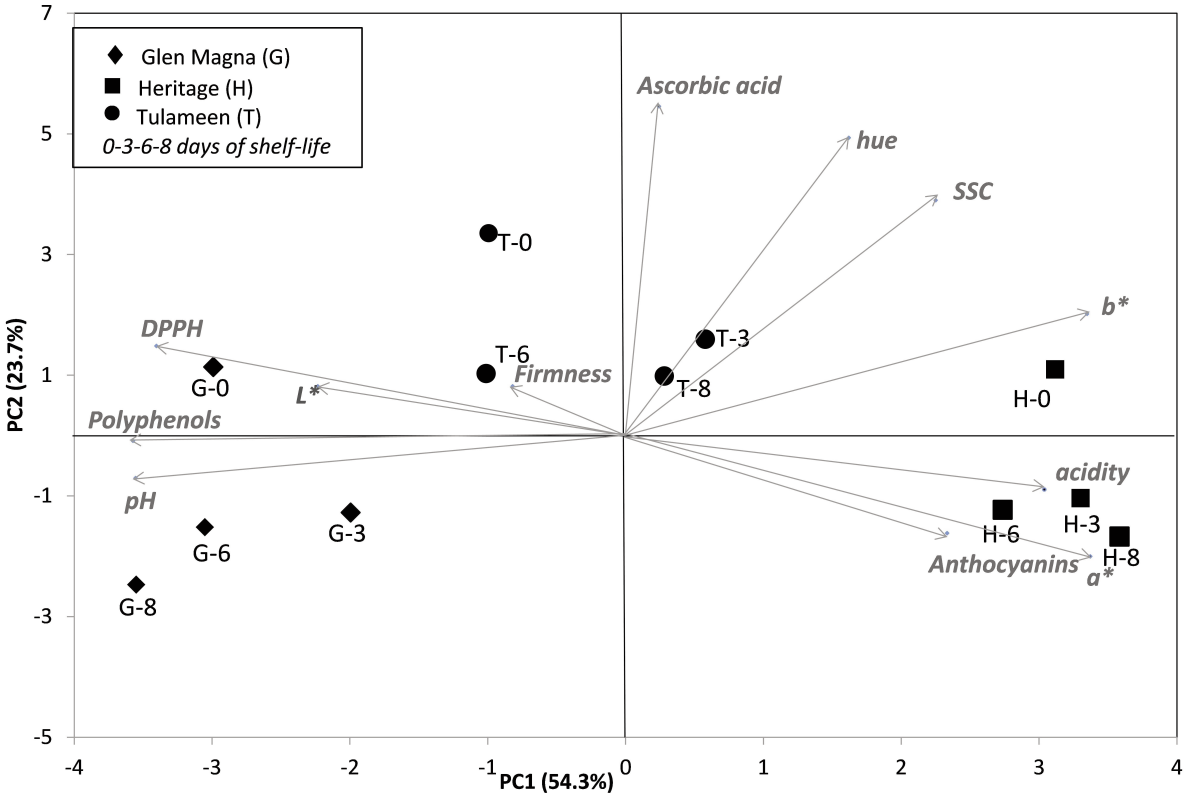

Fig. 2 - PCA biplot of quality and nutritional parameters during shelf-life of ready-to-eat raspberry fruit from the cultvars Heritage (H), Tulameen (T) and Glen Magna (G) after 0(raw)-3-6-8 days of shelf-life at 3°C (SSC=soluble solids content; L*, a*, b*, hue=colour parameters; DPPH=Scavenging activity).

'Heritage' reflected the findings of Cekic and Özgen (2010) whereas de Ancos *et al.* (2000) and Granelli *et al.* (2010) reported lower TPC values for this cultivar. TPC observed in the cv. Tulameen (Cekic and Özgen, 2010; Kruger *et al.,* 2011) and 'Glen Magna' (Mazur *et al.*, 2014 a) were in accordance with those reported in the literature. The stability of phenolics showed by the analyzed cultivar during shelf-life was also reported by Giovanelli *et al.* (2014) for fresh fruit and by de Ancos *et al.* (2000) for another type of processed raspberries product such as long-term frozen stored.

Ascorbic acid is one of the most important parameters used to control plant-derived food quality. Reduced temperature after harvest is considered to be important for its content in fruit (Krüger *et al.*, 2011). The decrease trend of the ascorbic acid of the analyzed cultivars during storage fully reflects the findings of de Ancos *et al.* (1999) and Krüger *et al.* (2011). Our results are, instead, not in accordance with Kalt *et al.* (1999) and Haffner *et al.* (2002) who found that AA level of raspberries, not washed and kept in normal atmosphere, was not reduced by several days of cold storage. A reasonable explanation for this discrepancy may be found in the washing step involved in the ready-to-eat processing: the hypochlorite contained in the sanitizing solution used could have oxidized the ascorbic acid, as reported by Bielski (1982).

It is well known that the scavenging activity of raspberry fruit is due to their high content of anthocyanins, phenolics and ascorbic acid (Krüger *et al.*, 2011). A significant correlation (R^2 = 0.857) was observed between the DPPH values and those of total phenol whereas the scavenging activity were not significantly correlated with total anthocyanins nor ascorbic acid. Our results, although lower than those found by Benvenuti *et al.* (2004), confirmed that the radical scavenging is related mainly to total phenol content.

The results of this study indicated the loss of firmness as the more problematic aspect of the ready-to-eat raspberries, followed by a less important change of the fruit colour from light red to dark red. The variations of chemical-physical parameters such as soluble solids content, pH and total titratable acidity, did not compromise the product quality. The processing (washing and packing) and the cold storage affected only slightly the nutraceutical profile, except for ascorbic acid, therefore the ready-to-eat raspberries could be considered a good source of compounds with potential health benefits. Some handling difficulties were highlighted during the processing steps of the samples (washing, drying and packing) due to the high fragility of fruit which caused a high percentage of waste. The hollow structure of raspberries made the drying step particularly difficult, however this is necessary as the residual water inside the fruit could significantly compromise the shelf-life of the product. In order to make this product more attractive to the consumers the loss of firmness and the darkening should be limited. For this purpose an alternative washing system like a fine spray and a packing in modified atmosphere could be adopted.

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Quality and nutraceutical properties of mango fruit: influence of cultivar and biological age assessed by Time-resolved Reflectance Spectroscopy

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Abstract: The content and composition of the main antioxidants in the pulp of mangoes depend also on cultivar and maturity degree, the latter being non-destructively evaluated by the absorption coefficient measured by Time-resolved Reflectance Spectroscopy (TRS) at 540 nm (μ_a540). Aiming at evaluating the levels of antioxidants [carotenoids (CAR), phenols (TPC), ascorbic acid (AA)] and antioxidant capacity (TAC) in relation to μ_a540 maturity class, selected 'Haden' and 'Palmer' mangoes were measured for μ_a540 by TRS, classified based on μ_a540 value as less (LeM), medium (MeM) and more (MoM) mature and analyzed for pulp firmness, pulp color (a*, h°, Yellowness Index), CAR (total and composition by HPLC-DAD), TPC, AA and TAC. 'Palmer' fruit had higher TPC, AA and TAC than 'Haden' mangoes. On average MoM fruit showed higher TPC, total CAR, total *all-trans*-violaxanthin esters and *all-trans*-β-carotene than MeM and LeM fruit. LeM fruit did not have compounds belonging to the 9-*cis*-violaxanthin group, while *cis*-β-cryptoxanthin was approx. 19% of total carotenoids. In MoM mangoes the main carotenoid was *all-trans*-β-carotene (53%), followed by total *all-trans*-violaxanthin esters (30%), 9-*cis*-violaxanthin group (8%) and *cis*-β-cryptoxanthin (6%). The μ_a540 significantly correlated (r=0.78-0.94) with total CAR, *all-trans*-β-carotene, *all-trans*-violaxanthin no.3 (both cultivars), TPC, *all-trans*-violaxanthin no.1, no.2, no.6 ('Haden'), and 9-*cis*-violaxanthin no.2, no.3 ('Palmer'). Our results indicate that TRS is suitable to non-destructively measure the pulp color of mangoes and to sort fruit with different ripening degree and nutraceutical properties.

1. Introduction

Mango (*Mangifera indica* L.) is a climacteric fruit belonging to the family of *Anacardiaceae* grown particularly in tropical and subtropical coun-

tries, with an estimated world production of 42 million tons per year (FAO, 2015). Brazil is one of the first ten largest mango producers, and more than 25% of its production is exported to Europe (Mitra, 2016). Appreciated for its excellent eating quality due to attractive flesh color, juicy texture and sweet flavor, mango provides high contents of bioactive compounds including carotenoids, phenolic compounds, ascorbic acid and reducing sugars (Rocha Ribeiro *et al.*, 2007; Manthey and Perkins-Veazie, 2009).

Carotenoids are responsible for the yellow-orange color of mango pulp and their content and composition depend mainly on cultivar and maturity degree, along with edaphic and climatic factors and postharvest handling, processing and storage conditions (Ornelas-Paz *et al.*, 2007, 2008; Manthey and Perkins-Veazie, 2009; Hewavitharana *et al.*, 2013; Liu *et al.*, 2013; Vásquez-Caicedo *et al.*, 2006; Vanoli *et al.*, 2016). During mango fruit ripening, biosynthesis of carotenoids occurs, due to chloroplasts differentiation into chromoplasts by disintegration of the thylacoid membranes and by the development of new pigment-bearing structures (Vásquez-Caicedo *et al.*, 2006). This process leads to carotenoids accumulation, which is usually accompanied by color changes of the pulp turning from white to yellow-orange (Ornelas-Paz *et al.*, 2008; Vásquez-Caicedo *et al.*, 2006). The accumulation of carotenoids in the mesocarp shows an exponential behavior during fruit ripening and cultivar-specific relationships between total or individual carotenoid (*all-trans*-β-carotene, *all-trans*-violaxanthin and 9-*cis*-violaxanthin) contents and mesocarp color (a^*, $h°$) were established in different mango cultivars (Vásquez-Caceido *et al.*, 2006; Ornelas-Paz *et al.*, 2008). Similarly to carotenes, ascorbic acid content and total phenolic content vary according to the cultivars, maturity stage and cultural practices (Rocha Ribeiro *et al.*, 2007; Valente *et al.*, 2011; Liu *et al.*, 2013; Oliveira *et al.*, 2016; Septembre-Malaterre *et al.*, 2016).

In order to withstand shipping to distant markets and at the same time to have the optimum eating quality when ripe, mango fruit are harvested at the hard green stage, after having reached the physiological maturity, but before the onset of the climacteric rise. If mango fruit are immature at harvest, they do not reach an eating quality when ripe and, hence, the discrimination between mature and immature fruit at harvest is very important from the marketing point of view in order to minimize qualitative losses during the supply chain. Fruit shape ("shoulders" should be full), pulp color and firmness are the most used maturity indices for mangoes, but differences among cultivars and growing conditions have precluded universal maturity indices. On the other hand, the current industry measurements of firmness and pulp color have the disadvantage of being destructive of fruit; hence, the development of a non-destructive technique could help the growers to pick fruit at the proper maturity degree for the different market destinations.

Among the non-destructive techniques able to assess the maturity degree of fruit, Time-resolved Reflectance Spectroscopy (TRS) is gaining increasing interest (Nicolai *et al.*, 2014). TRS is a non-destructive optical technique which can separate the effect of light absorption, due to chemical compounds such as pigments and water, and light scattering, due to microscopic changes in refractive index caused by membranes, vacuoles, starch granules, organelles, and air. By measuring photon time-of-flight distribution with picoseconds temporal resolution, the absorption (μ_a) and reduced scattering (μ_s) coefficients in the VIS-NIR wavelength range are quantified, by probing pulp at a depth of 1-2 cm with no or limited influence from the skin (Cubeddu *et al.*, 2001, Torricelli *et al.*, 2008). TRS has been used to study the internal fruit attributes related to maturity in apples, peaches, nectarines and pears (Rizzolo and Vanoli, 2016). In nectarines, the μ_a measured at harvest at 670 nm (μ_a670), near the chlorophyll-*a* peak, can be considered an effective maturity index as it is linked to the biological age of the fruit (Tijskens *et al.*, 2007) and has been successfully used to predict the softening rate during shelf life in nectarines and to select fruit for different market destinations (Eccher Zerbini *et al.*, 2009).

Previous studies carried out on mango fruit have demonstrated the potential of TRS for the non-destructive determination of pulp color (Vanoli *et al.*, 2013; Rizzolo *et al.*, 2016; Vanoli *et al.*, 2016), as well as the possibility of using TRS absorption in both the carotenoids (540 nm) and chlorophyll-*a* (670 nm) spectral regions to classify mango fruit according to maturity and to predict the ripening of individual fruit (Eccher Zerbini *et al.*, 2015).

The present work aimed at evaluating the levels of antioxidants and antioxidant capacity in the pulp of two cultivars of Brazilian mangoes in relation to fruit maturity class assigned according to the μ_a540 value, along with selected quality parameters. The relationships between μ_a540 maturity index and total carotenoid content, total phenolic compounds, ascorbic acid and the fourteen carotenoid com-

pounds identified were also studied.

2. Materials and Methods

Mango fruit

On November 2011, 'Haden' and 'Palmer' mangoes were picked in experimental orchards of EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais) in Minas Gerais state (Brazil) and transported to Milan (Italy) by plane soon after harvest. On arrival at CREA-IT lab (about 5-7 days from harvest), 60 fruits of 'Haden' and 90 fruits of 'Palmer' without defects were selected and measured by TRS at 650 nm ('Haden') or at 690 nm ('Palmer') on two opposite sides in the fruit equatorial region and ranked by decreasing μ_a averaged over the two sides, that is from less (high μ_a) to more mature fruit (low μ_a). 'Haden' fruit were put at 20°C for 2 days, while 'Palmer' mangoes were randomized into 3 batches of 30 fruits, corresponding to 0, 4 and 11 days of shelf life at 20°C, in order to have the whole range of μ_a690 in each batch. Sub-samples of 10 fruits, covering the whole range of μ_a, were selected for 'Haden' and for 'Palmer' batch held for 4 days at 20°C. Each selected fruit was measured by TRS at 540 nm on two opposite sides in the equatorial region; in the same positions of TRS measurements, skin was removed by a slicer, and, after measuring pulp color and firmness, the whole fruit were immediately deep frozen and kept at -30°C until carotenoid, ascorbic acid and total phenolic extractions.

Time-resolved Reflectance Spectroscopy (TRS)

A portable compact setup working at discrete wavelengths developed at Politecnico di Milano (Spinelli *et al.*, 2012) was used. The light source is a supercontinuum fiber laser (SC450-6W, Fianium, UK) providing white-light picoseconds pulses, with duration of few tens of picoseconds. A custom-made filter wheel loaded with 14 band-pass interference filters (NT-65 series; Edmund Optics) is used for spectral selection in the range 540-940 nm. Light is delivered to the sample by means of a multimode graded-index fiber and diffuse remitted light is collected by 1 mm fiber placed at 1.5 cm distance from the illumination point. A second filter wheel identical to the first one is used for cutting off the fluorescence signal originated when illuminating the fruit in the visible spectral region. The light then is detected with a photomultiplier (HPM-100-50, Becker & Hickl, Germany) and the photon time-of-flight distribution was measured by a time-correlated single-photon counting board (SPC-130, Becker & Hickl, Germany). The instrumental response function has a full width at half maximum of about 260 ps and the typical acquisition time is 1 s per wavelength. A model for photon diffusion in turbid media was used to analyze TRS data to assess the bulk optical properties of the samples (Martelli *et al.*, 2009) to obtain the estimates of μ_a and μ_s at each wavelength.

Firmness and pulp color

Flesh firmness was measured using a Instron UTM model 4301 penetrometer (crosshead speed 200 mm min^{-1}, 8 mm diameter plunger). Data were averaged per fruit.

Pulp color was measured with a spectrophotometer (CM-2600d, Minolta Co., Japan), using the primary illuminant D65 and 2° observer in the L^*, a^*, b^* color space. Hue ($h°$) was computed from a^* and b^* values according to:

$$h° = \text{arctangent}\,(b^*/a^*) \times 360/(23 \times 14)$$

Yellowness index (I_Y) was computed as:

$$I_Y = [81.2746X - 1.0574Z)/Y] \times 100$$

after converting $L^*a^*b^*$ parameters into the XYZ color space (Jha *et al.*, 2006). Data were averaged per fruit.

Carotenoids, ascorbic acid, total phenols and total antioxidant capacity analysis

Carotenoids, ascorbic acid, total phenols and total antioxidant capacity analysis were carried out on frozen fruit after 30 min thawing at ambient temperature, by slicing pulp portions without peel near the positions of TRS measurements and pooling the slices coming from the two fruit sides.

Carotenoids were extracted following the procedure described by Picchi *et al.* (2012) with slight modifications. Briefly, on individual fruit, 2 g of pulp (two replicates) was extracted with 10 mL of a solution of hexane:acetone:ethyl acetate (2:1:1 v/v/v) containing 100 µL of 1% butylhydroxytoluol (BHT) in methanol, to prevent carotenoid oxidative degradation, and centrifuged at 4°C at 15,000 rpm for 20 min. The extracts were stored at -80°C until spectrophotometric and high-performance liquid chromatographic (HPLC) analyses.

Total carotenoid content (CAR) was determined measuring absorbance at 450 nm using a spectrophotometer (UV-UVIDEC 320, Jasco, Japan). The hexane:acetone:ethyl acetate solution was used as the blank. Total carotenoid content was estimated from a standard curve of *all-trans*-β-carotene and data were expressed as milligrams of β-carotene

equivalent (β-car) per kilogram of fresh weight (mg β-car kg FW^{-1}).

Carotenoid composition was determined on extracts according to Azevedo-Meleiro and Rodriguez-Amaya (2004) with some modifications. A Jasco (Tokio, Japan) HPLC system consisting of a PU-1580 liquid chromatographic pump coupled to LG 1580-04 quaternary gradient unit, a model AS 2055-plus autosampler and an MD 2010-plus multi-wavelength detector was used. Separations were performed on an Inertsil ODS-3 column (4.6 mm i.d × 250 mm length, particle diameter 5 µm, GL Science) at the temperature of 40°C which was maintained using a Jasco Co-1560 Intelligent Column thermostat. The sample injection volume was 80 µL. The column was eluted with 20% methanol and 80% of a gradient mixture of acetonitrile (A) and ethyl acetate (B) at the flow rate of 0.6 mL min^{-1}, with 10% B at 0-25 min, 10-20% B at 25-35 min, 20-50% B at 35-40 min, 50% B at 40-45 min, 50-10% B at 45-50 min. Spectra of all peaks were recorded in the 200-600 nm wavelength range, and peak areas were monitored at 450 nm. Carotenoids (Table 1) were identified by comparing their retention times and spectral characteristics with those of standards (*all-trans*-β-carotene and violaxanthin, obtained by pansy petals) and with those reported in the literature (Ornelas-Paz *et al.*, 2007, 2008), considering the three maximum absorbance wavelengths (λ_{max}) and the spectral fine structure (% III/II), which is the percentage of the peak height of the longest wavelength absorption band (λ_{max} III) to that of the middle absorption band (λ_{max} II), taking the maximum of the valley between peak II and peak III as the baseline (Sajilata *et al.*, 2008). Carotenoids were quantified referring to the total carotenoid content estimated spectrophotometrically on the same extract in conjunction with the chromatogram percent composition and data were expressed as milligrams of β-carotene equivalent (β-car) per kilogram of fresh weight (mg β-car kg FW^{-1}). All the measurements were carried out in triplicate. The vitamin A value, expressed as retinol equivalent (RE) was estimated from *all-trans*-β-carotene and *cis*-β-cryptoxanthin amounts using as conversion figures 6 µg for Car and 12 µg for Crypt (Capra, 2006).

Ascorbic acid was extracted following the procedure described by Robles-Sánchez *et al.* (2009 a) with slight modifications. Briefly, on individual fruit, 2 g of pulp (two replicates) was homogenized with 10 mL of 6% (w/v) aqueous solution of metaphosphoric acid (MPA), vortexed for 30 s, and centrifuged at 4°C at 15,000 rpm for 20 min and the extracts were kept at -20°C till HPLC analysis. Ascorbic acid was determined on just thawed extracts according to the conditions reported by Rizzolo *et al.* (2002), using a Jasco (Tokio, Japan) HPLC system consisting of a PU-980 liquid chromatographic pump, a model AS 1055-10 autosampler and an UV-Vis 15770 detector set at 254 nm, coupled to an Inertsil ODS-3 column (4.6 mm i.d. × 250 mm length, particle diameter 5 µm, GL Science) at the temperature of 30°C, which was eluted with 0.02 M orthophosphoric acid at a flow rate of 0.7 mL min^{-1}. Ascorbic acid was estimated from a standard curve of L-ascorbic acid in 6% MPA and data were

Table 1 - Retention time (Rt, min), spectra characteristics [λ_{max} (nm) in the mobile phase, obtained by DAD, spectral fine structure (% III/II)] and name abbreviation of tentatively identified compounds according to Ornelas-Paz *et al.* (2007, 2008)

Peak no.	R_t	λ_{max} I	λ_{max} II	λ_{max} III	% III/II	Tentative identification	Abbreviation
1	5.24-5.89	419	439	471	82	unknown	UNK
2	5.92-5.97	415	439	471	82	*all-trans*-violaxanthin various esters	Viol no. 1
3	6.03-6.09	415	439	471	100	*all-trans*-violaxanthin various esters	Viol no. 2
4	6.11-6.19	415	443	471	90	*cis-β*-cryptoxanthin	Crypt
5	6.23-7.29	415	439	471	93	*all-trans*-violaxanthin various esters	Viol no. 3
6	7.40-7.83	411	435	463	75	9-*cis*-violaxanthin	9-viol no. 1
7	8.03-8.77	415	435	467	83	9-*cis*-violaxanthin	9-viol no. 2
8	9.40-10.31	415	439	467	84	*all-trans*-violaxanthin various esters	Viol no. 4
9	10.32-11.15	411	435	463	80	9-*cis*-violaxanthin	9-viol no. 3
10	33.48-36.19	419	439	471	n.c.	*all-trans*-violaxanthin various esters	Viol no. 5
11	37.97-40.61		451	479	23	*all-trans*-β-carotene	Car
12	40.90-43.60	419	439	471	100	*all-trans*-violaxanthin various esters	Viol no. 6
13	43.71-43.99	415	435	467	100	9-*cis*-violaxanthin	9-viol no. 4
14	44.00-44.60	415	439	467	100	*all-trans*-violaxanthin various esters	Viol no. 7

expressed as milligram per kilogram of fresh weight (mg kg FW^{-1}). All the measurements were carried out in triplicate.

Total phenol content (TPC) and total antioxidant capacity (TAC) were determined on the same extract (two replicates/fruit) obtained by homogenizing 2 g of pulp with 10 mL of acidic ethanol (ethanol:0.04 M HCl, 1:1 v/v), vortexed for 30 s and centrifuged at 4°C at 15,000 rpm for 20 min. Extracts were kept at -20°C till total phenol content and antioxidant capacity determinations. TPC was determined using the Folin-Ciocalteau method (Singleton *et al.*, 1999) based on the reduction of a phosphowolframate phosphomolibdate complex by phenolics to blue reaction products, and measuring absorbance at 730 nm using a spectrophotometer (UV-UVIDEC 320, Jasco, Japan). The TPC was estimated from a standard curve of gallic acid and data were expressed as milligrams of gallic acid equivalents (GAE) per kilogram of fresh weight (mg GAE kg FW^{-1}). All the measurements were performed in triplicate. TAC was evaluated using the free radical 1,1,-dyphenyl-2-picrylhydrazil (DPPH•) according to Brand-Williams *et al.* (1995) with modifications. Fifty microlitres of sample extract or Trolox standard solution (0.02-0.8 mM) were added to 2 mL of ethanol and 550 µL of DPPH• solution (0.05 mM in ethanol) and, during 5 min of incubation, the absorbance at 517 nm was measured with a Jasco 7800 UV/VIS spectrophotometer (Jasco Europe S.r.l., Cremella, LC, Italy). The DPPH scavenging capacity of the samples was calculated using a standard curve of Trolox, and expressed as micromoles of Trolox equivalents (TE) per kilogram of fresh weight (µmol TE kg FW^{-1}). All the measurements were performed in triplicate.

Statistical analysis

The Statgraphics v. 5.2 (Manugistic Inc., Rockville, MD, USA) software package was used. Data were submitted to multifactor analysis of variance (ANOVA) considering cultivar, TRS maturation class and their interaction as source of variation. In addition, one-way ANOVA was used to study the main factors (cultivar, TRS maturity class), and the TRS maturity class within each cultivar. Percentage data of carotenoids were analyzed after arcsine transformation. Means were compared by 95 percent Bonferroni's test. Relationships between μ_a540 and pulp color parameters and between μ_a540, a^*, $h°$, I_Y and ascorbic acid, TPC, TAC and carotenoids were studied using regression analysis. For each parameter, the model with the higher performance was considered.

3. Results

TRS optical properties

In 'Palmer' fruit, μ_a690 at harvest ranged from 0.074 cm^{-1} for the less mature fruit to 0.021 cm^{-1} for the more mature ones and decreased to 0.061 cm^{-1} and 0.019 cm^{-1}, respectively, after 4 days of shelf life at 20°C; concomitantly, after shelf life, μ_a540 ranged from 0.117 cm^{-1} for the least mature fruit to about 0.33 cm^{-1} for the most mature ones. In 'Haden' mangoes, μ_a650 ranged at harvest from 0.231 cm^{-1} to 0.030 cm^{-1}, with the majority of the fruit in the 0.030-0.065 cm^{-1} range; after 2 days of shelf life at 20°C, μ_a650 decreased only in less mature fruit, whereas in all the other mango fruit it increased to values ranging from 0.036 cm^{-1} to 0.053 cm^{-1}, while the μ_a540 values after shelf life ranged from 0.157 cm^{-1} for the least mature fruit to 0.835 cm^{-1} for the most mature ones.

The μ_a540 maturity index, related to carotenoids content, was then used to classify the selected fruit within each cultivar in three TRS maturity classes: less mature (LeM) with low μ_a540, more mature (MoM) with high μ_a540 and medium mature (MeM) with intermediate values of μ_a540. Cultivar and TRS maturity class influenced the value of μ_a540 maturity index (Table 2); on average μ_a540 was higher in cv. Haden ('Haden': 0.400±0.025 cm^{-1}; 'Palmer': 0.248±0.025 cm^{-1}) and in the MoM class in both cultivars, with MoM 'Haden' fruit being characterized by the highest μ_a540 value.

Quality parameters

TRS maturity class and cultivar greatly affected a^* and $h°$ pulp color parameters and had only a slight influence on firmness, probably due to the high standard error values, whereas I_Y depended only on maturity class (Table 2). On average, 'Palmer' fruit had lower firmness and a^* value, and higher $h°$ than 'Haden' fruit. In 'Palmer' mangoes firmness did not vary with maturity class, while in 'Haden' firmness showed the highest values in LeM fruit and the lowest in MeM and in MoM ones. MoM 'Palmer' fruit had higher a^* and I_Y and lower $h°$ than LeM and MeM maturity classes, whereas LeM 'Haden' fruit had lower I_Y than MoM fruit, and a^* increased and $h°$ decreased from LeM to MeM and MoM maturity classes.

Ascorbic acid, total phenolic content and total antioxidant capacity

AA content and TAC were significantly influenced only by cultivar, while TPC depended by both cultivar

and maturity class (Table 3). On average, 'Palmer' mangoes had higher AA, TPC and TAC than 'Haden' fruit, and LeM fruit had lower TPC than MoM mangoes (Fig. 1).

The average AA values were approx. 190 mg kg FW^{-1} for 'Haden' and 390 mg kg FW^{-1} for 'Palmer' and AA content did not change with maturity class in 'Palmer' mangoes while in 'Haden' showed the highest values in MeM fruit (Fig. 1). TPC was higher in 'Palmer' than in 'Haden' cv., being on average approx. 316 and 264 mg kg FW^{-1} in 'Palmer' and 'Haden' fruit, respectively, as well as it was higher in MoM fruit from both cultivars. 'Palmer' fruit were characterized by higher TAC showing on average 2.59 times greater than that from 'Haden'. TAC had significant positive correlations with TPC (r= 0.66, p= 0.002) and AA (r= 0.89, p<0.0001).

Carotenoids

Total carotenoids (CAR) depended only by maturity class (Table 3), with LeM fruit having less CAR than MoM ones (Fig. 1). The chromatographic carotenoid patterns of LeM and MoM maturity classes in both cultivars showed 9 common carotenoids (Fig. 2) out of 14 peaks tentatively identified by comparing spectral characteristics with those previously reported using a similar mobile phase (Table 1). The carotenoid pattern includes seven *all-trans*-violaxanthin (Viol) and four 9-*cis*-violaxanthin (9-Viol) containing compounds, *cis*-β-cryptoxanthin and *all-trans*-β-carotene.

The most abundant carotenoid in both cultivars was *all-trans*-β-carotene (Tables 4 and 5), representing 49-56% of the total carotenoid content, followed

Table 2 - Absorption coefficient at 540 nm (μa540, cm^{-1}), flesh firmness (N) and pulp colour parameters (a^*; hue, $h°$; yellowness index, I_Y) of 'Palmer' and 'Haden' mangoes of less mature (LeM), medium mature (MeM) and more mature (MoM) TRS maturity classes and results of multifactor ANOVA (F-ratio value and P-value)

Cultivar	Maturity class	μ_a540	Firmness	a^*	$h°$	I_Y
Palmer	LeM	0.196±0.027 b	8.33 ±0.55 a	0.86±0.79 b	89.13±0.75 a	130.7±1.7 b
	MeM	0.241±0.006 ab	7.70±0.45 a	3.64±1.03 b	86.85±0.89 a	153.3±1.6 ab
	MoM	0.310±0.017 a	6.12±0.63 a	9.84±1.75 a	81.72±1.30 b	165.7±9.5 a
Haden	LeM	0.191±0.011 b	37.29±9.89 a	2.98±1.62 c	87.18±1.55 a	130.0±6.0 b
	MeM	0.336±0.033 b	10.47±3.62 a	10.60±1.44 b	80.32±1.15 b	159.7±6.6 a
	MoM	0.677±0.158 a	5.85±0.65 b	20.46±1.12 a	72.37±0.84 c	191.2±6.1 a
ANOVA						
A: cultivar		18.88 ***	4.81*	28.85 ***	32.20 ***	3.94 NS
B: maturity class		23.52 ***	4.85 *	36.79 ***	32.25 ***	27.18 ***
A × B		9.21 **	3.98 *	3.94 *	4.16 *	2.01 NS

Mean±SE. Within each cultivar, means followed by different letters are statistically different (Bonferroni's test, P≤0.05). P-value of F-ratio: NS=not significant; *P<0.05; **P<0.01; ***P<0.001.

Table 3 - Multifactor analysis of variance (F-ratio and P-value) for ascorbic acid (AA), total carotenoids (CAR), total plenolic content (TPC) and total antioxidant capacity (TAC)

Factors	AA	CAR	TPC	TAC
		main factors		
Cultivar (A)	38.79 ***	0.48 NS	5.12 *	32.75 ***
Maturity class (B)	0.75 NS	7.28 **	4.20 *	1.85 NS
		interaction		
A × B	0.68 NS	0.98 NS	0.81 NS	0.53 NS

*P<0.05; **P<0.01; ***P<0.001; NS=not significant.

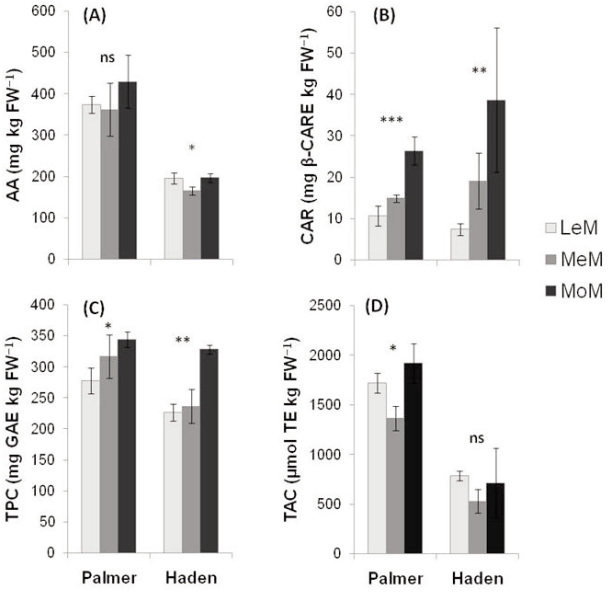

Fig. 1 - (A) Ascorbic acid (AA), (B) total carotenoids (CAR), total phenol content (TPC) and total antioxidant capacity (TAC) in 'Palmer' and 'Haden' mangoes in function of μ_a540 maturity class (LeM, less mature; MeM, medium mature; MoM, more mature). Bars refer to SE. Within each cultivar ANOVA results are indicated as follows: *, **, ***: significant at P≤0.05, 0.01, 0.001, respectively; NS, not significant.

by *cis*-β-cryptoxanthin (6-18%) and Viol no.3 (11-16%). The content of *all-trans*-violaxanthins was higher than that of 9-*cis*-violaxanthins in both cultivars.

The content of Viol no.3, no.4 and no.6, 9-Viol no.4 and *all-trans*-β-carotene, as well as the sums of *all-trans*-violaxanthins (∑Viol) and of 9-*cis*-violaxanthins (∑9-Viol) depended only on maturity class, that of 9-Viol no.1 only on cultivar, whereas those of 9-Viol no.2 and no.3 on both cultivar and maturity class (Table 4). In fact 'Haden' mangoes had higher amounts of 9-Viol no.3 (Table 4) and had lower proportion of 9-Viol no.4 than 'Palmer' fruit (Table 5). Viol no. 1 and 9-Viol no.1 were present only in 'Haden' and 9-Viol no. 2 only in 'Palmer' fruit.

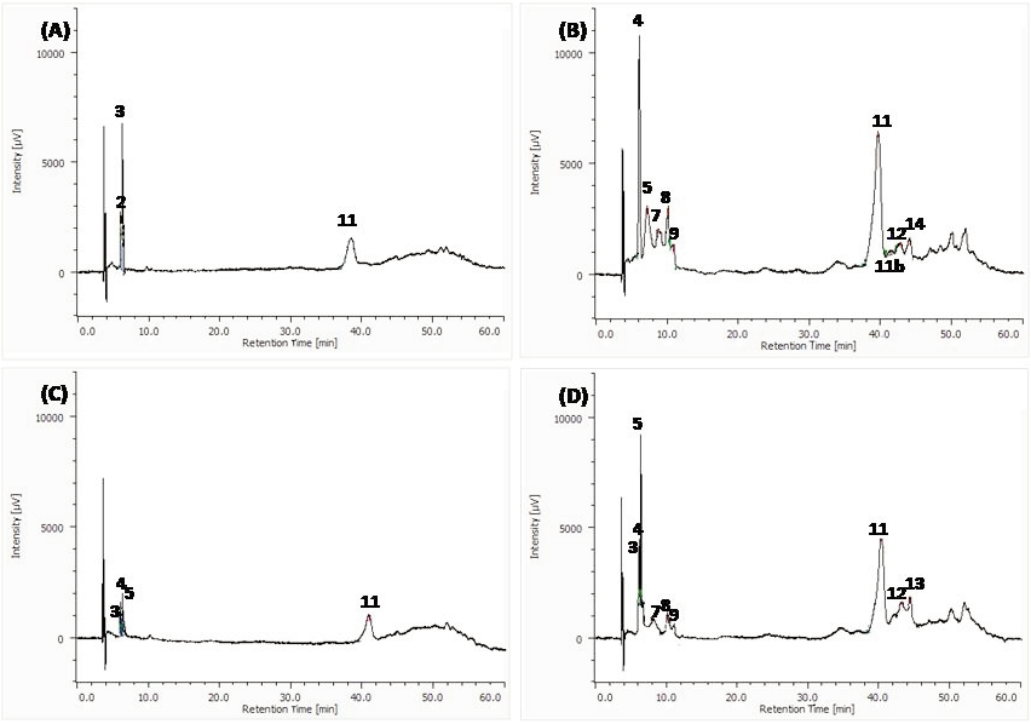

Fig. 2 - Typical chromatographic patterns at 450 nm of carotenoid extracts of (A) LeM 'Palmer', (B) MoM 'Palmer', (C) LeM 'Haden' and (D) MoM 'Haden' mangoes. For peak assignment see Table 1.

Table 4 - Carotenoid compounds of 'Palmer' and 'Haden' mangoes (mg β-CARE kg FW^{-1}) and vitamin A value (RE 100 g FW^{-1}) influence of cultivar and of TRS maturity class and results of multifactor ANOVA (F-ratio value and P-value). For identification data of each carotenoid see Table 1

	Cultivar		Maturity class			ANOVA		
	Palmer	Haden	Less mature	Medium mature	More mature	Cultivar (A)	Maturity class (B)	A × B
Viol no. 1	0±0 a	0.49±0.49 a	0 ±0 a	0±0 a	0.99±0.99 a	3.25 (*)	2.89 (*)	2.89 (*)
Viol no. 2	1.14±0.46 a	0.87±0.68 a	0.68 ±0.40 a	0.49±0.38 a	2.15±1.33 a	0.08 NS	2.11 NS	2.48 NS
Crypt	2.58±0.55 a	1.82±0.38 a	1.66 ±0.37 a	2.69±0.47 a	2.18 ±0.97 a	1.53 NS	0.89 NS	0.39 NS
Viol no. 3	2.43±0.31 a	2.49±0.55 a	1.53±0.28 b	2.82±0.67 ab	3.26±0.30 a	0.07 NS	3.08 (*)	2.37 NS
9-viol no. 1	0±0 b	0.81±0.42 a	0±0 a	0.67±0.42 a	0.68±0.68 a	4. 91 *	1.36 NS	1.36 NS
9-viol no. 2	0.33±0.16 a	0±0 b	0±0 b	0.06±0.06 ab	0.52±0.26 a	10.91 **	6.23 *	6.23 *
Viol no. 4	0.84±0.16 a	0.91±0.40 a	0.27±0.13 b	0.88±0.40 ab	1.72±0.37 a	0.65 NS	4.90 *	0.89 NS
9-viol no. 3	0.09±0.06 b	0.35±0.15 a	0±0 b	0.21±0.11 ab	0.56±0.25 a	7.62 *	7.57 **	2.35 NS
Car	9.36±1.14 a	9.63±2.72 a	5.05±0.67 b	8.85±1.81 ab	16.65±3.47 a	0.81 NS	8.37 **	1.03 NS
Viol no. 6	0.64±0.14 a	0.56±0.23 a	0.08±0.08 b	0.74±0.16 ab	1.12±0.25 a	0.12 NS	10.19 **	1.66 NS
9-viol no. 4	0.55±0.19 a	0.22±0.15 a	0±0 b	0.55± 0.21 a	0.67±0.28 a	1.58 NS	4.15 *	0.99 NS
Viol no. 7	0.15±0.10 a	0.19±0.10 a	0.07±0.07 a	0.17±0.11 a	0.30±0.18 a	0.19 NS	0.72 NS	0.97 NS
∑Viol	5.20±0.76 a	5.51±1.95 a	2.64±0.74 b	5.11±1.22 ab	9.54±2.82 a	0.88 NS	5.69 *	2.26 NS
∑9-viol	0.98±0.35 a	1.38±0.67 a	0±0 b	1.49±0.48 ab	2.43±1.06 a	1.06 NS	4.39 **	0.32 NS
Vitamin A value	258.2±22.3 a	162.0±45.3 a	85.6±11.4 b	149.7±30.5 ab	279.3±57.2 a	0.40 NS	8.38 **	1.01 NS

Mean±SE; 0=not detected. Within cultivar and within TRS maturity class means followed by different letters are statistically different (Bonferroni's test, (*) P<0.10; * P<0.05; **P<0.01; NS =not significant). ∑Viol= total all-trans-violaxanthin esters; ∑9-Viol= total 9-cis-violaxanthin.

Table 5 - Carotenoid composition (percent to total carotenoids) of 'Palmer' and 'Haden' mangoes: influence of cultivar and of TRS maturity class and results of ANOVA (F-ratio value and P-value). For identification data of each carotenoid see Table 1

	Cultivar		Maturity class			ANOVA		
	Palmer	Haden	Less mature	Medium mature	More mature	Cultivar (A)	Maturity class (B)	A × B
Viol no. 1	0±0 a	0.87±0.87 a	0±0 a	0±0 a	0.36±0.36 a	3.25 (*)	2.89 (*)	2.89 (*)
Viol no. 2	6.74±2.84 a	3.13±1.49 a	2.51±0.64 a	1.04±0.43 a	2.89±0.58 a	0.21 NS	0.36 NS	1.65 NS
Crypt	14.87±3.01 a	15.40±2.77 a	18.18±0.53 a	15.31±0.07 a	5.88±1.04 a	0.01 NS	1.80 NS	1.40 NS
Viol no. 3	14.32±2.05 a	16.30±2.07 a	16.33±0.18 a	15.96±0.09 a	11.31±0.07 a	0.04 NS	1.51 NS	2.63 NS
9-viol no. 1	0±0 b	2.44±1.30 a	0±0 a	1.122±0.26 a	0.25±0.25 a	7.84 *	2.48 NS	2. 48 NS
9-viol no. 2	1.36±0.60 a	0±0 b	0±0 b	0.06±0.06 ab	1.08±0.20 a	14.55 **	5.95 *	5.95 *
Viol no. 4	4.23±0.58 a	3.17±0.95 a	1.27±0.17 a	3.07±0.12 a	5.51±0.01 a	0.54 NS	2.49 NS	0.40 NS
9-viol no. 3	0.33±0.22 b	1.24±0.46 a	0±0 b	0.46±0.11 ab	1.21±0.08 a	7.51 *	8.04 **	2.49 NS
Car	50.62±1.03 a	54.25±2.31 a	55.65±0.08 a	48.86±0.02 b	53.26±0.02 ab	3.22 (*)	3.28 (*)	3.38 (*)
Viol no. 6	3.20±0.71 a	1.98±0.68 a	0.07±0.07 b	3.27±0.10 a	3.57±0.005 a	1.66 NS	11.66 **	0.62 NS
9-viol no. 4	2.63±0.91 a	0.71±0.52 b	0±0 b	1.73±0.23 a	1.14±0.20 ab	4.52 *	6.20 **	2.26 NS
Viol no. 7	0.77±0.51 a	0.95±0.51 a	0.06±0.06 a	0.25±0.11 a	0.56±0.21 a	0.19 NS	0.46 NS	1.24 NS
∑Viol	29.25±3.30 a	26.41±3.04 a	25.030±0.42 a	28.13±0.09 a	28.76±0.07 a	0.22 NS	0.17 NS	4.27 *
∑9-viol	4.31±1.33 a	4.39±1.71 a	0±0 b	6.07±0.19 a	5.79±0.21 a	0.01 NS	13.50 **	0.00 NS

Mean±SE; 0=not detected. Within cultivar and within TRS maturity class means followed by different letters are statistically different (Bonferroni's test, (*) P<0.10; * P<0.05; **P<0.01; NS =not significant). ∑Viol= total all-trans-violaxanthin esters; ∑9-Viol= total 9-cis-violaxanthin.

LeM mangoes had no 9-*cis*-violaxanthins and were characterized by lower contents of Viol no.3, Viol no.4, Viol no.6 and *all-trans*-β-carotene than MoM fruit, but higher proportion of *all-trans*-β-carotene than MeM ones (Tables 4 and 5). These carotenoids increased with advancing maturity degree, showing the highest contents in MoM mangoes. On average *all-trans*-β-carotene corresponded to 53% of total carotenoids; the *all-trans*-β-carotene proportion was not significantly affected by cultivar, whereas on average was lower in MeM fruit than in LeM ones, while MoM mangoes were not statistically different from fruit of the other two maturity classes (Table 5).

The vitamin A value did not differ between cultivar, but significantly increased with maturity class from 86 of LeM fruit to 279 RE 100 g^{-1} of MoM mangoes (Table 4).

Regression analysis

The results of regression analysis between μ_a540 and pulp color parameters and between μ_a540, *a**, *h*°, I_Y and ascorbic acid, TPC, TAC and carotenoids differed for the two cultivars and data are summarized in Tables 6 and 7, reporting the type of the model having the best performance.

The μ_a540 was positively related to *a** and I_Y and negatively to *h*° (Table 6) with *r* ranging from 0.83 to 0.87 for 'Palmer' fruit and approx. 0.98 for 'Haden' cultivar. In 'Palmer' mangoes (Table 7) μ_a540, *a**, *h*° and I_Y were related to total carotenoids, Viol no.3 and no.4, 9-Viol no.2 and no.3, *all-trans*-β-carotene, ∑9-Viol and vitamin A value with lower *r* values (0.62-0.84) for μ_a540 respect to those found for pulp color parameters (0.74-0.96). Only I_Y was related to Viol no.6, 9-Viol no.4 and ∑Viol with *r*≥0.7, and only *a** was related to TPC, but with *r*<0.6. In contrast, in 'Haden' fruit μ_a540, *a**, *h*° and I_Y were related to total

Table 6 - Results of regression analysis between absorption coefficient at 540 nm (μ_a540) and pulp color parameters

μ_a540	'Palmer'			'Haden'		
	r	P	MT	r	P	MT
*a**	0.831	**	L	0.975	***	E
h°	0.826	**	RX	0.976	***	Sc
I_Y	0.872	**	Ln	0.977	***	DR

For each regression, the following data are given: r = correlation coefficient, P = significance of the model (***, P<0.001; **, P< 0.01) and MT= model type (DR= doble reciprocal, E= exponential, L= linear, Ln= logarithmic-X, M= multiplicative, RX= reciprocal-X, Sc = S-curve).

carotenoids, Viol no.2, no.3, no.4 and no.6, 9-Viol no.1 and no.3, *all-trans*-β-carotene, ∑Viol, ∑9-Viol, vitamin A value and TPC, with higher *r* values (0.72-0.95) for μ_a540 than for pulp color parameters. In addition, only μ_a540 was related to 9-Viol no. 4.

No significant relationships were found between μ_a540, *a**, *h°* and I_Y and ascorbic acid and TAC, whatever the cultivar, suggesting that μ_a540 was able to reveal the carotenoids content in the pulp, as this wavelength corresponds to the tail of carotenoid absorption.

independently from cultivar. Rizzolo *et al.* (2016) also showed that μ_a540 maturity index, related to carotenoids content, successfully classified 'Tommy Atkins' mangoes at harvest.

As for quality parameters, 'Palmer' mangoes had firmness values typical of fully ripe fruit (Beaulieu and Lea, 2003), independently from maturity class, whereas in 'Haden' fruit LeM class showed firmness values typical of firm-ripe stage and MeM and MoM classes values typical of ready-to-eat or ripe fruit (Eccher Zerbini *et al.*, 2015). Pulp color parameters

Table 7 - Results of regression analysis between absorption coefficient at 540 nm (μ_a540), pulp color parameters and total carotenoid (CAR), total phenolic compounds (TPC), carotenoid compounds (for identification data see Table 1) and vitamin A value

	μ_a540			*a**			*h°*			I_Y		
	r	P	MT	r	P	MT	r	P	MT	r	P	MT
'Palmer'												
CAR	0.78	*	L	0.909	***	L	0.902	***	RX	0.897	***	L
Viol no.3	0.814	**	L	0.789	*	L	0.783	*	RX	0.735	*	L
9-Viol no.2	0.839	**	L	0.959	***	L	0.958	***	RX	0.823	**	L
Viol no.4	0.625	(*)	L	0.908	***	L	-0.912	***	L	0.784	*	L
9-Viol no.3	0.765	*	L	0.871	**	L	0.871	**	RX	0.778	*	L
Car	0.793	**	L	0.913	***	Sy	-0.909	***	Sy	0.888	**	Sy
Viol no.6	–			–			–			-0.745	*	RX
9-Viol no.4	–			–			–			-0.847	**	RX
∑Viol	0.576	(*)	Log	–			–			0.693	*	Log
∑9-Viol	0.812	**	L	0.873	**	L	0.86	**	RX	0.954	***	L
Vitamin A value	0.792	**	L	0.915	***	Sy	-0.912	***	Sy	0.887	**	L
TPC	–			0.575	(*)	Sy	–			–		
'Haden'												
CAR	0.912	***	L	0.855	**	E	0.854	**	Sc	0.876	***	E
Viol no.2	0.853	**	L	0.618	(*)	L	0.634	*	RX	0.601	(*)	L
Viol no.3	-0.824	**	Sc	0.723	*	E	-0.742	*	E	-0.805	**	Sc
9-Viol no.1	0.756	*	L	0.666	*	L	0.661	*	RX	0.718	*	L
Viol no.4	0.815	**	Sx	0.789	**	L	0.79	**	RX	0.823	**	L
9-Viol no.3	0.95	***	L	0.83	**	L	0.834	**	RX	0.848	**	L
Car	0.924	***	L	0.823	**	Sy	0.817	**	Sc	0.837	**	Sy
Viol no.6	0.944	***	L	0.862	**	L	0.862	**	RX	0.873	***	L
9-Viol no.4	0.700	*	L	–			–			–		
∑Viol	0.937	***	L	0.885	***	Sy	-0.886	***	E	0.905	***	Sy
∑9-Viol	0.848	**	L	0.723	*	L	0.721	*	RX	0.756	*	L
Vitamin A value	0.923	***	L	0.823	**	Sy	0.817	**	Sc	0.838	**	Sy
TPC	0.867	**	L	0.758	*	L	0.761	**	RX	0.766	**	L

For each significant regression, the following data are given: r= correlation coefficient, P= significance of the model (***, P< 0.001; **, P <0.01; *, P< 0.05; (*), P<0.10) and MT= model type (DR= doble reciprocal, E= exponential, L= linear, Ln= logarithmic-X, Log= logistic, RX= reciprocal-X, Sc= S-curve, Sx= square-root-X, Sy= square-root-Y).

4. Discussion and Conclusions

The absorption coefficient measured at 540 nm (μ_a540) showed different value ranges between 'Haden' and 'Palmer' mangoes and it was able to distinguish more mature fruit from less mature ones differed among maturity classes, confirming previous results obtained for 'Tommy Atkins' cultivar. In fact Rizzolo *et al.* (2011) and Vanoli *et al.* (2011) found that LeM 'Tommy Atkins' mangoes were characterized by higher *h°* and lower *a** and I_Y than MoM fruit. Moreover, Vanoli *et al.* (2011) found that with fruit

ripening at 20°C *h*° decreased and I_Y increased, confirming that the trend of pulp color observed in our work with the TRS maturity classes was actually due to a different ripening degree.

The average AA values found for 'Palmer' and 'Haden' fruit are comparable with the data by Rocha-Ribeiro *et al.* (2007) for the same cultivar, and with AA content reported for other cultivars by Liu *et al.* (2013) and Elsheshetawy *et al*. (2016). However, within the same variety, AA content may vary according to climatic conditions, cultural practices, maturity stage and postharvest factors. For 'Keitt' cultivar Ibarra-Garza *et al.* (2015) found that AA content varied from about 1300 mg kg FW^{-1} in fruit soon after harvest, to about 2500 mg kg FW^{-1} till 8 days of ripening at room temperature, followed by a 54% decrease in fully-ripe fruit. Similarly, Robles-Sánchez *et al.* (2009 b) reported for 'Ataulfo' fruit stored for 15 days at 12°C a 50% decrease of AA content at the end of shelf-life.

The TPC contents found in this work are in agreement with Rocha-Ribeiro *et al.* (2007), even if these Authors reported lower AA contents than those found in our work for the same cultivars. No data on TPC content in mangoes in relation to TRS maturity classes, having same harvest time and same postharvest management, are available in the literature. However, the TPC increasing trend from less to more mature fruit class found in this work is in agreement with the results for the final period of shelf life/storage reported by Ibarra-Garza *et al.* (2015) and Robles-Sánchez *et al.* (2009 b) when fruit are becoming softer and with a yellower pulp color. Ibarra-Garza *et al.* (2015) found higher TPC in 'Keitt' fruit at the beginning of a 10 d shelf life period at room temperature, with a sharp decrease from 2 to 4 days of shelf life, followed by a slight but significant TPC increase till the end of shelf life; a similar trend was also found by Robles-Sánchez *et al.* (2009 b) for whole and fresh-cut 'Ataulfo' mangoes stored at 5°C for 15 days.

In agreement with data obtained for TPC and AA, 'Palmer' fruit showed higher TAC than 'Haden' ones. The positive correlations of TAC with TPC and AA found in this work are in agreement with literature data. In fact Silva and Sirasa (2018) reported significant correlations between ascorbic acid and TPC and FRAP and DPPH scavenging activity measured for several fruit species, and Palafox-Carlos *et al.* (2012) between DPPH scavenging activity and TPC in 'Ataulfo' mangoes; on the other hand, Liu *et al.* (2013) and Ibarra-Garza *et al.* (2015) found in mangoes correlation between phenolic concentration and antioxidant activity measured with other methods (FRAP, ORAC), but not between antioxidant activity and ascorbic acid content; in contrast Rocha-Ribeiro *et al.* (2007) reported that DPPH radical scavenging activity of mango extracts was strongly correlated with ascorbic acid content, but not with phenolic content. Liu *et al.* (2013) suggested that the difference in relationships between antioxidant activity and ascorbic acid and phenolic compound contents found among authors could be due to a masking effect of phenolics present in far higher concentration than ascorbic acid. Our results suggest that in this work the antioxidant activity can be attributed more to ascorbic acid than to total phenols.

No data on total carotenoids in relation to TRS maturity classes are available in the literature. Vanoli *et al.* (2016) for 'Palmer' and 'Haden' mangoes reported a wide fruit-to-fruit variability in CAR content for both cultivars and that CAR content had an increasing trend with μ_a540 value measured on fruit belonging to the same harvest date, i.e. that CAR content increases with advancing maturity degree. Similarly, an exponential increase in the carotenoid content with fruit ripening has been reported for 'Ataulfo' mangoes (Ornelas-Paz *et al.*, 2008) and nine Thai cultivars (Vásquez-Caicedo *et al.*, 2005). As for the chromatographic carotenoid patterns, the tentative identification of peaks was carried out by comparing spectral characteristics with those previously reported using a similar mobile phase. Seven peaks were tentatively identified as *all-trans*-violaxanthin (439 nm maximum absorption wavelength) and four as 9-*cis*-violaxanthin (435 nm maximum absorption wavelength) containing compounds. The spectral maximum for peak 4 was similar to that reported for *cis*-β-cryptoxanthin. A standard mixture of *all-trans*-β-carotene was used for the identification of peak 11; the retention time and the spectroscopic characteristics of reference material were identical to those observed for peak 11 in all the samples. In general, the spectral fine structures (% III/II values in Table 1) found in this work are in agreement with the values reported in the literature (Ornelas-Paz *et al.*, 2007, 2008). In both cultivars the proportions of *all-trans*-β-carotene and *cis*-β-cryptoxanthin to total carotenoid content, as well as the higher content of *all-trans*-violaxanthins than that of 9-*cis*-violaxanthins are very similar to findings reported in 'Ataulfo', 'Keitt', 'Tommy Atkins' and 'Kent' mangoes (Mercadante and Rodriguez-Amaya, 1998; Pott *et al.*, 2003; Ornelas-Paz *et al.*, 2008). The differences in

carotenoid composition among the maturity classes found in this research are consistent with literature data. Previous researches on carotenoid composition of various mango cultivars carried out by Godoy and Rodriguez-Amaya (1989), Mercadante and Rodriguez-Amaya (1998), Yahia *et al.* (2006), and Ornelas-Paz *et al.* (2007) have shown that generally the most important carotenoid in mango is *all-trans*-β-carotene and its proportion to total carotenoids depends on cultivar and fruit maturity stage. Ibarra-Garza *et al.* (2015) reported for 'Keitt' fruit that *all-trans*-β-carotene corresponded to 33% of total carotenoids in unripe fruit at harvest, ranged from 37 to 44% during the first 6 day-period of ripening at room temperature and reached 61% in fully ripe fruit. The contents of *all-trans*-β-carotene in 'Haden' and 'Palmer' mangoes were similar to those reported by Rocha-Ribeiro *et al.* (2007) for the same cultivars and by Mercadante and Rodriguez-Amaya (1998) for 'Keitt' and 'Tommy Atkins' fruit, but lower than those found for 'Haden' and other cultivars by Ornelas-Paz *et al.* (2007). Also the amounts of *all-trans*- and 9-*cis*-violaxanthins in both cultivars were lower than those found by Ornelas-Paz *et al.* (2007) for 'Haden' and other cultivars, but similar to those reported by Low *et al.* (2015) for 'Kensington Pride' mangoes. The amounts of *cis*-β-cryptoxanthin in both cultivars were far higher than the maximum of 0.1 μg g^{-1} reported by Mercadante and Rodriduez-Amaya (1998) for 'Keitt' and 'Tommy Atkins' mangoes from mature-green to ripe stages, indicating that in the fruit of this experiment, not only β-carotene, but also *cis*-β-cryptoxanthin was a contributor to the vitamin A value for these fruit.

The differences in the single carotenoid concentrations respect to literature data for the same cultivar could be due to the different maturity degree of mango fruit. In fact, referring to pulp color parameters, the fruit used in our experiment had pulp *a** values similar to those reported by Rocha-Ribeiro *et al.* (2007) for the same cultivars and in this case the carotenoid amounts were similar, whereas pulp *h*° values for 'Haden' fruit were higher than those reported by Ornelas-Paz *et al.* (2007), indicating a less advanced ripening degree consistent with the lower carotenoid content of our results.

The results of regression analysis showed that μ_a540 was positively related to *a** and I_Y and negatively to *h*° pulp color parameters, confirming the results obtained by Spinelli *et al.* (2012, 2013) and Vanoli *et al.* (2016) for the same cultivars and by Vanoli *et al.* (2013) for 'Tommy Atkins' mangoes. Color changes in the pulp of mango fruit are usually accompanied by carotenoid accumulation. In this work significant correlations between μ_a540, pulp color parameters *a**, *h*°, I_Y and carotenoids were found for both cultivars. Vanoli *et al.* (2016) found an increasing trend of total carotenoids content with μ_a540 in 'Palmer' and 'Haden' mangoes; they also found high positive correlations between total carotenoids and *a** and I_Y and a higher negative correlation with *h*° following a logarithmic-law function with higher correlation in 'Palmer' than in 'Haden' cv., confirming the better relationships of pulp color in 'Palmer' than in 'Haden' fruits. High correlations between pulp color and *all-trans*-β-carotene, *all-trans*-violaxanthin and 9-*cis*-violaxanthin were also observed in 'Ataulfo' and in 'Manila' mangoes (Ornelas-Paz *et al*., 2008) with the highest correlation coefficients for *a** and *h*° parameters; in 'Manila' mangoes the best results were associated with the concentrations of *all-trans*-violaxanthin and 9-*cis*-violaxhantin, while in 'Ataulfo' with *all-trans*-β-carotene, confirming that there is a cultivar specific relationship between pulp color and carotenoids content. Similar correlations were also found by Vasquez-Caceido *et al.* (2005) in 9 Thai mango cultivars (power law functions) and by Bicanic *et al.* (2010) in 21 mango homogenates (second order polynomial dependence).

Differently from carotenoids, ascorbic acid content and total antioxidant capacity for both cultivars and TPC for 'Palmer' were not related to pulp color, measured both by *a**, I_Y and *h*° color parameters and μ_a540; this is not surprising as literature reported that AA and TPC contents with shelf life does not follow a well-defined increasing or decreasing trend (Robles-Sánchez *et al*., 2009 b; Ibarra-Garza *et al*., 2015).

In conclusion our results confirmed that the absorption coefficient at 540 nm (μ_a540) can be used as a non-destructive maturity index for mangoes. In fact it was able to classify intact fruit of two mango cultivars according to pulp color, the destructive maturity index commonly used for mangoes, as well as according to the contents of total carotenoids and of individual carotenoid compounds and vitamin A value. The good correlations between μ_a540, pulp color parameters and carotenoids indicate that TRS is a suitable tool to sort fruit with different ripening degree, having specific carotenoid pattern.

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Biochemical, physiological changes and antioxidant responses of cut gladiolus flower 'White Prosperity' induced by nitric oxide

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Key words: anthocyanin, catalase, cut gladiolus flower, enzymatic antioxidant system, nitric oxide (NO), sodium nitroprusside (SNP)

Abstract: Sodium nitroprusside (SNP), as nitric oxide (NO) donor, has been considered by postharvest researchers as one of the best option for slowing the processes controlling senescence in cut flowers. Here, we investigate the role of NO on postharvest physiology and vase life of the *Gladiolus grandiflorus* cv. White Prosperity. Vase life markedly extended by SNP at 150 µM from 3 day to 7.33 day and thus those inducer effects were dose- and time-dependent. SNP at 125 µM interdependent on vase life time period was observed to be the optimal dose for improving of relative fresh weight (RFW), peroxidase (POD), and total monomeric anthocyanin (TMA) in cut flowers. Supplementing vase solution with SNP indicated significant increase in water uptake of cut flowers and consequently protected to decline in RFW due to alleviate water losses stress. SNP was maintained the level of total soluble protein, lipid peroxidation, and POD, whereas it enhanced the level of catalase (CAT) and TMA in flower petals. Summary of our results revealed that SNP exogenous prolongs vase life via maintaining protein degrade, scavenging free radical in term of anthocyanin and enzymes antioxidant, decreasing polyphenol oxidase, inhibiting lipid peroxidation, and improving membrane stability in 'White Prosperity' cut flowers.

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1. Introduction

Floriculture is an emerging and fast expanding globalized market and subsequently studies on postharvest handling of cut flowers occupy a fundamental position (Gul and Tahir, 2013). Therefore, the postharvest longevity of flowers have a vital importance in evaluating the value of the each horticulture plant. This aspect can be particularly hold good with cut flowers and it is a necessity for extended handling and transportation periods. Cut flowers are greatly perishable, and consequently they have short vase life and also are exposed to early senescence processing, which restricts efficient marketing of economically significant ornamental plants

(Nasibi *et al*., 2014). However, postharvest senescence is a major restriction to the marketing of many species of cut flowers and so much appreciable efforts have been dedicated to developing postharvest treatments to extend the marketing period or increasing postharvest longevity (Vajari and Nalousi, 2013). Sodium nitroprusside (SNP), as donor nitric oxide (NO, is one of the postharvest treatments which recently using from it for improving postharvest life of horticulture crops has exceptionally increased. Postharvest application of SNP has been shown to be effective in extending the postharvest life of a range of flowers, fruits and vegetables when applied as a short term fumigation treatment at low concentrations (Wills *et al*., 2000). NO is a short-lived bioactive molecule, which is considered to function as prooxidant as well as antioxidant in plants. NO molecule is now documented as an important signaling molecule and reported to be involved in various key physiological processes such as plant defense mechanism, abiotic stress resistance, germination, stimulate antioxidant compounds, decrease lipid peroxidation, growth and development of plants etc. (Zhao *et al*., 2004). Furthermore, it was also revealed that plant response to such stress or like drought, high or low temperature, salinity, heavy metals and oxidative stress derived from reactive oxygen species (ROS), is moderate by NO (Mandal and Gupta, 2014). NO is recognized as a biological messenger in plants and it has been proved that NO is effective for increase the vase life of cut flowers because it can be may play role as anit-ethylene synthesized from wounded or non-wounded organ (Abasi, 2014). Liao *et al*. (2009) reported that NO may act as an antagonist of ethylene in cut rose flowers senescence. Optimum SNP levels could postponement the climacteric phase of many tropical fruits and elongate the post-harvest shelf life of a wide range of horticultural crops by inhibiting ripening and senescence (Singh *et al*., 2013).

Gladiolus is one of the four famous cut flowers in the world (Bai *et al*., 2009). Gladiolus cut flowers have extremely used to decorate graves and celebrate major life events in Iran. Likewise, the longevity of cut flowers is one of the main challenges of florists today. First data concerning about the effect of SNP on differential activity of antioxidants and expression of SAGs (senescence associated genes) in relation to vase life of gladiolus cut flowers (*Gladiolus grandiflora* cv. Snow Princess) has been reported by Dwivedi *et al*. (2016). Finding of their study suggested that the application of SNP increases vase life by increasing the scavenging mechanism of reactive oxygen species (ROS) in terms of antioxidants activity, membrane stability and down-regulation of *GgCyP1* gene expression in gladiolus cut flowers. Under condition in plants subjected to SNP, not only the responses of various genotypes or cultivars to SNP may be multi-response, but also the responses rely on dose-and cultivar-dependent, physiological growth state, and environmental factors status. The same trend has been stated by Naing *et al*. (2017) who found that SNP promoted the vase life of the cut gerbera flowers via a delay in the time to stem bending; however, all three gerbera cultivars responded to SNP and the effects were found to be dose- and cultivar-dependent. In the previous study, it has been demonstrated that the SNP dose that was best for one cultivar was not suitable for another; thus, variation in the optimal dose of SNP among cultivars for the enhancement of their vase life could result from differences in their genetic background (Naing *et al*., 2017).

Hence, whether SNP participates in improving of cut flowers of White Prosperity cultivar has not been yet reconnoitered. However, the purpose of the present study was to evaluate the effect induced by nitric oxide donor namely, SNP, on the enzymatic antioxidant activity, biochemical and physiological processes of cut gladiolus (*Gladiolus grandiflorus* cv. White Prosperity) flowers in order to extend their vase life and postharvest shelf-life.

2. Materials and Methods

Plant material and SNP treatments

Cut flowers used in the experiment were *G. grandiflorus* cv. White Prosperity. Cut gladiolus flowers were obtained from a commercial grower presented in Mahallat city, as famous central commercial production of ornamental plant, in Iran at normal harvest maturity and transferred immediately to laboratory of the Postharvest Physiology and Technology Research, Faculty of Agriculture and Natural Resources, Hormozgan University at Jun, 2017 and the experiments were established on the same day. Flowers stems ends were recut under tap water to eliminate air emboli, to inhibit vascular blockage, and to trim to a uniform length of 70 cm. Stock solutions of SNP (Enzo Life Sciences) were prepared following the manufacturer's instructions. Uniform cut flowers

were placed in holding solutions, that containing of SNP, Na_2 [Fe (CN) 5 NO]. $2H_2O$ (Sigma-Aldrich), as NO donor (0, 25, 50, 75, 100, 125 and 150 µM) plus 3% sucrose as carbohydrate supplement. For control set, flowers were dipped in distilled water plus 3% sucrose. Finally, the flowers stems were placed in 500 ml bottles with 250 ml of each mentioned solutions containing different concentrations of the SNP solutions + 3% sucrose and they were maintained at a temperature of 23±3°C, 60±5% relative humidity and under a 12 h photoperiod using cool-white fluorescent lamps (24 µmol m^{-2} s^{-1} irradiance) during experimental period. There were three bottles (21 flowers) per treatment and the experiment was done seven treatments. To escape from photodegradation of SNP (release of a nitrosyl ligand and a cyanide ion), the bottles were shielded with black nylons. SNP treatment was applied as a continuous treatment and flower stems were kept in solutions till the end of vase life.

Vase life and water uptake

The vase life was determined based on wilting of more than one-third of the petals of flower and vase life termination of each floret was considered as soon as the first symptom of wilting was observed. Indeed, it was defined as the number of days in vase life required for one-third of the florets of each spike to lose its ornamental value (lost turgor and wilted). Water uptake was measured by periodically weighting the vase of a control bottle without cut flowers and bottles containing flowers. Finally, vase water uptake was determined using the formula (Rezvanypour and Osfoori, 2011):

$$\text{Water uptake (ml day}^{-1}\text{ g}^{-1}\text{ fresh weight)} = (St^{-1}\text{-}St)/Wt$$

Where St= solution weight (g) at = days 1, 4, 8 and S_{t-1}= solution weight (g) on the preceding day, and Wt = fresh weight of the cut flower (g) on t days.

Number of opened, unopened florets and relative fresh weight

On each spike, the number of opened and unopened florets was recorded from the beginning of the experiment to until 20 days after SNP treatments. The fresh weight was measured every 4 days and relative fresh weight (RFW) of cut flowers was calculated by the following equation:

$$RFW\ (\%) = (Wt/W_{t=1}) \times 100$$

where Wt = weight of cut flowers (g) at t = days 1, 4, 8 and $W_{t=1}$ = the initial fresh weight of the same cut flower (g) on day 1 (Rezvanypour and Osfoori, 2011).

Antioxidant Enzyme assays

Antioxidant enzyme activities were determined in the third floret from the base of spike at three time points (days 1, 4, and 8). The 100 mg of floret tissue from controls and SNP treatments were removed, were homogenized with mortar and pestle in 1 mL 50 mM EPPS buffer (pH 7.8) containing 0.2 mM EDTA and 2% PVP, and were ice-covered for the analysis of antioxidant activity. The homogenates were centrifuged at 4°C for 20 min at 12 000×g and the obtaining supernatants were used to evaluate of antioxidant enzyme activities. Catalase (CAT) activity was assayed as described by Chance and Mahly (1995) as follows: the assay reaction mixture of CAT contained 50 mM phosphate buffer (pH 7.8), 15 mM H_2O_2, and crude enzyme. The decomposition of H_2O_2 was followed at 240 nm (E = 39.4 mM^{-1} cm^{-1}). Absorbance values were quantified using standard curve generated from known concentrations of H_2O_2. For the measurement of peroxidase (POD) activity, the reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM guaiacol, 5 mM H_2O_2 and enzyme. The reaction was started by adding 300 µl of H_2O_2 (0.03%). The POD activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation (E = 26.6 mM^{-1} cm^{-1}) (Chance and Mahly, 1995). The polyphenol oxidase (PPO) activity was assayed in 2.8 mL of reaction mixture comprised 2.5 mL of 50 mM potassium phosphate buffer (pH 7.8), 0.3 mL substrate containing 0.2 mL pyrogallol and 0.1 mL crude enzyme (Kar and Mishra, 1976). The reaction mixture was mixed and the PPO activity was determined in absorbance at 420 nm (6.2 mM^{-1} cm^{-1}). It's to be remembered that the blank cuvette consisted of 3.0 mL potassium phosphate buffer (pH 7.8). The results of antioxidant enzymes activitie was expressed as units (U) per mg FW.

Anthocyanin content assay

Petals were cut from controls and SNP treatments of at three time points (days 1, 4, and 8) and were frozen for the analysis of total monomeric anthocyanin (TMA). TMA content in petals extract was determined by the pH-differential method based on two buffer system described previously by Giusti and Wrolstad (2005). To measure the absorbance at pH 1.0 and 4.5, the samples were diluted 2 times with pH 1.0 potassium chloride buffer (0.025 M) and pH 4.5 sodium acetate buffer (0.4 M), respectively. Therefore, the TMA content analyses of prepared mixtures were performed following the methods of Giusti and Wrolstad (2005).

Lipid peroxidation

The level of lipid peroxidation in petals tissue was measured by determination of malondialdehyde (MDA), which is recognized to be breakdown products of lipid peroxidation, at the end of time points (days 8). The MDA content was determined with the thiobarbituric acid (TBA) reaction. Temporarily, 0.2 g of sample tissue was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10000 g for 5 min. 4 ml of 20% TCA containing 0.5% TBA were added to 1 ml aliquot of the obtained supernatant. The mixture was heated at 95 ºC for 15 min and cooled immediately on ice. The absorbance was measured at 532 nm by a spectrophotometer. The value for the non-specific absorption at 600 nm was subtracted from the above value. The level of lipid peroxidation was expressed as mmol of MDA formed using an extinction coefficient of 155 $mmol^{-1}$ cm^{-1} (Heath and Packer, 1968).

Total soluble proteins

Total soluble proteins (TSP) content of petals at the three time point (days 1, 4, and 8) was determined according to the method of Bradford (1976) using Bovine serum albumin as standard.

Statistical analysis

The experiment was carried out in completely randomized design (CRD) with three replications. Three flowers stems were used for each replication and thus, the experiment was done with seven treatments and three replication per treatment. The non-normalize date of the total soluble protein, POD enzyme activity and RFW of cut flowers were normalized with kurtosis and skewness test; so, their transformed date used for analyzing. Data were statistically analyzed using analysis of variance (ANOVA) in SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Correlations among the evaluated parameters were analyzed using Pearson's correlations (p<0.05 and p<0.01). Mean comparisons to identify significant differences between treatments were performed using Least Significant Difference (LSD) at the p<0.01 or 0.01 level of probability.

3. Results

Vase Life, RFW and water uptake

Application of SNP markedly enhanced the time to vase life for White Prosperity cultivar (p < 0.01). Results showed that the bottle solution containing SNP + sucrose, significantly increased the vase life of cut flowers compared to the control solution (distilled water), as maximum vase life with higher concentration of SNP treatments was verified near the end of storage (Table 1). However, it positive impacts on increasing vase life was dose-dependent: 150 and 125 µM were displayed to be the best concentration for vase life (7.33 and 5.66 days) of 'White Prosperity', respectively, whereas the other concentrations lower than the 125 µM did not markedly influenced vase life as compared to control (p<0.01). Generally, based on the results of vase life, 'White Prosperity' exhibited a longer vase life (4.33 days) when exposed to 150 µM NO as compared to control (3 days). The prolonged vase life in SNP-treated cut flowers were approximately associated with increasing in floral opening of cut flower by 150 µM treatment (Table 1). A direct significant relationship was detected between SNP and floral opening (%); however, increasing in SNP concentration resulted in increasing in floral opening percentage. Statistically, the floral abscission and un-opened flower did not affected by SNP treatments as compared to control (p<0.01).

Table 1 - Effect of different concentrations of sodium nitroprusside (as nitric oxide donor) on vase life, flower opening and floral abscission in cut Gladiolus flowers (*Gladiolus grandiflorus* cv. White Prosperity)

Treatments	Vase Life (days)	Full-opened flower (%)	Un-opened flower (%)	Floral abscission (%)
Sodium nitroprusside 0 µM	3.0 c	58.60 b	19.21 a	24.39 a
Sodium nitroprusside 25µM	3.0 c	60.33 ab	15.51 a	22.99 a
Sodium nitroprusside 50 µM	3.33 c	69.83 ab	14.83 a	18.02 a
Sodium nitroprusside 75 µM	4.0 bc	66.92 ab	15.87 a	17.19 a
Sodium nitroprusside 100 µM	3.33 c	72.38 ab	10.52 a	19.72 a
Sodium nitroprusside 125 µM	5.66 ab	74.81 ab	6.38 a	18.79 a
Sodium nitroprusside 150 µM	7.33 a	80.25 a	6.52 a	13.21 a

Values followed by the same letter within a column indicate they are not significantly different (p< 0.01) by Least Significant Difference (LSD).

As shown in figure 1A, RFW (data normalized; 4.6 is equal to 100%) in cut gladiolus flowers of control gradually was declined during the vase life period, while the decline in RFW was no observed by SNP treatments throughout the vase life. It is notable that the RFW of the SNP treatments solutions except to 125 µM at three point time did not significantly difference with those in control solutions at initial point time (day 1). So, the presence of SNP in vase solutions displayed a protective role to the inhibition of RFW decline in vase life period, even when the vase life of cut flowers ended. Only SNP with concentration 125 µM (5.60±0.08) in vase solution prolonged the RFW 22.55% higher than other concentrations on day 4 or day 8 in comparison to those in controls at initial time (Fig. 1A).

Senescence is a process characterized by water loss and desiccation of plant tissues. During vase life period, water uptake gradually was declined in both some of the SNP treatments (25, 50, and 75 µM) and control cut flowers (Fig. 1B). Generally, the water uptake with SNP concentration 100, 125, 150 µM were higher than those under control condition, respectively (p<0.01). At the initial point time (day 1) of vase life, the White Prosperity showed a rapid response to high SNP concentrations for promoting water uptake; however, the water loss was not observed during its vase life period. The vase solutions containing SNP at concentration 125, 150 µM significantly increased water uptake (12.33±0.56 and 12.69±1.51 ml day^{-1} FW) compared to the control (9.16±0.81 ml day^{-1} FW) resulted in 34.60% and 38.53% increase in vase solution uptake on day 1, respectively; however, they were also conserved the same manner on day 4 or day 8. In contrast, the low concentration did not sufficiently play protective role to inhibit water losses on 4 days, which was also detected that the 25 and 50 µM accelerated water losses, even faster than controls, especially on 8 days.

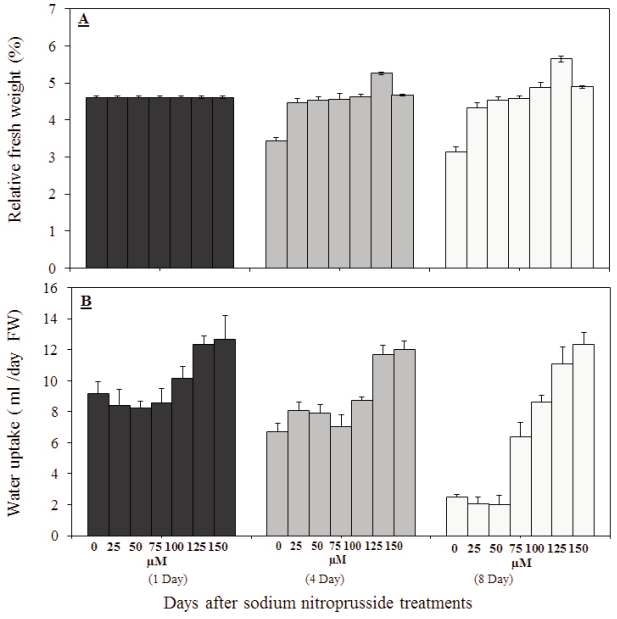

Fig. 1 - The effects of different concentrations of sodium nitroprusside (as nitric oxide donor) on physiological changes of *Gladiolus grandiflorus* cv. White Prosperity cut flowers during vase life. Data are means of three replications. Vertical bars indicate standard deviation.

Lipid peroxidation

The data, belong to MDA concentration of flowers petals representing the level of lipid peroxidation is revealed in figure 2A. Measurement of MDA demonstrated that vase solutions containing 125 and 150 µM SNP significantly decreased MDA production in comparison to control (p<0.01). Overall, it was predictable that White Prosperity without treatment (control) showed higher MDA concentration than the SNP treatments. Results found inversely correlation between lipid peroxidation and higher SNP concentration. At any specified level of SNP concentration, the production of lipid peroxidation product was lesser in treatments at the end experiment (8 days) comparison to control. Thus, the vase solutions having

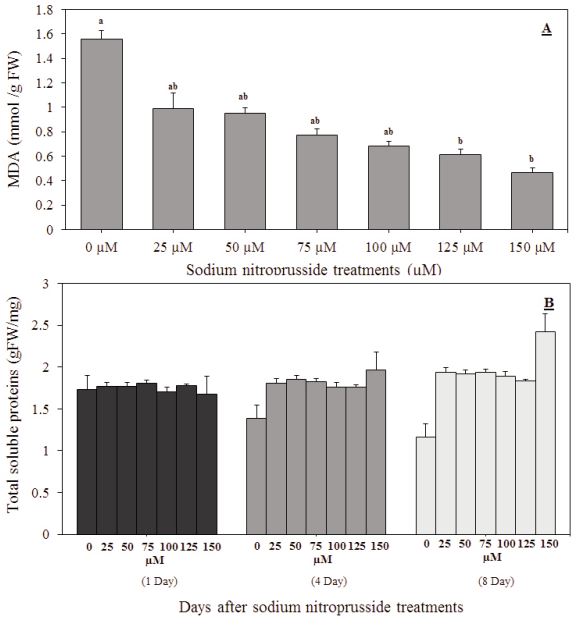

Fig. 2 - Effect of different concentrations of sodium nitroprusside (as nitric oxide donor) on Malondialdahyde (MDA), as an indicator of lipid peroxidation, total soluble protein in *Gladiolus grandiflorus* cv. White Prosperity cut flowers during vase life. Vertical bars with the same letters did not show significantly different using LSD method at P<0.01 significant level.

SNP at concentration 125, 150 µM significantly declined the lipid peroxidation of cell membrane (0.463±0.04 and 0.61±0.04 mmol g^{-1} FW) compared to the control (1.55±0.07 mmol g^{-1} FW) resulted in 70.13% and 60.65% decline in product induction of lipid peroxidation, MDA, on days 8 prior to senescence appearance in cut flowers.

Total soluble proteins

The chemical analysis for TSP of the flower petals exhibited that SNP significantly increased the TSP during vase life period in comparison with control (p<0.01) (Fig. 2B). The protein degradation of flower petals in control was higher than SNP treatments; however, the total soluble protein gradually was declined in control across days. Thus, not only SNP lead to help to the inhibition of protein degradation in flowers petals on day 4 or day 8, but also it caused in delaying the senescence of gladiolus flowers. So, all of the SNP treatments except to 150 µM displayed a protective or maintain role for protein degradation in cut flowers. Overall, only increase in TSP was observed with 150 µM SNP and also was recorded highest TSP for its on day 8; however, the protein degradation did not occurred by 150 µM SNP treatment during vase life period. Furthermore, the prolong vase life of White Prosperity flowers can be strongly associated with increasing in TSP and inhibiting from its degradation in flower petals during vase life.

Enzymatic antioxidant and non-enzymatic antioxidant activities

ANOVA analysis with mean comparison showed that antioxidant enzymes and non-enzymatic antioxidant activities in flower petals differed significantly between control and treatments in White Prosperity (p<0.01). As expected, the PPO activity (U/mg FW) was continually increased in control during vase life, which this tendency was also approximately found for 25 µM (Fig. 3A). The low concentration from 25 to 75 µM did not sufficiently decrease the PPO activity in flower petals comparison to control (p<0.01). So, the decrease in PPO activity was observed by 100, 125, and 150 µM treatments on day 4 or day 8, respectively; however, increasing SNP concentration in vase solution resulted in markedly decreasing PPO activity in comparison to controls at initial time of vase life (p<0.01). It can be predictable that the positive effect of SNP on maintaining or decreasing PPO activity was high dose-dependent. It is now well recognized that high PPO activity accelerate to senescence and to induce browning in plant tissues. Generally, the high concentrations of SNP to White Prosperity cut flowers, check the activity of PPO enzyme, lead to help in delaying the senescence of gladiolus flower via preventing the PPO activity compared to control (p<0.01). As shown in figure 3B, the POD activity (U/mg FW) significantly decreased in control flowers throughout vase life, while the CAT activity (U/mg FW) in control flowers displayed a constant tendency at all of the 3 point time of vase life (Fig. 3C) comparison to SNP treatments(p<0.01). It is appears that all of the treatments except to 125 µM significantly played a protective role to conserve the decrease of POD activity during vase life (p<0.01). The highest POD activity obtained by 125 µM on 8 days, according to LSD test at p<0.01. However, the positive effect of SNP on POD activity was dose- and time-dependent: 125 µM was observed to be the optimal concentration for POD activity on day 4. Thus, in White Prosperity, low concentrations did not adequately increase POD activity, which was also found for concentrations higher than the optimal levels (Fig. 3B). In concerning about CAT activity, the positive relation was detected between CAT activity and SNP treatments; however, increasing in SNP concentration resulted in increasing CAT activity, especially on day 4 or day 8, compared to control (p<0.01) (Fig. 3C). The results of the present study indicated that with more addition SNP concentration by 100 to 150 µM into vase solution was lead to positively increase in CAT activity at each of three time points during vase life.

The results of LSD test (p<0.01) indicated that TMA degradation was gradually happened in control flowers during vase life (Fig. 3D). The SNP treatments not only significantly prevented from the TMA degradation but also they were greatly enhanced the TMA production over 8 days, compared to control (p<0.01). At the during vase life, the low concentrations of SNP demonstrated a protective role to inhibit TMA degradation in flower petals in comparison to control (p<0.01). The furthest increase in the TMA production was archived for 125 µM (0.273 ± 0.037 mg l^{-1}), and 150 µM (0.193±0.015 mg l^{-1}) with a significant difference compared to control, respectively. Hence, improved TMA in flower petals likely to POD activity was dose- and time-dependent: 125 µM was observed to be the optimal concentration for TMA content. Thus, in White Prosperity, low concentrations did not adequately increase TMA content, which was also detected for concentrations higher than the optimal levels on day 4 or day 8.

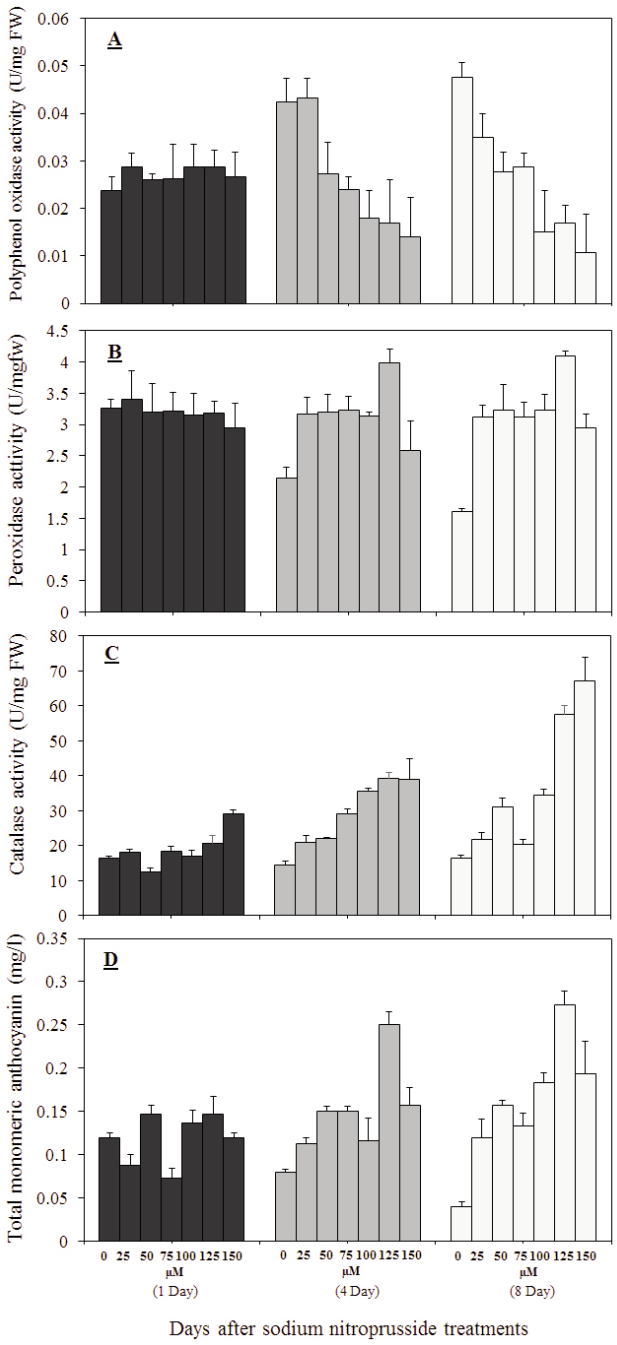

Fig. 3 - Evaluating the effects of different concentrations of sodium nitroprusside (as nitric oxide donor) on enzymatic and non-enzymatic antioxidant system changes during vase life period of *Gladiolus grandiflorus* cut flowers. Vertical bars indicate standard deviation.

Pearson correlation analysis reveals interactions between physiological, biochemical and antioxidant system related traits

In order to arrange for an overview of the associations between physiological, biochemical traits, and antioxidant system activity, the Pearson correlation test used for analyzing and thus was investigated all of the significant associations, as presented in Table 2. From this analysis 23 positive and 11 negative significant correlations was achieved. Among them, some correlations were expected, such as the positive and negative correlations observed between antioxidant system activity, for example, CAT activity and TMA content (r= 0.89, p<0.01), and PPO activity and TMA content (r= -0.95, p<0.01) on days 8, respectively. With regard to physiological traits, the results of paired linear correlation indicated that RFW was positively correlated with CAT (r= 0.85, p<0.05 on days 4), and POD activity (r= 0.86, p<0.05 on days 4 and r= 0.81, p<0.05 on days 8), and TMA content (r= 0.79, p<0.05 on days 8), while was negatively correlated with PPO activity on day 4 (r= -0.80, p<0.05) and day 8 (r= -0.87, p<0.05) of the White Prosperity vase life. Also, the Pearson correlation of water uptake with CAT activity (r= 0.80, p<0.05 on days 1; r= 0.82, p<0.05 on days 4; r= 0.86, p<0.05 on days 8) and with TMA content (r= 0.81, p<0.05 on days 8) was positive significant, whereas displayed a negative significant with PPO activity (r= -0.79, p<0.05 on days 4 and r= -0.85, p<0.05 on days 8) (Table 2). However, suggesting that the SNP treatment is a key inhibitor to water loss and an inducer to antioxidant system for delaying the senescence of gladiolus flowers during vase life, especially on 4 and days 8.

Total soluble protein had a positive correlation with TMA content and a negative correlation with PPO activity. TMA indicated a positive correlation with water uptake, RFW, total soluble protein, and CAT activity and a negative correlation with PPO and POD activity. CAT activity was positively correlated with physiological traits, TMA and negatively correlated with PPO and POD activity. However, according to the results of Pearson correlation, suggesting that SNP might be an important protective or inducer involved in the physiological, biochemical process and antioxidant system in White Prosperity vase life that can be alleviate to water loss, RFW, and to browning process, which lead to early senescence appearance.

4. Discussion and Conclusions

The postharvest longevity of cut flower has a critical importance in determining the value of crop. Recently, SNP, a NO donor known to be a signal molecule involved in biotic and abiotic stress tolerance, has been increasingly used to extend the vase life of

Table 2 - Pearson correlation between physiological and biochemical characteristics of Gladiolus grandiflorus cv. White Prosperity cut flowers affected by sodium nitroprusside during vase life period

Traits	WU1	WU2	WU3	RFW2	RFW3	TSP1	TSP2	TSP3	PPO1	PPO2	PPO3	POD1	POD2	POD3	CAT1	CAT2	CAT3	TMA1	TMA2	TMA3
WU1	1	0.932 **	0.919 **	0.499 NS	0.595 NS	-0.499 NS	0.261 NS	0.408 NS	0.288 NS	-0.584 NS	-0.713 NS	-0.741 NS	0.152 NS	0.152 NS	0.803 *	0.746 NS	0.927 **	0.421 NS	0.655 NS	0.732 NS
WU2		1	0.849 *	0.698 NS	0.753 NS	-0.383 NS	0.517 NS	0.616 NS	0.48 NS	-0.579 NS	-0.801*	-0.666 NS	0.347 NS	0.347 NS	0.752 NS	0.829 *	0.981 **	0.434 NS	0.752 NS	0.854 *
WU3			1	0.627 NS	0.722 NS	-0.388 NS	0.430 NS	0.553 NS	0.40 NS	-0.791*	-0.850*	-0.787*	0.271 NS	0.271 NS	0.780 *	0.871 *	0.862 *	0.25 NS	0.697 NS	0.811 *
RFW2				1	0.988 **	0.208 NS	0.747 NS	0.687 NS	0.802 *	-0.575 NS	-0.809*	-0.295 NS	0.866 *	0.866 *	0.296 NS	0.854 *	0.656 NS	0.233 NS	0.534 NS	0.745 NS
RFW3					1	0.100 NS	0.713 NS	0.677 NS	0.774 *	-0.663 NS	-0.872*	-0.406	0.817 *	0.817 *	0.352 NS	0.892 **	0.724 NS	0.311 NS	0.565 NS	0.794 *
TSP1						1	-0.009 NS	-0.208 NS	0.071 NS	0.36 NS	0.342 NS	0.615 NS	0.585 NS	0.585 NS	-0.514 NS	-0.215 NS	-0.436 NS	-0.333 NS	-0.319 NS	-0.405 NS
TSP2							1	0.971 **	0.558 NS	-0.64 NS	-0.75 NS	-0.432 NS	0.445 NS	0.445 NS	0.405 NS	0.685 NS	0.543 NS	-0.062 NS	0.771 *	0.820 *
TSP3								1	0.526 NS	-0.693 NS	-0799*	-0.561 NS	0.299 NS	0.299 NS	0.591 NS	0.751 NS	0.642 NS	-0.073 NS	0.851 *	0.889 **
PPO1									1	-0.272 NS	-0.615 NS	0.056 NS	0.747 NS	0.747 NS	0.16 NS	0.775 *	0.365 NS	0.068 NS	0.154 NS	0.507 NS
PPO2										1	0.897**	0.868*	-0.21 NS	-0.21 NS	-0.486 NS	-0.7 NS	-0.682 NS	-0.286 NS	-0.734 NS	-0.847*
PPO3											1	0.739 NS	-0.45 NS	-0.45 NS	-0.556 NS	-0.910**	-0.832*	-0.36 NS	-0.742 NS	-0.956**
POD1												1	0.152 NS	0.152 NS	-0.688 NS	-0.532 NS	-0.786*	-0.363 NS	-0.817*	-0.791*
POD2													1	1.000**	-0.14 NS	0.548 NS	0.269 NS	0.185 NS	0.086 NS	0.318 NS
POD3														1	-0.14 NS	0.548 NS	0.269 NS	0.185 NS	0.086 NS	0.318 NS
CAT1															1	0.661 NS	0.749 NS	-0.124 NS	0.779 *	0.682 NS
CAT2																1	0.786*	0.125 NS	0.646 NS	0.862 *
CAT3																	1	0.482 NS	0.823 *	0.894 **
TMA1																		1	0.084 NS	0.3 NS
TMA2																			1	0.887 **
TMA3																				1

WU= water uptake; RFW= relative fresh weight; TSP= total soluble protein; PPO= polyphenol oxidase activity; POD= peroxidase activity; CAT= catalase activity; TMA= total monomeric anthocyanin, the 1, 2, and 3 representing vase life time for each variable on day 1, day 4, and day 8.
NS, *, ** non-significant, correlation is significant at the 0.05 and the 0.01level, respectively. RFW1 has no computed because at least one of the variables was constant.

cut flowers, such as rose, gladiolus, and carnation (Naing *et al*., 2017). First data concerning about application exogenous SNP to improve vase life of G. *grandiflora* cv. Snow Princess cut flower has been reported by Dwivedi *et al*. (2016). It is generally accepted that different genotypes or cultivars might indicate different physiological or biochemical responses to exogenous SNP, which is the effects induced by it may be rely on dose- and cultivar-dependent. Some published evidences supports NO acting as a negative regulator during leaf senescence, but also there is opposite result in this regard; NO enhances flower abscission and senescence in cut racemes of *Lupinus havardii* Wats (Sankhla *et al*., 2003; Guo and Crawford, 2005). Thus, the properly effects of SNP on enhancing physiological and biochemical processes for one cultivar, may not be suitable for another, which is due to differences in their genetic background. This aspect has also been confirmed by Naing *et al*. (2017), who found that SNP dose that was best for one cultivar of Gerbera cut flower was not suitable for another; thus, variation in the optimal dose of SNP among cultivars for the enhancement of their vase life could result from differences in their genetic background. Hence, whether SNP participate in improving of cut flowers of White Prosperity cultivar has not been yet reconnoitered.

Therefore, in the current study, we investigated the role of SNP in the enhancement of physiological, biochemical responses, and antioxidant activity to extend vase life of *Gladiolus grandiflorus* cv. White Prosperity cut flower. Cut flower senescence is linked to a sequence of highly regulated physiological and biochemical processes such as degradation of proteins, DNA content, peroxidation lipids and membrane leakage, degradation of macromolecules, cellular decompartmentalization, floral abscission, color change, leaf yellowing, and weight loss (Buchanan-Wollaston *et al*., 2003; Nasibi *et al*., 2014). In this study, results of our findings revealed that the physiological, biochemical, and antioxidant activity induced by SNP in White Prosperity cultivar were more different than those induced in Snow Princess cultivar, a previous study by Dwivedi *et al*. (2016), which it may be due to differences in their genetic background. Hence, in present study, SNP was significantly promoted the vase life of 'White Prosperity' cut flowers through help to delay the senescence appearance and desiccation on tissue or organ level; however, it effects were discovered to be dose- and time-dependent. Vase life positively associated with

RFW, water uptake, TSP content, TMA, enzyme antioxidant activity and lipid peroxidation. At the start of vase life, there was a noticeably increase and then a constant tendency in water uptake of White Prosperity cut flowers during their vase life, which suggested that SNP might have a protective role in cut flowers against water losses stress (Fig. 1B). The rapid increase in initial water uptake was dose-and time-dependent, while increase in RFW was more dose-dependent (Fig. 1A). The 125 µM concentration was observed to be the optimal concentration for increasing in RFW. The RFW increase obtained in White Prosperity is in isagreement with results pronounced by Dwivedi *et al*. (2016) in Snow Princess. The inhibition or improvement in RFW across days under NO condition is probbly attributed to the excessive potential of water uptake, leading to a stability or promote in cell turgidity pressure, which restricts burning from reserved carbohydrates in respiration and limits fresh weight reduction. An association of improved water uptake and inhibited fresh weight reduction has been reported by Vajari and Nalousi (2013) in carnation and Naing *et al*. (2017) in gerbera cut flower. Overall, the 'White Prosperity' in SNP (150 µM) had prolonged vase life over control, which was strongly associated with increased water uptake and improved RFW.

The damage to the plant cell's biomembrane liable to senescence process, decrease in the ratio of unsaturated fatty acids, change mobility of the cell membrane, and generate free radicals are resulted in an increase in the concentration of Malondialdahyde (MDA), which is an indicator of lipid peroxidation and of injury to the plant cell membrane (Chen, 2009). So, the higher membrane stability plays a key role in inhibiting leakage of electrolytes, sugars, pigment, solute leakage, and also lipid peroxidation as well as in delay senescence during gladiolus cut flowers postharvest (Ezhilmathi *et al*., 2007; Ghadakchiasl *et al*., 2017). Our results showed that the change in membrane stability and lipid peroxidation occurrence resulting from the MDA production were alleviated by SNP concentrations (150, 125 µM) on day 7 after treatment and therefore protected and reduced White Prosperity MDA production in cell membrane (Fig. 2A). These results are in agreement with those reported earlier by Mansouri (2012), who suggested that SNP prolonged the vase life of chrysanthemum flowers, which was accompanied by decreasing in the electrolyte leakage, levels of MDA and lipid peroxidation. Indeed, the role of NO in prevention of lipid peroxidation is related to the ability of NO to react with lipid alcoxyl (LO•) and lipid peroxyl (LOO•) radicals and stop the chain of peroxidation in a direct fashion (Beligni and Lamatina, 1999). The role of SNP in reducing membrane lipid peroxidation has previously been stated by Liao *et al*. (2012) and Dwivedi *et al*. (2016).

Many researchers have been shown that protein degradation and also shortage protein due to consumption it instead of soluble carbohydrate during senescence process for respiration in petals are the most important causes for shortening cut flowers vase life (Rezvanypour and Osfoori, 2011). In addition, SNP significantly maintained the TSP degradation in cut flowers petals, while in absence SNP increased the TSP degradation to a greater rate than SNP treatments on day 4 and day 8 (Fig. 2B). The TSP measured in vase solution supplemented by 150 µM was distinctly higher than those placed in controls on day 8. The proteins are the basic components of all cell activities, their reduction degrades enzymes and causes higher production of free radicals, as well as reducing protein synthesis (Saed-Moucheshi *et al*., 2014). Therefore, it was clear that the proteins degradation during vase life significantly inhibited by SNP supplements in the vase life of 'White Prosperity'. The increase or protect of TSP degradation by SNP application in strawberry (Ghadachiasl *et al*., 2017) and in peanuts (Verma *et al*., 2010) has also been claimed.

Earlier studies have been confirmed that SNP may either be directly scavenging ROS and thus decreasing lipid peroxidation, or it may be modulating the activity of antioxidant system (Beligni and Lamatina, 1999; Saed-Moucheshi *et al*., 2014). Various studies have demonstrated that the vase life of cut flowers is modulated by antioxidant enzymes and non- enzymatic antioxidant activities (Vajari and Nalousi, 2013). Thus, supplemented vase solutions with SNP stimulated a higher enzymatic or non-enzymatic antioxidant activity in flower petals during vase life period. The PPO catalyzes the browning reaction and results in the formation of quinine, which is subsequently polymerized to varying degree leading to production of brown pigments (Dubravina *et al*., 2005). The PPO activity greatly was reduced by SNP, while the POD and CAT activity greatly promoted by SNP during progress senescence (Fig. 3). The results were in accordance with the findings of Ghadakchiasl *et al*. (2017) and Dwivedi *et al.* (2016). Indeed, the NO synthesized by SNP in tissue plant acts signaling

molecule to enhance the enzymatic antioxidant activity such as SOD and CAT and ultimately protects proteins degradation as well as lipid peroxidation against free radicals. So, POD and CAT high activity induced by SNP showed a negatively correlation with PPO activity and thus they blocked PPO activity during vase life (Table 2). Approximately, high and markedly negative between PPO and more traits examined in current study were also detected in Pearson correlation analysis. Furthermore, TMA degradation in flower petals protected by SNP, while TMA degradation increasable induced in flower petals without presence SNP during vase life period. However, positive effects induced by SNP in both POD activity and TMA were dose- and time-dependent, therefore, the 125 µM was selected as an optimal concentration for TMA and POD activity (Fig. 3B, D). Antioxidant compounds such as vitamin C, glutathione, and anthocyanin plays vital role, as non-enzymatic system, in protecting cell against destructive chemical compounds such as free radicals and reactive oxygen species (ROS) that are constantly produced by the cell metabolism and their concentration increases under stress conditions (Kazemzadeh *et al*., 2015). However, SNP increased TAM content at any time and level of SNP concentration in comparison to controls. The high and significantly positive correlation between TMA anthocyanin with CAT activity and total soluble protein was obtained by pearson analysis (Table 2). With progress senescence during vase life, TMA probably scavenged free radicals due to oxidative stress, consequently, inhibited more deterioration of membrane and protein degradation in flower petals.

In conclusion, vase life period in the *G. grandiflorus* cv. White Prosperity cut flower is likely to be associated with many parameters, particularly fresh weight content, water uptake, enzymatic or non-enzymatic antioxidant activities, membrane stability and lipid peroxidation. Furthermore, it was found that positive effects induced by SNP on vase life distinctly were dose- and time-dependent and were also genetic back ground cultivar-dependent in comparative responses between White Prosperity with Snow Princess, which has previously been reported by Dwivedi *et al*. (2016). Results showed that supplementing vase solution with SNP enhanced RFW and water uptake, maintained or increased antioxidant activity, leading to inhibit lipid peroxidation and protein degradation, scavenged free radical, and ultimately causing delay in the senescence of White Prosperity.

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Effect of wounding intensity on physiological and quality changes of strawberry fruit

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Abstract: Wounding makes fresh-cut product more perishable than whole fruit. The effect of wounding intensity on respiration rate and nutritional quality of fresh-cut 'Candonga' strawberries was investigated. Fruit were submitted to six levels of cutting intensity - whole fruit (WHO), 4, 16, 64, and 128 pieces and chopped (CHO) samples. Respiration rate, and the main nutritional parameters were evaluated at the processing day and after 2 days of storage at 5°C. Results showed that wounding intensity significantly influenced respiration rate, ascorbic and dehydroascorbic acids, total phenolic content, and antioxidant capacity. Respiration rate increased with wounding intensity up to the level of 64 pieces (10.01 μg kg^{-1} s^{-1}) compared to WHO (5.5 μg kg^{-1} s^{-1}) and then decreased in the CHO samples (2.81 μg kg^{-1} s^{-1}). At Day 2, the stress caused by the high intensity of cutting (64 pieces and CHO) induced a higher degradation of ascorbic acid, phenolic compounds, and antioxidant capacity. Stress-related changes decrease when the wounding damage was so high that it completely compromises the functionality of the cells (from 64 pieces up). These results should be considered for processing and packaging optimization of minimally processed strawberries-based products.

1. Introduction

The continuous physiological activity of living plant tissues induces severe compositional and structural variations, also associated to ripening and senescence of fresh produce during postharvest life. Tissue responses cannot be blocked but it is possible to delay them within certain limits in order to prolong fruit shelf life (El-Ramady *et al*., 2015). The physiological stresses due to physical damage and wounding occurring during minimal processing, make fresh-cut products more perishable than whole fruit (Nicola and Fontana, 2014). Immediate response of plant cells start from a wound signal formed in adjacent and distant tissues, which gives rise to a wide range of different physiological and biochemical reactions. Common are respiration rate and ethylene production increase, variation in product quality, synthesis and/or loss of phytochemicals with consequent decrease of nutritional content, stimulation of enzymatic activity

and bacterial spoilage (Brecht, 1995; Surjadinata and Cisneros-Zevallos, 2003). The complex interrelationship among the different effects of wounding on physiological processes of fresh-cut products, are comprehensively described by Saltveit (1997). Wound responses could vary depending on different factors: species and cultivar of the product, maturity stage, temperature of processing and storage, cutting-type and sharpness of the blades, but also on process temperature (El-Ramady *et al*., 2015), O_2 and CO_2 levels, and water vapor pressure (Brecht, 1995). The basis for the wound-induced changes include altered genes expression and changes in enzyme activities involved in an effort to heal the damaged tissues providing defense mechanisms of the plant aimed to prevent further and more serious damages (Chung *et al*., 2006). During storage time the increase in respiration rate is usually responsible for the aging of the products due to consumption of reserve energy during redox process. As a consequence, the higher the respiration rate, the shorter is the storage life and therefore the faster is the quality deterioration. Respiration is a function of the climacteric or non-climacteric behavior of the product and of the physiological age of climacteric fruit (Gunes and Lee, 1997). As already reported wounding induces an increase in enzyme activity, in particular higher activities of phenylalanine ammonia-lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO) are observed. (Saltveit *et al*., 2005 b). The main effect of the post-cutting interaction of substrates with enzymes, such as ascorbate oxidase, PPO, and POD, is the degradation of phytonutrients. Phenols oxidation and the resulting browning may induce a reduction in nutrient content resulting often in degradation of color, texture and flavor of fresh-cut products (Saltveit, 1997; Francis *et al*., 2012). It is possible to reduce wound-induced browning with the application of antioxidant or calcium-based active compounds and treatments that interfere with the synthesis or oxidation mechanisms of the phenolic compound precursors (Brecht, 1995; Saltveit, 1997; Saltveit *et al*., 2005 a). However, according to Francis *et al*. (2012), the induced synthesis of phenolic compounds after cutting caused an increase in nutritional value for lettuce, celery, carrot, parsnips, and sweet potato, while in the same study a decrease of phenols was observed in cut zucchini, radish, potato, and red cabbage, pointing out the influence of product on the wound-induced response type. Amodio *et al*. (2014) described the consecutive reaction mechanism that regulates the phenolic content in fresh-cut produce during storage and how their variation is related to cut intensity. They observed, for example, that there was an increase in k_1 values (the rate constant for the *de novo* synthesis of the phenols), when fresh-cut lemons were cut as half-slices rather than slices. This result was also in agreement with the increase in PAL activity resulting from the higher level of wounding on vegetable tissues. At the same time, the cut intensity did not affect the rate of phenolic oxidation. The authors stated that one of the most important factor affecting the phenolic content and synthesis is the biological variability; in fact, each product shows a particular combination of factors that can contribute to the amount and composition of wound-induced phenolics (Francis *et al*., 2012). Fernando Reyes *et al*. (2007) stated that the final concentration of phenolic compound in cut products is strongly affected also by the type of tissue and the initial level of reduced ascorbic acid.

Strawberry-based product attracted in the recent years the food industry due to the high amount of bioactive compounds (vitamin C, anthocyanins and flavonols). For this reason strawberries are one of the richest fruits in term of antioxidant capacity (Cordenunsi *et al*., 2002; Pertuzatti and Barcia, 2015). Understanding the stress-induced changes is important in order to develop reliable approaches to control the stress responses, and improve the quality of minimally processed fresh products, particularly when the fruit is subjected to a pronounced mechanical damage. This makes very interesting the investigation on the effect of wounding on soft and fresh fruit like strawberries. Therefore, the main objective of the present study was to determine the effect of wounding intensity, on physiological and quality changes of fresh strawberry fruit, with a particular focus on the respiration rate and nutritional compounds.

2. Materials and Methods

Sample preparation

'Candonga' strawberries (*Fragaria x ananassa* Duch.) were purchased from local stores in Foggia (South Italy) and stored at 5°C overnight. In the next morning, fruit with uniform color and size, free of physical defects and decay, were divided into six groups (treatments), each one corresponding to one different level of wounding intensity: whole fruit (no cutting), cutting into 4, 16, 64, 128, pieces and chopped defined as WHO, P4, P16, P64, P128 and

CHO, respectively. About 12-15 fruit were cut (300 g) for each replicate and 150 g of product were used for initial determinations, whereas the remaining samples were stored at 5°C under a continuous flow of humidified air for 2 days. Three replicates were used for each cutting intensity treatment and for each quality parameter determination. Vitamin C, total phenolic and anthocyanin content, antioxidant capacity, soluble solid content, pH value, titratable acidity and sugar/acid ratio were evaluated at the processing day (Day 0) and after storage (Day 2). Separate samples were used for respiration rate measurement.

Respiration rate

Respiration rate of fresh-cut strawberries was measured in static conditions as described in Kader (2002). Respiration rate (expressed as μg kg^{-1} s^{-1} of CO_2) was determined at 120 min after cutting. Separate samples (about 300 g each) of strawberries were placed in 5 L sealed glass jars with a plastic septum for sampling gas; jars were closed after an equilibration time of about 1 hours. From each jar, a gas sample (0.5 mL) was collected after the required time to accumulate CO_2 in the headspace up to a concentration of 0.1-0.2%, and injected into a gas chromatograph (Shimadzu, model 17 A, Kyoto, Japan), equipped with a thermal conductivity detector (200°C). Separation of CO_2 was achieved on a Carboxen 1006 plot (30 m × 0.53 mm, Supelco, Bellefonte, PA, USA), with a column flow of 7 mL min^{-1}, and an oven temperature of 180°C.

Compositional attributes

Vitamin C content was measured in 5 grams of fresh homogenized strawberry tissue as L-ascorbic acid (AA) and L-dehydroascorbic acid (DHA) contents expressed as g of AA, DHA or total vitamin C (AA + DHA) per 1 kg of fresh weight (g kg^{-1}) following the procedure by Zapata and Dufour (1992) with slight modifications.

Total phenolic content (TPC) was analyzed using the Folin-Ciocalteau method of Singleton and Rossi (1965), with some modifications where five grams of fresh tissue were homogenized in an Ultraturrax (IKA T18 basic, Wilmington, NC, USA) with 10 mL of extraction buffer containing 200 mL of distilled water, 800 mL of methanol and 2 mM (84 mg L^{-1}) of sodium fluoride (NaF). The absorbance was read at 725 nm compared with a blank (prepared in the same way, replacing the sample with 100 μL of distilled water) using a spectrophotometer (UV-1700 Shimadzu, Jiangsu, China). TPC was calculated based on the calibration curve of gallic acid and results were expressed as g gallic acid equivalents per 1 kg fresh weight (g kg^{-1}).

Total anthocyanin content (TAC) was determined following the protocol described by Cordenunsi *et al*. (2002), with small modifications using hydrochloric acid/methanol mixture as extraction medium. 700 μL of extract plus 300 μL of 1% HCl-MeOH solution were put in cuvettes and absorbance was read immediately in a spectrophotometer at 510 nm. Results were expressed g of pelargonidin-3-glucoside (PG-3-glu) equivalents per 1 kg of fresh weight (g kg^{-1}).

The antioxidant capacity (AC) assay was conducted on the same extract made for TPC, following the method of Brand-Williams *et al*. (1995), with slight modifications. Fifty μL of extract were mixed with 950 μL of DPPH (2, 2-Diphenylpicrylhydrazyl) solution and absorbance was read at 515 nm after 24 h. Trolox (6-Hydroxy-2, 5, 7, 8-tetramethlychromane-2-carbxylic acid) was used as a standard and results were expressed in g of Trolox equivalents per 1 kg of fresh weight (g kg^{-1}).

Total soluble solids (TSS) were obtained by measuring the refractive index of fresh strawberry juice using a digital refractometer (Atago RX-7000cx; Atago Co. Ltd., Japan) at 25°C and expressed as percentage. One gram of sample was used to determine the pH and titratable acidity (TA), with an automatic titrator (T50 M Terminal, METTLER TOLEDO, Switzerland) against a volume of 0.1 N NaOH until reaches the final pH of 8.2. TA was expressed as g of citric acid equivalent per 1 kg of product (g kg^{-1}). TSS/TA ratio was also calculated.

Statistical analysis

Data were subjected to a 2-way ANOVA (for treatment and sampling time); treatment means were separated by Tukey's test at $P<0.05$ using Stat Graphics Centurion XVI.I (Stat Point Technologies, Inc., Warrenton, VA USA) software.

3. Results

Wounding intensity had a significant effect on all parameters except pH; the same for storage time with exception of total phenolic content, while the interaction between the two factors showed a significant effect on respiration rate, ascorbic and dehydroascorbic acids, total vitamin C, total phenolic content and antioxidant capacity but no significant effect

on TAC, TSS, pH, TA and TSS/TA ratio. Following, these effects are described in detail. The effect of wounding intensity on the respiration rate of 'Candonga' strawberry fruits after 120 min post-cutting at 5°C is shown in figure 1. Wounding induced a significant raise in respiration of strawberries tissues that resulted to be increasing with cutting intensity up to a certain point from 5.5 (WHO) to 10.01 µg kg^{-1} s^{-1} of CO_2 (P64). Further increase of wounding beyond P128 did not stimulate respiration rate: a significant decrease up to a minimal level of 2.81 µg kg^{-1} s^{-1} was observed for chopped samples.

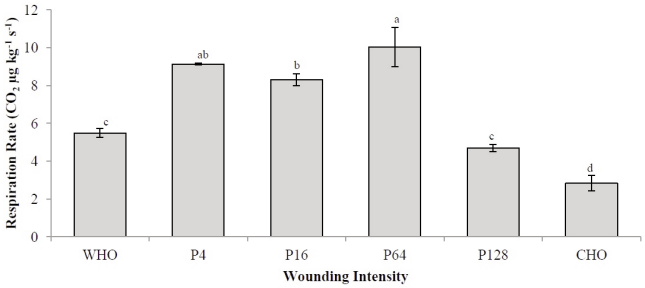

Fig. 1 - Effect of wounding intensity on respiration rate at 5°C of 'Candonga' strawberry fruit. WHO, P4, P16, P64, P128 and CHO stay for whole fruit, cut into 4, 16, 64, 128 pieces and chopped. Error bar represent st. dev of mean values (n=3). Different lowercase letters indicate significant difference among treatment according to Tukey's test (P≤0.05).

The effect of wounding intensity on AA, DHA and total vitamin C content (AA+DHA) of fresh-cut 'Candonga' strawberries is shown in figure 2. At Day 0, AA and total vitamin C were not significantly affected by wounding intensity, with almost similar values referred to ascorbic acid among treatments ranging from 0.37 (P64) to 0.44 g kg^{-1} (P4). On the other hand, an increase in DHA content could be observed already at Day 0 with a significant difference among treatments ranging between 0.067 g kg^{-1} in the WHO and 0.22 g kg^{-1} in the treatment P128. The mean values of total vitamin C content at Day 0 ranged from 0.51 (WHO) to 0.61 (P128) g kg^{-1}. P128 and CHO samples showed in fact significantly lower values of ascorbic acid (0.27 and 0.17 g kg^{-1}, respectively) than other treatments, with 64-piece sample showing an intermediate behavior (0.41 g kg^{-1}). Moreover a significant increase of DHA with the increase of wounding intensity was observed for all samples starting from P16. The lowest and highest DHA level at Day 2 were found with the WHO and CHO samples (0.12 and 0.55 g kg^{-1}, respectively). The highest level of wounding intensity (CHO) induced about a 5-fold increase in DHA content.

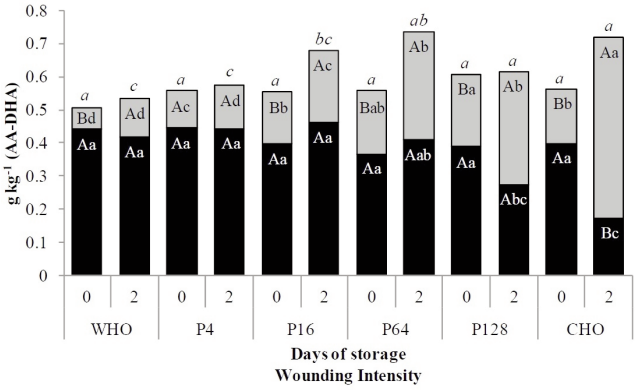

Fig. 2 - Effect of wounding intensity on ascorbic acid (AA, black bars, g kg^{-1}), dehydroascorbic acid (DHA, grey bars, g kg^{-1}) and total vitamin C content (sum of AA and DHA, g kg^{-1}) of fresh-cut 'Candonga' strawberries at day 0 and day 2 of storage at 5°C. WHO, P4, P16, P64, P128 and CHO stay for whole fruit, cut into 4, 16, 64, 128 pieces and chopped. Mean value (n=3) is reported. Different lowercase and uppercase letters, indicate significant differences among treatments and storage times, respectively, according to Tukey's test (P≤0.05). Different italics letters indicate significant differences among treatments for total vitamin C content for each storage time.

The effect of wounding intensity on TPC, TAC, and AC of fresh-cut 'Candonga' strawberries is shown in Table 1. The different cutting stress significantly affected the TPC of fresh-cut strawberries. At Day 0, P128 had significantly higher (P<0.05) total phenolic content compared to other treatments with the exception of P64, with P16 showing the lowest amount (2.27 g kg^{-1} of gallic acid equivalent) and, together with P4 (2.37 g kg^{-1}), resulting in significantly lower content of phenolic compounds than whole fruit. There was no significant difference in TAC both at Day 0 and 2 although the mean TAC value of sample P4 showed slight increase during storage time. The mean value of TAC ranged from 0.19 (P4) to 0.22 (CHO) g kg^{-1} of PG-3-glu (Table 1). Wounding intensity also significantly affected antioxidant capacity of fresh 'Candonga' strawberries. At Day 0, samples cut into 16 (6.35 g kg^{-1} of Trolox equivalent) and 64 (6.59 g kg^{-1}) pieces had significantly higher AC than the CHO (5.41 g kg^{-1}) and WHO (5.20 g kg^{-1}) samples. A similar trend was also observed after 2 days from cutting when P16 sample showed significantly higher AC value when compared to the CHO samples, although this difference was not significant if compared to the rest of the treatments. A particular behavior was observed for CHO strawberries (Table 1): no significant effect of storage time was observed on the TPC, TAC, and AC values of this sample indi-

cating that these compounds were quite preserved during cold storage, and did not show after two days of storage great variations if compared with other samples. In addition, this sample showed also the lowest respiration and no significant difference in total vitamin C if compared to the WHO samples. These characteristics may be therefore exploited to maintain the quality of fresh-blended products.

TSS, pH value, TA and TSS/TA ratio of fresh-cut strawberry subjected to different wounding intensity and stored for two days at 5°C were significantly affected by wounding intensity (with exception of pH) and storage time, but not by their interaction (data not shown). The mean values of TSS ranged from 7.6 (WHO at Day 0) to 8.6% for WHO, P16 and CHO samples at Day 2. Almost similar pH values (3.9 to 4.0) were determined for all treatments and sampling times. TA ranging from 0.07 to 0.08 g kg^{-1} of citric acid showed almost no differences among treatments and sampling time except for P64 which had a slightly lowest value after two days. Similarly, almost no differences in the TSS/TA ratio were recorded. The highest and lowest values were 9.4 (P4 at day 0) and 12.1 (P16 at Day 2).

4. Discussion and Conclusions

The effect of wounding intensity on respiration rate and compositional values of fresh 'Candonga' strawberry fruit was clearly determined. In general, high degree of wounding intensity (P128 and CHO) caused a significant decrease in respiration rate. In addition, the chopped sample did not show any significant difference in TPC, TAC and AC during storage time.

Respiration rate gives an immediate overview of the metabolism of a commodity (Fig. 1), where higher respiration is an indicator of accelerated metabolism which is usually inversely related to shelf-life. Strawberry is among the commodities with highest respiration rate (Saltveit, 2002), that can be further increased as a cut consequence. According to Surjadinata and Cisneros-Zevallos (2003), an increase in respiration may occur due to simultaneous enzyme synthesis and decrease. The newly synthesized enzymes could in fact be degradated by an inactivation system. It is possible that, after a certain cutting degree, the very high tissue damage compromised the cell functionality. The transition from respiring to non-respiring tissues after wounding is probably related to the damage of the membrane system or mitochondria and consequent disruption of oxidative phosphorylation. Changes in ammonium dihydrogen phosphate (ADP) and ammonium transferase phosphate (ATP) concentrations in wounded tissue indicate that oxidative phosphorylation failed to keep place with ATP utilization in injured tissues (Lafta and Fugate, 2011). According to these authors, a 41% reduction in ATP concentration and a simultaneous increase in ADP (31%) were observed between day 1 and day 4 after incremental injury of sugar beet root. Costa *et al*. (2011), reported that respiration rates of fresh-cut strawberries was higher than whole fruit and that low storage temperature significantly influenced this parameter. This is possibly due to the fact that cutting increases the surface exposed to the air, and as a consequence, oxygen is able to diffuse into the internal cells more rapidly. Moreover, injured cells show an increased metabolic activity (Nilsson and Hedenqvist, 2011; Saltveit, 1997). Thus, respiration is stimulated by physical damages given to the

Table 1 - Effect of wounding intensity on total phenolic ontent (TPC, in g kg^{-1} of gallic acid equivalent), total anthocyanin content (TAC, in g kg^{-1} of pelargonidin-3-glucoside), and Antioxidant Capacity (AC, in g kg^{-1} of Trolox equivalent) of fresh-cut 'Candonga' strawberries at day 0 and day 2 of storage at 5°C

	Days at	Wounding intensity					
	5 °C	WHO	P4	P16	P64	P128	CHO
Total phenolic content	0	2.88±0.07 Ab	2.37±0.16 Bc	2.27±0.04 Bc	2.95±0.02 Aab	3.23±0.08 Aa	2.94±0.05 Ab
	2	2.62±0.12 Bb	3.19±0.18 Aa	3.25±0.17 Aa	2.52±0.15 Bb	2.87±0.10 Bab	2.58±0.07 Ab
Total anthocyanin content	0	0.19±0.004 ns	0.19±0.007 Ans	0.20±0.004 ns	0.21±0.018 ns	0.20±0.019 ns	0.20±0.010 ns
	2	0.21±0.015 ns	0.21±0.002 Bns	0.21±0.014 ns	0.22±0.002 ns	0.22±0.021 ns	0.22±0.012 ns
Antioxidant capacity	0	5.89±0.03 Abc	6.02±0.05 Aabc	6.35±0.12 Aa	6.59±0.09 Aa	6.27±0.12 Aab	5.41±0.06 Ac
	2	5.20±0.22 Bab	5.98±0.44 Bab	6.16±0.02 Ba	5.44±0.03 Bab	5.67±0.09 Aab	4.62±0.10 Ab

IWHO, P4, P16, P64, P128 and CHO stay for whole fruit, cut into 4, 16, 64, 128 pieces and chopped; mean values (n=3) ± standard deviations are reported.

Different lowercase and uppercase letters, indicate significant differences among treatments and storage times, respectively, according to Tukey's test ($P \leq 0.05$).

fruits: the more the severity of damage, the more the degree of respiration rate increase (Kader, 1987; Zhu *et al*., 2001). The main consequence of an increase in CO_2 and ethylene production as a response to cutting process (Saltveit, 1997) could be a reduction of the fresh-cut product shelf life. Moreover, moisture in the cut surface may impede gas diffusion, and this, together with increased respiration, could possibly lead to anaerobiosis, causing further deterioration of the tissues (Saltveit, 1997; Surjadinata and Cisneros-Zevallos, 2003). However, wound-induced respiration depends on the type of tissue, temperature, controlled atmospheres and degree of cutting (Zhu *et al*. 2001). As described by Surjadinata and Cisneros-Zevallos (2003) respiration rate of carrot tissues after wounding showed a typical increase (resulting in a maximum peak) and then a decrease reaching steady-state respiration values similar to that of whole carrot. In some plant tissues, such as potato, this behavior may be related to the oxidation of fatty acids and carbon dioxide, being these reactions responsible for increased respiration after wounding (Gunes and Lee, 1997). According to Surjadinata and Cisneros-Zevallos (2003), wounding stimulates enzymatic activity of phosphofructokinase and cytochrome oxidase from the respiration pathway, which catalyse the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate (a key regulatory step in the glycolytic pathway) and the electrons transfer to oxygen, respectively. A higher enzymatic activity could be due to activation of already present enzyme or to *de novo* synthesis, as suggested by Surjadinata and Cisneros-Zevallos (2003). Their higher activity results in an increase of respiration rate in wounded tissues (Lafta and Fugate, 2011).

On the processing day total vitamin C content did not vary among the treatments, most probably because ascorbic acid was oxidized to dehydroascorbic. The same vitamin C content trend showed in figure 2 was observed by Costa *et al*. (2011), and may be ascribed to the fact that tissue had only slightly responded to the stress. After 2 days from cutting a slight increase in AA was observed for samples P16 and P64 while a noticeable reduction was found with the increase of cutting intensity. As a results of enzymatic and non enzymatic oxidation of ascorbic acid, prolonged storage period, mechanical damages or thermal treatment, DHA amount is known to increase. The oxidized form thus represents the majority of vitamin C (Davey *et al*., 2000; Lee and Kader, 2000). In the present study different behaviors were observed: as for P128 and CHO samples the decrease in AA amount due to oxidation was accompanied by an increase in DHA amount ending in a rise of total vitamin C content of the products. Since this increase was proportional with the decrease in AA concentration, it can be supposed that no further oxidation of DHA into 2,3-diketogulonic acid occurred. Regarding P16 and P64 samples at day 2, higher AA amounts were detected, suggesting the occurrence of new synthesis or the presence of different sources of ascorbic acid. Cordenunsi *et al*. (2005), reported that ascorbic acid synthesis in strawberries occurs during the storage period, and that temperature may affect it. As for sweet pepper fruit, it was observed that wounding stress activated both biosynthesis and metabolism reduction of ascorbic acid, leaving unaffected the level of AA in the product (Imahori *et al*., 1997). Moreover, Wolucka and Van Montagu (2003) proposed a new vitamin C biosynthesis pathway in which L-gulose and L-gulono-1,4-lactone act as direct precursors of ascorbic acid in plant tissue during storage. As for the present work it was possible to suppose that new synthesized ascorbic acid replaced the amount which was oxidized. As a result, ascorbic acid content did not decrease over time while a huge increment in DHA occurred for P64 sample. Some authors observed in different strawberry cultivars that DHA evolution with storage time, when associated with ascorbic acid retention, can be considered an evidence of a redox system (AA/DHA) triggered during cold storage, reported to be a cultivar-specific more than fruit-specific process (Cordenunsi *et al*., 2005). Moreover, to date, many of the products deriving from DHA degradation are still unclear, although 2,3-diketogulonic acid, threose and oxalic acid, glyoxal, methyl glyoxal and diacetyl have been identified or hypothesized as byproduct of dehydroascorbic acid decomposition (Fayle *et al*., 2000). Ascorbate, its product of oxidation (DHA) and consequently DHA metabolism play significant roles in the apoplast (Lin and Varner, 1991). For this reason DHA results to be a key-factor in ascorbate catabolism, and it could be oxidized to oxalate or hydrolyzed to 2,3-diketogulonate and downstream carboxypentonates. The prevalence of one of the two reactions (oxidation or hydrolysis) is dependent on the status of the reactive oxygen species (Parsons *et al*., 2011). In general, DHA/AA ratio tends to rise during storage time although the oxidized form is unstable and is easily decomposed, this leading to a decrease in its biological activity. The changes in the form of ascorbic acid result to be important from a technological and a

nutritional point of view (Lee and Kader, 2000; Cordenunsi *et al*., 2005).

A possible explanation for what is showed in Table 1 may be found in the prevalence of phenolics *de novo* synthesis with respect to their oxidation as response to cutting stress. As described by Kang and Saltveit (2002), the cutting related to processing of fresh-cut fruit and vegetables may induce an increase in their antioxidant capacity, enhancing synthesis and accumulation of phenols. In fact, wounding stimulates the activity of phenylalanine ammonia-lyase, which is responsible for the catalyzation of the first step of phenylpropanoid metabolism due to which tartaric acid is converted into chlorogenic acid. An increase in its activity leads to accumulation of phenols, enzymatic oxidation and tissue discoloration (Saltveit, 2000; Adams and Brown, 2007). In contrast, however, wounding also induces antioxidant degradation, resulting in oxidation of active compounds such as ascorbic acid and phenolic compounds. Their final content is the balance result between production and oxidation rates, being these rates affected by storage temperature or cutting intensity as modeled by Amodio *et al.* (2014). However, in the case of a more serious damage, as for CHO samples, TPC degradation was supposed to be faster than their production, ending in the lowest value of TPC. At Day 2 however, TPC increased more clearly in P4 and P16 samples, which showed significantly higher values than other treatments, including the WHO. This is supported by literature, Torres-Contreras *et al*. (2014) report that phenolic content in white potato tubers that were subjected to different wounding stresses showed an accumulation of 100% and 65% TPC for slices and pieces, respectively, whereas shredded potatoes stored at 10°C for 96 hours showed 40% lower phenolic content if compared to the starting product. Similarly, an accumulation of wounding-induced TPC (approximately 60%) and an increase in the activity of PAL enzyme were found in purple-flesh sliced potato tissues stored at 15°C for 48 hours (Reyes and Cisneros-Zevallos, 2003). According to Surjadinata and Cisneros-Zevallos (2003), phenolic antioxidant accumulation is dependent on the level of wounding intensity. The phenolic content increased with wounding intensity by 97, 76, and 252% when cut as slices, pieces and shreds, respectively compared to non-wounded carrots (0.45-0.52 g kg^{-1}). Moreover, the same behavior was observed by Hu *et al*. (2014) on fresh-cut lotus root. After 7 days of storage PAL activity of fresh-cut lotus root slices was 68% higher compared to control. At the same time 130% increase in phenolic content was detected. Antioxidant activity also increased due to total phenol accumulation; however, wounding resulted in a significantly higher browning by increasing the PPO activity of the slices. Phenols represent the main structural and defense-related functions in plant cells via the phenylpropanoid pathway. Wounding of fruit and vegetables tissues obviously causes rupture of the cell membrane and this induce several physiological responses causing the combination of phenolics with the oxidative enzymes and/or the synthesis of different classes of phenolics to repair the wounding damage. In strawberries, TAC was reported to be in the range between 0.15 to 0.80 g kg^{-1} (Padmanabhan *et al*., 2016) and cold storage may induce their biosynthesis and accumulation (Holcroft and Kader, 1999). Accordingly, about 60% accumulation of TAC in sliced potatoes was described by Reyes and Cisneros-Zevallos (2003), and a similar increase was reported for anthocyanin content in pomegranates stored at low temperatures (Arendse *et al*., 2014). The high amount of vitamin C, phenolics including anthocyanins and therefore antioxidant activity in strawberries, even after severe cutting stress, is very important for beneficial effects on consumer health (Giampieri *et al*., 2012). The relatively higher values of AC determined for CHO sample could probably be due to the higher values of total vitamin C and phenolics. Antioxidant capacity of fruit is, in fact, strictly related to the presence of vitamin C and phenolic compounds, being these effective systems to scavenge oxygen radical, AC level is influenced by the occurrence of different active phytochemical compounds (Giampieri *et al*., 2012). As reported by Tulipani *et al*. (2008), vitamin C in strawberries is the greatest contributor (>30%) to the total antioxidant capacity followed by anthocyanins (contributing for 25-40%). The remaining part was composed mainly of ellagic acid derivatives and flavonols (Padmanabhan *et al*., 2016). Antioxidant activity is therefore an expression of total vitamin C and total phenolic content of the product, including anthocyanin and ellagitannins groups (Giampieri *et al*., 2012).

As for pH, TSS and TA, no clear trend and very slight differences were observed, as also reported by Li *et al*. (2017) studying the effect of different cutting styles on postharvest quality of pitaya fruit. In this case, fresh-cut processing also showed little effect on the contents of vitamin C, TSS and TA. This behavior could be interesting since the nutritional quality of the products do not greatly change after treatments,

allowing to maintain their fresh-like state.

The results of the present study demonstrated that the application of wounding intensities could be used as simple emerging technology to induce the accumulation of TPC in plants (Torres-Contreras *et al*., 2014) and selection of appropriate wounding intensity and/or abiotic stress can enhance the nutritional and functional and health-related values of fresh produce (Reyes and Cisneros-Zevallos, 2003; Hu *et al*., 2014). These results should be taken into consideration for processing and packaging optimization of minimally processed products from fresh strawberries, although further investigations extending shelf life period would be necessary in order to make these results useful for industrial application.

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Short note

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Effects of ozonation on the phenolic fraction of olive oil mill wastewater (OOMW): a study case

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Key words: antioxidant capacity, biotoxicity, hydroxytyrosol, seeds germination test, tyrosol.

Abstract: Olive oil mill wastewater (OOMW) is considered the most polluting fraction of olive processing residues, due to its high content in polyphenols. In this study it has been examined the possibility of using an ozone source as a strong oxidant agent, to lower the phenolic fraction of OOMW, allowing its use in agriculture. The OOMW, coming from a continuous 3-phase olive oil mill located in the province of Arezzo (Italy) was submitted to ozonation for 1, 3, and 8 hours, using an ozone generator. Total polyphenols, antioxidant activity, and the amount of tyrosol and hydroxytyrosol were determined on the derived samples. To measure the biotoxicity of the treated OOMW the germination test of radish seeds was used. The results of the chemical determinations highlighted the effect of dephenolyzation performed by ozonation of OOMW, with a significant decrease of antioxidant activity. Hydroxytyrosol was significantly lowered, depending on the duration of treatment, while tyrosol resulted less affected. The germination test showed that, with a 50% dilution of OOMW, the biotoxicity decreases as the ozone treatment increases.

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1. Introduction

The production of olive oil in the world since 2010 has exceeded 3 million of tons; in the last decades olive growing has expanded beyond its traditional cultivation areas, characterized by warm and arid climate (Marone and Fiorino, 2012), thanks to the development of continuous oil separation techniques during the 1960s and 1970s, determining a qualitative improvement and a faster processing of the product (Kapellakis *et al.*, 2008), the harvesting mechanization (Fiorino *et al.*, 2010), the improvement of cultivation techniques, as well as new training systems and canopy management (Tous *et al.*, 2010). As processing by-product, important amounts of pomace (solid residues) and olive oil mill wastewater

(OOMW) are obtained (Table 1) (Di Giovacchino and Preziuso, 2006), both considered environmental pollutants.

In the past, the olive pomace obtained from pressure and continuous three-phase mills was used only to produce oil by means of chemical solvents (Di Giovacchino and Preziuso, 2006), whereas with the evolution of agriculture practices, and the search for new renewable sources of energy, it was employed as raw material for biogas production and, more recently, it started to be considered a dietary component of dairy cattle in order to improve the characteristics of both milk (Castellani *et al.*, 2017) and derived products (Castellani *et al.*, 2018).

The major problem related to the widespread use of pomace in agriculture depends on the high presence of phenolic compounds, with strong antimicrobial activity that determine a remarkable biotoxicity. Phenols show antioxidant properties but also phytotoxic actions in soil and are credited to reduce microbial growth in both anaerobic and aerobic digester. This aspect was explained by Girardi *et al.* (2014), who investigated different oxidative chemical treatments able to reduce both the phenolic content and the triglyceride fraction in various olive solid residues obtained through different extraction processes. In this way it was possible to obtain combined phenol-free compounds and reduced triglyceride, for a semi-finished product appropriated for agricultural purposes, using Hydrogen peroxide (H_2O_2) alone or combined with Fe^{2+} (the Fenton system).

The OOMW is probably the most polluting and biotoxic by-products of the olive processing because of their remarkable antioxidant capacity deriving from the phenolic compounds (De Marco *et al.*, 2007), even if it represents a promising resource for agriculture, especially considering their contribution in terms of water intake, and the high quantities of nutritive minerals directly deriving from the fruit juices.

An INCO-MED research project entitled ‘‘New Technologies for Olive Mill Waste Water Detoxification and Product Recovery’’ (NewTech OMW, Contract ICA3-CT-2002-10033) has been granted by the European Commission to valorize the OOMW. Started in 2003 and involving four Mediterranean countries, it deals with the treatment of OOMW with the aim to develop low-cost, low technology, and environment-friendly treatments to recover high added-value products for different agricultural uses. Among the explored solutions can be listed the addiction of fungal cells, soil minerals or pure oxidation catalysts (Gianfreda *et al.*, 2006).

Recently, Kerasioti *et al.* (2017) presented a work focused on the investigation of the effects of livestock feed supplemented with OMW on the enzymatic activity and protein expression of antioxidants enzymes, in liver and spleen tissue of sheeps. Therefore, in this case was presented a solution for the development of a low-cost intervention for pathological conditions associated with oxidative stress, while at the same reducing the potential risk of environmental pollution.

Among the oxidant agents commonly distributed in nature, the strongest is certainly represented by ozone (O_3), a component of the atmosphere, generated by the absorption of UV radiation by oxygen. It is considered a strong oxidant which guarantees a broad spectrum of bactericidal effects both in the gaseous and aqueous states. High reactivity, penetrability, and spontaneous decomposition without leaving any harmful by-product, justify the numerous potential uses and make it preferable compared to other oxidizing agents, such as sodium hypochlorite and hydrogen peroxide (Brodowska *et al.*, 2017). In food industry, the ozone application is mostly related to decontamination of water and detoxification of product surface, such as the elimination of mycotoxins and pesticide residues from some agricultural products (Jin-Gab *et al.*, 1999).

Because of its high oxidizing potential and the minimal environmental impact, this compound has been used for the first time in this work for the detoxification of OOMW through the control of the phenolic fraction in order to induce a decrease of the antioxidant activity. The aim of this study was therefore the development of a simple, cheap and low environmental impact methodology, transferable on

Table 1 - Quantity of olive pomace and olive oil mill wastewater (OOMW) obtained in olive processing by different mechanical systems

	Pressure	Decanter 3-phase	Decanter 2-phase and half	Decanter 2-phase
Olive pomace (kg t^{-1})	250-350	450-550	550-650	800-850
Olive oil mill wastewater (L t^{-1})	400-500	600-800	150-300	-

From Di Giovacchino and Preziuso, 2006.

an industrial scale, for oxidative detoxification of OOMW through ozonation.

2. Materials and Methods

Reagents and standards

Hydroxytyrosol, tyrosol, gallic acid, ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox [(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], Folin-Ciocalteu's reagent, sodium carbonate, potassium persulfate, $FeCl_3 \cdot 6H_2O$, methanol and HPLC-grade ethanol were purchased from Sigma-Aldrich (Milan, Italy), while ethyl acetate was obtained from Carlo Erba (Milan, Italy). All chemicals and reagent grade used in the present research were used without further purification steps.

Olive oil mill wastewater (OOMW) collection

The used OOMW from season 2017 (from October to December) was obtained in march of the present year from a decanting tank of a continuous three-phase olive oil extraction system located in the province of Arezzo (Italy), where the main cultivars are, in order, Moraiolo, Leccino and Frantoio.

Ozonation of OOMW

The OOMW was treated with ozone for 1, 3 and 8 h. For each condition, 500 mL of crude OOMW were placed in a glass beaker with a total capacity of 800 mL (base diameter: 10 cm; height: 13.5 cm), and ozone was insufflated at room temperature by using a OZOsteril generator (P.M.G. Depurazione, Vercelli, Italy) with a production capacity of 250 mg/h and an emission flow equal to 62±0.3 mL/min (Power: 30 W max). Samples were then aliquoted and stored at -20°C until use.

Seeds germination test

To evaluate the effect of ozonation on the biotoxicity of OOMW, a germination test was performed using radish seeds (*Raphanus sativus* L.), according to the methodology used for assessing compost maturity (Warman, 1999). Comparison was made among the germination of seeds in three different substrates: distilled water (W), untreated OOMW (C), and OOMW treated with ozone for 1, 3, and 8 hours (without dilution and after a 50% dilution (v/ v) with distilled water).

For each testing condition were prepared 10 Petri dishes (diameter 9.0 cm, height 1.5 cm) with a double layer of filter paper (Whatman® 41) soaked in 10 mL of each substrate, each dish containing 10 radish seeds appropriately arranged (Fig. 1).

The dishes were placed in a climatic chamber in the dark and at a temperature of 25±1°C. The surveys on germination were carried out after 1 day (T1), 3 days (T2) and 6 days (T3), counting the seeds for which it was possible to observe the appearance of the *plumula*. The obtained data were submitted to one way and multiway analysis of variance (ANOVA). Separation of means was performed by the Fisher's LSD test ($p<0.05$). Computations were performed by Statgraphics Centurion XV v. 15.0.04.

Extraction of phenolic compounds from OOMW

The recovery of OOMW phenolic compounds was performed according to the procedure reported by Dammak *et al.* (2016) with slight modifications. Ethyl acetate was added to OOMW samples (1:1; v/v) and the resulting mixture was stirred for 30 min at room temperature. After centrifugation at 4000 rpm for 5 min, the organic phase was separated, filtered with paper filters (Whatman® 41) and concentrated in a rotary vacuum evaporator (40°C). The residual was dissolved in methanol and filtered through a syringe filter (0.45 µm) before subsequent analysis.

Determination of total phenolic content

The total polyphenol content in samples of treated OOMW was estimated by the Folin-Ciocalteu colorimetric method, according to the procedure of Singleton and Rossi (1965). 10 µL of each sample were mixed with 90 µL of distilled water and 500 µL of freshly prepared 0.2 N Folin-Ciocalteu's reagent (1:10 v/v with water). After 10 min, 400 µL of satured

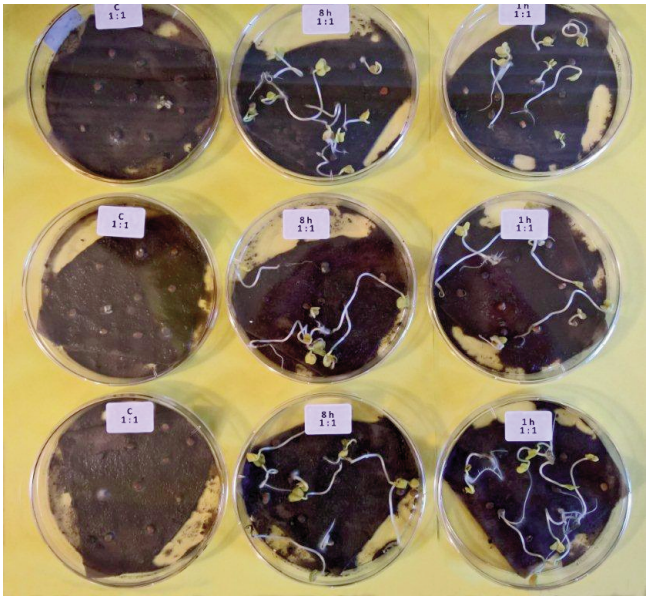

Fig. 1 - Radish seeds germination at T3; on the left, Petri dishes with untreated OOMW; in the middle, OOMW 8h; on the right, OOMW 1h (diluted 1:1 v/v).

sodium carbonate (75 g/L) were added. After incubation at 23°C (room temperature) for 1.5 h, the absorbance of the resulting blue coloured solution was measured at 765 nm with JENWAY 6305 UV/vis spectrophotometer. Quantitative evaluations were performed by using a standard calibration curve of six points (R^2 = 0.9944) ranging from 0 to 50 µg/mL of gallic acid in 80% methanol. The total phenolic content was expressed as gallic acid equivalents (GAE), in milligrammes per milliliter of sample.

Evaluation of total antioxidant capacity

The estimation of total antioxidant capacity (TAC) in OOMW extracts was carried by comparing two different approaches: the ABTS method and the ferric reducing antioxidant power (FRAP). With regard to the ABTS was used a modified methodology previously reported by Ozgen *et al.* (2006). ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) 7 mM was incubated at room temperature for 12-14 h with an oxidant (2.45 mM potassium persulfate) to obtain a stable, dark blue-green radical solution. The solution was than diluted with 80% HPLC-grade ethanol to an absorbance of 0.70±0.2 at 734 nm to form the test reagent. Reaction mixtures containing 10 µL of sample and 1.990 µL of reagent were incubated in the dark at room temperature for 6 min, and the reduction of color deriving from the antioxidants action was measured at 734 nm with JENWAY 6305 UV/vis spectrophotometer. The absorbance was compared to that of the calibrated Trolox standard (range 0-16 µM). Additional dilution was needed if the absorbance values were over the linear range of the standard curve. Results were expressed in terms of Trolox Equivalent Antioxydant Capacity (TEAC; mmol/mL).

The FRAP assay was done according to Thaipong *et al.* (2006). The fresh working solution was prepared by mixing in a 10:1:1 ratio 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution, and $FeCl_3 \cdot 6H_2O$ solution. 10 µL of each OOMW extract were allowed to react with 1.990 µL of the FRAP solution for 30 min in dark condition. The formation of colored product (ferrous tripyridyltriazine complex) was recorded at 593 nm. Results are expressed in mM TEAC per ml of OOMW extract.

HPLC analysis

The evaluation of phenolic profile was performed by using a HPLC method; attention was focused on the relative amounts of hydroxytyrosol and tyrosol before and after the oxidative treatments. The HPLC instrument was a Varian system coupled to a UV/Vis detector set at 280 nm and equipped with a SUPELCOSIL LC-18 column (5 µm particle size, 25 cm x 4.6 mm; Sigma Aldrich, Milan, Italy) maintained at 40°C in an oven. The eluent flow was fixed at 1 mL/min and the used HPLC grade solvents were water and methanol. The elution gradient starts with 80% of water and 20% of methanol; after 30 minutes the water starts to be excluded in favor of the methanol, until reaching the 100% of methanol after 50 minutes from the beginning of the analysis. This condition is then kept constant for further 20 minutes. At the end of the analysis (70 min) the ratio between the solvents was turned back to the initial 80:20 and kept for other 10 min (equilibration of the instrument). The biophenols of interest were identified by comparing the elution times of chromatograms obtained for OOMW extracts with those of standards.

3. Results

Chemical effects of OOMW ozonation

Following the ozonation of OOMW, a simple and efficient extraction of phenolic component from each sample was obtained by using ethyl acetate, allowing to analyze in an accurate way the total phenolic content, the antioxidant activity and relative quantities of two biophenols of particular interest: hydroxytyrosol and tyrosol.

The first noteworthy finding concerns the fact that the OOMW used in this study is characterized by a higher phenolic content than that reported in other studies (Ochando-Pulido *et al.*, 2015; Dammak *et al.*, 2016).

After treatment with ozone, a marked and time-dependent reduction of the phenolic content was evidenced. As reported in Table 2, the reduction of TPC is significant after 3 hours of ozonation (from 54.60 mg/mL to 35.34 mg/mL; $P<0.01$), and reaches the minimum value (16.19 mg/mL; $P<0.01$) after 8 hours of treatment.

These results correlates with the TAC which was evaluated in each sample through two different approaches; both the ABTS and the FRAP assay showed the lowest antioxidant potential after 8 hours of ozonation ($P<0.01$), that is precisely in the samples poorest in terms of phenolic compounds.

The HPLC analysis was performed with the aim of determining the influence of the ozone oxidation

Table 2 - Analytical parameters collected after oxidative treatments of OOMW with ozone for 1, 3 and 8 hours

	C	1h	3h	8h
Total phenolic compounds (GAE mg/mL)	54.60±2.98 a	51.10±2.59 a	35.34±4.68 b	16.19±0.42 c
Total antioxidant capacity				
ABTS (TEAC µmol/mL)	6.26±0.41 a	5.16±0.63 a	4.52±0.49 b	1.75±0.35 c
FRAP (TEAC µmol/mL)	3.72±0.12 a	3.03±0.06 b	2.13±0.02 c	1.62±0.14 d
Hydroxytyrosol*,§	741 a	639 b (81.0)	471 c (61.4)	490 c (66.1)
Tyrosol*,§	630 a	595 a (88.1)	511 b (78.0)	518 b (78.8)
pH	4.52±0.03 a	4.49±0.02 a	4.51±0.04 a	4.48±0.03 a

Values in the same row followed by different letters differ significantly (significance was set at P<0.05).
* Arbitrary unit.
§ % residual inside the round brackets.

treatments on the relative concentrations of three biophenols. In particular the attention was focused on hydroxytyrosol and tyrosol, two compounds commonly found in the by-products of the olive oil production industry, to which a high antioxidant activity is associated (De Marco *et al.*, 2007). The analysis showed only a partial reduction of hydroxytirosol and tyrosol content; as reported in Table 2, hydroxytyrosol showed a minimum residual of 61% after 3 h of ozonation, while tyrosol did not fall below the 78% even after 8 hours of treatment. This datum is not in full agreement with the marked reduction of the TPC previously reported.

Unexpected is the data concerning pH (Table 2), which does not seem to undergo significant changes.

Seed germination test

Table 3 shows the germination ability of the radish seeds, placed on different germination substrates: solutions of untreated OOMW (C) and OOMW derived from different ozonation times (1h, 3h, and 8h, respectively) diluted to 50%; as control, distilled water (W) was used. For each test, the number of seeds germinated after 1, 3 and 6 days (T1, T2, and T3, respectively), was counted. The trial was carried out also on the undiluted OOMW, but in this case no germination was obtained: therefore these data have not been reported.

From Table 3, it is clear that the radish has a high germination ability and, after 1 day, all the seeds placed in distilled water (W) germinated. In the 50% diluted OOMW (C) an inhibition is still measurable, and only after 6 days of staying on the substrate it was possible to observe the beginning of germination (on average 6.0%). It is also evident the "time effect" of ozonation, which allowed a germination percentage of 30% already on the first day for OOMW treated for 8 hours (T1) and 17% for OOMW treated for 3 hours (T1). In the case of the best result (8 hours of ozonation, T3), the percentage of germination is statistically equal to that obtained by using distilled water as a substrate (Fig. 2).

Table 3 - Average number of seed germinated per Petri dish (ten seeds) with different treatments (W, C, OOMW 1h, 3h, 8h) after 1 (T1), 3 (T2), and 6 days (T3), respectively

	Ozone treatment				
	W	C	1h	3h	8h
T1	10.0±0.0 d	0.0±0.0 a	1.0±0.0 b	1.7±1.49 b	3.0±1.15 c
T2	10.0±0.0 c	0.0±0.0 a	6.7±1.57 b	7.0±2.83 b	7.7±0.67 b
T3	10.0±0.0 c	0.6±0.52 a	7.0±1.49 b	7.3±2.83 b	8.7±0.82 c

Values in the same row followed by different letters differ significantly (significance was set at P<0.05).

The effect of the different ozonation times on the germination is highlighted in figure 2, that clearly shows as in OOMW (C) germination starts only after the third day of incubation, while the seeds placed on ozonated substrates arrange in a sequence (OOMW 1h, OOMW 3h, and OOMW 8h) which reflects the increases in the ozonation. The number of seeds that germinate continues to increase after the third day practically only in the OOMW 8h test, and on the sixth day (T3) 87% of the incubated seeds germinated.

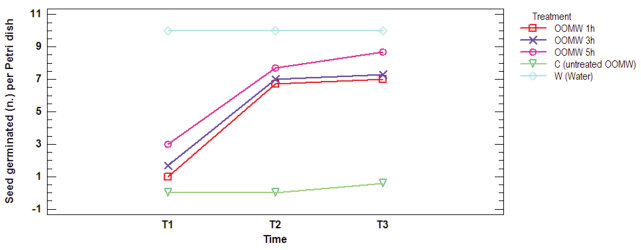

Fig. 2 - Interaction plot (treatment x time) from multiway ANOVA.

4. Discussion and Conclusions

Ozonation has proven to be a valuable tool for lowering the phenolic fraction in the OOMW. In the present study, using a laboratory instrument, after 8 hours of ozonation the phenolic fraction decreased to less than one third of the initial amount, with an almost equivalent lowering of the antioxidant activity, according to previous works; hydroxytyrosol showed to be reduced in a more marked way with respect to the tyrosol. The findings concerning the germination essay evidenced the detoxifying effect of ozonation; after 8 hours of ozonation the percentage of germinated seeds is statistically equal to that obtained using only distilled water as a substrate, while in the untreated solution the seeds germination is still inhibited after 6 days. These results are also confirmed by comparing the succession of the final germination percentages obtained using different ozonation times: 6% for untreated OOMW, 70% after 1, 73% after 3, and 87% after 8 hours of ozonation, respectively.

Further investigations should be performed to deeply investigate the biochemical effects of ozonation on OOMW, but the treatment is really promising for an effective dephenolyzation of the OOMW, even for oil mill industrial plants allowing to transform this by-product, currently considered highly polluting, in a crop product suitable for energy production in bioreactors, as natural fertilizer, or foodstuff supplement.

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