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Effects of Salicylic Acid, Jasmonic Acid, and Calcium Chloride on Reducing Chilling Injury of Pomegranate (*Punica granatum* L.) Fruit

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ABSTRACT

The pomegranate fruits of cvs. Malas Yazdi and Malas Ashkezar were immersed in SA (1 and 2 mM from source of acytyl salicylic acid) for five minutes, in JA (0.3 and 0.4 mM from source of n-propyl dihydrojasmonate) for fifteen minutes, in $CaCl_2$ (1 and 2%) for five minutes, and in distilled water for five minutes as control. Then, the fruits were stored in cold storage at 1.5±0.5°C and 85±5% relative humidity for 2 months. Every 21 days, samples were taken out of the cold storage and were kept at 20°C for 3 days before analysis. The analysis results revealed that treatments with SA, JA, and $CaCl_2$ significantly reduced the chilling injury of pomegranate fruits. The lowest chilling injury index was observed in 0.4 mM of JA-treated fruits and the highest was for untreated fruits. Electrolyte leakage of fruits increased upon treatments of fruits with 0.3 mM JA and 2 mM SA, but it was not significantly affected by other treatments. Treatments of fruits with SA, JA, and $CaCl_2$ had no significant effect on phenolic compounds, but the total soluble solids of fruit juices was increased. Our findings indicated that total antioxidant activity decreased in treatments with 1, 2 mM SA and 0.3 mM JA , but it was not significantly affected by other treatments.

Keywords*:* Arils, Browning, Cold storage, Ion leakage, Shelf life.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family of Punicaceae and is one of the favorite fruits of tropical and subtropical regions that, in recent decades, has attracted interest in all aspects of cultivation, extension, and human health (Pezeshki Rad, *et al.,* 2009). The edible part of the fruit is called arils, which contain around 80% juice and 20% seed. The fresh juice contains 85.4% moisture and considerable amounts of total soluble solids, total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid, and proteins (Yasoubi, *et al.*, 2007; El-Nemr *et al.*, 1990) and has also been reported to be a rich source of antioxidants (Sadeghi *et al.,* 2009; Gil *et al.*, 2000; Kulkarni *et al.*, 2004). It has been suggested that compounds that possess antioxidant activity can inhibit mutation and cancer because they can scavenge a free radical or induce antioxidant enzymes (Hochstein and Atallah, 1988).

According to Elyatem and Kader (1984), pomegranate is susceptible to chilling injury (CI) when exposed to temperatures below 5°C for 5 weeks. In their study, chilling injury symptoms, which became more visible after storage at 20°C for 3 days, included brown discoloration of the skin, surface pitting, and increased susceptibility to decay organisms. Internal symptoms were manifested as pale colour of the arils and brown discoloration of the white segments separating the arils (Elyatem and Kader,

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1984). To reduce the occurrence of CI in pomegranate, several techniques have been applied including intermittent warming (Artes *et al.*, 2000; Mirdehghan *et al.*, 2007b), polyamine (Mirdehghan *et al.*, 2007a), salicylic acid (Sayyari *et al.*, 2009) and methyl jasmonate (Ranjbar *et al.*, 2007) treatments.

Acetyl salicylic acid (ASA) is a derivative of salicylic acid (SA) and, when applied exogenously, it undergoes spontaneous hydrolysis and is converted to SA (Popova *et al.*, 1997). SA is a natural phenolic compound involved in regulation of many processes in plant growth and development. SA is also known for its induction of plant defense against biotic and abiotic stress and is reported to increase chilling tolerance in peach (Wang *et al.*, 2006), tomato (Ding *et al.*, 2002) and sweet peppers (Fung *et al.*, 2004). Also, it plays an important role in modulating redox balance across the membranes, thereby counteracting the negative effects of reactive oxygen intermediates caused by oxidative stress (Yang *et al.*, 2004) by increasing the activity of antioxidant enzymes such as superoxide dismutase (Wang *et al.*, 2004). Exogenous SA treatment may also induce the expression of pathogenesis-related protein (Malamy *et al.*, 1990) and establish systemic acquired resistance (Gaffney *et al.*, 1993).

Jasmonic acid and its volatile methyl ester, methyl jasmonate (MeJA), are a class of cyclopentanone compounds regarded as endogenous regulators that play an important role in regulating the stress response, plant growth, and development (Creelman and Mullet, 1997). In recent research, MeJA has been applied to reduce the development of chilling injury symptoms in a number of horticultural crops, including zucchini squash (Wang and Buta, 1994), mango (Gonzalez-Aguilar *et al.*, 2000), avocado, grapefruit, and peppers (Meir *et al.*, 1996). Application of n-propyldihydrojasmonate (PDJ), which is a JA derivative, also reduces chilling injury of mangosteens (Kondo *et al.*, 2004). Reduction of chilling injury by MeJA might be due to enhanced antioxidant enzyme activity and a higher unsaturated/saturated fatty acid ratio (Cao *et al.*, 2009). Recently, MeJA has shown promising signs in preventing postharvest disease and disorders in horticultural crops and application of MeJA has been reported to effectively suppress gray mold rot caused by *Botrytis cinerea* in strawberry (Moline *et al.*, 1997). Very recently, Cao *et al.* (2012) have shown that MeJA could induve chilling tolerance in loquat fruit by increasing proline and γ-aminobutyric acid contents.

Calcium is a divalent cation that readily enters the apoplast and is bound in exchangeable form to cell wall and exterior surface of plasma membrane. Calcium maintains the cell wall structure in fruit by interacting with the pectic acid in the cell walls to form calcium pectate. Ca^{2+} forms cross-links between pairs of negatively charged homogalacturonans, thus tightening the cell wall (Picchioni *et al.*, 1998). Postharvest calcium application maintains cell turgor, membrane integrity, tissue firmness, and delays membrane lipid catabolism, extending storage life of fresh fruits and reduce the physiological disorders (Garcia *et al.*, 1996; Picchioni *et al.*, 1998). The postharvest application of calcium to some horticultural commodities has been demonstrated to reduce the incidence of chilling-induced disorders. Application of calcium significantly reduces the severity of chilling injury in avocados (Chaplin and scott, 1980), peaches (Wade, 1981) and tomatoes (Moline and Teasdale, 1981). Lester and Grusak (1999) have shown that calcium application in plums was effective in terms of membrane functionality and integrity maintenance, with lower losses of phospholipids and proteins and reduced ion leakage. Cold stress induced Ca^{2+} movement from vacuoles and intercellular space to the cytoplasm. It is speculated that increased Ca^{2+} content in the cytoplasm may help maintaining plasma membrane integrity to improve cold stress tolerance.

The aim of this study was to determine the effects of SA, JA, and $CaCl_2$ on reducing

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chilling injury and qualitative characteristics of pomegranate fruits of cvs. Malas Yazdi and Malas Ashkezar stored at 1.5±0.5°C.

MATERIALS AND METHODS

Plant Material and Treatment

Pomegranate fruits of cvs. Malas Yazdi and Malas Ashkezar were harvested at horticultural maturity in the orchard of Agricultural Research Center of Yazd province. Fruits were immediately transported to the postharvest laboratory and sorted based on size and absence of physical injuries or sunburn, then, they were randomly divided into 7 groups of 128 fruits for the following treatments: lot 1 and 2 were immersed into solution of 1 and 2 mM ASA, pH 3.5 for 5 min, lot 3 and 4 were immersed into the solution of 0.3 and 0.4 mM PDJ at 25°C for 15 minutes, lot 5 and lot 6 were dipped in 1 and 2% $CaCl_2$ solution for 5 min, and the last lot of fruits was dipped for 5 minutes in distilled water and served as control. All solutions contained tween-20 (2 ml L^{-1}). There were 4 replications in the experiment and each experimental unit contained 5 fruits. After immersion, the fruits were air-dried and stored at 1.5±0.5°C and 85±5% relative humidity (RH) for 63 days. Fruit samples were taken after immersion (day 0) and at 21-day intervals during storage, and were finally stored at 20°C for 3 days. Subsequently, characteristics such as chilling injury (CI), total electrolyte leakage, total soluble solids (TSS), titratable acidity (TA), ascorbic acid (AA), total antioxidant activity (TAA), and total phenolic compounds were evaluated.

Evaluation of Chilling Injury and Electrolyte Leakage

CI index was scored according to external skin browning as follows:

0 (no symptom); 1 (20% browning lesion, BL); 2 (40% BL); 3 (60% BL); 4 (80% BL); 5 (100% BL). The severity of CI was calculated by the following formula:

CI index (%) = Σ [(CI level)×(Number of fruit at the CI level)]/(5×Total number of fruit in the treatment)

The rate of electrolyte leakage was determined as described by McCollum and McDonald (1991). For each husk, six discs (10 mm) of the peel tissue were cut with a cork borer. After incubation in 25 ml of 0.4M manitol, conductivity was measured with a conductivity meter after 4 hours of incubation under constant shaking. Following the initial reading, the vials were autoclaved at 121°C for 20 minutes, held overnight, and the conductivity was measured again for total electrolytes leakage. The rate of electrolyte leakage was expressed as a percentage of total: *(Initial/Total)×100.*

Measurement of Total Soluble Solids, Titratable Acidity, and Ascorbic Acid

Total soluble solids concentrations (TSS) were measured with refractometer and expressed as (%) or ºBrix. Titratable acidity (TA) was assayed by titration of 5 ml of fruit juice with 0.2N NaOH to pH 8.2 and expressed as gram of citric acid equivalent per 100 g fresh weight. Ascorbic acid content was determined by titration with iodine and expressed as (mg100g^{-1} fresh weight). Five ml of the freshly prepared aliquot was titrated with iodine solution until a permanent bluing of the starch indicator resulted. Since 1 ml of normal iodine solution reacts with 0.88 mg. of ascorbic acid, the ascorbic acid equivalent of the dye solution was calculated.

Measurement of Total Antioxidant Activity and Total Phenolic Compounds

The arils of each replicate were combined and frozen in liquid N_2, were milled to

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obtain homogeneous samples, and were stored at -20°C until analysis. For each sample, 5 g of arils was homogenized in 10 ml of 50 mM phosphate buffer at pH 7.8 and centrifuged at 4,800 rpm for 15 minutes at 4°C. The supernatant was used for total antioxidant activity (TAA) and total phenolic compounds quantification in duplicate, as previously described (Serrano *et al.*, 2005) with some slight changes. Briefly, TAA was determined using the enzymatic system composed of the chromophore 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horse radish peroxidase enzyme (HRP), and its oxidant substrate (hydrogen peroxide), in which $ABTS^{\cdot}+$ radicals are generated and monitored at 730 nm. The decrease in absorbance after adding the aril extract was proportional to TAA of the sample. For (TAA), L-ascorbic acid was used for calibration curve, and the results were expressed as mg ascorbic acid equivalent per 100 g fresh weight (fw). The total phenolic compounds were quantified using the Folin-Ciocalteu reagent and results were expressed as mg gallic acid equivalent per 100 g fw. The extracts were appropriately diluted and then oxidized with 2.5 ml of freshly diluted (1:10) 2N Folin–Ciocalteau reagent. This reaction was neutralized by adding 2.0 ml of 7.5% w/v sodium carbonate, and the samples were vortexed for 20 seconds. The samples were then incubated at 50°C for 5 minutes and the absorbance was measured at 760 nm on an UV–Vis recording spectrophotometer.

Statistical Analysis

The experiment was factorial with completely randomized design and four replications. Data for the analytical determinations were subjected to analysis of variance (ANOVA). Mean comparisons were performed by Duncan's Multiple Rang Test. Differences at $P< 0.05$ were considered as significant. All analyses were performed with MSTATC.

RESULTS AND DISCUSSION

Since the interaction of cultivars and treatments was not significant in important characteristics, the results represent the means of treatments and the days after storage.

Occurrence of Chilling Injury Symptoms and Electrolyte Leakage

In pomegranate fruits, chilling injury manifested as skin browning increased during storage, but was affected by the applied treatment. The highest chilling injury was observed for the control fruits after 63 days, whereas the lowest value was for 0.4 mMeJA and 1 mM SA at 21 days (Figure 1). These results were in agreement with those of Ding *et al.* (2001) who reported that MeSA and MeJA treatments reduced chilling injury in tomato fruit. The effect of MeSA and MeJA on alleviating chilling injury of fruits during cold storage may be attributed to its ability to induce the accumulation of heat shock protein (HSP) (Ding *et al.*, 2001) and antioxidant systems (Wang *et al.*, 2006; Evans *et al.*, 1991). The $CaCl_2$ in both concentrations could also reduce the browning of pomegranate fruit. After 40 and 60 days of storage, $CaCl_2$-treated fruits showed significantly lower percentage of chilling injury index compared to the control. It has been shown in many studies (Bitencourt De Souza *et al.*, 1999; Chaplin and Scott, 1980) that Ca^{2+} could improve the integrity of plasma membrane and, consequently, lower chilling damage. In an experiment on avocado, Chaplin and Scott (1980) found that the severity of observed chilling symptom was reduced by application of Ca ion through vacuum infiltration of $CaCl_2$. In pomegranate fruit, Ramezanian and Rahemi (2011) found that Ca-treated fruit, compared to the control,

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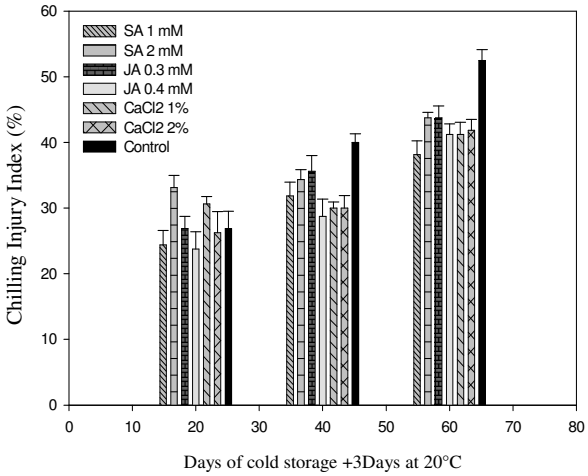

Figure 1. Chilling injury index of the control and treated pomegranate fruits after several periods of cold storage and 3 days at 20°C (shelf-life). Data are mean±SE. SA= Salicylic acid; JA=Jasmonic acid

showed significantly higher activities of catalase and superoxide dismutase and a lower activity in peroxidase. They concluded that antioxidant enzymes were responsible for inducing chilling tolerance of pomegranate fruits.

With respect to electrolyte leakage (EL), there were no significant differences between treated fruits and the control, whereas EL in fruits treated with 0.3 m MeJA and 2 mM SA was higher than that of control (Figure 2). As shown in Figure 2, EL gradually increased within 42 days, followed by a decrease from 42 to 63 days. Contrary to our findings, Meng *et al.* (2009) found that MeJA treatment in peach fruit could reduce electrolyte leakage of cells by maintaining the membrane integrity. Also, Sayyari *et al.* (2009) have reported that SA treatment were effective in reducing

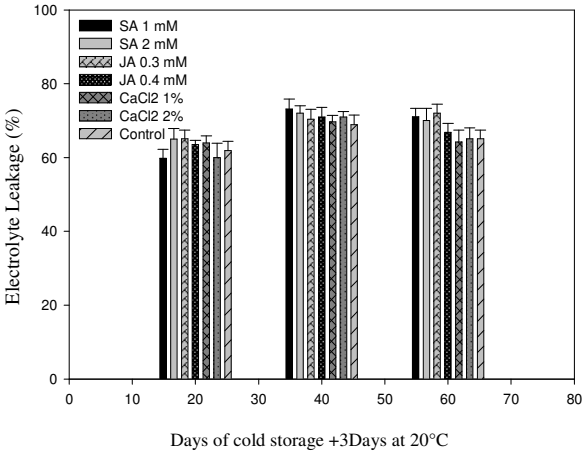

Figure 2. Electrolyte leakage of the control and treated pomegranate fruits after several periods of cold storage and 3 days at 20°C (shelf-life). Data are mean±SE. SA= Salicylic acid; JA=Jasmonic acid

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electrolyte leakage of pomegranate fruit during cold storage. Thus, these contradictory results concerning the precise role of MeJA and SA in regulating chilling indices are yet to be fully explained, and probably other mechanisms such as antioxidant defense systems and factors affecting membrane properties could be potentially more important in conferring chilling tolerance.

The injury induced by low temperature accelerates the deterioration of chilling-sensitive fruits. Consequently, any treatment such as MeJA and SA that increases chilling tolerance of these commodities also retards degenerative processes. It was postulated that jasmonates (Meir *et al.*, 1996) and SA (Qin *et al.*, 2003) may play an integral role in the signal transduction cascade through chemical changes involved in CI tolerance.

Total Soluble Solids, Total Acidity and Ascorbic Acid

The influence of all treatments on total soluble solids of fruit juice (TSS) is shown in Table 1. SA applied at 1 mM significantly increased the TSS compared to untreated fruits. These results are in line with Srivastava and Dwivedi (2000) who reported that treatment with SA increased TSS in banana fruits. The effects of SA treatments on the sugar content of fruits and vegetables reported in the literature are controversial. Lower contents of TSS were reported in Kiwifruit treated with 32 µl L^{-1} of MeSA at the end of cold storage (Asghari and Aghdam, 2010). The authors proposed that MeSA reduced ethylene production and that might result in decreased sucrose-phosphate synthase enzyme activity leading to decrease in sucrose synthesis. On the other hand, cell walls contain large amounts of polysaccharides, mainly pectins and cellulose, and are digested due to the activity of the cell wall degrading enzymes leading to a significant increase in TSS content. However, the amount and retention of these sugars might not be a direct effect of MeSA treatment and could differ among fruits, as well as with the maturity stage at the time of application.

The results showed that total acidity (TA) was not influenced by any treatment (Table 1). Similar observation has been reported by Ding *et al.* (2007), Ranjbar *et al.* (2007), and Biten Court De Souza *et al.* (1999) who had described that TA were not affected by SA or MeJA or $CaCl_2$. Results evidenced that pomegranate fruits treated with 2 mM SA had the lowest ascorbic acid (AA), although difference between other treatments and the untreated fruits was not significant (Table 1). Our results are consistent with the finding of Gonzalez-Aguilar *et al.* (2004) who reported that ascorbic acid was not affected by the MeJA treatment.

Table 1. Changes in total soluble solids (TSS, ^{o}Brix), total acidity (TA, per 100 mg FW), ascorbic acid (AA, per 100 mg FW) and total phenolic compounds (mg eq. gallic acid per 100 mg FW) in the control and treated fruits during storage[a].

	TSS	TA	AA	Total phenolic compounds
SA 1 mM	13.27 a	0.88 a	15.62 ab	65.69 a
SA 2 mM	13.00 ab	0.90 a	14.01 b	62.79 a
JA 0.3 mM	12.92 ab	0.89 a	15.68 ab	59.44 a
JA 0.4 mM	12.83 ab	0.90 a	15.75 ab	63.13 a
$CaCl_2$ 1%	13.09 ab	0.92 a	15.75 ab	62.78 a
$CaCl_2$ 2%	13.13 ab	0.89 a	16.48 a	62.62 a
Control	12.75 b	0.90 a	15.57 ab	62.71 a

[a] For each parameter, similar letter within rows are not significantly different at $P< 0.05$ level.

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Total Phenolic Compounds and Total Antioxidant Activity

Total phenolic compounds were not influenced by all treatments (Table 1). These results are in agreement with Gonzalez-Aguilar *et al.* (2004) who described that total phenolics were not affected by the MeJA treatment, while, Rudell *et al.* (2002) reported that treatments of MeJA in apple 'Fuji' induced the accumulation of chlorogenic acid. The physiological mechanism by which MeJA reduces CI impact in plant tissues is not known in detail, although it has been shown in many studies that MeJA could play an important role in the tolerance to chilling stress, where phenolics compounds are involved. However, these might not be a direct effect of MeJA treatment and could differ among different fruits and with the maturity stage at the time of application and other factors (Wang and Buta, 1994). In the present study, we measured the changes of total phenols and, according to previous reports, punicalagin has been described as the major compound in pomegranate arils contributing to total antioxidant activity (Kulkarni *et al.*, 2004). Therefore, further research is necessary to assay the amount of punicalagin separately. TAA increased at the midpoint of cold storage, then, gradually decreased (Figure 3). The results revealed that TAA decreased in treatments with 1, 2 mM SA and 0.3 mM MeJA, but it was not significantly affected by other treatments. Contrary to our findings, Huang *et al.* (2008) reported that application of SA could increase antioxidant enzyme activity and thus delay membrane lipid peroxidation. They suggested that pretreatment with SA in combination with low temperature may be a useful strategy for prolonging orange postharvest life and maintaining nutritional conditions during storage. In pomegranate cultivars, anthocyanin, ascorbic acid, and phenolics are responsible for the TAA, alone or in combination with other compounds (Kulkarni *et al.*, 2004). In our experiment, the content of total phenolics did not change during storage, while the amount of ascorbic acid diminished throughout storage period. However, TAA increased with prolonging storage time, probably due to the increased anthocyanin or punicalagin as the major phenolic compound that contributes to TAA.

Fruits and vegetables contain many different antioxidant components (Cao *et al.*, 2009). Prior *et al.* (1998) found different

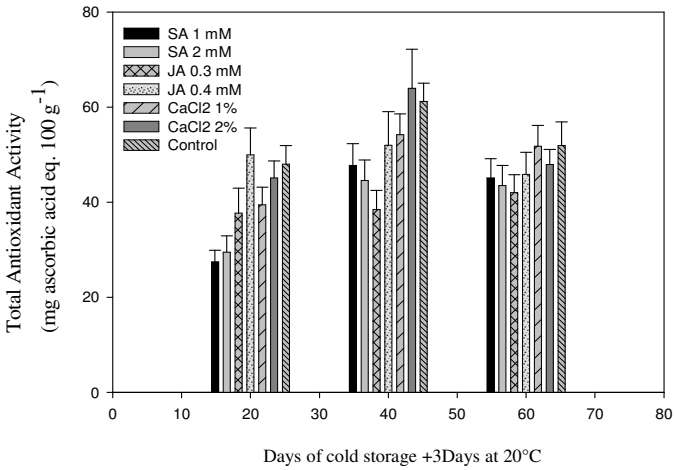

Figure 3. Total antioxidant activity of arils during cold storage+3 days at 20°C (shelf-life). Data are mean±SE. SA= Salicylic acid; JA=Jasmonic acid

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antioxidant capacities in various species and cultivars of produce. MeJA treatment was shown to increase raspberry antioxidant enzyme activities, including SOD, GPX, APX, GR, DHAR, and MDHAR (Chanjirakul *et al.*, 2006). However, more experimental evidence is necessary to support this statement in pomegranate fruit and others.

It has been shown that 2 mmol L^{-1} of SA could enhance the TAA of strawberry fruit. This concentration was the most effective, while SA at 4 mmol L^{-1} caused a slight increase in fruit TAA (Asghari and Aghdam, 2010). Chilling injury is a type of damage caused by low temperature as a result of oxidative burst. Although there are many methods to reduce CI in various horticultural crops, SA treatments are inexpensive, easy to set up, and applicable to various horticultural crops (Asghari and Aghdam, 2010).

Calcium treatments at different concentration could increase and preserve TAA of pomegranate fruit during storage (Figure 3). These effects of calcium, found in a range of fruits (Bitencourt De Souza *et al.*, 1999; Chaplin and Scott, 1980), are not clearly explained but are likely to reside in the interaction of calcium with cell wall polymers and enzymes, and with membrane function. It is likely that effects of calcium on enzymes and metabolism are indirect, associated with calcium maintaining membrane function under conditions where permeability changes are naturally occurring. Therefore, these effects may cause higher activity of different antioxidants enzyme and eventually lead to lower chilling index and injury of pomegranate fruit compared to the control (Figure 1).

Based on the data it was concluded that treatment with 0.4 m MeJA was the most effective treatment for reducing CI. The reduction in chilling injury by MeJA may be due to enhanced antioxidant enzyme activity (Cao *et al.*, 2009). The multiple biochemical effects caused by MeJA suggest pleiotropic action of these compounds. The signals generated by MeJA in plant cells induce the activation of several enzymatic reactions and synthesis of proteins that play different roles in the postharvest life of horticultural crops (González-Aguilar *et al.*, 2004). However, further research using more than two cultivars is necessary to carry out with different postharvest applications (pressure-infiltration and vacuum infiltration) and different concentrations for reducing CI of pomegranate fruits and maintaining antioxidant activity and nutritional conditions during storage.

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تاثیر اسید سالیسیلیک، اسید جاسمونیک و کلرید کلسیم بر کاهش خسارت سرمازدگی میوه انار(*Punica granatum* L)

س. ح. میر دهقان ، ف. قطبی

چکیده

میوه ارقام ملس یزدی و ملس اشکذر انار به مدت ۵ دقیقه در محلول اسید سالیسیلیک (۱ و ۲ میلی مولار از منبع استیل سالیسیلیک اسید)، در اسید جاسمونیک (۰.۳ و ۰.۴ میلی مولار از منبع ان- پروپیل دی هیدرو جاسمونات) به مدت ۱۵ دقیقه)، کلرید کلسیم (۱ و ۲ ٪) به مدت ۵ دقیقه و آب مقطر (کنترل) غوطه ور شده و سپس در انبار سرد با دمای ۱.۵±۰.۵ درجه سانتی گراد و رطوبت نسبی ۸۵±۵ ٪به مدت ۲ ماه نگهداری شدند. نمونه ها هر ۲۱ روز از انبار خارج و ۳ روز در دمای ۲۰ درجه سانتی گراد نکهداری و مورد تجزیه قرار گرفتند. نتایج نشان داد که تیمار اسید سالیسیلیک، اسید جاسمونیک و کلرید کلسیم به طور معنی داری خسارت سرمازدگی میوه انار را کاهش داد. کمترین شاخص سرمازدگی در میوه های تیمار شده با غلظت ۰.۴ میلی مولار اسیدجاسمونیک و بیشترین مقدار در میوه های تیمار نشده مشاهده شد. تیمار میوه ها با غلظت ۰.۳ میلی مولار اسید جاسمونیک و ۲ میلی مولار اسید سالیسیلیک نشت الکترولیت میوه ها را افزایش داد ولی تاثیر معنی داری در دیگر تیمار ها مشاهده نشد. همجنین نشان داده شد که تیمار با اسید سالیسیلیک، اسید جاسمونیک و کلرید کلسیم تاثیر معنی داری بر ترکیبات فنلی ندارد، ولی مواد جامد محلول افزایش می یابد. یافته های ما نشان می دهد که تیمار با اسید سالیسیلیک ۱، ۲ میلی مولار و ۰.۳ میلی مولار اسید جاسمونیک فالیت آنتی اکسیدانی کل را کاهش می دهد ولی به طور معنی داری تحت تاثیر دیگر تیمارها قرار نمی گیرد.

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