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Effect of Putresine and Methyl Jasmonate on Antioxidant Responses in Peel and Pulp of Orange (*Citrus sinensis* L. var. Valencia) Fruit

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Abstract
This experiment was carried out to determine the effects of putrescine and methyl jasmonate on lipid peroxidation and altering the peroxide hydrogen of peel and pulp of Valencia orange fruit. Orange fruits were treated with 0 (control), 2.5 and 5 mM putrescine, and 0 (control), 10 and 20 µM methyl jasmonate, then stored at 5±1 °C, 85-90% relative humidity for four months. Oranges treated with 5 mM putrescine and 10 µM methyl jasmonate had significantly lower lipid peroxidation and peroxide hydrogen of peel and pulp and lower chilling injury percentage than the non-treated fruits. Polyphenol oxidase (PPO) activity was considerably increased in the treated and non-treated fruits but, the treated fruits exhibited significantly lower activity of PPO. Peroxidase (POD) activity increased at first and then decreased. However, the treated fruits exhibited significantly higher activity of POD than the control fruits during the storage period. In conclusion, fruits treated with 5 mM putrescine combined with 10 µM methyl jasmonate showed the best effect.

Keywords: Browning; Lipid peroxidation; Peroxide hydrogen

Introduction

The Valencia orange (*Citrus sinensis* L.) is widely cultivated throughout the world due to its taste and nutritional value and is the major citrus crop in Iran. Chilling injury (CI) is a serious problem during storage of subtropical fruits at low temperatures such as 2-5 $^{\circ}$C (Ritenour *et al*. 2004). One of the biochemical changes occurring when plants are subjected to low temperature stress is the production of reactive oxygen species (ROS) which are the result of disruption of normal metabolism associated with the oxidative damage of macro molecules (Allen 1995). ROS-induced stress is thought to be a fundamental cause of cell death in cryopreserved samples (Benson 1990). Lipids are the major class of biomolecules targeted by ROS in the membrane. The main lipids targeted by ROS are the polyunsaturated fatty acids (PUFA) (Esterbauer *et al*. 1991). PUFA constitutes approximately 50–90% of the membrane lipids (Douce *et al*. 1973). Lipid peroxidation can be used as a biochemical marker for the estimation of free radical mediated injury.

ROS include superoxide radicals (O^{2-}), hydroxyl radicals (OH^{-}) and hydrogen peroxides (H_2O_2) that are produced as byproducts during membrane linked electron transport activities as well as by a number of metabolic pathways (Becana *et al*. 2000; Kanazawa *et al*. 2000). Environmental stressors are known to induce hydrogen peroxide and other toxic oxygen species in cellular compartments and result in acceleration of leaf senescence through lipid peroxidation and other oxidative damages. Hydrogen peroxide as a

strong oxidant can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity that result in senescence. It also changes the redox status of surrounding cells where it initiates an antioxidative response by acting as a signal of oxidative stress (Sairam and Srivastava 2000). Many vegetables and fruits are discolored during storage by the polyphenol oxidase enzyme (Broothaerts *et al*. 2000). The polyphenol oxidase enzyme activity is widespread in plants which occurs early in the tissue development and is stored in the chloroplast (van Gelder *et al.* 1997). Enzymatic browning reaction by polyphenols oxidase and peroxidase occur in plant cells (Onsa *et al*. 2000). Brown reactions are caused when the cells are destroyed, and indigenous phenolic compounds oxidized in the presence of molecular oxygen (Mayer 1986). Peroxidase activity increased when plants exposed to stresses such as low temperature, pathogen infections, UV radiation, poisonous gases and heavy metals (Grover and Sinha 1985). Polyphenol oxidase is involved in the biosynthesis of melanin in animals and browning of plants (Lee *et al*. 2007). Peroxidase and polyphenol oxidase activity are main enzymes involved in phenolic oxidation of many fruits and vegetables (Ye *et al*. 2007). Environmental stresses induce the synthesis of different plant hormones. These phytohormones are required at various developmental stages of the plants and help in their defense responses (Denance *et al.* 2013). Rosahl and Feussner (2005) demonstrated that jasmonic acid and their derivatives regulate the gene expression involved in defense responses. Jasmonic acid dependent defense responses are activated as a result of necrotrophic pathogen infection (Avanci *et al.* 2010). Polyamines take part in the regulation of many basic cellular processes, including DNA replication, modulation of enzyme activities, cellular cation-anion balance and membrane stability (Singh Gill and Tuteja 2010). Exogenous application of polyamines at different concentrations has enhanced the tolerance to various stresses in different plants (Duan *et al.* 2008). The objective of this study was to determine the effect of putresine (Put) and methyl jasmonate (MJ) on antioxidative system, lipid peroxidation and hydrogen peroxide levels of orange fruits during chilling stress, which is perhaps one of the mechanisms of improved chilling tolerance.

Materials and Methods

Plant materials and treatments

Orange fruits (*Citrus sinensis* L. var. Valencia) were harvested at full maturity from a commercial orchard at Jiroft (Kerman Province, Iran) on 15 March, 2011 and transported to laboratory. Fruits were selected for uniform size and color, and without any mechanical and pathogen damage. Then, oranges were treated with 0 (control), 2.5 and 5 mM putrescine and 0 (control), 10 and 20 µmol methyl jasmonate and stored at 5±1 °C, 85-90% relative humidity for four months. Treated fruits were used for determination of visible chilling injury symptoms, lipid peroxidation and hydrogen peroxide levels, and activities of peroxidase and polyphenol oxidase.

Chilling injury assessment

CI was evaluated visually by surface area scale of surface pitting and dark patches (browning). CI (%) was calculated for each treatment using the following formula (Roberts *et al.* 2002):

[(Total number of fruits in each treatment - number of fruits with no chilling injury/(total number of fruits in the treatment)]× 100.

Assays of lipid peroxidation and hydrogen peroxide

Lipid peroxidation was estimated by determining the malondialdehyde (MDA) content according to the method of Dhindsa *et al*. (1981). 100 mg of fruit samples was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 g for 5 min at 4 °C. Aliquot of 0.3 mL supernatant mixed with 1.2 mL of 0.5% thiobarbituric acid (TBA) was prepared in TCA 20%, and incubated at 95 °C for 30 min. After stopping the reaction in an ice bath for 5 min, samples were centrifuged at 10000 g for 10 min at 25 °C. Absorbance was measured at 532 nm using a Beckman UV-DU 520 spectrophotometer (USA). After subtracting the non-specific absorbance at 600 nm, the MDA concentration was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

The assay for hydrogen peroxide content was carried out according to *Patterson et al.* (1984). Fresh tissues (2 g) were homogenized with 10 mL of acetone at 0 °C. After centrifugation for 15 min at 6000 g and 4 °C, the supernatant phase was collected. The supernatant (1 mL) was mixed with 0.1 mL of 5% titanium sulphate and 0.2 mL ammonia, and then centrifuged for 10 min at 6000 g and 4 °C. The pellets were dissolved in 3 mL of 10% (v.v) H_2SO_4 and centrifuged for 10 min at 5000 g. Absorbance of the supernatant phase was measured at 410 nm. Furthermore, the hydrogen peroxide content was calculated using hydrogen peroxide as the standard solution and then was expressed as µmol g^{-1} on fresh weight basis.

Assays of enzyme activity

Polyphenol oxidase was extracted and assayed by the method of Luh and Phithakpol (1972). The assay medium contained 0.1 ml of enzyme extract, 5 ml of catechol and 2 ml of phosphate buffer (pH 6.8). PPO activity was determined by measuring absorbance at 420 nm. One unit of activity was defined as the amount of enzyme required to increase one absorbance unit in the optical density of 420 nm Min^{-1}. Peroxidase activity was assayed according to Kochba *et al.* (1977). The POD reaction solution (3 ml) contained 20 mmol.l phosphate buffer (pH 6.0), 20 mmol.l guaiacol, 40 mmol.l H2O2, and 40 µl enzyme extract. One unit of activity was defined as the amount of enzyme required to increase one absorbance unit in the optical density of 470 nm min^{-1}. Protein content in the enzyme extract was estimated using the Bradford (1976) method. Specific activity of the enzymes was expressed as units per milligram protein.

Results

Chilling injury

Chilling stress symptoms increased during storage in the treated and control fruits. However, the chilling injury symptoms in the peel of treated fruits was significantly decreased as compared to the control fruits. There was a significant decreased in the browning of the fruits treated by 5 mM putrescine and 10 µmol methyl jasmonate (Figure 1). Therefore, putrescine treatment significantly ($p \leq 0.05$) alleviated the chilling injury severity of the orange fruits during storage (Figure 1).

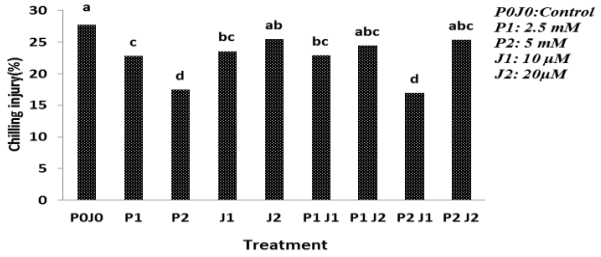

Figure 1. Effect of putrescine and methyl jasmonate on chilling injury of orange fruit

Hydrogen peroxide and Lipid peroxidation

The amount of hydrogen peroxide of peel and pulp as a function of low temperature for different months are shown in Figure 2 and Table 1. The hydrogen peroxide values increased until the 4th month of the experiment in both treated and non-treated fruits. Hydrogen peroxide content of fruits was lower in fruits treated with putresine and methyl jasminate than controls (Table 1 and Figure 2). There was a significant decrease in hydrogen peroxide content in fruits treated with 5 mM putresine and 10 µM methyl jasminate. The effects of putresine and methyl jasminate on lipid peroxidation content in peel and pulp of orange fruits subjected to low temperature are shown in Figures 3 and 4. In general, lipid peroxidation content increased linearly and reached to the highest level in the 4th month of storage in the low temperature in controls as compared to the fruits treated with 5 mM putresine and 10 µM methyl jasminate treatments. Therefore, by decreasing the chilling stress symptoms during storage, lipid

Table 1. The effect of putrescine and methyl jasmonate on peel hydrogen peroxide values of orange fruit

Treatment		Hydrogen peroxide value (µg gFW^{-1})			
		Time (month)			
P (mM)	J (µM)	1	2	3	4
0	0	13.4^{e-j}	15.6^{cd}	16.4^{bc}	18.4^{a}
	10	11.6^{i-n}	12.2^{h-m}	12.6^{g-l}	14.5^{def}
	20	12.5^{g-l}	13.5^{e-i}	13.9^{d-h}	15.2^{cde}
2.5	0	11.0^{o}	1.5^{j-m}	12.2^{h-m}	2.1^{l-k}
	10	8.1^{i-n}	12.5^{g-l}	13.1^{l-k}	13.8^{d-h}
	20	12.0^{h-m}	13.2^{l-k}	14.2^{d-g}	15.1^{cde}
5	0	10.5^{mno}	11.0^{l-o}	11.6^{i-n}	12.4^{g-m}
	10	9.3^{o}	10.0^{no}	10.7^{l-o}	11.3^{k-n}
	20	12.1^{hm}	14.5^{def}	16.5^{bc}	17.9^{ab}

*Means with different letters are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

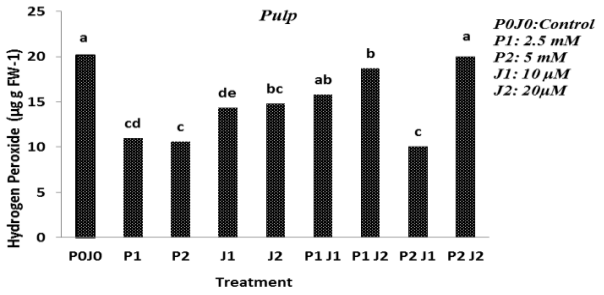

Figure 2. Effect of putrescine and methyl jasmonate on pulp hydrogen peroxide of orange fruit

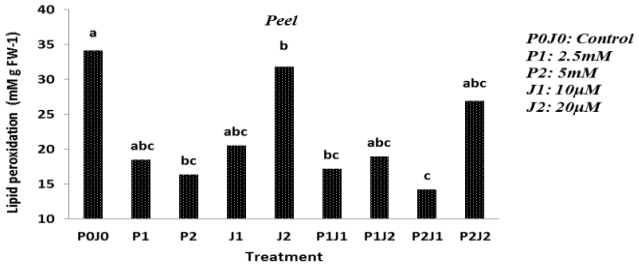

Figure 3. Effect of putrescine and methyl jasmonate on peel lipid peroxidation of orange fruit. P_0J_0: Control

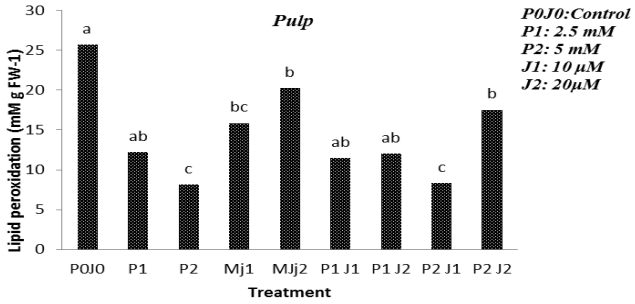

Figure 4. Effect of putrescine and methyl jasmonate on pulp lipid peroxidation of orange fruit

peroxidation content was also increased.

Effect of putrescine and methyl jasmonate on activities of enzyme

The POD is very important enzyme for scavenging active oxygen species, thus protecting biological membranes. Effects of putrescine and methyl jasmonate on cold induced changes in the activities of POD is shown in Table 2, 3. Changes in POD activities in orange fruits showed a pattern similar to the chilling injury during cold storage. The activity of POD first increased, reached a peak value on the second month of storage and then decreased up to the end of storage. Exogenous putrescine and methyl jasmonate enhanced the activity of POD. The activity of POD was significantly ($p \leq 0.05$) higher in 5 mM putrescine and 10 µmol methyl

jasmonate treated fruits as compared to the control fruits (Tables 2 and 3).

PPO activity was affected by putrescine and methyl jasmonate. The significant increase in PPO activity from tissue excised from the orange fruit exposed to chilling temperature can be regarded as a qualitative indicator of chilling injury. As it is observed in Tables 4 and 5, the rapid increase in the PPO activity appeared during storage, and the control fruits had a higher PPO activity than the putrescine and methyl jasmonate treated samples.

Table 2. Effect of putrescine and methyl jasmonate on peel peroxidase values of orange

Treatment		Peroxidase value (unit.mg protein)			
		Time (month)			
P (mM)	J (µM)	1	2	3	4
0	0	29.5^{r}	30.12^{qr}	31.0^{pqr}	32.1^{n-r}
	10	35.0^{h-n}	35.5^{g-l}	36.4^{d-j}	37.5^{c-i}
	20	31.2^{o-r}	32.6^{l-q}	33.0^{l-q}	34.5^{i-n}
2.5	0	35.2^{h-m}	36.2^{e-j}	37.4^{c-i}	39.0^{b-f}
	10	36.0^{t-k}	36.9^{d-j}	37.8^{c-h}	38.5^{b-g}
	20	36.1^{e-j}	38.0^{c-h}	38.5^{b-g}	39.0^{b-f}
5	0	38.4^{b-g}	39.4^{bgd}	40.1^{abc}	41.2^{ab}
	10	39.1^{b-e}	40.2^{abc}	41.2^{ab}	42.8^{a}
	20	32.5^{m-q}	33.1^{k-p}	34.1^{k-p}	35.2^{h-m}

*Means with different letters are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 3. Effect of putrescine and methyl jasmonate on pulp peroxidase values of orange

Treatment		Polyphenol oxidase value (unit.mg protein)			
		Time (month)			
P (mM)	J (µM)	1	2	3	4
0	0	23.5^{a}	23.1^{ab}	22.5^{abc}	21.5^{a-d}
	10	20.5^{a-f}	19.8^{a-h}	19.4^{b-h}	18.2^{d-i}
	20	22.5^{abc}	21.4^{a-e}	19.9^{a-h}	19.1^{c-h}
2.5	0	19.6^{a-h}	19.0^{c-i}	18.5^{b-i}	17.4^{f-j}
	10	20.2^{a-g}	19.2^{b-h}	19.1^{c-h}	18.5^{d-i}
	20	20.8^{a-f}	19.5^{b-h}	18.5^{b-i}	17.5^{e-j}
5	0	17.5^{e-j}	16.1^{hij}	15.2^{ijk}	14.0^{jkl}
	10	16.4^{g-j}	12.5^{kl}	12.0^{kl}	11.2^{l}
	20	22.1^{a-d}	21.0^{a-f}	20.0^{a-h}	19.4^{b-h}

*Means with different letters are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 4. Effect of putrescine and methyl jasmonate on peel polyphenol oxidase values of orange

Treatment		Polyphenol oxidase value (unit.mg protein)			
		Time (month)			
P (mM)	J (µM)	1	2	3	4
0	0	22.5^{a}	21.5^{a-d}	20.2^{b-g}	19.8^{c-g}
	10	19.8^{c-g}	19.2^{e-i}	20.8^{a-f}	17.4^{hij}
	20	21.9^{abc}	21.0^{a-e}	20.8^{a-f}	20.2^{b-g}
2.5	0	19.6^{d-g}	19.0^{e-i}	18.2^{g-j}	18.0^{g-i}
	10	20.2^{b-g}	19.6^{d-g}	19.0^{e-i}	18.2^{g-j}
	20	21.0^{a-e}	20.1^{b-g}	19.7^{d-g}	19.0^{e-i}
5	0	18.5^{g-j}	18.0^{g-j}	17.4^{hij}	16.5^{jk}
	10	17.3^{ij}	16.5^{jk}	15.2^{kl}	14.5^{l}
	20	22.0^{ab}	21.6^{a-b}	21.1^{a-e}	19.5^{d-h}

*Means with different letters are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 5. effect of putrescine and methyl jasmonate on pulp polyphenol oxidase values of orange

Treatment		Peroxidase value (units.mg protein)			
		Time (month)			
P (mM)	J (µM)	1	2	3	4
0	0	34.0^{f}	35.8^{def}	36.2^{c-f}	37.0^{b-f}
	10	36.0^{c-f}	36.5^{c-f}	37.5^{b-f}	38.2^{a-f}
	20	34.5^{ef}	35.2^{def}	36.9^{b-f}	37.3^{b-f}
2.5	0	37.0^{b-f}	38.0^{a-f}	38.5^{a-f}	39.5^{a-f}
	10	36.8^{c-f}	37.8^{a-f}	38.1^{a-f}	38.5^{a-f}
	20	36.2^{c-f}	37.1^{b-f}	37.8^{a-f}	38.2^{a-f}
5	0	38.5^{a-f}	39.9^{a-e}	40.1^{a-f}	41.5^{abc}
	10	39.9^{a-e}	40.5^{a-d}	42.5^{ab}	43.1^{a}
	20	35.0^{def}	36.1^{c-f}	37.0^{b-f}	38.0^{a-f}

*Means with different letters are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Discussion

In the present study, exogenous pre-treatment with putrescine and methyl jasmonate significantly decreased the chilling injury in 'Valencia' orange as compared to the untreated fruits (Figure 1). Thus putrescine and methyl jasmonate have potential application in postharvest treatment by alleviating chilling injury and maintaining quality. Polyamines are important small molecules involved in many plant physiological processes (Vams-Vigyazo 1981). It has been indicated that polyamines protect plant

cells against oxidative stress by reducing ROS accumulation (Oliveia *et al.* 2009). The toxicity of ROS is due to their reactions with numerous cell components causing a cascade of oxidative reactions and the consequent inactivation of enzymes, lipid peroxidation, protein degradation and DNA damage (Hodges *et al*. 2004). Plants are protected against ROS effects by a complex antioxidant system (Apel and Hirt 2004). This involves both nonenzyme antioxidants (ascorbate and α-tocopherol) and enzymes such as catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), POD and PPO (Lamikanra and Watson 2000). Oxidative damage only ensues when this complex system fails to limit ROS accumulation (Xu *et al*. 2010).

Putrescine and methyl jasmonate have been applied to reduce the development of chilling injury symptoms in a number of horticultural crops (Parkin *et al.* 1989; Duan *et al.* 2008; Avanci *et al.* 2010; Gill and Tuteja 2010), maintaining quality (Sanchez-Ballesta *et al.* 2000; Hisaminato *et al*. 2001) and decrease the lipid peroxidation and hydrogen peroxide production (Serrano *et al*. 2003). However, the mode of action of polyamines and jasmonic acid in reducing chilling injury and quality deterioration have not been clearly elucidated (Wang 1995; Nguyen *et al.* 2003). In many studies, it was found that polyamines (putrescine) and methyl jasmonate could counteract oxidative damage and have protective effect against various stress conditions (Espin *et al.* 1998). Lipid peroxidation and protective enzyme systems are often evaluated in studies of plant mechanisms under various stresses (Onsa *et al.* 2000; Gawlik-Dziki *et al.* 2007). Low temperature disrupts the balance of active oxygen species metabolism, such as hydrogen peroxide, leading to their accumulation and destruction of scavenging enzymes such as SOD, CAT, POD and APX (Gawlik-Dziki *et al.* 2007). Alternatively, the accumulation of ROS would also induce lipid peroxidation, damage membrane structure and cause solute leaking (Lee *et al.* 2007). Similar to results of our experiment, it has been reported that the improvement of chilling tolerance in the harvested horticultural crops is related to enhancement in the activities of antioxidant enzymes (Lamikanra *et al.* 2001; Apel and Hirt 2004). Sala (1998) found that the chilling tolerant mandarins have a higher antioxidant enzyme activity than the chilling sensitive cultivars. A number of postharvest treatments that induce chilling tolerance and alleviate chilling injury (e.g. heat shocks, low temperature conditioning, atmospheric oxygen treatment) also enhance antioxidant enzyme activity (Ye *et al.* 2007).

Orange fruits are relatively sensitive to chilling injury, i.e. they show some symptoms at temperatures 2-5 ˚C and at these temperatures the symptoms of chilling injury are serious. But orange must be stored at the temperature of 2-7 ˚C (Sanchez-Ballesta *et al.* 2000). The chilling injury symptoms in orange are usually apparent first in the peel rather than the pulp. The peroxidase and polyphenol oxidase enzymes primarily affect the ability of fresh and processed fruits and vegetables to retain their characteristic flavor and color (Hung *et al.* 2007). POD activity may result in oxidative actions that involve hydrogen donors in horticultural crops (Amako *et*

al. 1994). Stored fruits at low temperature are accompanied by the disruption of surface cells and injury of underlying tissues (Hung *et al.* 2007). Enzymatic activities increase as the consequence of the increased permeability that results from tissue disruption and mixing of enzymes and substrates that are otherwise sequestered within vacuoles. The properties of enzymes such as POD and PPO that affect fruit flavor and texture would significantly affect storage qualities of storage fruits (Lee *et al.* 2007). POD is characterized by broad specificity with respect to electron donors and participates in many physiological processes such as biosynthesis of lignin and ethylene and scavenging potentially harmful H_2O_2 (Lamikanra *et al.* 2001). POD activity could be indicative of oxidative stress in plant tissues. POD under conditions of cold storage would act to reduce potential oxidative damage to the fruit (Nguyen *et al.* 2003). Presence of putrescine and methyl jasmonate effectively increased POD activity in "Volencia" orange in this study. Changes in enzyme activity in orange as a result of the presence of putrescine and methyl jasmonate might thus be too low (Vamos-Vigyazo 1981). The low PPO activity with increase in concentration of putrescine, indicates that enzymatic browning percentage significantly was reduced in orange fruits. Oxidation of phenolic substrates by polyphenol oxidase is believed to be a major cause of the brown discoloration of many fruits and vegetables (Ye *et al.* 2007). Degree of browning was correlated with PPO activity and the concentration of free phenolic substrates. PPO activity may be a main factor in the browning reaction. PPO enzymes have often been found to localize in the chloroplasts, where they are associated with the internal thylakoid membranes (Ye *et al.* 2007).

The results of the present study showed that oranges treated with 5 mM putrescine and 10 µM methyl jasmonate had significantly lower amount of chilling injury, lipid peroxidation and hydrogen peroxide levels in the peel and pulp as compared to the non-treated fruits. Cell membrane stability has been widely used to express stress tolerance and higher membrane stability could be correlated with abiotic stress tolerance such as chilling stress (Premachandra *et al.* 1992). Uemura *et al.* (2006) indicated the necessity of an increase in membrane stability during cold-acclimation both under natural and artificial conditions. Moreover, it was stated that there are compositional, structural and functional changes occurring in the plasma membrane, which result in an increased stability of the plasma membrane under cold conditions.

It has been reported that many of the changes during acclimation to the temperature stress are reversible. However, if the stress is too high, irreversible changes can occur and these can lead to the plant death (Lester 1985). According to Shilpi and Narendra (2005) symptoms of stress induced injury in plants appear after 48 to 72 h. However, this duration varies and depends upon the sensitivity of individual plants to cold-stress. In the present study, MDA content as an expression of lipid peroxidation was increased by the low temperature treatment. However, polyamines protect plant cells against oxidative stress by reducing ROS accumulation (Wang

1995) a finding that was also shown in the present study.

Conclusions

Our results indicated that putrescine and methyl jasmonate could improve the chilling resistance of orange by improving protective enzyme activities and decreasing the accumulation of ROS to protect membranes from chilling damage. This induction of cold resistance by putrescine and methyl jasmonate treatment may result from the stimulation of antioxidant enzyme activities, protection against membrane oxidative damage, decreased lipid peroxidation levels and reduced H_2O_2 content in the orange fruit. These results may have implications for the use of putrescine and methyl jasmonate in managing postharvest CI in other subtropical fruits that are stored at low temperatures.

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