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Exogenous polyamines alleviate chilling injury of *Citrus limon* fruit

[1]Kowaleguet, M. G., [1]Chen, F. Y., [1]Shi, W. L., [2]Wu, Z. B., [3]Wang, L. Y., [1]*Ban, Z. J., [1]Liu, L. L., [4]Wang, L. J. and [1]Wu, Y. F.

[1]*Zhejiang Provincial Key Laboratory of Chemical and Biological Processing Technology of Farm Products, Zhejiang Provincial Collaborative Innovation Centre of Agricultural Biological Resources Biochemical Manufacturing, School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou 310023, China*
[2]*Economic Forest Research Institute, Xinjiang Academy of Forestry Sciences, Urumqi 830000, China*
[3]*Aksu Youneng Agricultural Technology Group Co., Ltd., Aksu 843001, China*
[4]*School of Environmental and Natural Resources, Zhejiang University of Science and Technology, Hangzhou 310023, China*

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Abstract

The present work investigated the alleviation of chilling injury in response to exogenous polyamines in “Eureka” lemon (*Citrus limon*) fruits stored at low temperature. The lemon fruits were immersed either in polyamine solutions [1 mmol/L putrescine (PUT), 1 mmol/L spermidine (SPD), or 0.5 mmol/L PUT + 0.5 mmol/L SPD (combined)] or in distilled water (control). The morphology, cellular structure (using transmission electron microscopy), chilling injury (CI) index, total soluble solids (TSS), titratable acid (TA), malondialdehyde contents, and membrane permeability, as well as the peroxidase (POD) and polyphenol oxidase (PPO) activities of the lemon fruits were measured after 0, 15, 30, and 45 days of storage at -2 ± 0.5°C. Results showed that lemon fruits treated with polyamine had higher amounts of TSS and TA, as well as POD and PPO activities. The PUT, SPD, and combined treatments exhibited significantly reduced electrolyte leakage and less evidence of chilling injury. This indicated that the synergistic effects of PUT and SPD protected the fruit from chilling injury and maintained the postharvest quality of the lemon fruits better than PUT or SPD alone did.

Introduction

"Eureka" lemon (*Citrus limon*) fruits, which are thought to have originated from Argentina, are the most widely cultivated lemon cultivar in the world (Orjuela-Palacio *et al.*, 2019). Lemon fruits, like other citrus species, contain water, acids (citric and malic), soluble sugars (fructose, glucose, and sucrose), pectin, carotenoids, vitamin C, and flavonoids (Baruah and Kotoky, 2018). Due to its unique chemical composition, lemon juice is often preferred by consumers and nutritionists (El-Otmani *et al.*, 2011).

Chilling injury (CI) is a physiological disorder that affects subtropical and tropical fruits stored at lower than optimum temperatures (Valero and Serrano, 2010). Chilling injury symptoms vary depending on the species, intensity of the cold storage conditions, cultivar, and farming conditions. For example, a significant increase in CI was observed in bell pepper stored at 4°C (Wang *et al.*, 2019). Moreover, eggplant fruit exhibited either flesh browning or seed blackening when it was subjected to prolonged storage at 2°C, and then transferred to room temperature conditions (20°C) for one day (Tsouvaltzis *et al.*, 2020). Banana fruit is also susceptible to CI, mainly developing pitting and browning after being exposed to temperatures lower than 13°C (Guo *et al.*, 2018). In recent years, some exogenous treatments to alleviate horticultural product post-harvest CI have been reported. Treating banana fruit with hydrogen sulphide using a technique called fumigation, for instance, could increase P5CS activity and proline content in it, thereby inhibiting the development of cold damage during cold storage and ripening stage (Luo *et al.*, 2015; Li *et al.*, 2016b). In addition, methyl jasmonate, salicylic acid, and polyamines have been shown to

*Corresponding author.
Email: banzhaojun@zust.edu.cn

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alleviate CI symptoms (Cao *et al.*, 2010; Saini *et al.*, 2017).

Polyamines (PA) are polycationic molecules that stabilise the cell membrane, thereby minimising permeability, damage, and the loss of fluidity in the membrane during storage at cold temperatures (Martínez-Téllez and Lafuente, 1993; Palma *et al.*, 2014). Exogenous PA have been shown to inhibit the decrease of TSS and TA during storage. Additionally, Martínez-Téllez *et al.* (2002) demonstrated that exogenous PA inhibited the electrolyte leakage and *in vitro* polygalacturonase (PG) activity of zucchini. These findings suggest that there is a molecular mechanism controlling CI resistance (Davarynejad *et al.*, 2013). Exogenous PA are important factors in other kinds of stress responses too, including ozone (Singh *et al.*, 2015) and cold tolerance (Patel *et al.*, 2019; Phornvillay *et al.*, 2019).

Therefore, the present work's objective was to elucidate the role of exogenous PA in reducing CI and maintaining lemon quality during storage at low temperatures. To this end, the CI index, TSS content, TA, MDA concentration, and antioxidant-related enzymes activity levels of lemon fruits treated with PA were compared against those untreated with PA following cold storage.

Materials and methods

Plant materials

"Eureka" lemon (*Citrus limon*) fruits were harvested in September 2019 by the Sichuan Ningdian Agriculture Co., Ltd. from an orchard in Anyue County, Sichuan Province, China. Fully matured lemon fruits (yellow/yellow-green coloured skin) with a uniform size (5 to 7 cm) and appearance (slightly glossy) were selected from the batch, and immediately transported to the laboratory at the Zhejiang University of Science and Technology, Hangzhou, China. Lemon fruits without injuries, mechanical damages, diseases, blemishes, and pests were selected for the trial. Three replicates with five lemon fruits per replicate were immediately sampled to evaluate their quality attributes at harvest (day 0). The lemon fruits were then randomly divided into four groups, and completely submerged in either distilled water (control), 1 mmol/L putrescine (PUT) solution, 1 mmol/L spermidine (SPD) solution, or 0.5 mmol/L PUT + 0.5 mmol/L SPD solution (PUT + SPD) for 6 min. The treated lemon fruits were left for 3 h to air dry at 25°C. Thereafter, the lemon fruits were stored at -2 ± 0.5°C with a relative humidity of 85%. Quality attributes were examined after 0, 15, 30, and 45 days of storage. The experiments were carried out for each treatment and sampling date using three biological replicates with five lemon fruits per replicate.

Microstructure analysis

The microstructures of the lemon fruits' mesocarp and pericarp were observed using transmission electron microscopy (TEM, Hittachi Model H-7650, Japan) (Bu *et al.*, 2013).

Chilling injury (CI) development

The CI index was evaluated based on a rating scale depending on the total area of CI on the peel surface (Li *et al.*, 2016a). The rating scale used was 0 = normal (no injury); 1 = ≤ 10% chilling injury area; 2 = 10 - 20% chilling injury area; 3 = 20 - 30% chilling injury area; and 4 = ≥ 30% chilling injury area. The CI index was calculated using Eq. 1:

CI (%) = Σ (chilling scale rating × number of lemon fruits in the group) / (total number of lemon fruits × highest chilling scale rating) × 100% (Eq. 1)

Total soluble solids (TSS) and titratable acidity (TA)

The lemon pulp was ground with a mortar, crushed, and sieved to obtain its juice. A digital refractometer was used to measure the total soluble solids (TSS) content of the juice, and a digital acidity assay was performed to measure the titratable acidity (TA). Results were expressed as a percentage.

Malondialdehyde content and membrane permeability (electrolyte leakage)

The malondialdehyde (MDA) content was determined following a method described by Siboza and Bertling (2013). Briefly, lemon pulp (0.2 g) was obtained by homogenising the pulp from five lemon fruits in 1.8 mL of 5% trichloroacetic acid (TCA). The homogenate was centrifuged for 20 min at 12,000 rpm. The supernatant was mixed with 1.5 mL of 0.67% thiobarbituric acid (TBA), heated for 30 min at 100°C, immediately cooled in ice, and then centrifuged for another 10 min at 3,000 rpm. The absorbance of the solution was measured at 450, 532, and 600 nm, and the MDA content was expressed in nmol/g FW.

The membrane permeability was determined by measuring electrolyte leakage (EL) according to

Huang *et al*. (2012) with some modifications. Briefly, the lemon rind (2 mm thick granules) was dipped in distilled water, and incubated with continual shaking at 23°C for 3 h. The initial EL level was measured using a conductivity meter. The solution was then incubated with steady oscillation at 100°C for 1 h before the total EL was estimated. The EL was expressed as a percentage (%) of the total electrolytes.

Peroxidase (POD) and polyphenol oxidase (PPO) activities

The POD and PPO activities in lemon fruits were determined using a Micro POD Assay Kit (Solarbio BC0095-100T/96S, Beijing, China) and Micro PPO Assay Kit (Solarbio BC0190-50T/24S, Beijing, China), respectively. The extraction was carried out by homogenising 0.1 g of frozen powder with 1 mL of extraction solution, and centrifugation at 8,000 *g* and 4°C for 10 min following the manufacturer's instructions. The absorbance of the reaction solution was measured at 470 nm for POD, and 410 nm for PPO. The results were expressed in U/g FW.

Statistical analysis

The experiment was conducted in a completely randomised design. All data were reported as means with standard deviations (± SD), and analysed using the One-way analysis of variance (ANOVA) test in SPSS version 20.0 (SPSS Inc., Chicago, USA). The differences were considered significant if the *p*-value was smaller than the alpha value ($\alpha = 0.05$).

Results

Lemon morphology and microstructure

Lemon fruits are susceptible to chilling injury (CI), especially when stored at low temperatures. As shown in Figure 1a, all the symptoms of decay decreased significantly with the PUT, SPD, and PUT + SPD treatments relative to the control treatment. The treated lemon fruits developed less brown skin and had better visual morphology scores than the control lemon fruits after being stored for 45 days.

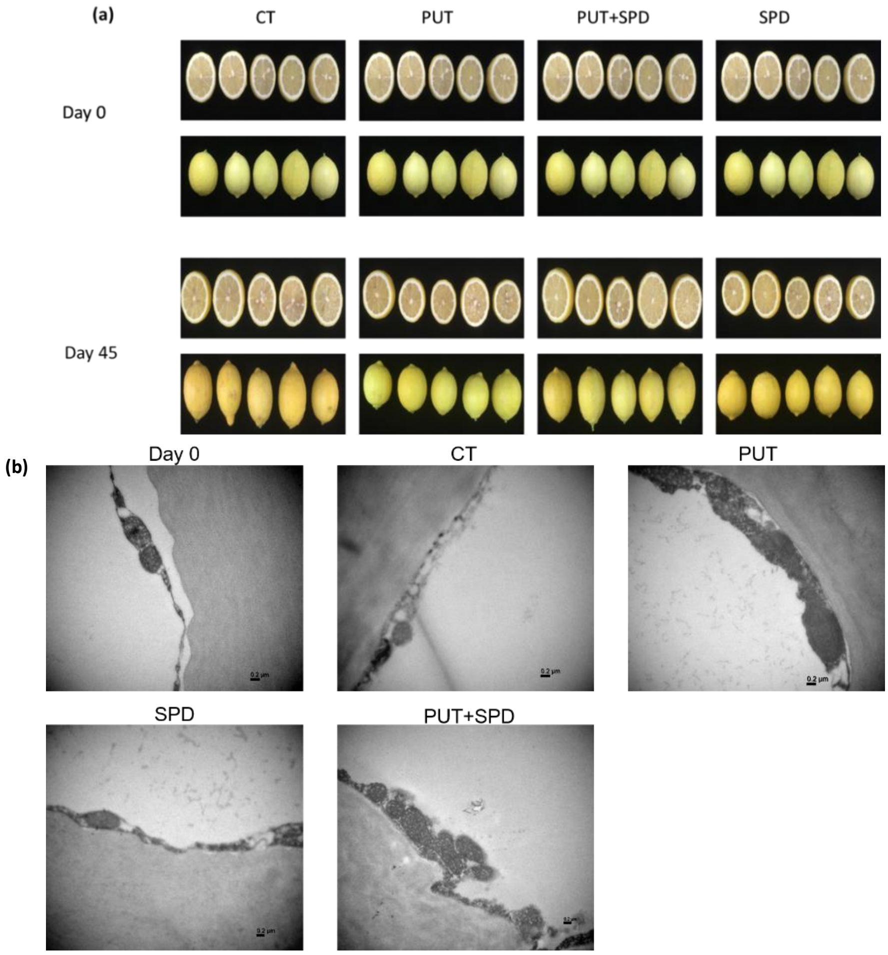

Figure 1. Morphology of lemon fruits **(a)** and TEM images of lemon pericarp **(b)** before (0 days) and after (45 days) being stored at -2°C. The lemon fruits were either treated with water (CT), 1 mM putrescine (PUT), 1 mM spermidine (SPD), or 0.5 mM putrescine (PUT) + 0.5 mM spermidine (SPD).

TEM was used to observe changes in the microstructure of the cell membrane before (0 day) and after (45 days) cold storage (Figure 1b). At day 0, the plasma membranes remained within the confines of the cell membrane, and cell wall integrity was high in the lemon fruits. After 45 days of storage, the integrity of the cell membrane decreased, and the cell wall appeared partially detached in the control lemon fruits. In the treated lemon fruits, however, cell membrane and cell wall integrity were almost intact.

Chilling injury (CI)

The CI index of lemon fruits was evaluated after 0, 15, 30, and 45 days of storage at -2 ± 0.5°C as shown in Figure 2.

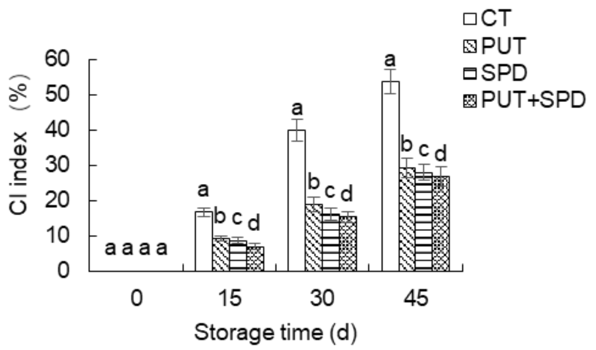

Figure 2. The chilling injury (CI) indexes of lemon fruits stored at -2°C. The lemon fruits were either treated with water (CT), 1 mM PUT, 1 mM SPD, or 0.5 mM PUT + 0.5 mM SPD. Values are mean of three replicates (n = 3) with error bars indicating standard deviation (± SD). Means that do not share the same lowercase letter are significantly different from each other ($p < 0.05$).

The CI severity increased with the storage time. After 15 days of storage, all treated lemon fruits exhibited symptoms of CI, but the control lemon fruits had a significantly higher CI index. After 30 days, the CI index of the treated lemon fruits was almost the same as that of the control lemon fruits at 15 days. The treated lemon fruits had significantly reduced CI index scores after 15, 30, and 45 days of storage relative to control lemon fruits ($p < 0.05$), and combining the PUT and SPD treatments was more effective than PUT or SPD alone.

TSS and TA

The TSS was significantly affected by the treatments (PUT, SPD, and PUT + SPD) administrated prior to fruit storage (Figure 3a). The amount of TSS obtained from the lemon fruits increased in the first 15 days regardless of treatment, but gradually decreased in control lemon fruits and SPD as storage progressed. In lemon fruits treated with PUT, the amount of TSS remained constant until day 30 of storage, at which point TSS began to slightly increase. TSS increased in PUT + SPD within the first 15 days, reached its peak by day 30, then gradually declined during the remaining storage period.

The TA changed during storage in all the treatments (Figure 3b). TA decreased dramatically in control lemon fruits within the first 15 days, stayed constant from day 15 - 30, and then declined during the remaining storage period. TA remained constant in PUT and PUT + SPD from 0 - 15 days, and then decreased during the remaining storage period. The rate of TA degradation was faster in the SPD-treated lemon fruits by day 30 of storage, but SPD still had a higher TA than control lemon fruits did by day 15.

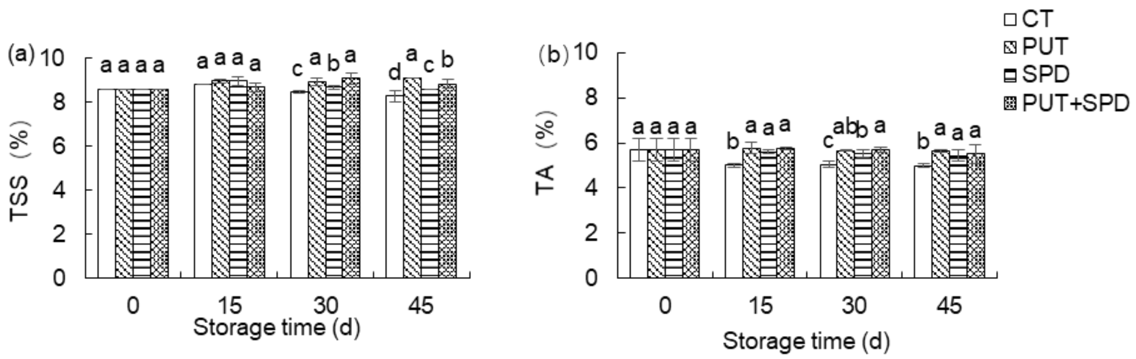

Figure 3. Total soluble solid (TSS) **(a)** and titratable acidity (TA) **(b)** contents of lemon fruits stored at -2°C. The lemon fruits were either treated with water (CT), 1 mM PUT, 1 mM SPD, and 0.5 mM PUT + 0.5 mM SPD. Values are mean of three replicates (n = 3) with error bars indicating standard deviation (± SD). Means that do not share the same lowercase letter are significantly different from each other ($p < 0.05$).

MDA content and membrane permeability

As shown in Figure 4a, the MDA content increased in all treatments; but lemon fruits treated with PA exhibited only slight increase in MDA content during storage, whereas the control lemon fruits showed a remarkable increase, particularly after 15 and 30 days. The results showed that PA treatment postponed MDA content increase in lemon fruits at chilling temperature during storage.

A similar result was also observed for electrolyte leakage (EL), wherein treated lemon fruits had lower EL values than control lemon fruits after 15 and 30 days of storage at -2 ± 0.5°C (Figure 4b).

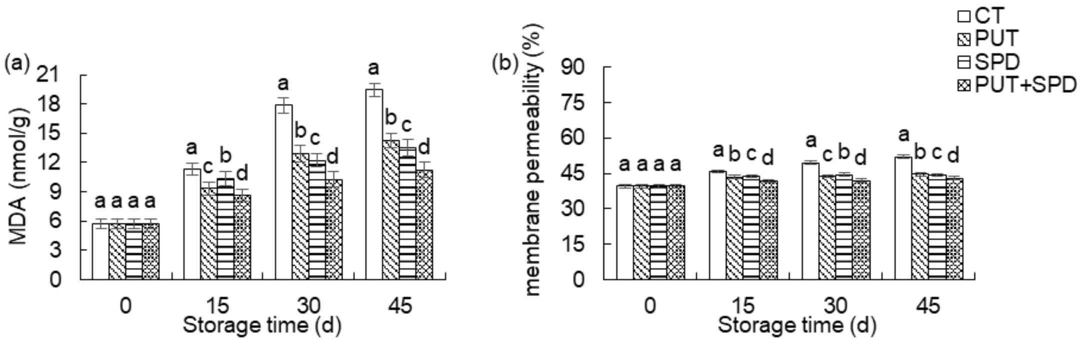

Figure 4. Malondialdehyde (MDA) contents **(a)** and membrane permeability **(b)** of lemon fruits stored at -2°C. The lemon fruits were either treated with water (CT), 1 mM PUT, 1 mM SPD, or 0.5 mM PUT + 0.5 mM SPD. Values are mean of three replicates ($n = 3$) with error bars indicating standard deviation (± SD). Means that do not share the same lowercase letter are significantly different from each other ($p < 0.05$).

POD and PPO activities

The POD activity increased in control lemon fruits, and SPD- and PUT-treated lemon fruits within 15 days of storage at -2 ± 0.5°C (Figure 5a). From day 15 - 30, an increase in POD activity in the PUT and PUT + SPD treatments, and a decrease in POD activity in the control lemon fruits and SPD treatments were observed. From day 30 - 40, POD activity decreased at a similar rate in all treatments.

As shown in Figure 5b, 1 mmol/L PUT, 1 mmol/L SPD, and 0.5 mmol/L PUT + 0.5 mmol/L SPD increased PPO activity in lemon fruits after 45 days of storage at -2 ± 0.5°C. PPO activity increased within the first 15 days of storage in all the treatments. PPO activity reached its peak in the SPD treatment on day 15. Activity in SPD then dramatically decreased by day 30, and remained constant until the end of the storage period. PPO activity decreased in PUT but increased in the PUT + SPD treatment throughout the storage period. PPO activity slightly increased in the control lemon fruits by day 30, then decreased prior to day 45.

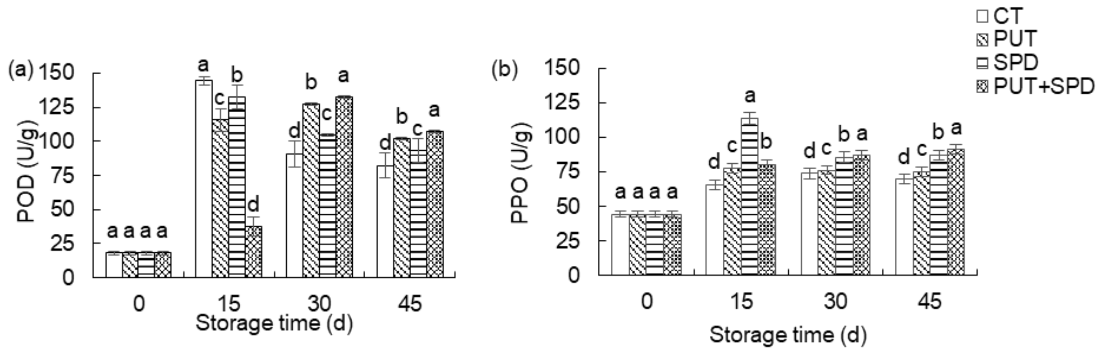

Figure 5. Peroxidase (POD) **(a)** and polyphenol oxidase (PPO) **(b)** activities of lemon fruits stored at -2°C. The lemon fruits were either treated with water (CT), 1 mM PUT, 1 mM SPD, or 0.5 mM PUT + 0.5 mM SPD. Values are mean of three replicates ($n = 3$) with error bars indicating standard deviation (± SD). Means that do not share the same lowercase letter are significantly different from each other ($p < 0.05$).

Discussion

CI symptoms appeared on untreated lemon fruits after two weeks of storage at -2 ± 0.5°C. Lemon fruits that were treated with PA, on the other hand, exhibited less CI. Our findings have been confirmed by previous studies. For instance, Raeisi *et al.* (2013) demonstrated fruits treated with PA showed no sign of CI symptoms after being stored at 3°C and SPD. Patel *et al.* (2019), meanwhile, studied the effect of PA application on green bell peppers stored for 40 days at 4 ± 1°C. They found that 20 μmol/L SPD combined with 20 μmol/L PUT preserved the quality of green bell pepper the best. Furthermore, Valero *et al.* (2002) reported that the exogenous application of PA and abscisic acid maintained the firmness of lemon fruits. Of all they tested, PUT resulted in the lowest amount of weight loss and high level of firmness relative to calcium treatment and control.

In fruits stored below their chilling point, the cell membrane is converted from liquid-crystalline into solid-gel state. This directly influences ion leakage and membrane permeability which could have provided protection from CI (Galindo *et al.*, 2004). Then, in a later study by Shu *et al.* (2015), it was shown that PUT reduced salt-induced breakage of cell membranes in cucumber seedlings. Zhang *et al.* (2020) demonstrated that the appearance of CI symptoms on jujube fruit stored at low temperature storage was delayed by PA. The treatment also increased the cold tolerance and expanded the shelf life of the jujube fruit. Another study found that CI enhanced the degree of lipid peroxidation in bell pepper by triggering the production of H_2O_2, eventually causing fluid leakage (Ge *et al.*, 2019). In the present work, PA treatment inhibited the development of CI in lemon fruits. Moreover, the lemon fruits showed fewer symptoms of CI, maintained better cell structure, and maintained better cell membrane integrity.

Total soluble solids (TSS) are the main substrate consumed during cellular respiration. The utilisation of sugars previously caused TSS levels to decrease in pomegranates (cv. Wonderful) stored at 5, 7.5, and 10°C for five months (Fawole *et al.*, 2020). Interestingly, storing *'Malas Yazdi'* pomegranate treated with 4% calcium chloride + 1 mmol/L SPD at 2°C for 4.5 months also significantly decreased TSS levels (Ramezanian *et al.*, 2010). PUT application has been reported to affect TSS in many fruits. For example, in strawberry fruits and *Aloe vera*, TSS decreased following PUT treatment (Zafari *et al.*, 2015). On the other hand, Khan *et al.* (2008) reported that PUT treatment did not decrease the TSS content in 'Angelino' plums stored at low temperatures (0°C). Our results agree with Kou *et al.* (2019) who showed that increases in TSS content were related to hydrolytic transformations which are crucial for fruit ripening; we reasoned that a decrease in respiration rate and/or an increase in primary and secondary metabolism could have caused TSS levels to increase in the lemon fruits. In PUT-treated 'Langra' mango fruit stored for four weeks at 13°C, TSS increased and acidity decreased (Jaw *et al.*, 2012). Serrano *et al.* (2003) observed that TA levels decreased in four plum cultivars stored at 20°C following PUT treatment. Furthermore, Barman *et al.* (2011) showed that TA levels were reduced following PUT treatment in pomegranate fruit (cv. *Mridula*) stored for 60 days at 3°C. In the present work, the results showed that TA of lemon fruits in the control group decreased significantly, and that those in the PA-treated group remained essentially unchanged during storage. It may be that the lemon fruit quality was maintained by PA treatment because the pH was lower, since this would have prevented fungal infections and modulated the production of ethylene.

MDA is produced during lipid peroxidation, and directly related to the stability and integrity of cell membranes of fresh produce. Zhang *et al.* (2020) showed that PUT and SPD treatment inhibited MDA accumulation in jujube fruit. Moreover, Gupta *et al.* (2013) reported that oxidative stress in okra stored at low temperatures was improved by PUT treatment because of its antioxidant properties. MDA accumulation was, likewise, inhibited by PUT treatment (2 mmol/L) in okra pods (Phornvillay *et al.*, 2019). The present work demonstrated that MDA and EL levels were lower in PA-treated lemon fruits than they were in control lemon fruits. The reduction of membrane leakage was due to PA treatments. PUT and SPD protect chilling-sensitive plant tissues by reducing the amount of ROS accumulated during chilling.

In jujube fruits stored at -2°C, a combination of PUT and SPD dramatically improved and stimulated the POD and PPO activity (Zhang *et al.*, 2020). Our research results also showed that the combined treatment of PUT and SPD has a significant effect on the activities of POD and PPO.

Conclusion

The present work demonstrated that the application of 1 mmol/L PUT, 1 mmol/L SPD, and especially a combination of 0.5 mmol/L PUT and 0.5 mmol/L SPD could alleviate chilling injury of lemon fruits. Exogenous PA maintained TSS and TA levels in the fruits by maintaining higher levels of PPO and POD activities, thereby maintaining cell membrane integrity, delaying senescence, enhancing cold tolerance, alleviating CI, and reducing MDA accumulation during storage. The present work also demonstrated once more that exogenous PA applications could prevent chilling injury during postharvest cold storage of chilling-sensitive fruits and vegetables. Further molecular studies are needed to fully understand the complex effects of exogenous PA on chilling stress.

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