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ORIGINAL ARTICLE

Effect of 24-epibrassinolide treatment on the metabolism of eggplant fruits in relation to development of pulp browning under chilling stress

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Abstract This study aims to investigate the effect of 24-epibrassinolide (EBR) on the metabolism in relation to development of chilling injury-induced pulp browning of eggplant fruit. The fruits were dipped for 10 min in solutions containing 10 μmM EBR and then stored at 1 °C for 15 days. Chilling injury index, weight loss, electrolyte leakage and malondialdehyde (MDA) content of control fruit increased during storage. Chilling injury improved phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD) activities, which are correlated with the increase of total phenolic content and pulp browning of eggplant fruit. The inhibition of pulp browning by EBR treatment was possibly attributed to preserving the cell membrane integrity, reducing total phenolic content, and decreasing PAL, PPO, and POD activities. These results suggest that EBR may inhibit chilling injury and pulp browning in eggplant fruit during cold storage.

Keywords Eggplant fruit · Brassinosteroids · Chilling injury · Pulp browning · Phenolic metabolism

Introduction

Eggplant fruit is a rich source of vitamins and dietary fibers, as well as phytonutrients, mainly phenolic compounds such as caffeic and chlorogenic acids, and flavonoids (Hanson et al. 2006; Singh et al. 2009; Mennella et al. 2010; Das et al. 2011). The established health-promoting properties have led to an increase in the consumption of eggplant fruits in recent years (Akanitapichat et al. 2010). Fresh eggplant fruits deteriorate rapidly after harvest and have a very limited shelf life at ambient temperatures. Low temperature storage is an effective method to prolong the postharvest life, however, eggplant fruits are chilling sensitive and can develop some chilling injury symptoms when stored below 7–10 °C (Fallik et al. 1995). Pulp and seed browning is the most common chilling injury symptom in eggplant fruits accompanied by the development of off-flavour (Pérez-Gilabert and García-Carmona 2000; Concellón et al. 2004). In China, eggplant fruits harvested in winter and early spring show higher levels of pulp browning compared to those harvested in other months of the year, which severely restricts the economic importance of the eggplant on account of its negative impact on palatability and consumer acceptability in terms of appearance, freshness and taste. Therefore, it is urgent to find methods to extend the storage life and maintain storage quality, especially reducing the occurrence of pulp browning for eggplant fruits.

Brassinosteroids (BRs), a group of naturally occurring plant hormones, exhibit unique biological effects on plant growth and development and resistance to biotic and abiotic stresses (Bajguz and Tretyn 2003; Kagale et al. 2007; Bajguz and Hayat 2009; Krishna 2003). Recently, BRs have also attracted much attention in the preservation of postharvest horticultural products. For example, brassinolide, the first isolated BR species, can effectively protect jujube fruit from senescence by reducing ethylene production (Zhu et al. 2010). The application of brassinolide is conductive to the better maintenance of membrane integrity and alleviation of chilling injury in tomato and mango fruits, and thus improving the storage quality during cold storage (Aghdam et al. 2012; Li et al. 2012). In addition, treatment with exogenous brassinolide increased resistance of green bell pepper fruit to low temperature stress through a mechanism that improves the activity of antioxidant enzymes (Wang et al. 2012). 24-

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Epibrassinolide (EBR) is one of the most active forms of BRs available commercially, and has a favorable safety profile (Kuzmitsky and Mizulo 1991; Malikova et al. 2008; Hu et al. 2010). The use of EBR to enhance tolerance of eggplant seedlings to salt and cold stresses has been demonstrated (Wu et al. 2014; Ding et al. 2012). However, little is known about the effects of EBR applied after harvest on the chilling injury-induced pulp browning of eggplant fruit.

Therefore, this study aims to investigate the physiological and biochemical responses of eggplant fruit to EBR treatment and to evaluate the capability of EBR as a postharvest tool to alleviate chilling injury and pulp browning during cold storage.

Materials and methods

Plant material and treatments

Eggplants (*Solanum melongena* L. cv. Brigitte) were grown in a standardized greenhouse during the spring season in a commercial farm at Xi'an in Shaanxi Province, China. The eggplant fruit used in the experiment is a variety with elongated shapes. The length and the diameter of the thickest part of the eggplant fruits were respectively about 23 cm and 6 cm. Fruits were harvested at a commercially mature stage with uniform size, sharp and color, and without mechanical damage. The harvested eggplants were divided into 25 lots of 10 fruits each. Twelve lots were immersed in 10 μM EBR for 10 min according to our preliminary experiments at ambient temperature (20 °C). Another twelve lots were immersed in water for 10 min as controls. The eggplants were cool air-dried for approximately 30 min, packed in low-density polyethylene bags, and then placed at 1 °C in a temperature-controlled storeroom. Samples were taken after 0, 3, 5, 10, and 15 days of storage at 1 °C.

Weight loss

Thirty fruits in each treatment were weighed at the beginning of the experiment and after 3, 5, 10, and 15 days of storage at 1 °C. Weight loss was calculated as Weight loss (%)=100 × $(W_0 - W_1)/W_0$, where W_0 and W_1 are the initial and final sample weights, respectively. The results were expressed as the percentage of weight loss.

Chilling injury index

The chilling injury index was evaluated following the method described by Concellón et al. (2007) with slight modifications. Symptoms of external and internal chilling injury were analyzed using 30 fruits in each treatment after 3, 5, 10, and 15 days of storage at 1 °C. The chilling injury index was determined in accordance with the following scale: 1, no damage; 2, low damage; 3, regular damage; 4, moderate damage; and 5, severe damage. The chilling injury index was calculated as chilling injury index = (Injury level × Number of fruit at that level)/Total number of fruits.

Electrolyte leakage and MDA content

Electrolyte leakage was determined using the method of Wang et al. (2012) with some modifications. Discs (3 mm×5 mm) from the pulp tissues weighing approximately 2 g from 6 fruits were obtained with a cork borer and incubated in 20 mL of 0.6 mol L^{-1} mannitol. The initial conductivity of the bathing solution at 25 °C was measured with a conductimeter (DDS-11A, Youke Instrument Co., Ltd., Shanghai, China). The solutions were boiled at 95 °C for 30 min and then cooled quickly before measuring the total electrolyte leakage. The relative electrolyte leakage was defined as a percentage of the initial electrolyte leakage.

MDA content was measured following the method described by Dhindsa et al. (1981) with some modifications. Pulp tissue (2 g) from 6 fruits was homogenized with 10 mL of 10 % trichloroacetic acid containing 0.5 % (*w*/*v*) thiobarbituric acid. The mixture was then heated at 100 °C for 10 min. After the rapid cooling of the sample to room temperature and centrifugation at 4,000 g for 15 min, the absorbance of the supernatant was measured at 450, 532, and 600 nm. MDA content was calculated as $6.45\times(OD_{532}-OD_{600})-0.56\times OD_{450}$.

Browning of pulp tissue

The browning of pulp tissue was evaluated using the method of Concellón et al. (2007). A cross section (thickness=0.5 cm) was excised from the fruit central section. The pulp lightness (L^*) was immediately measured using a Chroma Meter (SC-80C, Kangguang Instrument Co., Ltd., Beijing, China). Thirty fruits were evaluated for each treatment after 3, 5, 10, and 15 days of storage at 1 °C.

Total pehnolics

Total phenolic content was determined according to the method of Hinneburg et al. (2006). Pulp tissue (2 g) from 6 fruits was homogenized in 80 % methanol containing 0.5 N hydrochloric acid and extracted overnight for 24 h at 4 °C in the dark (Tomás-Barberán et al. 2001). The extract (0.5 mL) was added to 1.0 mL of Folin − Ciocalteu reagent. Next, 3 mL of sodium carbonate (1 M) was added to the mixture and was mixed gently. The total volume of the mixture was adjusted to 10 mL with distilled water. After the mixture had been kept at room temperature for 60 min, the absorbance was read at 760 nm. Results are expressed as the gallic acid (GA) equivalents.

Enzyme activities

Polyphenol oxidase (PPO) activity was assayed according to the method of Murr and Morris (1974). Pulp tissue (1 g) from 6 fruits was homogenized with 5 mL of 0.05 M phosphate buffer (pH 6.8). The homogenate was centrifuged at 10,000 g for 20 min at 4 °C, and the supernatant was collected as enzyme extract. The reaction mixture contained 0.5 mL of enzyme extract and 2.5 mL of 0.1 % catechol. The increase in absorbance at 420 nm was recorded for 3 min. PPO activity was defined as a 0.01 change in OD_{420} per min.

Peroxidase (POD) activity was measured using the modified method of Kochba et al. (1997) with slight modifications. The crude POD enzyme was extracted in the same manner as the crude PPO enzyme. The reaction mixture contained 2 mL of 0.05 M phosphate buffer (pH 6.8), 0.25 mL of 0.16 M guaiacol, 0.25 mL of 0.88 M H_2O_2, and 0.5 mL of crude enzyme extract. POD activity was measured by an increase in absorbance at 470 nm. One unit of POD activity was defined as a 0.01 increase in absorbance at 470 nm per min.

Phenylalanine ammonia-lyase (PAL) assay was performed according to a modified method of Assis et al. (2001). Approximately 1 g of pulp tissue from 6 fruits was homogenized with 5 mL of 0.2 M borate buffer (pH 8.8) containing 6 g of polyvinylpyrrolidone. The homogenate was centrifuged for 20 min at 10,000 g, and the supernatant was collected for enzyme activity determination. PAL activity was measured by incubating 0.5 mL of the supernatant with 2 mL of 0.2 M borate buffer (pH 8.8) and 1.0 mL L-phenylalanine (0.6 mM) for 1 h at 37 °C. An increase in absorbance at 290 nm caused by the formation of trans-cinnamate was measured spectrophotometrically. PAL activity was expressed as a 0.01 change in OD_{290} per min.

Statistical analysis

The experiments were conducted in a completely randomized design. All experiments were carried out in triplicate and average values with standard errors are reported. Analysis of variance was performed, and means were compared using Tukey's multiple range tests at a significance level of 0.05 with Origin (Version 8.0).

Results and discussion

Weight loss

It has been demonstrated that weight loss is a nondestructive indicator of chilling injury in chilling-sensitive horticultural products (Cohen et al. 1994). The weight loss in both control and EBR-treated eggplant fruits increased gradually with storage duration. EBR treatment significantly delayed the increase of weight loss, and the eggplants treated with EBR showed relatively lower weight loss in comparison with the control fruit after 5 days of storage (Fig. 1a). Storage conditions or treatments that reduce fruit water loss can alleviate chilling injury (Wang 1993). Zaharah and Singh (2011) reported that nitric oxide fumigation suppressed weight loss in mango fruits and led to the reduction of chilling injury. Taking into account the development of chilling injury in this study (Fig. 1b), EBR treatment alleviated chilling injury in eggplant fruits in relation to its positive impact on the better maintenance of moisture content is evident.

The major pathway for weight loss in eggplant is through the calyx (Díaz Pérez 1998). Calyx deterioration is one of the external chilling injury symptoms in eggplant during cold storage (Fallik et al. 1995). EBR treatment notably inhibited calyx deterioration, which might be responsible for the higher resistance to transpiration of water from the fruit. Similar

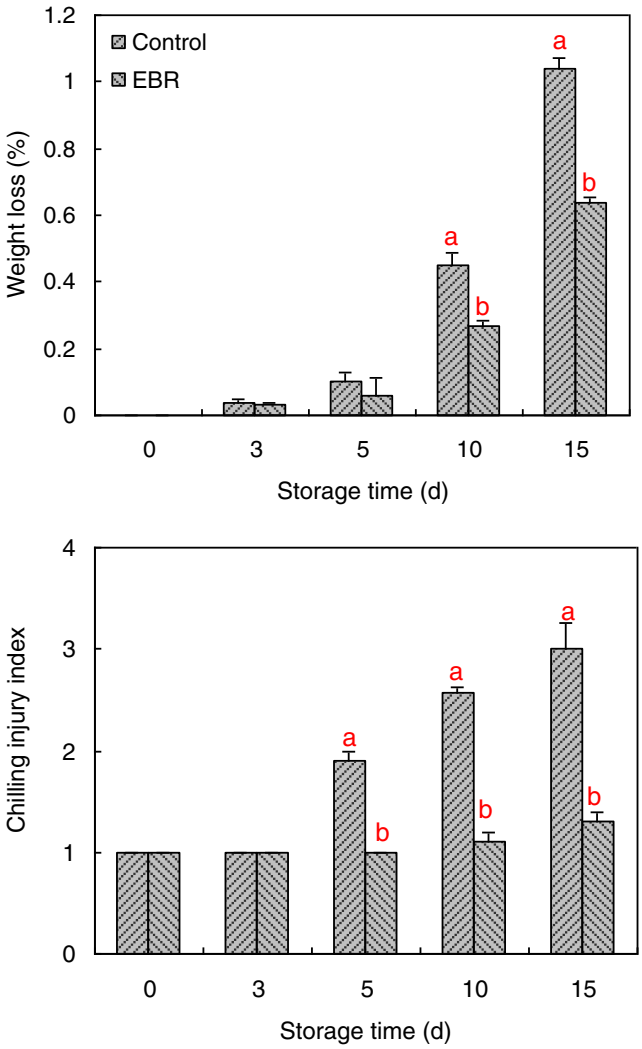

Fig. 1 Effect of EBR treatment on weight loss (**a**) and chilling injury index (**b**) of eggplant fruit during storage at 1 °C for 15 days. Values with different letters for each day were significantly different at $P<0.05$

result was also reported for eggplant fruits treated with 1-MCP during cold storage (Massolo et al. 2011).

Chilling injury index

Chilling injury easily occurred in the eggplant fruit when stored at low temperatures. In this study, chilling injury symptom was first visible as irregular skin pitting in the control fruit within 5 days of storage at 1 °C. Calyx deterioration and pulp browning were then observed. At the end of storage, the chilling injury index was as high as 3.0. The exogenous application of EBR treatment effectively retarded the increase of chilling injury index. In particular, there showed no visible chilling injury symptoms in the EBR-treated fruit during the first 5 days of storage. In addition, a 57 % decrease in chilling injury index compared with the control was observed on day 15 (Fig. 1b). These results suggested that EBR treatment activates a strong defensive response to chilling in eggplant fruits. Our results are consistent with previous reports that brassinosteroids can protect green bell pepper, tomato, and mango fruits against chilling stress (Aghdam et al. 2012; Wang et al. 2012; Li et al. 2012). In addition, Massolo et al. (2011) suggested that eggplant pulp browning can be prevented by 1-methylcyclopropene (1-MCP) during cold storage. 1-MCP may possibly act differently from EBR; but an early research showed that heat and modified atmosphere treatments can reduce chilling injury in eggplant fruit by decreasing putrescine and spermidine concentrations (Rodriguez et al. 2001).

Electrolyte leakage and MDA content

Chilling injury firstly happens in cell membrane and then membrane damage activates a series of secondary reactions finally leading to disruption of cellular and sub-cellular structures. So a significant increase in electrolyte leakage reflects the occurrence of chilling injury when fruits and vegetables are placed to chilling stress. As shown in Fig. 2a, electrolyte leakage increased rapidly for control fruits in the first 5 days of storage due to destruction of membrane integrity. Afterwards, electrolyte leakage kept constant until day 10 and then increased greatly again because of the development of irreversible chilling injury (Wang et al. 2005). Electrolyte leakage in EBR-treated fruits presented a similar trend, but EBR inhibited the electrolyte leakage increase, being about 80 % of that in the control on day 15. This demonstrated that EBR alleviates chilling-induced membrane damage in eggplant fruits. The same result was also reported for green bell pepper, tomato, and mango fruits treated with BRs during cold storage (Wang et al. 2012; Aghdam et al. 2012; Li et al. 2012). A possible mechanism recently suggested that the BRs-induced expression of membrane protein and unsaturation degree of

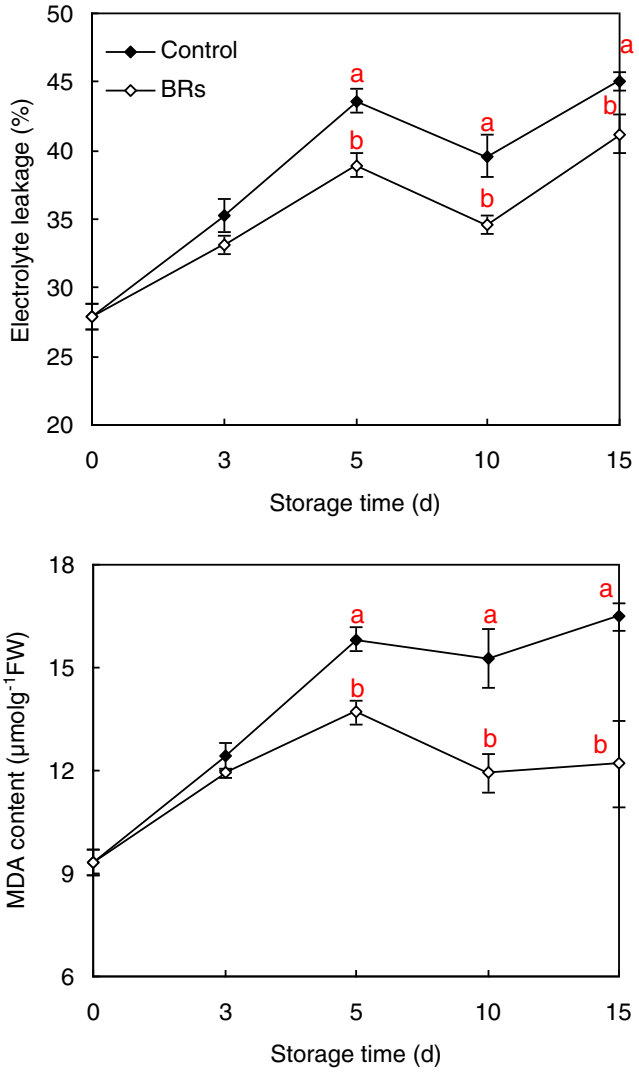

Fig. 2 Effect of EBR treatment on electrolyte leakage (**a**) and MDA (**b**) of eggplant fruit during storage at 1 °C for 15 days. Values with different letters for each day were significantly different at $P<0.05$

plasma membrane lipids is responsible for increased membrane integrity (Li et al. 2012).

MDA, another indicator of membrane damage, is a secondary end product of polyunsaturated fatty acid oxidation. Cold exposure can destroy the membrane structure because of lipid peroxidation, so an increase of MDA has been considered a marker for chilling injury. It has been demonstrated that lipid peroxidation and MDA content induced by cold stress can be suppressed by EBR treatment in cucumber and eggplant plants (Hu et al. 2010; Wu et al. 2014). In this study, the increase of MDA content in the controls progressed rapidly within the first 5 d of storage and then maintained constant. EBR treatment significantly decreased MDA content during storage except on day 3. At the end of storage, MDA content in EBR-treated fruits was 26 % lower than that in control fruits (Fig. 3b). These data showed that chilling-induced membrane

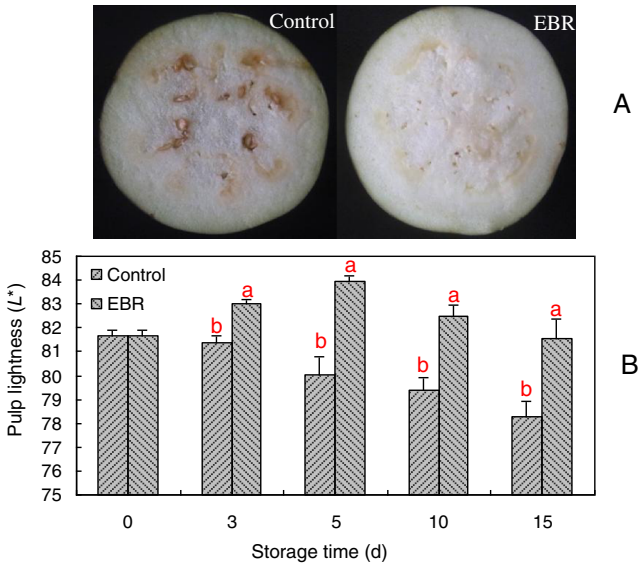

Fig. 3 Effect of EBR treatment on pulp browning appearance (**a**) and pulp lightness (**b**) of eggplant fruit during storage at 1 °C for 15 days. Values with different letters for each day were significantly different at $P<0.05$

lipid peroxidation in eggplant fruit is also effectively inhibited by EBR treatment. Browning is associated with loss of membrane integrity and cellular compartmentation that can occur during the development of chilling injury in cold-stored tissue (Luo et al. 2012). EBR treatment inhibits both ion leakage and MDA production, and thus reducing cellular decompartmentation; and finally avoided browning.

Pulp browning

Pulp and seed browning is the most common chilling injury symptom of eggplant fruit (Pérez-Gilabert and García-Carmona 2000). The same phenomenon was detected by visual inspection in this study (Fig. 3a). Present work chose the L^* values as an indicator to evaluate the pulp browning of eggplant fruits. The lower L^* values means the more serious pulp browning. As shown in Fig. 3b, the control fruits pulp had constant L^* values during the first 3 days of storage and showed a significant drop thereafter. By contrast, the L^* values of the EBR-treated fruits pulp showed a significant increment during the first 5 days of storage resulting from chlorophyll degradation, and then obviously declined during the remain time of storage. Moreover, EBR-treatment resulted in higher L^* values during the whole storage. This indicated that EBR treatment contributes to the better maintenance of eggplant pulp color under chilling stress. There was a relation between L^* values and electrolyte leakage and MDA content, which reconfirms the conclusion that EBR has protective effects against membrane disintegration.

PPO and POD activities

PPO plays a pivotal role in the process of enzymatic browning in several horticultural products (Kumar et al. 2011). It promotes enzymatic browning by catalyzing the oxidation of phenolic compounds in the presence of oxygen. The PPO activity in the control fruits was constant with the exception of a significant decrease on day 3 during the first 10 day of storage. Afterwards, it increased and reached the maximum activity on day 15, with increases of 1.6-folds in comparison with the initial activity (Fig. 4a). Fruits treated with EBR showed a similar trend, but EBR treatment significantly suppressed PPO activity during the whole storage. At the end of storage, the PPO activity of the EBR-treated eggplants was about 37 % lower than those of the controls. These results indicated that the chilling-induced PPO activity can be inhibited by EBR treatment. In this study, PPO activity

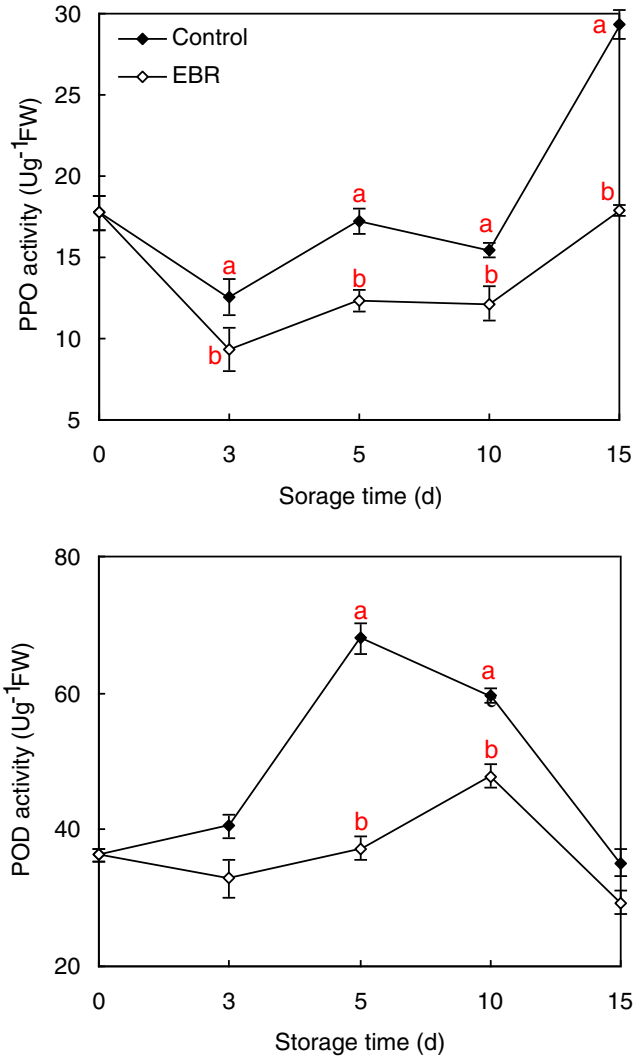

Fig. 4 Effect of EBR treatment on PPO (**a**) and POD (**b**) activities of eggplant fruit during storage at 1 °C for 15 days. Values with different letters for each day were significantly different at $P<0.05$

changed in parallel with pulp browning, which seems to suggest that chilling injury-induced pulp browning in eggplant fruits directly results from the oxidation of phenolic compounds. The result is in agreement with that previous study, where soluble PPO activity in eggplant fruits stored at 0 °C was directly related to the value for L^* (Concellón et al. 2004).

POD is also capable of oxidizing phenolic compounds to brown-colored pigments; its involvement in browning of various fruits and vegetables has been reported (Graham-Acquaah et al. 2012). According to results presented in Fig. 4B, the POD activity in the control fruits sharply increased during the first 5 days of storage, showing about 47 % increase compared with the initial level, then rapidly declined. By contrast, POD activity was significantly inhibited by the EBR treatment during the 5 and 10 day of storage. Taken POD activity and pulp L^* values together, we found that POD activity increased along with constant L^* values in the first 3 days of storage, which might be due to phenolic radicals that were formed in the process of POD-catalyzed phenolic compounds oxidation. These phenolic radicals would bleach chlorophyll to colorless compounds and thus no pulp browning was recorded. It, therefore, was suggested that POD takes part in the pulp browning of eggplant fruits. Retardation of POD activity is responsible for better maintenance of pulp color has also been reported for 1-MCP-treated eggplant fruits (Massolo et al. 2011).

Total phenolic content and PAL activity

Phenolic compounds are usually accumulated under cold stress and these contribute to pulp browning (Luo et al. 2012). The total phenolic content in the control and EBR-treated eggplants fruits displayed similar decreasing patterns during the first 3 d of storage (Fig. 5a). EBR treatment significantly accelerated this decrease in comparison with control, and the total phenolic content in EBR-treated was significantly lower than that in controls over storage. This behavior indicated that the accumulation of phenolic compounds induced by chilling injury is effectively suppressed by EBR treatment. However, Aghdam et al. (2012) reported that there was an enhancing total phenol content in brassinolide-treated tomato fruit under chilling stress. The different response might be due to the fact of difference in experimental materials, BRs species or storage methods. Phenolic compounds are substrates for enzymatic browning. Zhou et al. (2012) also showed that increase in total phenolic content occurred in peaches during the development of pulp browning.

PAL is a key enzyme for the biosynthesis of phenolic compounds, and is rapidly synthesized de novo with various stress condition, including the chilling injury (Stewart et al. 2001). In this study, the activity of PAL really increased during storage, but it was significantly lower in EBR-treated eggplants than in controls (Fig. 5b). At the end of storage, PAL activity in EBR-treated fruits was 26 % lower than that in control fruits. These data showed that EBR treatment restrains the increase of PAL activity induced by chilling stress. A similar finding was reported by Luo et al. (2012) for bamboo shoot, of which PAL activity in samples treated with salicylic acid was significant low. The participation of PAL in enzymatic browning of horticultural products has been reported (Nguyen et al. 2003). Our data appeared that lower levels of pulp browning correlated fairly well with the PAL activity, which confirms the conclusion that EBR has protective effects against pulp browning in eggplant fruit s during cold storage. It has been reported that hydrogen peroxide can induce the expression of PAL (Desikan et al. 1998). Lin et al. (2005) suggested that inhibiting the production of hydrogen peroxide by dimethylthiourea can decrease the PAL activity in rice

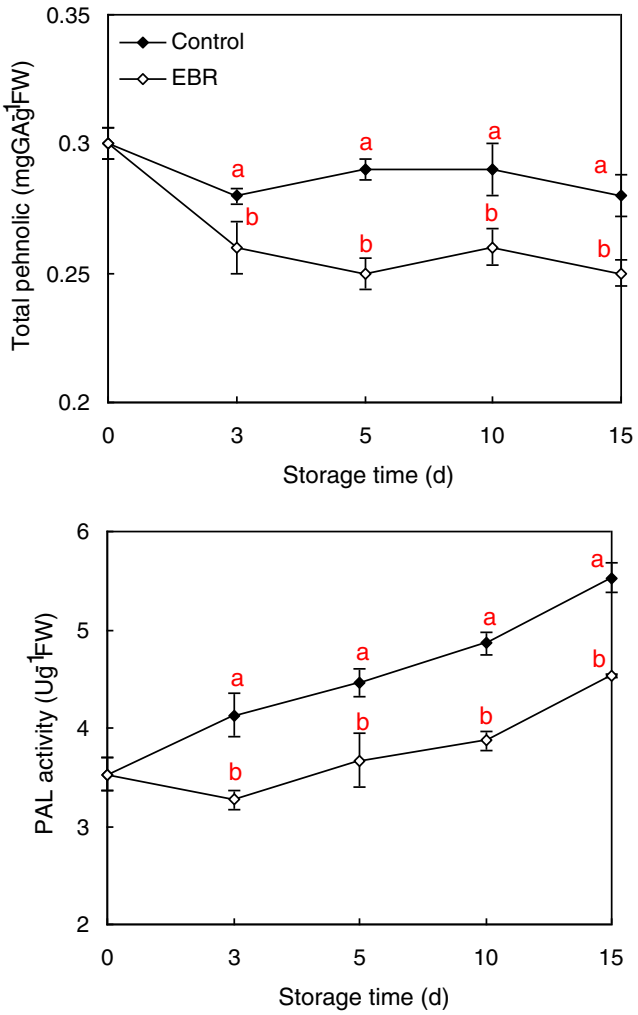

Fig. 5 Effect of EBR treatment on total phenolics (**a**) and PAL activity (**b**) of eggplant fruit during storage at 1 °C for 15 days. Values with different letters for each day were significantly different at $P<0.05$

cells. Wang et al. (2012) found that the activities of superoxidase dismutase, catalase and asscorbate peroxidase were stimulated by BRs, and these enhanced three enzymes would contribute to elimination of hydrogen peroxide. Therefore, it is reasonable to assume that the inbibitional effect of EBR on PAL activity in eggplant fruits might be due to the small amount of hydrogen peroxide production during cold storage.

Conclusions

EBR treatment significantly induced chilling tolerance in eggplants. Chilling injury, weight loss, electrolyte leakage, MDA and total phenolic contents were inhibited by EBR treatment. The increases in PPO, POD, and PAL activities were also significantly suppressed by EBR treatment, which contributes to the better maintenance of eggplant pulp color. These data suggest that EBR can be used as a possible strategy to alleviate chilling injury and pulp browning in eggplant fruit.

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