Effect of exogenous spermine on chilling injury and antioxidant defense system of immature vegetable soybean during cold storage

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Abstract The effect of exogenous spermine on chilling injury (CI) and antioxidant defense system of immature vegetable soybean (*Glycine max* L.) during cold storage were investigated. Freshly harvested immature soybeans were treated with 0.8 mmol/L spermine at room temperature for 20 min and then stored at 5 ± 1 °C or 1 ± 1 °C and 85–95% relative humidity for up to 60 days. Results showed that exogenous spermine alleviated the CI, delayed the gradual decreasing activities of superoxide dismutase (SOD) and catalase, and maintained a favourable balance in reactive oxygen species levels at storage period. Although cold temperature (1 ± 1 °C) inhibited the synthesis of 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC), raised ethylene production, and resulted in membrane damage, exogenous spermine obviously hindered the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC). It was concluded that exogenous spermine alleviated CI of cold-stored immature soybeans through regulating the antioxidant system and ACC metabolism.

Keywords Exogenous spermine · Immature vegetable soybean · Cold storage · Chilling injury · Antioxidant system

Introduction

Vegetable soybean [*Glycine max* (L.) Merrill] green pods are widely consumed as a special vegetable in China, Japan and Korea. After harvest, it deteriorates rather quickly at ambient temperature. Although, low temperature storage has been reported to delay postharvest quality deterioration and storage life extension of immature soybean, the beneficial effects may be limited pertaining to chilling injury (CI) development during extended cold storage (Wang 1989; Song et al. 2015; Liu et al. 2015). Further, the extreme range of cold storage temperature worsen the visual quality by developing symptoms such as surface pitting, pods yellowing and increased lignification (Wang 1989). CI considered as an oxidative stress related to a decrease in the scavenging activity of enzymes, which remove reactive oxygen species (ROS), such as catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) (Zhou et al. 2005). The generated ROS induce cell damage, including loss of membrane integrity in the tissue and oxidative damage to lipid, DNA and proteins (Lacan and Baccou, 1998). The development of CI undergoes a series of biochemical changes that lead to events concluding in cell death and tissue deterioration (Marangoni et al. 1996; Cai et al. 2006). Because these events also occur in ripening/senescing tissues (Lester and Stein 1993; Ramezani and Rahemi 2011), it has been hypothesized that the plant hormone ethylene could play a role in CI triggering. Earlier studies had already documented that ethylene acts in conjunction with low temperature storage, inducing metabolic shifts participating in CI development correlated with lower activity of ROS scavenging enzymes (Ben-Amor et al. 1999).

Polyamines (PA) are a new group of plant growth regulators, which are known for delaying ripening process and

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extending shelf life of several fruits and vegetables. The highly active PAs, putrescine (PUT), spermidine (SPD), spermine (SPM) and cadaverine (CAD) have been reported to affect cell division, fruit development, ripening, softening and senescence (Valero et al. 2002; Khan et al. 2007; Raeisi et al. 2013). In ripening/senescing plant tissues, both PAs and ethylene exhibit antagonistic effects pertaining to a common precursor S-adenosyl methionine (Pandey et al. 2000). Therefore, reduced levels of PAs have been correlated with increased ethylene production during ripening and vice versa (Kusano et al. 2008).

In recent years, several conservation techniques have applied to perishable soybeans. Su et al. (2003) investigated the effects of 1-methylcyclopropene (1-MCP) on decay and senescence in vegetable soybean pods during storage. 1-MCP significantly inhibited the senescence process of harvested vegetable soybean pods, as manifested in lower levels of ethylene production, respiratory rate, malondialdehyde and superoxide contents and higher levels of superoxide dismutase (SOD), ascorbate peroxidase (ASA-POD) activities, ascorbic acid and chlorophyll contents. Moreover, 1-MCP promoted phenylalanine ammonia-lyase (PAL) activity and lignin synthesis, inhibited decay incidence. Huang et al. (2011) also aimed to validate the preservation effect of 1-MCP, methyl jasmonate and chitosan under low temperature on vegetable soybean. However, no information was given about spermine-induced postharvest physiological response and improving the antioxidant system of immature vegetable soybeans. According to previous studies (Song et al. 2015), exogenous spermine could prolong shelf life of cold-stored immature soybeans, inhibit the increase of weight loss and decay index and retain the colour. Protection of cells from CI under cold stress was thought to be a major mechanism; this resistance was likely to depend on the competence of the antioxidant system (Lelievre et al. 1997). However, as far as we know, there were no reports on assessing the physiological responses that affect cold-induced changes of immature soybeans quality with spermine treatment. Thus, the aim of this work was further to evaluate the effect of exogenous spermine on the ethylene and ROS metabolism of immature vegetable soybean during cold storage.

Materials and methods

Plant material, treatment, and storage

Immature vegetable soybean (*Glycine max* L. ‘Xindali No.1’) pods were harvested at 45 days after anthesis from Luhe Animal Science Base, Jiangsu Academy of Agricultural Sciences (Nanjing, China) situated at 32.08°N

118.40°E. Vegetable soybean pods with similar size, maturity free from physical injuries, disease and insect attack were selected. Pods were immediately brought to the laboratory, washed, and weighed.

The homogeneous pods were randomized and divided into 3 lots for following treatments in three replicates: (1) 5 ± 1 °C, distilled water; (2) 1 ± 1 °C, distilled water; (3) 1 ± 1 °C, 0.8 mmol/L spermine which was found to be more effective in maintaining storage quality in immature soybeans as previously reported by Song et al. (2015). Treatments were performed by immersing the pods (500 g) in a 5 L solution for 20 min, then transferring into the baskets to drain the surface water at room temperature. The samples were packed in micro-perforated (hole diameter 0.5 mm) plastic bags (500 g/bag) and stored at 5 ± 1 or 1 ± 1 °C and 85–95% relative humidity (RH) for up to 60 days. After 0, 15, 30, 45 and 60 days (15 days intervals), pods from each replicate and for each treatment were immediately analyzed for related indicators.

Chilling injury index

Chilling injury (CI) symptoms manifested as surface pitting, the severity of the symptoms was assessed using a 4-stage scale: 0 = no pitting; 1 = a few scattered pits; 2 = pitting covering up to 25% but < 50% of the pod surface; 3 = extensive pitting covering > 50% but < 75% of the pod surface; and 4 = extensive pitting covering > 75% of the pod surface, as previously described by Liu et al. (2015). The average extent of CI damage was evaluated in 3 replicates containing 20 stored pods. CI damage was expressed as the CI index (between 0 and 4), which was calculated using the following formula:

$$\text{CI index} = \frac{\sum [\text{the injury score of individual pod} \times \text{the number of pod affected}]}{[4 \times \text{the total number of pod recorded}]}$$

Ethylene production

Samples of vegetable soybean pods (100 g) were placed and sealed in 2 L air tight glass vessels fitted with gas sampling ports. The vessels were kept at 25 ± 1 °C for 1 h. Gas samples (1 mL) were withdrawn from the headspace of vessels for ethylene determination. Ethylene content of the samples was quantitatively analyzed by gas chromatography at days 0, 15, 30, 45 and 60 from the beginning of the experiment using a GC, which was equipped with a GDX-502 resin-packed column (4 mm × 2 m) and a flame ionization detector. The column and detector temperatures were 60 and 110 °C, respectively. Ethylene values were indicated as $\mu\text{L.kg}^{-1} \text{h}^{-1}$.

Extraction and determination of ACC and MACC

1.5–2.0 g fresh grains were weighed into a mortar, and then 5 mL of 95% ethanol were added, the homogenate was centrifuged at $1000\times g$ for 10 min at 4 °C. After pouring out the supernatant, the residue was then extracted with 10 mL of 80% ethanol at 70 °C and centrifuged at $1000\times g$ for 5 min, the supernatant were finally merged. The combined supernatant solutions were concentrated in a high-speed rotary vacuum concentrator to dryness, and resolved in 2.0 mL distilled water. The 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-(malonylamino)-cyclopropane-1-carboxylic acid (MACC) contents was then determined according to Hoffman et al. (1983), ACC and MACC contents were calculated in terms of the amount of generated ethylene, and expressed as $\text{nmol.g}^{-1}\text{FW}$.

Extraction and assay of aminocyclopropane-1-carboxylic acid oxidase (ACO) activity

Aliquots (0.5–1.0 g) of flesh tissue were ground with 3 mL of 100 mmol/L phosphate buffer (pH 6.2) containing 2% sucrose, 1 mmol/L cycloheximide and 10 mmol/L ACC. After standing for 6 h, gas samples (1 mL) were withdrawn for ethylene determination. The activities of ACC oxidase (ACO) were determined in accordance with the method of Moya-León and John (1994) and expressed as $\text{nl g}^{-1}\text{FW h}^{-1}$.

Extraction and assay of aminocyclopropane-1-carboxylic acid synthase (ACS) activity

2.0 g of fresh soybean grains was homogenized with 3 mL of 100 mmol/L N-2-hydroxyethylpiperazine-N-ethane-sulphonic acid (HEPES) (pH 8.5) buffer, containing 4 mmol/L DTT, 0.5 $\mu\text{mol/L}$ pyridoxal phosphate (PLP) and 10 mmol/L EDTA. The homogenate was centrifuged at $12,000\times g$ for 10 min, the supernatant was collected and dialyzed against 2 mmol/L HEPES (pH 8.5) containing 0.1 mmol/L DTT and 0.2 $\mu\text{mol/L}$ PLP, then used as a source of enzyme assay.

The reaction system contained 0.5 mL of 60 $\mu\text{mol/L}$ S-Adenosyl methionine and 1.0 mL enzyme solution, after incubated at 30 °C for 2 h, 0.2 mL of 10 mmol/L HgCl_2 was added to terminate the reaction, the production of ACC was then determined, the activities of ACS were expressed as $\text{nl g}^{-1}\text{FW h}^{-1}$.

H_2O_2 content

The method of Jin et al. (2012) was used for the determination of hydrogen peroxide (H_2O_2). The H_2O_2 content was expressed as $\mu\text{mol g}^{-1}\text{FW}$.

Enzyme extraction and assays of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) enzymes

Superoxide dismutase (SOD) activity was determined by nitroblue-tetrazolium (NBT) photo-oxidation as described previously (Hassan and Mahfouz 2012); a unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in 1 min. Catalase (CAT) activity was assayed based on the UV–visible absorption spectroscopy following the method of Ramachandra Reddy et al. (2004); The UV absorbance detection at 240 nm, and a decrease of 0.01 units per minute in absorbance was considered. Peroxidase (POX) activity was determined from the crude extract according to the method of Morsy et al. (2007) using guaiacol as a common substrate for peroxidases. The absorbance was measured at 470 nm, and an increase of 0.01 units per minute in absorbance was considered.

Data analysis

Data were processed with Excel 2003 or GraphPad Prism 6. Duncan's multiple range tests was used to identify significant ($P < 0.05$) differences between treatments. All data represented in the figures were the means of three replicates $\pm$ standard deviation.

Results

Ethylene production

At the beginning of storage (0 day), the immature vegetable soybeans did not have most of ethylene produced. During prolonged storage at 1 °C, ethylene released from the soybean grains quickly, a climacteric-like peak in

Fig. 1 Effects of exogenous spermine (SPM) on ethylene production in immature vegetable soybean. Data are mean $\pm$ SD of three replications

ethylene production was observed on the 15th day of storage (Fig. 1). Thereafter, ethylene production gradually decreased. Whereas no obvious peak of ethylene concentration was found at 5 °C in the soybean grains, and samples treated with spermine inhibited the appearance.

ACC and MACC content

ACC levels gradually declined in immature soybeans stored at 5 °C after 15 days (Fig. 2). On the other hand, ACC levels in immature soybeans stored at 1 °C, with or without application of spermine treatment, continuously elevated up to 60 days after harvest. Nevertheless, spermine treatment significantly inhibited the ACC accumulation as compared to the immature soybeans stored at 1 °C, especially during late storage.

In the immature soybean grains stored at 5 °C, the MACC level showed an obvious (threefold) increase from initial 0.22 nmol g⁻¹ FW to 0.91 nmol g⁻¹ FW at the end of storage time. At 1 °C the similar change was detected until the 30th day of storage and a slight decrease was observed afterwards. No significant variation ($P > 0.05$) was observed between spermine treatment and the control samples.

Fig. 2 Effects of exogenous spermine (SPM) on ACC and MACC levels in immature vegetable soybean. Data are mean $\pm$ SD of three replications

ACO and ACS activities

It was found that ACO activity increased rapidly when the immature soybeans stored at 1 °C, and reached the highest value on the 45th day (Fig. 3). In contrast, spermine treatment eliminated this increase in activity; the spermine-treated immature soybeans exhibited a similar trend to that of the soybeans kept at 5 °C.

The changes of ACS activity were similar among treatments within the first 30 days during storage, and reached the highest value on the 15th day. Thereafter, the ACS activity rapidly increased at 1 °C, and reached the second peak on the 45th day, then kept at a higher level at the end of storage. However, it decreased rapidly for both 5 °C storage and spermine-treated groups during late storage, especially in the spermine-treated group, the ACS activity fell even faster.

CI index and H₂O₂ content

The CI index indicated the degree of damage to the surface subjected to cold conditions. As shown in Fig. 4, the immature soybeans did not exhibit CI symptoms during the first 15 days of storage at 1 or 5 °C. However, immature soybeans in all groups exhibited varying degrees of cold damage after 30 days of storage. The CI index of the immature soybeans stored at 1 °C was significantly higher than that of the other treatments after 30 days of storage

Fig. 3 Effects of exogenous spermine (SPM) on activities of ACC synthase and ACC oxidase in immature vegetable soybean. Data are mean $\pm$ SD of three replications

Fig. 4 Effect of exogenous spermine (SPM) on chilling injuries. Data are mean $\pm$ SD of three replications

($P < 0.05$). The spermine-treated and 5 °C of stored groups had a similar CI index at 30 days after storage. The spermine-treated group had a lower CI index than that stored at 5 °C. It revealed that spermine reduced the chilling injury incidence. Similar results were reported in squash where spermine and spermidine can act to prevent chilling injury (Kramer and Wang 1989).

Mostly, no significant change in H_2O_2 content was noticed during the whole storage period. Only a higher H_2O_2 level was observed during cold storage after 30 days at 5 °C (Fig. 5).

Antioxidant enzyme activities (SOD, CAT and POX)

The activities of SOD and CAT slightly decreased during storage, but there were no significant differences ($P > 0.05$) among all treatments. In contrast, significant differences ($P < 0.05$) for POX activity were found (Fig. 6). In each treatment group during the first 15 days, POX activity increased. On the 30th days of storage at 1 °C, there had the highest activity of POX, which increased almost twice times, and then the POX activity slowly decreased. However, spermine inhibited the trend towards increased. Activity of POX in immature soybeans treated with spermine showed similar trends with the

Fig. 5 Effects of exogenous spermine (SPM) on H_2O_2 levels in immature vegetable soybean. Data are mean $\pm$ SD of three replications

Fig. 6 Effects of exogenous spermine (SPM) on antioxidant enzyme activity in immature vegetable soybean. Data are mean $\pm$ SD of three replications

control group, but was higher than the control group during late storage.

Discussion

1 °C storage induced the release of ethylene, and the ethylene production of immature vegetable soybeans stored at 1 °C for 15 days was about twice that of 5 °C storage, this result was in accordance with previous reports (Su et al. 2003), the storage time discrepancy might be due to the planting year, harvest period, harvest location and degree of aging caused by changes in environmental conditions.

Ethylene plays an important role in regulating ripening and senescence. In some fruits and vegetables, cold storage induced ethylene production, which attributed to the

accumulation of ACS and ACO mRNAs (Dong et al. 2001). Although this study did not directly determine the transcriptional level, higher levels of ACC and MACC could prove the fact. At 5 °C during storage, the lower level of ACC was mainly related with the higher level of MACC, it was suggested that the regulation mechanism of propylene acylation was potential, which could regulate the amount of ethylene production in immature soybeans.

ACS and ACO are the two kinds of most important regulatory enzymes in ethylene biosynthesis pathway (Zhu and Zhou 2007; Gómez-Jiménez et al. 2001). Ethylene biosynthesis was under the control of the levels of ACS and ACO activities. Due to the combination with ethylene receptor, spermine blockaded the combination of endogenous and exogenous ethylene with ethylene receptors, inhibited the physiological effect of ethylene, so as to defer aging. Previous reports showed that exogenous polyamine treatments significantly reduced ACS and ACO activities in plum fruit, and reduced ethylene production (Khan et al. 2007). The result also indicated that exogenous spermine was effective in suppressing ethylene biosynthesis and extending storage life of immature vegetable soybean.

ACC content in immature soybean grains was low in early storage, after storage for 15 days, ACC content increased gradually, but ACS activity gradually decreased, it was presumable that ethylene signaling pathway in the regulation mechanism of different species had different adaptabilities affected by exogenous spermine. The study also found that spermine and cold storage did not lead to a lower level of malonyl ACC produced, by comparison with non-treated immature soybeans, MACC of the soybean grains increased significantly at the end of storage, suggesting that ACS was partially inhibited by spermine (Pandey et al. 2000).

Environmental change could induce the accumulation of reactive oxygen species (ROS), and particularly H_2O_2 in plants. In Blanquilla pear (Larrigaudiere et al. 2004), cold storage was shown to induce a significant increase ($P < 0.05$) in H_2O_2 during the initial period, similar phenomena were also reported in cucumber and pumpkin (Lei et al. 2010; Sevengor et al. 2011). However, there was no obvious difference between 1 °C of storage and spermine treatment groups, and both treatments did not induce large increase of H_2O_2 content in immature soybeans. The results implied that little oxidative stress produced, a small increase of H_2O_2 content might be due to the increase of ROS scavenging enzyme activities. The effective destruction of ROS may require the action of several antioxidant enzymes acting concomitantly with non-enzymatic antioxidants. The superoxide radical ($O_2^{\cdot-}$) is effectively converted to H_2O_2 by the action of SOD. The main enzymes responsible for converting H_2O_2 to H_2O are CAT and POX. Only a few changes in SOD activity were

observed for the immature soybeans in both control and spermine-treated groups. In contrast, a transitory but sharp peak in CAT activity was observed in spermine-treated soybean grains during storage after 30 days. POX activity significantly increased ($P < 0.05$) during storage at 1 °C after 30 days, such an increase in POX activity was consistent with the greater resistance to oxidative stress, while spermine completely inhibited the trend. The results were in accordance with Golden Smoothee apples (Asrey et al. 2008); it suggested that the change of POX activity was related to ethylene signal transduction pathway being regulated by spermine.

Conclusion

The treatment with exogenous spermine has an important role in alleviating the chilling injury (CI) during cold storage of immature vegetable soybeans, exogenous spermine may limit aging and extend storage life of immature soybeans not only by delaying the decreased activities of SOD and catalase and maintaining the favourable balance in ROS levels, but also through the better control of immature soybean's ACC metabolic system. Although cold temperature (1 ± 1 °C) inhibited the synthesis of MACC, made ethylene production increased, which might result in membrane damage, exogenous spermine undoubtedly inhibited the accumulation of ACC during cold storage, it significantly inhibited the peroxidase activity, it was therefore concluded that spermine treatment was a promising tool to delay quality deterioration of cold-stored immature soybeans.

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