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Effect of putrescine application on maintenance of zucchini fruit quality during cold storage: Contribution of GABA shunt and other related nitrogen metabolites

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ABSTRACT

Polyamine metabolism has been suggested to be a crucial factor in the response of plants to several abiotic stresses, including low temperature. Zucchini fruit is susceptible to develop chilling injury when stored at low temperature. In this study, the effects of putrescine, spermidine and spermine treatment (1 mM) on the physiological behavior of zucchini fruit during cold storage were investigated, focusing on the changes in polyamine metabolism and in alterations of polyamine-related nitrogen metabolites and hormones. Among the polyamines used, exogenous application of putrescine was found to be the best treatment to improve postharvest cold tolerance. Treated fruit were of better quality, and in general after cold storage they showed reduced weight loss, chilling injury, and malondialdehyde and hydrogen peroxide contents. Putrescine treatment induced betaine and proline accumulation, fatty acid desaturase expression, and also changes in the biochemical GABA shunt pathway during cold storage. These responses may contribute to increased energy production in fruit treated with putrescine. In general, the putrescine treatment induced different pathways that are considered stress defense mechanisms, and we hypothesize that they could contribute to improve postharvest quality of zucchini fruit after storage at 4 °C.

1. Introduction

The implication of polyamines (PAs) in mechanisms to cope with different types of stress has been the object of much research. The capacity of PAs to bind to negatively charged molecules such as phospholipids, proteins and nucleic acids due to their polycationic nature, as well as their antioxidant properties, could explain their ability to protect the cell from abiotic stresses and to avoid the appearance of symptoms (Gupta et al., 2013). In addition to these modes of protection, polyamine metabolism is closely linked to other compounds such as glutamate, γ-aminobutyric acid (GABA), alanine and proline, all known to be involved in stress tolerance (Gupta et al., 2013).

Putrescine (Put) can be catabolized by the enzyme diamine oxidase (DAO) to GABA that is formed via pyrroline or 4-aminobutyraldehyde (Shelp et al., 2012a). Ornithine and arginine are substrates for the synthesis of Put, but they are also intermediates in the synthesis of proline and GABA, glutamate also being a precursor of proline, ornithine, arginine and GABA (Shelp et al., 2012a). Glutamate is converted into proline by two reactions, catalysis by glutamate dehydrogenase and Δ^1-pyrroline-5-carboxylate synthetase. Another precursor of proline synthesis is ornithine, which is transaminated by ornithine-δ-aminotransferase (Roosens et al., 2002). Under abiotic stress conditions it has been shown that polyamines interact with other hormones, such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) (Zheng and Zhang, 2004; Yoshikawa et al., 2007; Bitrian et al., 2012; Gupta et al., 2013), although the way this is accomplished depends on the stress, the species and the organ analyzed.

Zucchini (*Cucurbita pepo* L. morphotype *Zucchini*) is a non-climacteric fruit which is harvested immature. Its subtropical origin makes it susceptible to chilling disorders when stored at low non-freezing temperatures (Sevillano et al., 2009). The development of these symptoms reduces consumer acceptance and thus determines storage time.

Postharvest treatments, which reduce chilling injury in zucchini squash, such as temperature pre-conditioning (Wang, 1995)

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and heat shock (Wang, 1994), were able to increase endogenous levels of polyamines. In previous studies we have demonstrated that although Put content increases during cold storage, fruit of tolerant varieties of zucchini accumulated lower levels of Put than fruit of sensitive varieties (Palma et al., 2014). However, treatments with exogenous Put have been reported to improve fruit postharvest quality in lemons (Valero et al., 1998), apples (Wang et al., 1993), and apricots (Martínez-Romero et al., 2002). In squash, exogenous treatment with polyamines by pressure infiltration also increased the tolerance to chilling injury (Martínez-Téllez et al., 2002).

The aim of this work was in the first place to confirm the effect of polyamine treatments on the improvement of the cold storage behavior in zucchini fruit, and at the same time, to unveil the mechanisms by which Put triggers the tolerance response in zucchini fruit, by studying the changes in Put metabolism, and the implications of the changes of other related nitrogen metabolites and hormones in cold tolerance.

2. Materials and methods

2.1. Plant material and storage conditions

Zucchini fruit (*C. pepo* L. morphotype *Zucchini*) of the commercial variety 'Sinatra' (Clause-Tezier) were provided by E.H. FEMAGO S.A. Fruit were selected for uniformity in size, and without any mechanical damage or disease. A total of 18 fruit were sampled for immediate analysis to monitor fruit characteristics at harvest before application of treatments (day 0). They were then grouped at random into 4 lots of 18 fruit for the following treatments: fruit were submerged at 20 °C for 20 min in 1 mM putrescine (Put), 1 mM spermidine (Spd), 1 mM spermine (Spm) or distilled water as the control. All solutions contained Tween-20 (0.2% v/v) to improve the absorption of the polyamines. The fruit were then placed on desiccant paper and allowed to dry before storage in a temperature-controlled chamber and in permanent darkness. All of the control and treated fruit were stored at 4 °C in 85–90% RH during 3, 5, 10 and 14 days.

After the storage period, for each replicate the exocarp of the whole fruit were separated, mixed, pulverized in liquid nitrogen, and stored at −80 °C. RNA, metabolites and enzyme activities were performed in the exocarp.

2.2. Weight loss and chilling-injury index

Loss of fresh weight and chilling-injury index (non-destructive determinations) were determined after 3, 5, 10, and 14 days of storage at 4 °C. The percentage of weight loss of each fruit was calculated as: % weight loss = $(W_i - W_f)/W_i \times 100$, being W_i the initial fruit weight and W_f the final fruit weight. Chilling-injury index (CI) of the surface of the fruit was evaluated using a subjective scale of visual symptoms previously described by Martínez-Téllez et al. (2002): 0 = no pitting, 1 = slight (10% or less), 2 = medium (10–20%), and 3 = severe pitting (>20%). CI index was determined using the following formula: Σ (pitting scale (0–3) × number of corresponding fruit within each class)/total number of fruit estimated.

2.3. Measurement of TBARS

Malondialdehyde (MDA) content was determined using the thiobarbituric acid reactive species (TBARS) procedure described by Heath and Packer (1968), with some modifications. Exocarp ground in liquid nitrogen was homogenized (1:4, w/v) in 20% (w/v) trichloroacetic acid (TCA), 0.2 mL of 4% (w/v) butylated hydroxytoluene was added during the process. The homogenate was centrifuged at 4 °C and 10,000 *g* during 15 min. The supernatant was mixed with 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA in proportion 1:4 (v/v). The mixture was heated at 95 °C in a water bath for 30 min, cooled immediately in ice to stop the reaction, and centrifuged at 4 °C and 4000 *g* for 10 min. Absorbance of the supernatant was measured at 532 and 600 nm. TBARS were calculated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm and using a standard curve of MDA (0–20 μM). Results were expressed as nmol MDA g^{-1} of fresh weight.

2.4. H_2O_2 content

The hydrogen peroxide content was assayed spectrophotometrically by the procedure previously described by Alexieva et al. (2001). Zucchini exocarp was ground in liquid nitrogen and homogenized with 0.1% (w/v) trichloroacetic acid (TCA) (1:4, w/v). After centrifugation at 4 °C and 12,000 *g* for 15 min, the supernatant was collected. The reaction mixture consisted 0.25 mL supernatant, 0.25 mL 100 mM potassium phosphate buffer (pH 7) and 1 mL 1 M KI. The reaction was developed for 1 h in darkness and absorbance measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve of H_2O_2 (0–150 μM) and expressed as μmol H_2O_2 g^{-1} of fresh weight.

2.5. Polyamine content

0.5 g of exocarp was used for polyamine analysis. Extracts were prepared grinding the exocarp with 1.5 mL of 5% cold perchloric acid and 1,7-diaminoheptane (60 nmol mL^{-1}) as internal standard, and incubated 24 h at 4 °C. The homogenate was centrifuged (3000 *g*, 5 min at 4 °C) and 0.2 mL aliquot from the supernatant was used to determine free polyamines. The supernatants were mixed with 0.4 mL of dansyl chloride (prepared fresh in acetone, 10 mg mL^{-1}) and 0.2 mL of saturated sodium carbonate. After brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess of dansyl reagent was removed by a reaction with 0.1 mL (100 mg/mL) of added proline, after incubation for 30 min at room temperature. Dansylpolyamines were extracted in 0.4 mL toluene. The organic phase was collected and evaporated to dryness under a stream of air, and redissolved in 0.1 mL acetonitrile.

Polyamines were analyzed by HPLC using a Hewlett-Packard system equipped with a 4.6 mm × 250 mm C18 column. Solvent flow was 1.5 mL min^{-1} and the elution gradient was prepared with eluent A (water) and eluent B (acetonitrile). The gradient profile was applied as follow (*t* (min); %A): (0; 30%), (4.5; 30%), (9; 0%), (14; 0%), (15; 30%). The final step was held for 2 min before regenerating the column. Detection was with a fluorometer using excitation and emission wavelengths of 415 and 510 nm, respectively, according to Flores and Galston (1982).

A relative calibration procedure was used to determine the polyamines in the samples, using 1,7-diaminoheptane (60 nmol mL^{-1}) as internal standard and polyamine concentrations ranging from 0 to 150 nmol mL^{-1}. Results were expressed as nmol g^{-1} fresh weight.

2.6. Glutamic acid, GABA, and betaine content

Exocarp tissue (0.25 g) was homogenized in 2.4 mL of cold extraction medium (ethanol/chloroform/HCl 0.1 N) (12/5/1; v/v/v) and at this time 15 μL of internal standard (betaine-trimethyl-d9-hydrochloride, 5 μg mL^{-1}) was also added. The homogenate was centrifuged at 4 °C and 8000 *g* for 10 min. The supernatant was separated into aqueous and chloroform phases by the addition of chloroform (1.5 mL), 0.1 N HCl (0.15 mL) and water (0.3 mL) and incubated 24 h at 4 °C. The aqueous phase was evaporated under