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Research article

Contribution of polyamines and other related metabolites to the maintenance of zucchini fruit quality during cold storage

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ABSTRACT

In order to investigate the contribution of polyamines and related amino acids in the maintenance of zucchini fruit quality during cold storage, two varieties of *Cucurbita pepo* with different degrees of chilling tolerance were used, Natura (more tolerant) and Sinatra (moresensitive). After harvest, free putrescine levels decreased during storage at 20 °C, whereas in fruit kept at 4 °C this polyamine accumulated in both varieties, but with higher levels in the sensitive variety (Sinatra). This behavior suggests that putrescine is accumulated as a response to low temperature in zucchini fruit by stress-induced chilling injury, and not due to the postharvest storage itself. ADC activity responds quickly to chilling but sharply decreases after 14 days, whereas its expression remains high in both varieties. ODC activity takes over when the cold stress is relatively severe, as this activity was found to be much higher in Sinatra. ODCexpression also correlated with ODC activity. DAO activity increased in Natura fruit, and conversely decreased in Sinatra fruit during storage at 4 °C, whereas the proline content was higher in Natura and lower in Sinatra. Therefore, we suggest that putrescine degradation and proline accumulation contribute to the acquisition of chilling tolerance in zucchini fruit. GABA content decreased in both varieties, with a greater reduction in Natura fruit and less in Sinatra fruit. In addition, GABA transaminase showed a higher activity in Natura fruit than in Sinatra fruit during cold storage, suggesting that GABA catabolism could be involved in the tolerance to postharvest cold storage in zucchini fruit.

1. Introduction

Polyamines are low molecular weight, aliphatic polycations found in the cells of all living organisms. Due to their positive charges, polyamines bind to macromolecules such as DNA, RNA, and proteins (Kusano et al., 2008). Putrescine can be formed either directly from ornithine in a single reaction catalyzed by ornithine decarboxylase (ODC) or by decarboxylation of arginine via the arginine decarboxylase (ADC), with agmatine and *N*carbamoylputrescine as intermediates. Spermidine and spermine are formed by sequential addition of aminopropyl groups to putrescine and spermidine, respectively, by spermidine synthase and spermine synthase. The aminopropyl donor is generated from S-adenosylmethionine (SAM) by the action of SAM decarboxylase (Tiburcio et al., 1997). SAM is a common intermediate of polyamines and ethylene (Cantoni, 1975), being a substrate for the enzymes 1-aminocyclopropane-l-carboxylic acid (ACC) synthase during ethylene synthesis, and SAM decarboxylase in the formation of the polyamines spermidine and spermine. Diamine oxidase (DAO) catalyzes the degradation of putrescine and 1,3-diaminopropane generating 4-aminobutyraldehyde/Δ1-Pyrroline and 3-aminopropionaldehyde, respectively, which are converted to 4-aminobutyrate (GABA) and β-alanine. O_2 dependent polyamine oxidases (PAO) are responsible for catalysing the oxidation or back-conversion of spermine and spermidine, resulting in the formation of spermidine, putrescine, 3-aminopropionaldehyde, and their degradation to 1-(3-aminopropyl)-pyrroline, 1,3-diaminopropane and 4-aminobutyraldehyde (Shelp et al., 2012a). The pathway for polyamine synthesis is tightly connected to the metabolism of several amino acids. Ornithine and arginine are substrates for the synthesis of putrescine, but they are also intermediates in the synthesis of proline and GABA, being glutamate also a precursor of ornithine and GABA (Shelp et al., 2012a). In stressed tissues, putrescine and proline are connected by a precursor–product relationship via the activity of DAO and GABA metabolism (Bouchereau

Abbreviations: (ACC), 1-aminocyclopropane-l-carboxylic acid; (ADC), arginine decarboxylase; (DAO), diamine oxidase; (GABA), 4-aminobutyrate; (GABA-T), GABA transaminase; (ODC), ornithine decarboxylase; (PAO), polyamine oxidase; (SAM), S-adenosylmethionine.

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et al., 1999). In plants, GABA is directly derived from glutamate via calcium/calmodulin- or low pH-mediated glutamate decarboxylase activity, or indirectly from putrescine via a combination of diamine oxidase and γ-aminobutyraldehyde dehydrogenase activities (Shelp et al., 2012a, 2012b, 2012c). Therefore, a high number of compounds, most of which are present in conditions of stress, are imbricated and share common metabolic pathways, making difficult to elucidate the role and importance of each compound in the stress response.

Low-temperature storage is used to prolong shelf life of fruits and vegetables, although in tropical and subtropical fruit storage below critical temperatures can often cause severe losses (Muñoz et al., 2001; Wang, 2010). Zucchini (*Cucurbita pepo* L. morphotype *Zucchini*), bears fruits that are marketed at an immature stage. Its subtropical origin makes zucchini fruit susceptible to chilling disorders when stored at low non-freezing temperatures. Chilling injury symptoms in zucchini fruit are weight loss, softening, and the appearance of pits on the fruit surface (Martínez-Téllez et al., 2002; Serrano et al., 1998). In addition to the ultra structural changes, chilling also results in a series of physiological, biochemical and molecular modification, such as an accumulation of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), changes in the levels of endogenous abscisic acid (Anderson et al., 1994), sugars (Palma et al., 2014) and polyamines (Groppa and Benavides, 2008; Moschou et al., 2008a; Zhang et al., 2009). The polyamine anabolic/catabolic regulation has recently been suggested to be the crucial factor in polyamine mediated stress tolerance (Moschou et al., 2008b). In a previous work analysing the chilling resistance in fruit of different commercial varieties of *Cucurbita pepo* grown in the south-eastern Spain, we detected differences in chilling damage among varieties after 14 days of cold storage (Carvajal et al., 2011), and these results offer the possibility of comparing physiological changes that take place during fruit storage at different temperatures as well as changes among varieties with different chilling sensitivities.

The aim of this work has been the study of the changes in polyamines and other related nitrogen metabolites in the zucchini fruit during cold storage, and to unveil the contributions of these compounds to the maintenance of zucchini fruit quality during cold storage. For this, we have selected two varieties of zucchini fruit with different chilling sensitivities, and after storage at 4 °C we have measured and compared several nitrogen metabolites as well as enzymes involved in polyamine anabolism and catabolism.

2. Materials and methods

2.1. Plant material and storage conditions

Zucchini fruit (*Cucurbita pepo* L. morphotype *Zucchini*) of the commercial varieties Natura (Enza Zaden), variety more tolerant to cold storage, and Sinatra (Clause-Tezier), variety more sensitive, were provided by E.H. FEMAGO S.A. After harvest, fruit were stored in chambers at 4 °C and 20 °C. Three replicates were prepared per variety (Natura and Sinatra), storage period (0, 7 and 14 days) and storage temperature (4 °C and 20 °C), each consisting in 18 fruit of similar size. After the storage period, weight loss and the chilling-injury index were determined in the whole fruit. For each replicate, the exocarp was separated, mixed and grinded in liquid nitrogen, and stored it 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 was determined after 7 and 14 days of storage at 4 °C and 20 °C and the percentage of weight loss of each fruit was calculated. The chilling-injury index (CI) of surface fruit was evaluated using a subjective scale of visual symptoms previously described Martínez-Téllez, Ramos-Clamont, Gardea and Vargas-Arispuro (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. 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 from the supernatant was used to determine free polyamines. For soluble conjugated polyamines, 0.2 mL from supernatant was hydrolysed with 12 N HCl (1:1, v/v) at 110 °C for 24 h in flame-sealed glass ampoules. The hydrolysates were filtered, dried, and resuspended in 0.2 mL of 5% cold perchloric acid. Both samples (free and conjugated polyamines) were mixed with 0.4 mL of dansyl chloride (fresh prepared in acetone, 10 mg/mL) and 0.2 mL of saturated sodium carbonate. After brief vortexing, the mixture was incubated in darkness overnight at room temperature. Excess of dansyl reagent was removed with 0.1 mL of proline (100 mg/mL) 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 dissolved in 0.1 mL acetonitrile.

Polyamines were analyzed by HPLC using a Hewlett–Packard system equipped with a 4.6 × 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.

2.4. Glutamic acid and GABA content

Exocarp tissue (0.25 g) was homogenized in 2.4 mL of cold ethanol/chloroform/water (12/5/1) and 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 a flow of nitrogen until dry, in order to measure the glutamic acid and GABA. Finally, dry residues were solubilized in 0.3 mL methanol and 1.2 mL of acetonitrile, and filtered through nylon filter (0.22 μm).

An Acquity UPLC class system was used for solvent delivery and sample introduction. Samples were injected into a column (Acquity UPLC BEH C18 1.7 μm, 2.1 × 50 mm) and the column was eluted at a flow rate of 0.4 mL/min and developed with isocratic chromatographic conditions as follows: 25% A (water containing 0.01% formic acid and 0.05% ammonium) and 75% B (acetonitrile) with a run time of 2 min.

Eluates were detected using a Xevo TQ-S triple quadrupole mass spectrometer (Waters) in the positive electrospray ionisation (ESI)