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Research article

Exogenous γ-aminobutyric acid treatment improves the cold tolerance of zucchini fruit during postharvest storage

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ABSTRACT

This work examines the effect of a treatment with 1 mM of γ-aminobutyric acid (GABA) on zucchini fruit during postharvest cold storage. Specifically, the effect of GABA on postharvest quality was measured, as well as its implication in the GABA shunt and other related metabolic pathways. The treatments were performed in Sinatra, a variety of zucchini highly sensitive to low-temperature storage. The application of GABA improved the quality of zucchini fruit stored at 4 °C, with a reduction of chilling-injury index, weight loss, and cell death, as well as a lower rate of electrolyte leakage. GABA content was significantly higher in the treated fruit than in the control fruit at all times analyzed. At the end of the storage period, GABA-treated fruit had higher contents of both proline and putrescine. The catabolism of this polyamine was not affected by exogenous GABA. Also, over the long term, the treatment induced the GABA shunt by increasing the activities of the enzymes GABA transaminase (GABA-T) and glutamate decarboxylase (GAD). GABA-treated fruit contained higher levels of fumarate and malate than did non-treated fruit, as well as higher ATP and NADH contents. These results imply that the GABA shunt is involved in providing metabolites to produce energy, reduce power, and help the fruit to cope with cold stress over the long term.

1. Introduction

γ-aminobutyric acid (GABA), a non-proteinogenic amino acid, has different functions in plant growth and development (Li et al., 2018). Its accumulation has been described under several abiotic stress conditions such as heat and cold, salinity, drought, hypoxia, and mechanical damage (Kinnersley and Turano, 2000; Ramesh et al., 2015; Shelp et al., 1999). Under cold storage, an accumulation of GABA correlates with a higher capacity to cope with adverse postharvest conditions in several crops such as loquat (Cao et al., 2012), banana (Wang et al., 2016), and bamboo shoot (Wang et al., 2017). GABA acts as an inhibitor of malondialdehyde formation during lipid peroxidation (Deng et al., 2010), maintains membrane integrity (Aghdam et al., 2015), increases antioxidant metabolism and GABA-shunt pathway (Aghdam et al., 2016; Li et al., 2016; Malekzadeh et al., 2017), and helps to maintain osmotic adjustment (Li et al., 2016). In the case of zucchini (*Cucurbita pepo* L. morphotype Zucchini), we have previously reported a decrease of endogenous GABA content in fruit during cold storage along with an induction of the GABA-shunt pathway for long-term storage (Palma et al., 2014). The GABA shunt is the conversion of glutamate to GABA and its catabolism to succinate by three enzymatic reactions (Shelp et al., 1999). First, glutamate is α-decarboxylated to form GABA by the cytosolic enzyme glutamate decarboxylase (GAD). GABA is then transported to the mitochondria and converted to succinic semialdehyde via transamination by GABA transaminase (GABA-T). Finally, the oxidation of succinic semialdehyde to succinate coupled with the production of NADH is catalyzed by succinic semialdehyde dehydrogenase (SSADH). Succinate produced by this short pathway can enter the tricarboxylic acid (TCA) cycle.

Zucchini fruit is highly sensitive to cold storage, developing damage on the exocarp surface and suffering a weight and firmness loss when kept at temperatures below 8-10 °C. Sensitivity to cold storage in zucchini is variety dependent (Carvajal et al., 2011; Megías et al., 2016) with Sinatra being among the most chilling-sensitive varieties analyzed

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for this crop (Carvajal et al., 2018). Over the long term, a greater induction of the GABA shunt has been detected in cold-tolerant varieties of this species, and also in cold-sensitive fruit after treatments that improve chilling tolerance during fruit postharvest, such as a preconditioning treatment at moderate temperature before cold storage or an exogenous application of putrescine (Carvajal et al., 2015b; Palma et al., 2015). The induction of the GABA shunt reportedly correlates with greater oxidative deamination of the polyamine putrescine catalyzed by the diamine oxidase (DAO), an enzyme whose end product is a precursor of GABA different from glutamate, 4-aminobutyraldehyde (Shelp et al., 2012).

The aim of this work was in the first place to elucidate the effect of a GABA treatment on postharvest quality of a chilling sensitive variety of zucchini, and to study the implications of the GABA-shunt pathway in cold tolerance when exogenous GABA is applied to the fruit.

2. Materials and methods

2.1. Postharvest treatment and plant material

Zucchini fruit (*Cucurbita pepo* L. morphotype Zucchini) of the commercial variety Sinatra (Clause-Tezier) were supplied by La Ñeca S.L. The fruit were separated randomly into replicates before treatment, and each replicate was treated independently. Three replicates were prepared per treatment (control and GABA 1 mM), and storage period (0, 1, 3, 5, 10, 14 days), each consisting of 6 fruits (198 fruits in total). Based on previous studies (Malekzadeh et al., 2012, 2014; Shang et al., 2011), and on a preliminary experiment (data not shown), we selected 1 mM GABA as the most suitable concentration. Fruit were submerged during 20 min at 20 °C in 1 mM of γ-aminobutyric acid (GABA) or in the case of the control, distilled water. Each replicate was submerged in a volume of 5 L. Finally, fruit were dried 2 h before being stored in a controlled environmental chamber in darkness at 4 °C and 85–90% RH during 1, 3, 5, 10, 14 days. After weight loss and chilling-injury index were evaluated according to Martínez-Téllez et al. (2002), in each biological replicate the exocarp was separated, frozen in liquid nitrogen, pulverized, and stored at −80 °C.

2.2. Electrolyte leakage and cell-death assay

Electrolyte leakage was determined following Mao et al. (2007). From each treatment, four replicates were measured, each consisting of 10 discs excised from exocarp with a cork borer. After 3 rinses of 3 min each, discs were incubated in 50 mL of deionized water at room temperature with constant shaking at 100 rpm for 30 min. The conductivity was determined with a conductimeter. Finally, the samples were boiled for 10 min and measured to calculate total conductivity.

2.3. Cell-death assay

This parameter was measured according to Qu et al. (2009) with some changes reported in Carvajal et al. (2015a). The exocarp of the fruit was separated and, for each replicate, five discs (11 mm diameter) were used. For each treatment, 4 replicates were used. The discs were submerged in trypan blue (0.25%, w/v) for 10 min, then washed with deionized water, and dried. Afterwards, the discs were weighed, ground in ethanol (50%. v/v), and centrifuged for 10 min at 4 °C and 10000 *g*. Finally, the absorbance of the supernatant was measured at 585 nm.

2.4. Putrescine content

Free putrescine was detected using the method of Palma et al. (2014). Exocarp tissue was extracted for 24 h at 4 °C with 5% (v/v) perchloric acid at a proportion of 1:3 (w/v). 1,7-diaminoheptane (60 nmol mL^{-1}) was used as an internal standard. This extract was centrifuged for 10 min at 4 °C and 8000 *g*. The supernatants were derivatized with dansyl chloride following the method of Flores and Galston (1982). Dansylputrescine was measured by HPLC (Agilent 1260 Infinity) with a 4.6 mm × 250 mm C18 column and a fluorometer (excitation/emission wavelengths 415/510 nm). Dansylputrescine was eluted at a flow rate of 1.5 mL min^{-1} using a elution gradient with water (A) and acetonitrile (B). The gradient profile, expressed as (t [min]; %B), was: (0; 70%), (4.5; 70%), (9; 100%), (14; 100%), (15; 70%).

2.5. Alanine, glutamate, and proline content

Alanine, glutamate, and proline were analyzed following Palma et al. (2015). The amino acids were extracted with ethanol/chloroform/HCl 0.1 N (12/5/1; v/v/v) at a proportion of 1:7 (w/v). Norvaline and sarcosine (50 nmol mL^{-1}) were used as internal standards. The extract was centrifuged for 10 min at 4 °C and 3500 *g*, and the supernatant was separated into chloroform and aqueous phases by the addition of HCl 0.1 N and $CHCl_3$. The aqueous phase was filtered and derivatized with o-phthalaldehyde (OPA) and fluorenylmethylchlo-roformate (FMOC). These amino acids were measured by HPLC (Agilent 1260 Infinity) with a Zorbax Eclipse-AAA4.6 mm × 150 mm column and a fluorometer using excitation and emission wavelengths of 340 and 450 nm (0–15 min) and 266 and 305 nm (15–26 min). Alanine, glutamate, and proline were eluted at a flow rate of 2 mL min^{-1} using an elution gradient with acetonitrile/methanol/water mix (45/45/10, v/v/v) (A) and sodium phosphate 40 mM pH 7.8 (B). The gradient profile, expressed as (t [min]; %A), was: (0; 0%), (1.9; 0%), (18.1; 57%), (18.6; 100%), (22.3; 100%), (23.2; 0%), and (26; 0%).

2.6. GABA content

GABA extraction and quantification was conducted as in Palma et al. (2014). Exocarp tissue was homogenized with ethanol/chloroform/HCl 0.1 N (12/5/1; v/v/v) at a proportion of 1:10 (w/v) and centrifuged at 4 °C and 8000 *g* for 10 min. The supernatant was separated into chloroform and aqueous phases by the addition of HCl 0.1 N and $CHCl_3$. The aqueous phase was filtered and evaporated using a nitrogen flow. Dry residues were resuspended in acetonitrile/methanol (1:4). GABA was separated by Acquity UPLC with a Acquity UPLC BEH C18 1.7 µm, 2.1 mm × 50 mm column and quantified by Xevo TQ-S triple quadrupole mass spectrometer in the positive electro-spray ionization mode with the following transitions: m/z 103.84 → 62.78 (cone voltage 22 and collision energy 10) and 103.84 → 68.81 (cone voltage 22 and collision energy 12). GABA was eluted at a flow rate of 0.4 mL min^{-1} for 2 min using a isocratic gradient with 25% of water containing 0.05% ammonium and 0.01% formic acid, and 75% of acetonitrile.

2.7. Fumarate and malate content

Fumarate and malate were extracted and quantified following Del-Saz et al. (2018). The exocarp was ground in ethanol/chloroform/water (12/5/1, v/v/v) at a proportion of 1:7 (w/v). Trifluoroacetic acid was used as an internal standard. The homogenate was centrifuged for 15 min at 4 °C and 12000 *g*. Supernatant was separated into chloroform and aqueous phases by the addition of HCl 0.1 N and $CHCl_3$. The aqueous phase was filtered and evaporated using a nitrogen flow. Dry residues were resuspended in Milli-Q water, centrifuged for 15 min at 4 °C and 10000 *g*. Finally, the supernatant was filtered through nylon filter. Fumarate and malate were separated and quantified by ion chromatography (DionexICS-3000) with a AG11-HC guard column (50 mm × 4 mm), a AS11-HC separation column (250 mm × 4 mm) and a conductivity detector. Both organic acids were eluted at a flow rate of 1.5 mL min^{-1} using a elution gradient with Milli-Q water (A) and NaOH 100 mM (B). The gradient profile, expressed as (t [min]; % B), was: (0; 1%), (15; 1%), (25; 15%), (35; 30%), (45; 60%), (46; 1%),

and (50; 1%).

2.8. ATP content

ATP was determined as in Palma et al. (2014). The exocarp was homogenized with perchloric acid 5% (v/v) at a proportion of 1:2.4 (w/v). The extract was centrifuged for 10 min at 4 °C and 6000 *g*. The supernatant was neutralized with KOH (pH 6.5–6.8), incubated at 4 °C for 15 min, and centrifuged for 10 min at 4 °C and 6000 *g*. Then this supernatant was filtered and analyzed by HPLC (Agilent 1260 Infinity) with a ACE column (4.6 mm × 150 mm C18). ATP was eluted at a flow rate of 1.3 mL min^{-1} using a elution gradient with A (40 mM of potassium phosphate monobasic and 60 mM of potassium phosphate dibasic in Milli-Q water pH 7) and eluent B (acetonitrile). The gradient profile, expressed as (t [min]; %B), was: (0; 0%), (2; 5%), (4; 15%), (5; 25%), (7; 30%), and (8; 0%). Finally, ATP was detected with a diode array at 260 nm.

2.9. NADH content

NADH content was extracted according to Ortmayr et al. (2014) with modifications. The exocarp was ground (1:2; w/v) with preheated 5 mM ammonium acetate buffer pH 8 containing 5 mM sodium ascorbate, and incubated at 85 °C for 3 min with intermediate mixing, cooled on dry ice, and centrifuged 10 min at 5000 *g* and 4 °C. After centrifugation, the supernatant was filtered and used to analyze NADH content in an Acquity UPLC with an Acquity UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm). NADH was eluted at a flow rate of 0.3 mL min^{-1} using an elution gradient prepared with 5 mM ammonium acetate buffer pH 6 (A) and methanol (B). The gradient profile expressed as (t [min]; %B), was: (0; 0%), (2; 0%), (3.5; 17%), (6; 90%), (6.10; 0%). Finally, NADH was detected with the following transitions: m/z 666.35 → 107.83 (cone voltage 10 and collision energy 54) and 666.35 → 135.93 (cone voltage 10 and collision energy 56) using a Xevo TQ-S triple quadrupole mass spectrometer (Waters) in the positive electro-spray ionization (ESI) mode.

2.10. Assay of diamine oxidase (DAO, EC 1.4.3.22) activity

DAO activity was measured as in Su et al. (2005a) with modifications (Palma et al., 2014). The exocarp was ground with 0.1 M sodium phosphate buffer (pH 6.5) (1:2; w/v) and centrifuged for 20 min at 4 °C and 20000 *g*. Then, the supernatant was precipitated with ammonium sulfate until saturation and the protein was separated by centrifugation for 15 min at 4 °C and 15000 *g*. Finally, the pellet was resuspended in sodium phosphate buffer (0.1 M, pH 6.5) and dialyzed. The reaction mixture contained 20 mM putrescine, 4-aminoantipyrine/N,N-dimethylaniline solution, horseradish peroxidase, and dialyzed extract. Then, the mixture was incubated for 120 min at 40 °C, and the absorbance was measured at 555 nm. The protein content was measured by the Lowry method (Lowry et al., 1951).

2.11. Assays of glutamate decarboxylase (GAD, EC 4.1.1.15) and GABA transaminase (GABA-T, EC 2.6.1.19) activities

GAD and GABA-T activities were measured following the method of Deewatthanawong et al. (2010) with modifications. The exocarp was ground with Trizma–HCl buffer (0.1 M, pH 9.1) containing 1 mM dithiothreitol (DTT), 0.5 mM pyridoxal phosphate (PLP), 5 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% polyvinylpolypyrrolidone (w/v) (PVPP) at a proportion of 1:3 (w/v). The homogenate was centrifuged for 15 min at 4 °C and 10000 *g* and the supernatant was concentrated with Amicon Ultra centrifugal filters. Sodium phosphate buffer (0.2 M, pH 5.8) containing 0.04 mM PLP was added for GAD activity, or Trizma–HCl buffer (50 mM, pH 8.2) containing 1.5 mM DTT, 0.75 mM EDTA, 0.2 mM PLP and 10% (v/v) glycerol for GABA-T activity. GAD and GABA-T activities were performed with 0.6 mM sodium glutamate or 1 mM pyruvate and 3 mM GABA, respectively. The reaction mixtures were incubated for 120 min at 30 °C and stopped by adding HCl (1 M) and $CHCl_3$. Finally, GAD activity was quantified by measuring the production of GABA, as described above (Section 2.7), and GABA-T activity by measuring the production of alanine as described above (Section 2.6).

2.12. Statistical analysis

This experiment was totally randomized. The statistical analysis was performed by ANOVA using the SPSS15.0 program (SPSS Inc.). The means were compared by Fisher's least significant differences test ($P < 0.05$). All data in this paper are expressed as mean ± standard error (SE). Principal component analysis (PCA) was performed on mean-centered data scaled to have standard deviation 1 using MatLab software, R2017b (The MathWorks Inc.).

3. Results and discussion

3.1. Effect of exogenous GABA application on postharvest quality of zucchini fruit during cold storage

Zucchini fruit of the chilling-sensitive variety Sinatra were treated with 1 mM of GABA to test their response to cold storage. The quality parameters of weight loss, CI index, cell-death rate, and electrolyte leakage were evaluated over the storage period at low temperature, and the results are shown in Fig. 1. GABA treatment improved the physiological response of chilling-sensitive fruit to cold, reducing both weight loss and the development of damages. Differences in weight loss between control and treated fruit were significant for long-term storage (10 and 14 days), whereas in the case of CI these differences became significant from day 3 on. After 14 days of cold storage, the GABA treatment halved the injuries to Sinatra fruit. GABA treatment significantly reduced electrolyte leakage and cell-death rate over long-term storage. These parameters have been shown to be good indicators of zucchini fruit quality in relation with chilling resistance (Carvajal et al., 2011, 2015a). GABA has been applied exogenously in different fruit to extend their postharvest life (Sheng et al., 2017) and to increase their tolerance to chilling (Aghdam et al., 2015; Malekzadeh et al., 2017), with similar results as the found in this work with zucchini fruit.

3.2. Effect of exogenous GABA application on putrescine catabolism in cold-stored zucchini fruit

Due to the relationship between putrescine catabolism and GABA, DAO activity and putrescine content were quantified in control and GABA-treated fruit after 1, 3, 5, 10, and 14 d of storage at 4 °C (Fig. 2). Significant differences were detected in putrescine levels only at the end of the storage time, the GABA-treated fruit having higher contents of this polyamine than in control fruit. A higher amount of putrescine could help to maintain the quality of GABA-treated fruit during long-term storage, since it may have a protective role as a compatible solute, as a scavenger of oxygen and hydroxyl radicals, or as a stimulator of the production of antioxidant enzymes and metabolites (Minocha et al., 2014). It has been previously reported that cold tolerance in zucchini fruit is correlated with a lower level of putrescine by the induction of its catabolism via DAO, rendering more metabolites to the GABA shunt (Carvajal et al., 2015b; Palma et al., 2015). In the present work, no differences in DAO activity were detected among control and GABA-treated fruit, in both cases the activity diminished during cold storage. Thus, exogenous application of GABA showed no effect on putrescine catabolism, that would not be necessary to induce probably due to the high availability of GABA.

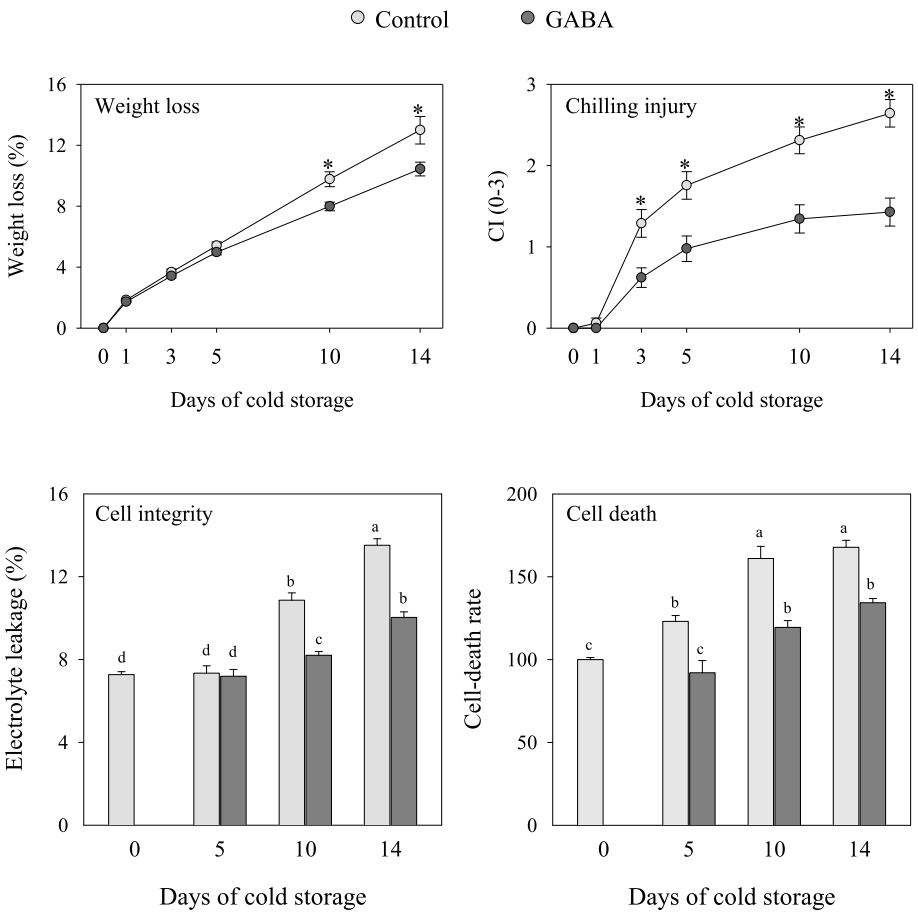

Fig. 1. Effect of exogenous application of GABA (1 mM) on weight loss, chilling-injury index, electrolyte leakage, and cell-death rate in fruit of the variety Sinatra stored at 4 °C. Data presented are means ± SE of triplicate samples of six fruit each. Asterisks indicate significant differences among treatments for the same storage period and different letters indicate significant differences according to Fisher's test ($p < 0.05$).

3.3. Effect of exogenous GABA application on proline content in cold-stored zucchini fruit

Proline is a molecule related to both putrescine and GABA, since it can be synthesized either from ornithine, Δ1-pyrroline via DAO, or from glutamate (Kaur and Asthir, 2015). The content of proline in control and GABA-treated fruit are shown in Fig. 3. During low-temperature storage, zucchini fruit accumulated proline, presumably to cope with the adverse environment. Significant differences in control and treated fruit were detected only after 10 and 14 days of storage at 4 °C; the GABA treated fruit contained more proline than control fruit. Accumulation of proline is part of the defense mechanism in plants when they are exposed to stress, and this accumulation has also been reported in crops after treatments that improve chilling performance during their later postharvest life at low temperature, such as pears (Li et al., 2017), mangos (Zhang et al., 2017), loquats (Zhang et al., 2016), bamboo shoot (Liu et al., 2016), banana (Luo et al., 2015), and zucchini (Carvajal et al., 2015b; Palma et al., 2014, 2015).

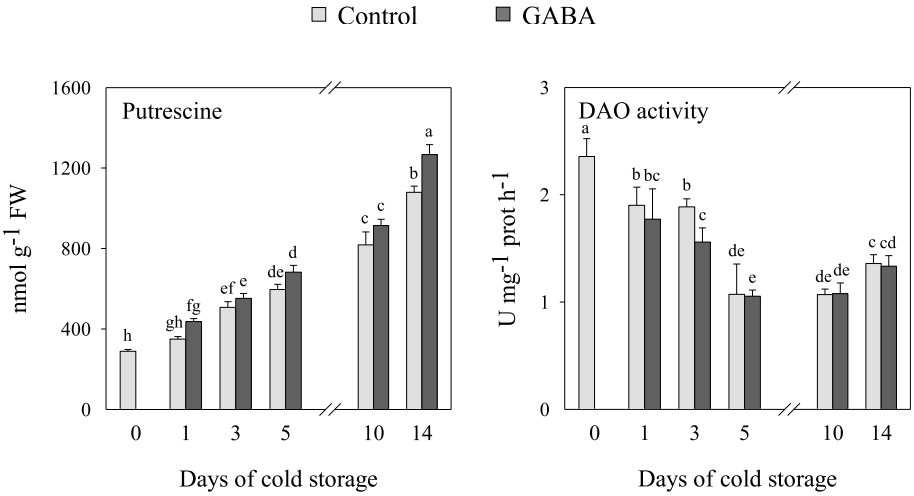

Fig. 2. Effect of exogenous application of GABA (1 mM) on putrescine content and diamine oxidase (DAO) activity in exocarp of Sinatra fruit stored at 4 °C. Data presented are means ± SE of triplicate samples of six fruit each. Different letters indicate significant differences according to Fisher's test ($p < 0.05$).

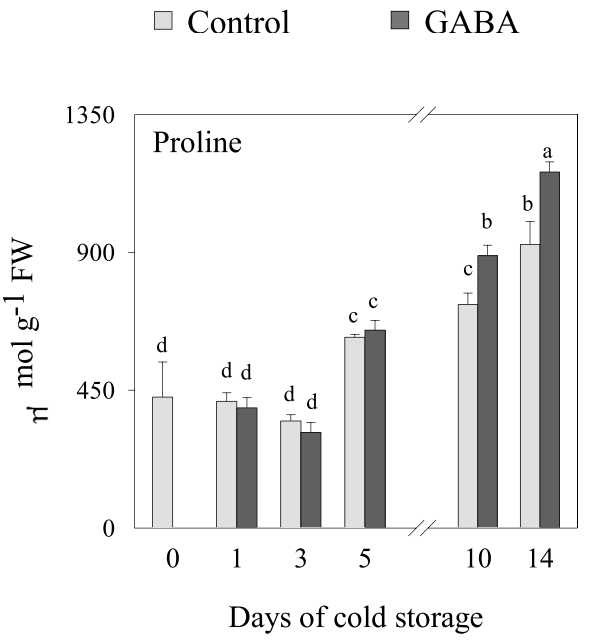

Fig. 3. Effect of exogenous application of GABA (1 mM) on proline content in exocarp of Sinatra fruit stored at 4 °C. Data presented are means ± SE of triplicate samples of six fruit each. Different letters indicate significant differences according to Fisher's test ($p < 0.05$).

3.4. Effect of exogenous GABA application on GABA content and GABA shunt in cold-stored zucchini fruit

The GABA content and the related amino acids alanine and glutamate, as well as the enzymatic activities of GABA transaminase (GABA-T) and glutamate decarboxylase (GAD) were measured, and the results are summarized in Fig. 4. GABA accumulated in treated fruit for the first 24 h of cold exposure in comparison with control and freshly harvested fruit. Thereafter, levels of this amino acid dropped in both control and treated fruit during cold storage but were always significantly higher in GABA treated fruit. In addition to the importance of the GABA-shunt pathway, GABA by itself is a compatible osmolyte that can act as cryoprotective molecule and also as a hydroxyl radical scavenger (Heber et al., 1971; Shelp et al., 1999; Smirnoff and Cumbes, 1989). Under stress conditions, it has also been described that GABA collaborates maintaining the membrane integrity (Aghdam et al., 2015; Deng et al., 2010) and the osmotic adjustment (Li et al., 2016), and increasing the antioxidant defense (Aghdam et al., 2016; Malekzadeh et al., 2017; Wang et al., 2014). All these properties could help zucchini fruit to cope with chilling conditions during the storage period, improving their postharvest quality as it happens in the fruit with the GABA treatment.

Glutamate is the precursor of GABA via GAD activity, and alanine is a product derived of GABA via GABA-T activity. Both alanine and glutamate content tended to increase at the end of the storage period and the differences between treatments were detected only over long-term of storage. After 14 days of cold storage the fruit treated with GABA contained less glutamate and higher amount of alanine that control fruit. Both GAD and GABA-T activities increased significantly in treated fruit after 14 d, indicating an induction of the GABA shunt over long-term storage that parallels the higher resistance to cold stress

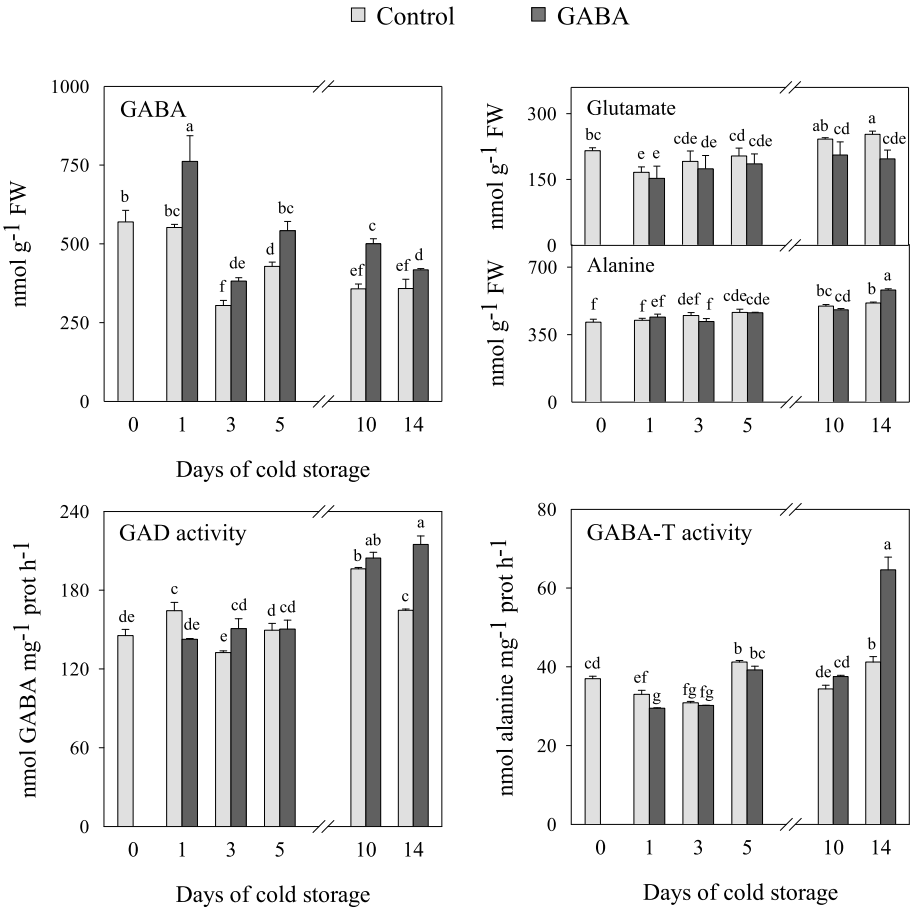

Fig. 4. Effect of exogenous application of GABA (1 mM) on GABA, glutamate, and alanine content, as well as on glutamate decarboxylase (GAD) and GABA transaminase (GABA-T) activities in exocarp of Sinatra fruit stored at 4 °C. Data presented are means ± SE of triplicate samples of six fruit each. Different letters indicate significant differences according to Fisher's test ($p < 0.05$).

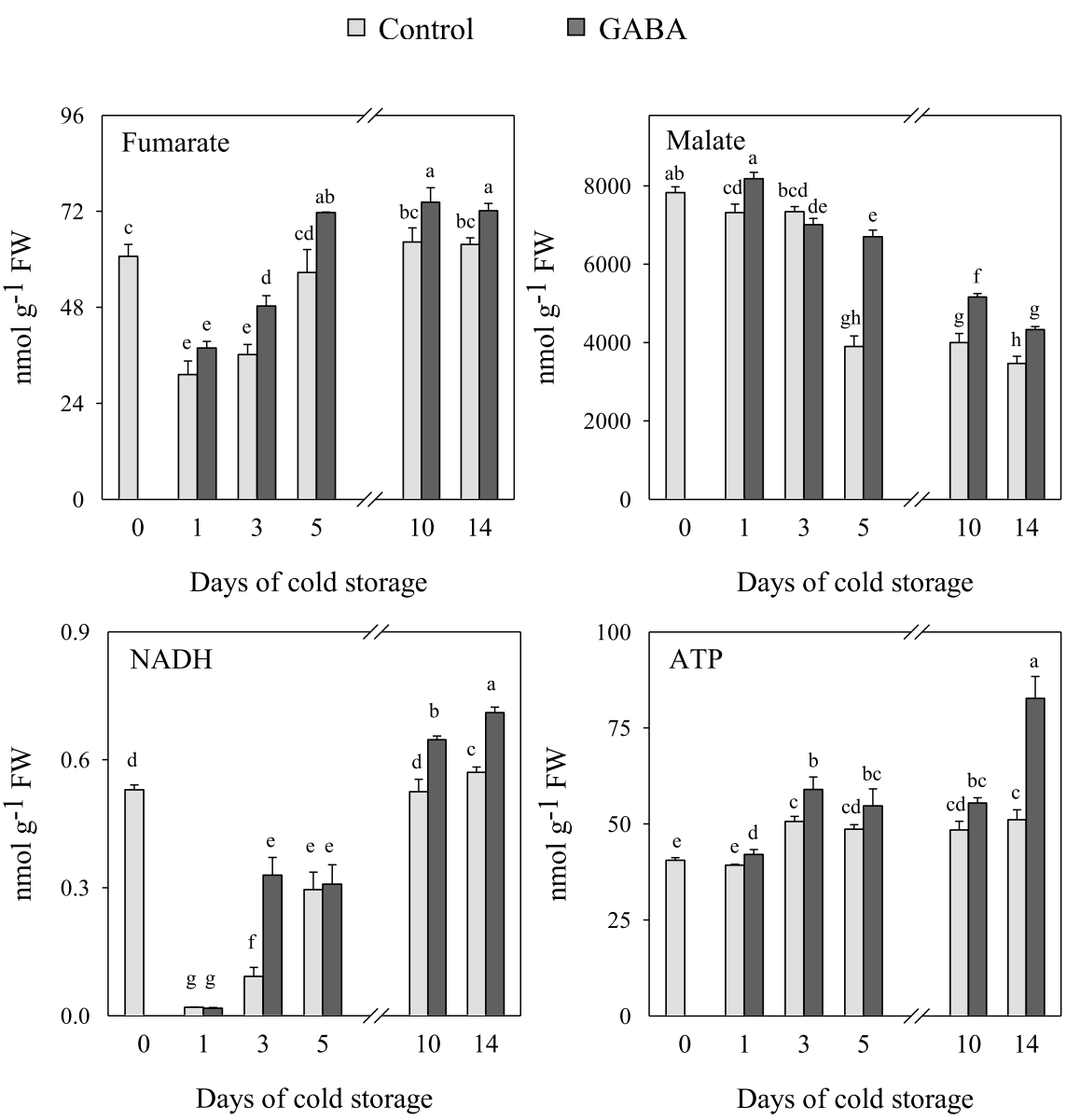

Fig. 5. Effect of exogenous application of GABA (1 mM) on fumarate and malate as well as ATP and NADH content in exocarp of Sinatra fruit stored at 4 °C. Data presented are means ± SE of triplicate samples of six fruit each. Different letters indicate significant differences according to Fisher's test ($p < 0.05$).

measured in the treated fruit. An exogenous application of putrescine to cold-sensitive zucchini fruit also induced the GABA shunt via putrescine catabolism at the end of postharvest cold storage (Palma et al., 2015) and similar results were detected in the cold-tolerant variety Natura during cold storage but without treatment (Palma et al., 2014). All these findings highlight the important role of the GABA and the GABA-shunt pathway in postharvest cold responses in zucchini fruit.

3.5. Effects of exogenous GABA application on organic acids, NADH, and ATP in cold-stored zucchini fruit

The GABA shunt renders succinate, which can directly enter the TCA cycle, bypassing two of its steps. Thereafter, succinate dehydrogenase (SDH) oxidizes succinate to fumarate, this being converted to malate by fumarase or fumarate hydratase (Araujo et al., 2012). The content of both organic acids fumarate and malate were quantified in the exocarp of control and GABA-treated fruit over the storage period at 4 °C, and the results are presented in Fig. 5. The fumarate content dropped immediately after transferring the fruit to low temperature, but after 1 day the values increased steadily in both control and GABA-treated fruit. Treated fruit contained significantly higher levels of fumarate from day 3 of storage. On the contrary, the malate content decreased progressively during postharvest cold storage, but levels were consistently higher in treated fruit from day 5. In the cell, the TCA cycle generates NADH and supports the production of ATP via the mitochondrial electron-transport chain (Araujo et al., 2012). Both molecules, NADH and ATP, were analyzed in the exocarp of zucchini fruit, and the results are also summarized in Fig. 5. The NADH levels dropped when zucchini fruit were transferred to 4 °C, but these levels recovered to reach values similar to those detected in freshly harvested fruit. Over long-term storage, 10 and 14 days, The fruit treated with GABA contained higher level of NADH that control fruit. The application of GABA also increased ATP content in Sinatra fruit, becoming significant at the end of the storage and coinciding with the GABA-shunt induction. The energy status of the tissue has been demonstrated to be an essential factor in chilling tolerance in zucchini fruit (Carvajal et al., 2015b; Palma et al., 2014, 2015). The presence of ATP seems to be important in fruit for the attenuation of CI and the preservation of

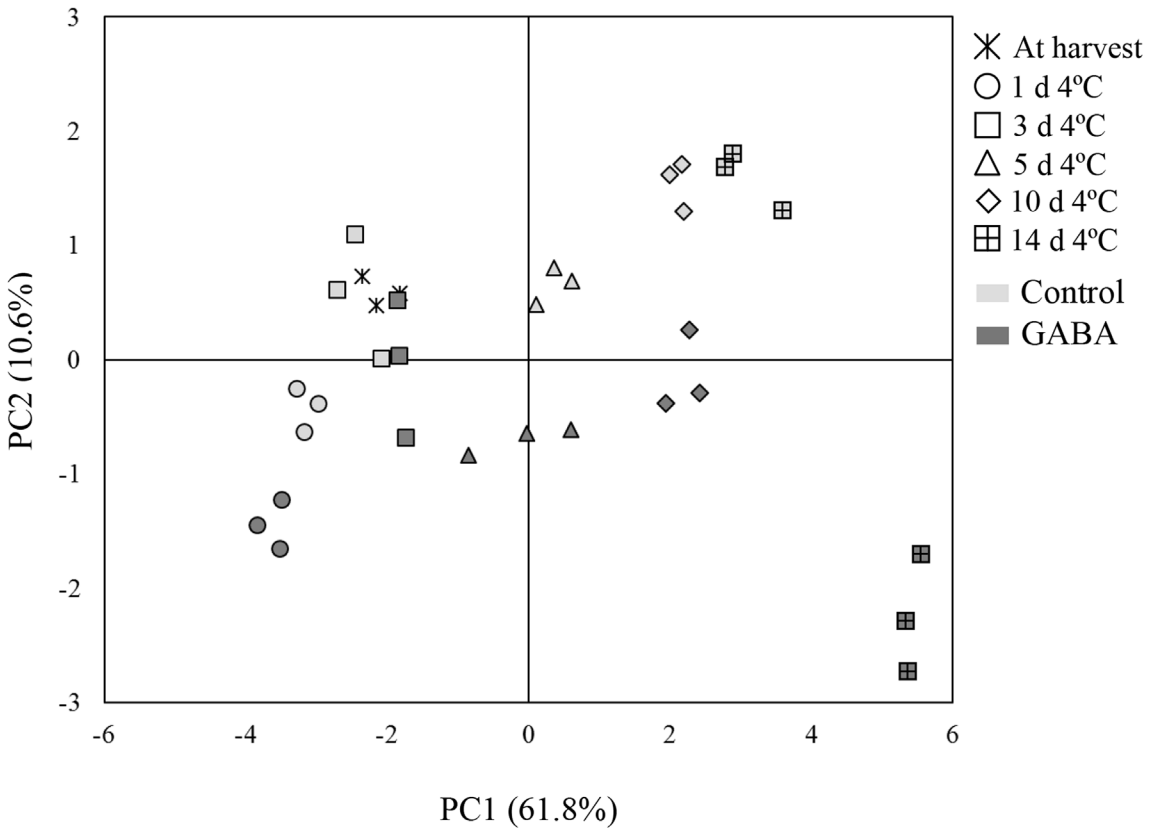

Fig. 6. Principal component analysis (PCA) scores plot of metabolites (putrescine, proline, GABA, glutamate, alanine, fumarate, malate, ATP, and NADH) and enzymes (DAO, GAD, and GABA-T) related with GABA metabolism measured in exocarp of Sinatra control and GABA fruit stored at 4 °C. Three independent biological replicates of each storage time and treatment were used for the different analysis. PC1 and PC2 accounted for 61.8% and 10.6% of the total explained variance, respectively.

fruit quality. During the postharvest life of longan fruit, Su et al. (2005b) reported that a treatment with pure oxygen increased ATP content and maintained high level of energy charge, which maintained better integrity of cell membranes. In different plants ATP deficit could induce lipid peroxidation under various stress conditions (Crawford and Brändle, 1996).

3.6. Principal component analysis (PCA) for the comparison of metabolites and enzymes related to GABA metabolism in treated and non-treated fruit during cold storage

Principal components analysis (PCA) was performed on metabolites and enzymes data to visualize possible grouping associated with storage time and treatment (Fig. 6). First two dimensions explained more than 70% of total variation found in the analysis. PCA scores plot showed a separation between control and GABA-treated fruit already after 1 day of storage at 4 °C, indicating an early response to chilling. From fifth day, the separation between treatments increased until reaching the maximum differences at 14 days of storage, time at what the induction of the GABA-shunt pathway was more prominent in the treated fruit and the quality parameters more differentiated.

4. Conclusion

The exogenous application of GABA improves the postharvest quality of zucchini fruit stored at low temperature. In the exocarp, this treatment induces the accumulation of GABA and putrescine, although putrescine catabolism is not affected. This response could be due to the greater availability of GABA as a consequence of the treatment. The GABA treatment also leads to a greater induction of the GABA-shunt pathway over long-term storage at 4 °C, which provides intermediates of the TCA cycle and, accordingly, higher ATP and NADH contents. Moreover, the availability of the substrate of GABA-shunt pathway allows the accumulation of other related metabolites such as proline. Overall, the changes that occur after exogenous application of GABA provide the fruit with a higher capability to withstand low temperature during postharvest storage, reflected in a reduction of weight loss, chilling injury, electrolyte leakage, and cell-death rate. These results highlight the importance of GABA in postharvest cold responses in zucchini fruit.

Contribution

Francisco Palma, Manuel Jamilena and Dolores Garrido designed research; Francisco Palma, Fatima Carvajal, Raquel Jimenez-Muñoz performed research; Dolores Garrido and Amada Pulido analyzed data; Francisco Palma, Fatima Carvajal and Dolores Garrido wrote the paper.

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References

Aghdam, M.S., Naderi, R., Jannatizadeh, A., Sarcheshmeh, M.A.A., Babalar, M., 2016. Enhancement of postharvest chilling tolerance of anthurium cut flowers by γ-

aminobutyric acid (GABA) treatments. Sci. Hortic. 198, 52–60.
Aghdam, M.S., Naderi, R., Sarcheshmeh, M.A.A., Babalar, M., 2015. Amelioration of postharvest chilling injury in anthurium cut flowers by γ-aminobutyric acid (GABA) treatments. Postharvest Biol. Technol. 110, 70–76.
Araujo, W.L., Nunes-Nesi, A., Nikoloski, Z., Sweetlove, L.J., Fernie, A.R., 2012. Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant Cell Environ. 35, 1–21.
Cao, S., Cai, Y., Yang, Z., Zheng, Y., 2012. MeJA induces chilling tolerance in loquat fruit by regulating proline and gamma-aminobutyric acid contents. Food Chem. 133, 1466–1470.
Carvajal, F., Martínez, C., Jamilena, M., Garrido, D., 2011. Differential response of zucchini varieties to low storage temperature. Sci. Hortic. 130, 90–96.
Carvajal, F., Palma, F., Jamilena, M., Garrido, D., 2015a. Cell wall metabolism and chilling injury during postharvest cold storage in zucchini fruit. Postharvest Biol. Technol. 108, 68–77.
Carvajal, F., Palma, F., Jamilena, M., Garrido, D., 2015b. Preconditioning treatment induces chilling tolerance in zucchini fruit improving different physiological mechanisms against cold injury. Ann. Appl. Biol. 166, 340–354.
Carvajal, F., Rosales, R., Palma, F., Manzano, S., Cañizares, J., Jamilena, M., Garrido, D., 2018. Transcriptomic changes in Cucurbita pepo fruit after cold storage: differential response between two cultivars contrasting in chilling sensitivity. BMC Genomics 19, 125.
Crawford, R., Brändle, R., 1996. Oxygen deprivation stress in a changing environment. J. Exp. Bot. 47, 145–159.
Deewatthanawong, R., Rowell, P., Watkins, C.B., 2010. γ-Aminobutyric acid (GABA) metabolism in CO_2 treated tomatoes. Postharvest Biol. Technol. 57, 97–105.
Del-Saz, N.F., Romero-Munar, A., Cawthray, G.R., Palma, F., Aroca, R., Baraza, E., Florez-Sarasa, I., Lambers, H., Ribas-Carbó, M., 2018. Phosphorus concentration coordinates a respiratory bypass, synthesis and exudation of citrate, and the expression of high-affinity phosphorus transporters in Solanum lycopersicum. Plant Cell Environ. 41, 865–875.
Deng, Y., Xu, L., Zeng, X., Li, Z., Qin, B., He, N., 2010. New perspective of GABA as an inhibitor of formation of advanced lipoxidation end-products: it's interaction with malondiadehyde. J. Biomed. Nanotechnol. 6, 318–324.
Flores, H.E., Galston, A.W., 1982. Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol. 69, 701–706.
Heber, U., Tyankova, L., Santarius, K.A., 1971. Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. Biochim. Biophys. Acta Biomembr. 241, 578–592.
Kaur, G., Asthir, B., 2015. Proline: a key player in plant abiotic stress tolerance. Biol. Plant. 59, 609–619.
Kinnersley, A.M., Turano, F.J., 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. Crit. Rev. Plant Sci. 19, 479–509.
Li, D., Cheng, Y., Dong, Y., Shang, Z., Guan, J., 2017. Effects of low temperature conditioning on fruit quality and peel browning spot in 'Huangguan'pears during cold storage. Postharvest Biol. Technol. 131, 68–73.
Li, D., Li, L., Xiao, G., Limwachiranon, J., Xu, Y., Lu, H., Yang, D., Luo, Z., 2018. Effects of elevated CO_2 on energy metabolism and γ-aminobutyric acid shunt pathway in postharvest strawberry fruit. Food Chem. 265, 281–289.
Li, Z., Yu, J., Peng, Y., Huang, B., 2016. Metabolic pathways regulated by γ-aminobutyric acid (GABA) contributing to heat tolerance in creeping bentgrass (*Agrostis stolonifera*). Sci. Rep. 6, 30338.
Liu, Z., Li, L., Luo, Z., Zeng, F., Jiang, L., Tang, K., 2016. Effect of brassinolide on energy status and proline metabolism in postharvest bamboo shoot during chilling stress. Postharvest Biol. Technol. 111, 240–246.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Luo, Z., Li, D., Du, R., Mou, W., 2015. Hydrogen sulfide alleviates chilling injury of banana fruit by enhanced antioxidant system and proline content. Sci. Hortic. 183, 144–151.
Malekzadeh, P., Khara, J., Heidari, R., 2012. Effect of exogenous Gama-aminobutyric acid on physiological tolerance of wheat seedlings exposed to chilling stress. Iranian J. Plant Physiol. 3, 611–617.
Malekzadeh, P., Khara, J., Heydari, R., 2014. Alleviating effects of exogenous Gamma-aminobutiric acid on tomato seedling under chilling stress. Physiol. Mol. Biol. Plants 20, 133–137.
Malekzadeh, P., Khosravi-Nejad, F., Hatamnia, A.A., Mehr, R.S., 2017. Impact of postharvest exogenous γ-aminobutyric acid treatment on cucumber fruit in response to chilling tolerance. Physiol. Mol. Biol. Plants 23, 827–836.
Mao, L., Pang, H., Wang, G., Zhu, C., 2007. Phospholipase D and lipoxygenase activity of cucumber fruit in response to chilling stress. Postharvest Biol. Technol. 44, 42–47.
Martínez-Téllez, M.A., Ramos-Clamont, M.G., Gardea, A.A., Vargas-Arispuro, I., 2002. Effect of infiltrated polyamines on polygalacturonase activity and chilling injury responses in zucchini squash (*Cucurbita pepo* L.). Biochem. Bioph. Res. Co. 295, 98–101.
Megías, Z., Manzano, S., Martínez, C., García, A., Aguado, E., Garrido, D., del Mar Rebolloso, M., Valenzuela, J.L., Jamilena, M., 2016. Postharvest cold tolerance in summer squash and its association with reduced cold-induced ethylene production. Euphytica 213, 9.
Minocha, R., Majumdar, R., Minocha, S.C., 2014. Polyamines and abiotic stress in plants: a complex relationship. Front. Plant Sci. 5.
Ortmayr, K., Nocon, J., Gasser, B., Mattanovich, D., Hann, S., Koellensperger, G., 2014. Sample preparation workflow for the liquid chromatography tandem mass spectrometry based analysis of nicotinamide adenine dinucleotide phosphate cofactors in yeast. J. Separ. Sci. 37, 2185–2191.
Palma, F., Carvajal, F., Jamilena, M., Garrido, D., 2014. Contribution of polyamines and other related metabolites to the maintenance of zucchini fruit quality during cold storage. Plant Physiol. Biochem. 82, 161–171.
Palma, F., Carvajal, F., Ramos, J.M., Jamilena, M., Garrido, D., 2015. Effect of putrescine application on maintenance of zucchini fruit quality during cold storage: contribution of GABA shunt and other related nitrogen metabolites. Postharvest Biol. Technol. 99, 131–140.
Qu, G.-Q., Liu, X., Zhang, Y.-L., Yao, D., Ma, Q.-M., Yang, M.-Y., Zhu, W.-H., Yu, S., Luo, Y.-B., 2009. Evidence for programmed cell death and activation of specific caspase-like enzymes in the tomato fruit heat stress response. Planta 229, 1269–1279.
Ramesh, S.A., Tyerman, S.D., Xu, B., Bose, J., Kaur, S., Conn, V., Domingos, P., Ullah, S., Wege, S., Shabala, S., 2015. GABA signalling modulates plant growth by directly regulating the activity of plant-specific anion transporters. Nat. Commun. 6, 7879.
Shang, H., Cao, S., Yang, Z., Cai, Y., Zheng, Y., 2011. Effect of exogenous gamma-aminobutyric acid treatment on proline accumulation and chilling injury in peach fruit after long-term cold storage. J. Agric. Food Chem. 59, 1264–1268.
Shelp, B.J., Bown, A.W., McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446–452.
Shelp, B.J., Bozzo, G.G., Trobacher, C.P., Zarei, A., Deyman, K.L., Brikis, C.J., 2012. Hypothesis/review: contribution of putrescine to 4-aminobutyrate (GABA) production in response to abiotic stress. Plant Sci. 193, 130–135.
Sheng, L., Shen, D., Luo, Y., Sun, X., Wang, J., Luo, T., Zeng, Y., Xu, J., Deng, X., Cheng, Y., 2017. Exogenous γ-aminobutyric acid treatment affects citrate and amino acid accumulation to improve fruit quality and storage performance of postharvest citrus fruit. Food Chem. 216, 138–145.
Smirnoff, N., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060.
Su, G.X., An, Z.F., Zhang, W.H., Liu, Y.L., 2005a. Light promotes the synthesis of lignin through the production of H_2O_2 mediated by diamine oxidases in soybean hypocotyls. J. Plant Physiol. 162, 1297–1303.
Su, X., Jiang, Y., Duan, X., Liu, H., Li, Y., Lin, W., Zheng, Y., 2005b. Effects of pure oxygen on the rate of skin browning and energy status in longan fruit. Food Technol. Biotechnol. 43, 359.
Wang, D., Li, L., Xu, Y., Limwachiranon, J., Li, D., Ban, Z., Luo, Z., 2017. Effect of exogenous nitro oxide on chilling tolerance, polyamine, proline, and γ-aminobutyric acid in Bamboo Shoots (*Phyllostachys praecox* f. prevernalis). J. Agric. Food Chem. 65, 5607–5613.
Wang, Y., Luo, Z., Huang, X., Yang, K., Gao, S., Du, R., 2014. Effect of exogenous gamma-aminobutyric acid (GABA) treatment on chilling injury and antioxidant capacity in banana peel. Sci. Hortic. 168, 132–137.
Wang, Y., Luo, Z., Mao, L., Ying, T., 2016. Contribution of polyamines metabolism and GABA shunt to chilling tolerance induced by nitric oxide in cold-stored banana fruit. Food Chem. 197, 333–339.
Zhang, Y., Jin, P., Huang, Y., Shan, T., Wang, L., Li, Y., Zheng, Y., 2016. Effect of hot water combined with glycine betaine alleviates chilling injury in cold-stored loquat fruit. Postharvest Biol. Technol. 118, 141–147.
Zhang, Z., Zhu, Q., Hu, M., Gao, Z., An, F., Li, M., Jiang, Y., 2017. Low-temperature conditioning induces chilling tolerance in stored mango fruit. Food Chem. 219, 76–84.