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Biochemical and Biophysical Research Communications 295 (2002) 98–101

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Effect of infiltrated polyamines on polygalacturonase activity and chilling injury responses in zucchini squash (*Cucurbita pepo* L.)

M.A. Martínez-Téllez,* M.G. Ramos-Clamont, A.A. Gardea, and I. Vargas-Arispuro

Centro de Investigación en Alimentación y Desarrollo, A.C., P.O. Box 1735, 83000, Hermosillo, Sonora, Mexico

Received 22 May 2002

Abstract

The effect of exogenous polyamines on electrolyte leakage, chilling index, polygalacturonase activity (PG), ethylene production, and firmness in zucchini squash fruits stored for 12 days at 2 °C or 10 °C, 85–90% RH was evaluated. Fruits were infiltrated with putrescine (PUT) spermidine (SPD) and spermine (SPM) at 0.1, 0.25, 0.5, 2.0, and 4.0 mM. All polyamines exerted a protective effect on cell and organelle membranes. The most effective was SPD, which reduced electrolyte leakage between 62% and 82%, compared to control fruits stored at 2 °C. At 10 °C they did not exhibit chilling injury (CI) symptoms, while at 2 °C SPM (0.5 mM) and SPD (0.5 mM) diminished them 92% and 100%, respectively; which extended storage life for 8–10 days at 2 °C. High concentrations of polyamines (> 2.0 mM) caused the appearance of CI symptoms. PG activity diminished proportionally to the concentration of polyamine except for the concentration at 4.0 mM. No significant changes were observed in ethylene production.

Keywords: Polyamines; Polygalacturonase-activity; Firmness; Chilling-injury

Chilling injury (CI) is the physiological damage induced in tissues of tropical and subtropical origin when they are exposed to low, but nonfreezing temperatures [1]. Zucchini squash sensitive to CI [2–4], cannot be stored more than 1–2 days at 5 °C without showing irreversible CI symptoms [5]. CI in zucchini squash is characterized by circular to longitudinal pits on the surface [2,4], and may be the result of a loss of cellular integrity caused by damage to membranes [6–8] and to cell walls [9].

Polyamines (PA) such as PUT, SPD, and SPM have been shown to reduce CI, thus increasing tolerance of chilling-sensitive tissues to low temperatures. It is believed that these polycationic molecules stabilize the cellular membranes, therefore minimizing changes in permeability and loss of fluid [10,11]. In addition, these compounds can act as free radical scavengers, protecting cellular membranes from oxidation [12,13].

Exogenous polyamines reduce stress symptoms caused by ozone [14], and increase tolerance of vegetables to low temperatures [10,15,16]. Treatments with exogenous polyamines after harvest, but before cold storage, increase endogenous polyamine levels and reduce CI in apples [1]. Exogenous polyamines also have been shown to inhibit in vitro polygalacturonase activity (PG) in "Golden Delicious" apples inoculated with *Penicillium expansum* and decrease apple softening [1,17]. The objective of this study was to determine the effect of exogenous PA's on ion leakage, flesh firmness, and PG activity, and to evaluate the relationship of PG activity and CI index in zucchini squash.

Materials and methods

'Raben' zucchini squash was harvested from a local farm near Hermosillo, Sonora, Mexico. Samples were selected according to size (16–22 cm long) and absence of damage, washed and randomly divided into two lots. Controls (lot one) were held at 2 and 10 °C in 85–90% RH for 12 days. Fruits of lot two were pressure infiltrated with three different PA (putrescine, spermine, and spermidine) at five different concentrations (0.1, 0.25, 0.5, 2.0, and 4.0 mM) and then storage at 2 and 10 °C, 85–90% RH for 12 days. Controls were infiltrated with distilled water. After storage, electrolyte leakage, flesh firmness, PG activity, and ethylene production were determined using three

* Corresponding author. Fax: +662-280-00-55.
E-mail address: norawa@cascabel.ciad.mx (M.A. Martínez-Téllez).

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00631-9

replicates of 18-fruit samples from each group. The extent of surface pitting was determined by CI index. An exploratory factorial experiment was run to evaluate the effect of temperatures and a mixture of PA. The experimental design used was completely randomized with two temperatures (2 and 10 °C) and with or without PA mixture application (PUT + SPD + SPM at 4 mM each). Later, individual PA concentrations were applied at either 2 or 10 °C to test their putative protective effect. Data were analyzed by ANOVA (NCSS, 1995).

Polyamines infiltration. Three polyamines at five different concentrations were used. Putrescine (PUT), spermidine (SPD), and spermine (SPM) (Sigma Chem., St. Louis, MO) were used at 0.1, 0.25, 0.5, 2.0, and 4.0 mM [18]. Infiltration was done by immersing the fruits in a 2-L of polyamine solution in a desiccator under an air pressure of 0.9 kg/cm^2/3 min [1].

Ion leakage. Only uniform, healthy fruit without external defects was used. Fruits from each treatment were held at 20 °C for 0.5 h before 1 cm diameter pericarp discs (1 cm diameter/2 mm thickness) were excised with a stainless steel cork borer. Discs were washed three times in deionized water and incubated in 20 ml of 0.3 M mannitol (Sigma Chem., St. Louis, MO) in 30 ml plastic tubes. The tubes were shaken at 65 cycles per min and solution conductivity was measured after 1 h with a Cole Palmer 1481–61 conductivity meter. Preliminary experiments showed that the rate of ion leakage was constant between 1 and 2 h. Tubes containing mannitol solution and tissue were heated in boiling water for 30 min, and total conductivity was measured after cooling down to room temperature. Rate of ion leakage was expressed as percentage of the total conductivity in an hour [19].

PG activity. PG activity was determined with the method described by Gross [20]. PG was extracted from 50 g of peel tissue 2 mm thick with 300 ml of a 1% sodium bisulfite solution at pH 6.0 and 150 ml of a 1 M sodium chloride solution. The number of reducing groups formed was calculated by a standard curve of 10–900 nmol of galacturonic acid/0.2 ml. Protein was estimated by Bradford [21] with bovine serum albumin (Bio-Rad CA, USA) as standard. Activity was expressed in Units, where a Unit is defined as the μmol of galacturonic acid/mg of protein produced in 1 h.

Chilling injury index. CI symptoms in "Zuchinni squash" are small dark pite-like depression along the fruit. CI index was evaluated by means of a subjective scale of visual symptoms based on necrotic surface and intensity of pitting determined: 0 = no pitting, 1 = slight (10% or less), 2 = medium (10–20%), and 3 = severe pitting (> 20%). A CI index was determined using the following formula: Σ (pitting scale (0–3) × number corresponding fruits within each class)/total number of fruits estimated (20 fruits) [22].

The CI symptoms of zucchini squash were determined 3 h after transferring the squash from 2 and 10 °C storage chambers to room temperature (25 °C).

Firmness. Flesh fruit firmness was measured on three replicates on centers without skin, in three fruits per treatment with a DFG-50 Chatillon firmness tester using a 10 mm tip. Fruits from all lots were tested for firmness at the end of the 12-day period at 2 and 10 °C.

Ethylene production. Three fruits were sealed in 3.5-L glass jars at 20 °C for 4 h. One-ml gas sample was withdrawn from the headspace and injected into a Varian Star Model 3400 CX gas chromatograph, equipped with an activated alumina column (80/100 mesh), and a flame ionization detector. Column temperature was maintained constant at 100 °C and nitrogen was used as a carrier gas.

Results

The average ion leakage from discs of squash peel tissue stored at 2 or 10 °C for 12 days is shown in Fig. 1A. Leakage was considerably lower on discs held at 10 °C than those kept at 2 °C. Infiltrated PA had no significant effect ($p < 0.05$) on ion leakage from discs held at 10 °C (Fig. 1A). PA infiltration affected tissue susceptibility to chilling injury. Leakage from control tissue held at 2 °C was greater than leakage from PA-treated discs. SPD at 0.25 mM was the most effective in preventing injury. Tissue leakage from this treatment was about four times lower than leakage from the control tissues (Fig. 1B).

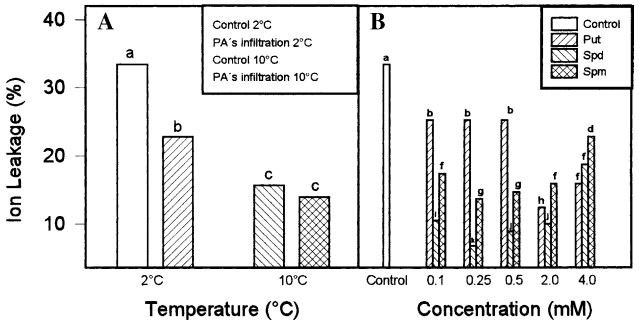

Fig. 1. Ion leakage (%) of zucchini squash treated with PUT, SPD, or SPM and storage for 12 days at 2 °C. (A) Effect of polyamine temperature. (B) Effect of polyamine concentration. Different letters show a statistical significant difference.

Data presented in Fig. 2A indicates that the effect of the storage temperature on PG activity was significant ($p < 0.05$). PA infiltration decreased PG activity in stored fruits, independently of temperatures. The activity of this enzyme in fruits stored at 10 °C was higher than in fruits stored at 2 °C. At 2 °C SPD and SPM treatments were the most effective in a global evaluation (Fig. 2B). SPD, SPM, and PUT at 2.0 mM were the most effective since their average value of PG activity was 60% lower than those of the control. SPD and SPM at 0.1 mM and PUT at 0.5 mM had no effect over enzyme activity. PG activity was higher at 4.0 mM concentration of polyamines compared to 2.0 mM (Fig. 2B).

CI in zucchini squash is characterized by circular to longitudinal pits on the surface. No CI developed during storage at 10 °C, however severe pitting was detected on

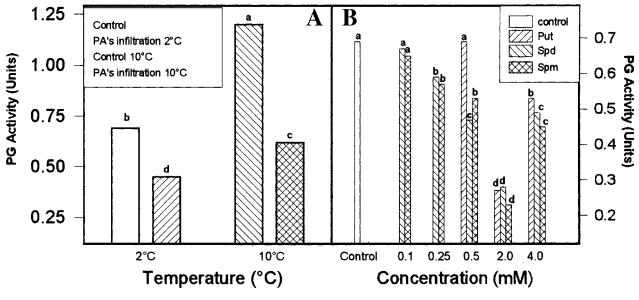

Fig. 2. Polygalacturonase activity (Units) of zucchini squash treated with PUT, SPD, or SPM and storage for 12 days at 2 °C. (A) Effect of temperature. (B) Effect of polyamine concentration. Different letters show statistical significant difference. Activity Unit = a μmol of galacturonic acid/mg of protein/h.

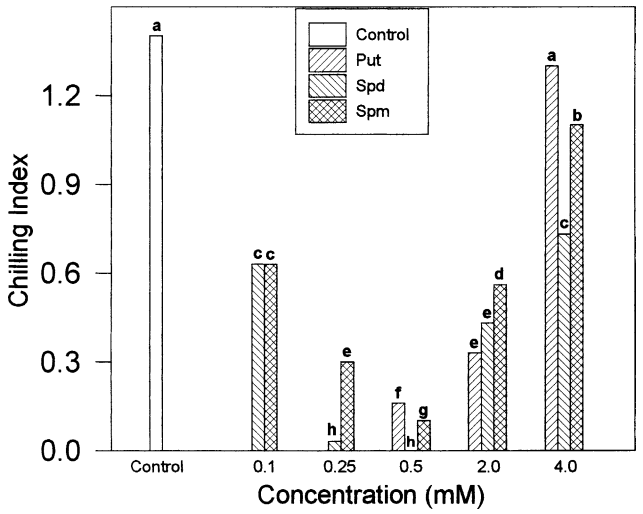

Fig. 3. Chilling index of zucchini squash treated with PUT, SPD, or SPM and storage for 12 days at 2 °C. Different letters show statistical significant difference.

the skin of squash from the 2 °C control group. Fig. 3 shows CI index after 12 days of storage at 2 °C. The CI symptoms in squash were reduced by PA infiltration. SPD at 0.5 mM was the most effective; its average CI value was 80% lower than that in controls. Squash treated with 0.5 mM SPD did not develop chilling injury during storage at 2 °C, implying an extension of the storage time up to 12 days.

At 2 °C, PA treatments had higher values of firmness than control (Table 1). The difference in firmness between control and treated fruit was significant ($p < 0.05$). After 12 days of storage at 2 °C, the difference between PA's treated and untreated fruits, ranged from 1.5 kgf (0.5 mM) to 1.98 kgf (4.0 mM), in squash treated with PUT. In squash treated with SPD, the range went from 0.9 kgf (0.1 mM) to 1.98 kgf (4.0 mM). Squash treated with SPM ranged from 1.43 kgf (4 mM) to 1.81 kgf (0.1 mM) in squash treated with SPM.

Results of the ethylene measurements showed that PA influence on ethylene production was negligent and independent of polyamine or its concentration at both storage temperatures for 12 days of storage (data not shown).

Table 1
Firmness (kgf) of zucchini squash infiltrated with PUT, SPD, or SPM and storage for 12 days at 2 °C

Concentration	PUT	SPD	SPM
Control	4.2[d]	4.2[d]	4.2[d]
0.10 (mM)	5.7[b]	5.1[c]	5.0[c]
0.25 (mM)	5.8[a]	6.0[a]	6.0[a]
0.50 (mM)	6.0[a]	5.1[c]	5.2[c]
2.00 (mM)	6.0[a]	5.5[b]	5.8[a]
4.00 (mM)	5.5[b]	6.0[a]	5.5[b]

Different letters show statistical difference ($p < 0.05$).

Discussion

Ion leakage was a good qualitative indicator of chilling sensitivity in zucchini squash. Electrolyte leakage, used as a gross measurement of membrane permeability, increased in fruits after 12 days of chilling at 2 °C in a water-saturated atmosphere, as compared to leakage rate from fruits held at 10 °C.

Ion leakage measurement can also be used to study the effectiveness of treatments that alter the susceptibility of sensitive plants to chilling induced injury [23,24]. At chilling temperature, PA treatments decreased leakage at all concentrations. SPD was more effective in fruits kept at 2 °C, since it reduced ion leakage 67–82% in comparison to controls. Sanchez et al. [25] observed that the increase in endogenous PUT, SPD, and SPM induced by thermal treatments showed a concomitant relation with a decrease of leakage in cucumber cotyledons. Lee et al. [26] found a strong correlation between increase of endogenous PUT and decrease of leakage in seven rice cultivars subjected to CI. The mechanism by which PUT, SPD, and SPM can reduce ion leakage is still unknown, although it has been suggested that these compounds can reduce or delay the effects of different stress by its ability to stabilize and protect cellular membranes [27,28].

The increase in ion leakage following chilling was probably an early manifestation tissue collapse, which produced the visual symptoms [29]. At 10 °C there were no CI symptoms and ion leakage was significantly lower ($p < 0.05$) than in squashes stored at 2 °C. Chilling index at 2 °C was moderate (damage from 10% to 20%) in controls after 12 days. At 2 °C, PA's treatments decreased the CI. SPD was again the most effective, since SPD at 0.5 mM completely eliminated CI symptoms. At this concentration SPM and PUT reduces CI index at 92% and 87%, respectively.

PA infiltration decreased zucchini PG activity. SPD and SPM were the most effective at 2.0 mM concentrations. PAs are able to bind negatively charged molecules like pectic polysaccharides [30–32]. The formation of pectin–PA complexes in the cell wall would make pectin less accessible to PG attack. PA also inhibit biosynthesis of ethylene in plants [33] and the activation of transcription of the PG gene occurs after ethylene synthesis [34]. Nevertheless, PA application in squash does not have a significant effect on ethylene production. Thus it is possible that PA action is more related to cell wall protection than to ethylene metabolism.

Conclusions

PA application significantly reduced the CI, ion leakage, and PG activity in zucchini squash fruits stored for 12 days at 2 °C.

SPD was the most effective polyamine treatment at concentrations of 0.25 and 0.5 mM. Higher concentrations (2 mM) increased CI, ion leakage, and PG activity.

Polyamines had no significant effect on ethylene production. This is contrary to the opinion that PA influence ethylene production. It is possible that formation of pectin–PA complexes in cell walls would make pectin less accessible to PG action.

Acknowledgments

We thank Olivia Briceño-Torres for her technical assistance. Gabriela Ramos-Clamont M. received a fellowship from CONACYT-México.

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