CO_2 Treatment of Zucchini Squash Reduces Chilling-Induced Physiological Changes

María Serrano,[†] M. Teresa Pretel,[†] M. Concepción Martínez-Madrid,[†] Félix Romojaro,[‡] and Fernando Riquelme*[,‡]

Escuela Politécnica Superior (Universidad Miguel Hernández), Ctra. Beniel Km 3.2, Orihuela, Alicante, Spain, and Centro de Edafología y Biología Aplicada del Segura (CSIC), Avenida La Fama 1, 30003 Murcia, Spain

Zucchini squash showed chilling injury (CI) damage as pitting on 93% of their surface after 12 days at 2 °C, and a 2−2.5-fold increase in putrescine levels in both skin and pulp was detected during storage (from 162 and 38 to 320 and 98 nmol g^{-1} of fresh weight, respectively). Abscisic acid concentration increased mainly in the skin. Treatments with CO_2 prior to storage at 2 °C were found to be effective in reducing CI. With 5% CO_2, increases in putrescine and abscisic acid levels were lower than in control fruits, while exposure to 40% CO_2 resulted in a decrease of these values. Spermidine and spermine levels decreased during storage, regardless of treatment. Thus, the 40% CO_2 treatment was more effective than the 5% CO_2 one in reducing both CI and the putrescine and abscisic acid changes found in control chilling-injured squash.

Keywords: *Zucchini; chilling injury; polyamines; abscisic acid*

INTRODUCTION

Many tropical and subtropical fruits develop chilling injury (CI) when stored at temperatures below 12 °C. CI symptoms include surface pitting, discoloration, and decay (Saltveit and Morris, 1990). CI, like senescence, is thought to involve alteration of the membrane structure, leading to increases in membrane permeability and alteration of membrane proteins' activity (Raison and Orr, 1990). It has long been known that zucchini squash is prone to CI (Mencarelli et al., 1983) because it shows severe pitting and slight decay after 10−12 days of exposure to chilling temperatures (Kramer and Wang, 1989; Wang and Ji, 1989). Several types of treatment have been reported as reducing such injury (Wang, 1993). These include prestorage treatment with hot water (Wang, 1994) and preconditioning the fruit for 2 days at 10 °C (Kramer and Wang, 1989). The severity of CI in zucchini squash has also been reduced by using controlled atmospheres with 5% CO_2 (Mencarelli, 1987) or low (1−4%) oxygen concentration (Mencarelli et al., 1983; Wang and Ji, 1989) during storage. In addition, prestorage treatment of avocado fruit with low O_2 (Pesis et al., 1994) or high CO_2 (Marcellin and Chaves, 1983) reduced CI symptoms during and/or after cold storage, as did prestorage treatments of citrus fruit with 10−40% of CO_2 in air (Bertolini et al., 1991). However, the mechanism(s) by which these treatments alter chilling sensitivity is (are) not clear.

CI in a variety of fruit is manifested by significant increases in polyamine levels (Serrano et al., 1996). It has been proposed that these substances can act to prevent CI by protecting membrane lipids from peroxidation (Kramer and Wang, 1989; Wang, 1994). In addition, the supply of putrescine decreased the negative effect of several kinds of stress (Reggiani et al., 1990; Songstad et al., 1990). It has also been proposed that the phytohormone abscisic acid (ABA) might be involved in increasing tolerance to stress. Indeed, it has been shown to increase plant tolerance to adverse environmental conditions, such as chilling (Mohapatra et al., 1988; Xin and Li, 1992; Anderson et al., 1994; Fanowiak and Dörffling, 1996), probably because it protects mitochondria from irreversible oxidative damage by inducing antioxidant enzymes (Prasad et al., 1994).

In this study we describe the effect of treatments involving high CO_2 concentrations on the appearance of CI and alterations in polyamine and ABA levels during storage.

MATERIALS AND METHODS

Plant Material, Gas Treatments, and Storage Conditions. Zucchini squash fruit (*Cucurbita pepo* L. cv. Elite) were obtained from a greenhouse in Murcia (Spain) when they were in exponential growth phase with an approximate weight of 200 g (2 weeks of development). They were then taken immediately to the laboratory and sorted for freedom from visual defects and uniformity of weight and shape. This day was considered as day 0 of the experiment. The fruit were weighed and divided into five lots, the first lot (of five fruits) corresponding to fruit analyzed at day 0. Another lot (20 fruits) was placed in a chamber with a controlled temperature of 20 °C and a relative humidity (RH) of 85% [standard (STD)]. The three remaining lots (each of 20 fruit) were placed in a chamber at 2 °C and 85% RH but each within a glass cabinet (A−C) where the fruit were exposed to the following gas concentrations for 24 h: normal air [control (CTL), 20 ± 1% O_2 and 0.04 ± 0.02% CO_2] in box A, 20 ± 1% O_2 and 5 ± 0.5% CO_2 in box B, and 20 ± 1% O_2 and 40 ± 2% CO_2 in box C. After 24 h, the fruit of these three lots were taken from the cabinets and left on carboard trays in the same cold room at 2 °C. On the 4th, 8th, and 12th days of the experiment, five

* Author to whom correspondence should be addressed (telephone 34-68-215717; fax 34-68-266613; e-mail friquelme@natura.cebas.csic.es).

[†] Escuela Politécnica Superior.

[‡] Centro de Edafología y Biología Aplicada del Segura.

S0021-8561(97)00864-9 CCC: $15.00
Published on Web 07/01/1998

Table 1. Weight Loss (Percent of Initial Weight) of Zucchini Squash Stored at 20 and 2 °C (Treated with 5 or 40% CO_2 and Untreated Control)[a]

	storage temperature			
days of	20 °C	2 °C		
storage	STD	5% CO_2	40% CO_2	control
4	2.7 ± 0.6a	0.14 ± 0.04a	0.12 ± 0.03a	0.16 ± 0.03a
8	5.3 ± 0.8b	0.31 ± 0.05b	0.28 ± 0.05b	0.34 ± 0.06b
12	10.9 ± 1.10c	0.48 ± 0.04c	0.52 ± 0.06c	0.58 ± 0.07c

[a] Mean initial weight of the fruit: 200 g. Data are the mean ± SE of the measures carried out in five fruits. Means within a column not followed by the same letter are different ($p \leq 0.05$).

fruit from each lot were weighed to calculate the weight loss, evaluate the degree of CI, and to make analytical determinations.

Degree of CI Evaluation. The degree of CI, as judged by the extent of surface pitting, was evaluated 24 h after transfer of squash from storage chambers to room temperature (20 °C) by rating on a scale of 0 to 100 (percent of surface fruit affected by CI). Since this estimation is subjective, it was carried out independently by three trained panelists, and the data represent the mean of their subjective measurements. After that, squash were peeled using a peeler and the skin was scraped to remove the remaining pulp. The skin and pulp were cut into small pieces (0.2 × 0.5 cm), frozen separately in liquid N_2, and ground to obtain homogeneous samples of each fruit. These samples were then stored at −70 °C until determinations of the ABA and polyamines were carried out.

Polyamine Quantification. Polyamines were extracted by homogenizing 1.0 g of tissue in 10 mL of 5% $HClO_4$ using a Polytron and analyzed by the benzoylation method as previously reported by Serrano et al. (1995). 1,6-Hexanediamine (100 nmol g^{-1} of fresh weight of tissue) was used as an internal standard, and standard curves of putrescine, spermidine, and spermine (Sigma) were made. Two extractions of polyamines were made from both skin and pulp of each fruit, and data are the mean ± SE. These determinations were made independently on the five fruits of each sample. Results were expressed as nanomoles per gram of fresh weight.

ABA Quantification. ABA was extracted from 1 g of tissue samples with 5 mL of a solution of 80% acetone containing 100 mg L^{-1} of butylated hydroxytoluene and 0.5 g L^{-1} of citric acid. The extracts were diluted suitably with a 50 mM Tris buffer, pH 7.8, containing 1 mM $MgCl_2$ and 150 mM NaCl and then quantified by an enzyme-linked immunosorbent assay, using an IgG monoclonal antibody (Idetek, Inc., San Bruno, CA) as previously reported (Martínez-Madrid et al., 1996). For each fruit, two extractions were made of both pulp and skin tissues. ABA content was estimated from the standard curve prepared for each particular plate. For each extract four dilutions were made and at least three of them fell on the standard curve. The ABA levels were consistent with the dilution made, and no interference from impurities was detected when ABA standards were added to diluted extracts. Results were expressed as nanomoles per gram of fresh weight and are the mean ± SE of determinations made independently in five fruit.

Statistical Analysis. Experimental data are the mean ± SE of the determinations made independently in five fruits. A variance analysis using the Student *t* test was performed to determine if differences between means were significant at the level of $p \leq 0.05$.

RESULTS AND DISCCUSSION

Weight Loss and CI. Weight loss of the STD was substantial ($p \leq 0.05$), reaching 10.9% after 12 days (Table 1). Such losses were much less in the CTL, which was not different ($p > 0.05$) from the two treatments. In all cases, weight loss increased ($p \leq 0.05$) with storage time.

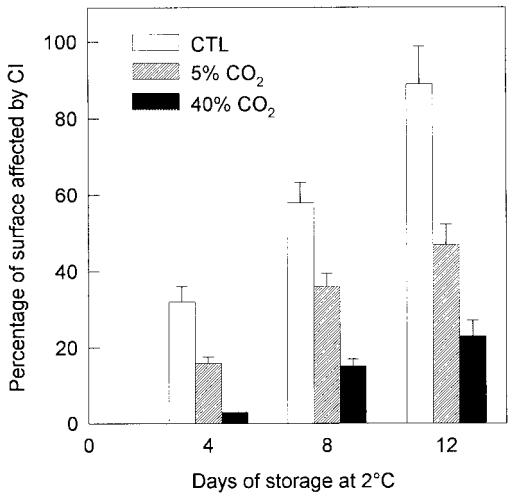

Figure 1. Surface of zucchinis affected by CI during preservation at 2 °C, in the control fruits (CTL) and in those previously treated with 5 or 40% CO_2 for 24 h. Data represent the mean ± SE of the estimates carried out independently by three people in five fruits.

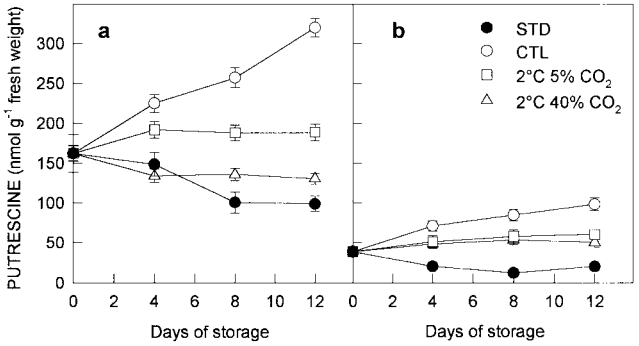

Figure 2. Changes in the putrescine levels in the skin (a) and pulp (b) of zucchinis stored at 20 °C (STD) (●), at 2 °C (CTL) (○), and at 2 °C pretreated with 5% CO_2 (□) or 40% CO_2 (△) for 24 h. Data represent the mean ± SE of two quantifications carried out independently in each of the five fruits.

CI symptoms were observed after 4 days in all fruit stored at 2 °C, being 32% in the CTL. Such damage increased during storage time, reaching 89% of the surface after 12 days (Figure 1). However, pretreatment with 5 or 40% CO_2 decreased CI, and after 12 days of storage at 2 °C the surface area of the zucchini squash affected was 47 and 23%, respectively (Figure 1). Thus, CO_2 pretreatment for 24 h (especially at high concentration, 40%), diminished or delayed the appearance of CI in squash for up to 12 days. Other authors have shown that keeping the squash in a constant atmosphere of 5% CO_2 or with 1% O_2 will reduce CI (Mencarelli, 1987; Wang and Ji, 1989).

Polyamine Levels. Putrescine levels in the skin rose gradually from 162 nmol g^{-1} of fresh weight at day 0 to 320 nmol g^{-1} of fresh weight at day 12 in the CTL but fell to 98 nmol g^{-1} of fresh weight at day 12 in the STD (Figure 2a). A similar trend was noted in the pulp (Figure 2b). These results indicate that CI induces a physiological alteration in putrescine metabolism in the whole fruit, since putrescine levels increased 2−2.5-fold during cold storage in both skin and pulp, although visible manifestation of CI is observed only in the skin. Pretreatment of fruit stored at 2 °C with 40% CO_2 kept levels of putrescine in the skin close to those observed in fruit stored at 20 °C, whereas pretreatment with 5% CO_2 led to a slight increase in the skin putrescine levels,

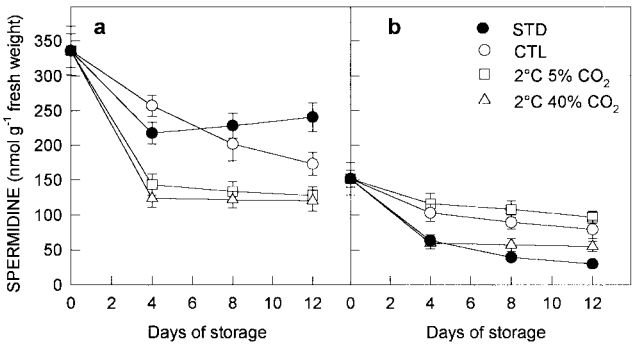

Figure 3. Changes in the spermidine levels in the skin (a) and pulp (b) of zucchinis stored at 20 °C (STD) (●), at 2 °C (CTL) (○), and at 2 °C pretreated with 5% CO_2 (□) or 40% CO_2 (△) for 24 h. Data represent the mean ± SE of two quantifications carried out independently in each of the five fruits.

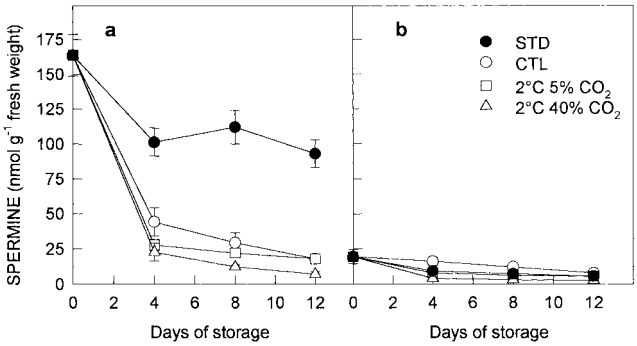

Figure 4. Changes in the spermine levels in the skin (a) and pulp (b) of zucchinis stored at 20 °C (STD) (●), at 2 °C (CTL) (○), and at 2 °C pretreated with 5% CO_2 (□) or 40% CO_2 (△) for 24 h. Data represent the mean ± SE of two quantifications carried out independently in each of the five fruits.

but this increase was not significant (Figure 2a). However, from the eighth day skin putrescine levels were significantly different in all fruits. The levels of putrescine in the pulp of squash treated with both 5 and 40% CO_2 did not show significant variations during storage at 2 °C (Figure 2b) and there were not significant differences between them.

Spermidine levels in the skin and pulp of recently harvested squash were 349 and 150 nmol g^{-1} of fresh weight, respectively, falling in both tissues after 4 days at 2 and 20 °C (Figure 3). However, the decrease in skin spermidine levels was more pronounced in fruit stored at 2 °C, particularly if pretreated with 5 and 40% CO_2 when the concentration fell sharply during the first few days and remained consistently below the levels of untreated fruit (Figure 3a), although significant differences were not found between them. Spermidine levels also fell in the pulp, although in this case they were lower in the squash stored at 20 °C than in both treated and untreated fruit at 2 °C (Figure 3b).

Spermine levels fell sharply in the skin of squash during the first days of storage at 20 °C and even more so in the fruit stored at 2 °C (with and without CO_2 treatment) (Figure 4a), and no significant differences were found in the spermidine levels in the skin of the fruit stored at 2 °C whether treated or not with CO_2. The levels of this polyamine were very low in the pulp and fell slightly during storage, with no significant differences between treatments (Figure 4b).

Kramer and Wang (1989) showed that CI in zucchini squash resulted in an increase in putrescine levels and decreases in spermidine and spermine in the skin. We

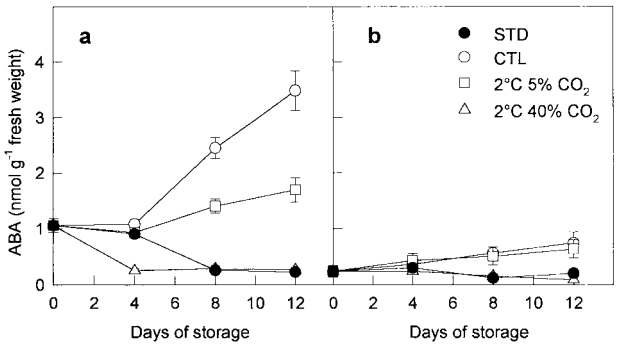

Figure 5. Changes in the ABA levels in the skin (a) and pulp (b) of zucchinis stored at 20 °C (STD) (●), at 2 °C (CTL) (○), and at 2 °C pretreated with 5% (□) or 40% CO_2 (△) for 24 h. Data represent the mean ± SE of two quantifications carried out independently in each of the five fruits.

have found similar results in both skin and mesocarp, which suggests that although CI visibly affects only the skin, the modification of polyamine levels brought about by low temperatures can also be detected in the pulp, although there is no visible sign of CI. However, pretreatment with CO_2 alleviates CI and also results in smaller increases in putrescine.

Kramer and Wang (1989) showed that CI in zucchini squash was reduced by preconditioning the fruit for 2 days at 10 °C, which was correlated with higher spermine and spermidine levels in the skin tissue. Also, zucchini squash stored in a low-O_2 atmosphere maintained higher levels of spermidine and spermine than those stored in air, and they also showed less CI (Wang and Ji, 1989). Wang (1994) found that both high- and low-temperature conditioning were effective in alleviating CI in zucchini squash, which was correlated with higher spermidine and spermine levels in the skin tissue caused by induction of the enzyme *S*-adenosylmethionine decarboxylase. These results could indicate that these polyamines might be involved in reducing CI in zucchini squash. However, our results show that pretreatment with CO_2 reduces CI in zucchini squash and prevents increased putrescine levels, especially at a concentration of 40%, with no additional increase in spermidine and spermine levels over those observed in untreated fruit stored at 2 °C. This seems to suggest that the increase in putrescine levels might be the result of cold-induced stress rather than a protection mechanism against it. Similar results have been obtained in peppers stored at 2 °C in modified atmospheres; an atmosphere of 5% CO_2 diminished CI and led to lower increases in putrescine levels than those observed in fruit stored in air (Serrano et al., 1997).

ABA Levels. There was also a very close relationship between the appearance of CI and increases in ABA levels both in the skin and in the pulp. In squash stored at 20 °C the ABA levels fell gradually in both skin and pulp, while in squash stored at 2 °C the levels rose from 1.06 nmol g^{-1} of fresh weight at day 0 to 3.48 nmol g^{-1} of fresh weight after 12 days in the skin, with a smaller increase in the pulp (Figure 5). In squash treated with 5% CO_2, ABA levels rose only slightly during the first week of storage at 2 °C, showing significant increases from the 8th day, whereas in fruit pretreated with 40% CO_2 ABA followed a pattern similar to the that of STD. These results point to a direct relationship between CI and increased ABA level, both in the skin and in the pulp. This increase could not be related with tissue dehydration because this was much greater in the fruit

stored at 20 °C than in those stored at 2 °C (and weight loss was on the order of 10 and 0.5%, respectively) and it was at 2 °C that ABA levels rose.

In the same fruit, a temperature conditioning treatment of 2 days at 10 °C reduced the severity of CI during subsequent storage at 2.5 °C and the ABA levels remained higher in treated squash than in untreated samples (Wang, 1990). In addition, direct treatments of zucchini squash with ABA before storage were also effective in reducing CI (Wang, 1990). Also, in maize seedlings, chilling led to an accumulation of ABA, and the higher chilling tolerance of acclimatized seedlings was accompanied by even greater ABA accumulation during chilling (Anderson et al., 1994; Fanowiak and Dörffling, 1996). These results suggest that increases in ABA might be the tissue defense mechanism against this kind of stress. However, our results seem to indicate that increased ABA levels, like those of putrescine, might be the consequence of the stress caused by cold storage since there was no CI damage in fruit pretreated with 40% CO_2 and ABA levels did not rise. On the other hand, the stress resulting from cold might provoke the increase in ABA, which, in turn, might induce higher putrescine levels because ABA treatment has been shown to increase putrescine levels in wheat seedlings (Aurisano et al., 1993). However, the concentration of spermidine and spermine does not seem to be related with the appearance of CI but rather with the storage temperature, since these polyamines show a similar trend in both treated and untreated fruit stored at 2 °C but a different trend from that observed in fruit stored at 20 °C.

The relationship found between CI and increased putrescine and ABA level is in agreement with the results obtained with pepper fruits, in which CI temperatures led to a rise in the levels of these growth regulators. However, CI was reduced when pepper fruit were stored in modified atmosphere with 4.5% CO_2, and no increases in ABA and putrescine were found (Serrano et al., 1997).

LITERATURE CITED

Anderson, M. D.; Prasad, T. K.; Martin, B. A.; Steward, C. R. Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisic acid in chilling tolerance. *Plant Physiol.* **1994**, *105*, 331−339.

Aurisano, N.; Bertani, A.; Mattana, M.; Reggiani, R. Abscisic acid induced stress-like polyamine pattern in wheat seedlings, and its reversal by potassium ions. *Physiol. Plant.* **1993**, *89*, 687−692.

Bertolini, P.; Lanza, G.; Tonini, G. Effect of pre-storage carbon dioxide treatments and storage temperatures on membranes of "Femminello comune" lemons. *Sci. Hortic.* **1991**, *46*, 89−95.

Fanowiak, F.; Dörffling, K. Chilling of maize seedling: changes in water status and abscisic acid content in ten genotypes differing in chilling tolerance. *J. Plant Physiol.* **1996**, *147*, 582−588.

Kramer, G. F.; Wang, C. Y. Correlation of reduced chilling injury with increased spermine and spermidine levels in Zucchini squash. *Physiol. Plant.* **1989**, *76*, 479−484.

Marcellin, P.; Chaves, A. Effect of intermittent high CO_2 treatment on storage life of avocado fruit in relation to respiration and ethylene production. *Acta Hortic.* **1983**, *138*, 155−163.

Martínez-Madrid, M. C.; Serrano, M.; Riquelme, F.; Romojaro, F. Polyamines, abscisic acid and ethylene production in tomato fruit. *Phytochemistry* **1996**, *43*, 323−326.

Mencarelli, F. Effect of high CO_2 atmospheres on stored zucchini squash. *J. Am. Soc. Hortic. Sci.* **1987**, *112*, 985−988.

Mencarelli, F.; Lipton, W. J.; Peterson, S. J. Responses of "zucchini" squash to storage in low-O_2 atmospheres at chilling and nonchilling temperatures. *J. Am. Soc. Hortic. Sci.* **1983**, *108*, 884−889.

Mohapatra, S. S.; Poole, R. J.; Dhindsa, R. S. Abscisic acid-regulated gene expression in relation to freezing tolerance in alfalfa. *Plant Physiol.* **1988**, *87*, 468−473.

Pesis, E.; Marinansky, R.; Zauberman, G.; Euchs, Y. Prestorage low-oxygen atmosphere treatment reduces chilling injury symptoms in "Fuerte" avocado fruit. *HortScience* **1994**, *29*, 1042−1046.

Prasad, T. K.; Anderson, M. D.; Stewart, C. R. Acclimation, hydrogen peroxide, and abscisic acid protect mitochondria against irreversible chilling injury in maize seedlings. *Plant Physiol.* **1994**, *105*, 619−627.

Raison, J. K.; Orr, G. R. Proposal for a better understanding of the molecular basis of chilling injury. In *Chilling Injury of Horticultural Crops*; Wang, C. Y., Ed.; CRC Pres: Boca Raton, FL, 1990; pp 145−164.

Reggiani, R.; Giussani, P.; Bertani, A. Relationship between the accumulation of putrescine and the tolerance to oxygen-deficit stress in gramineae seedlings. *Plant Cell Physiol.* **1990**, *31*, 489−494.

Saltveit, M. E. J.; Morris, L. L. Overview on chilling injury of horticultural crops. In *Chilling Injury of Horticultural Crops*; Wang, C. Y., Ed.; CRC Press: Boca Raton, FL, 1990; pp 3−15.

Serrano, M.; Martínez-Madrid, M. C.; Riquelme, F.; Romojaro, F. Endogenous levels of polyamines and abscisic acid in pepper fruits during growth and ripening. *Physiol. Plant.* **1995**, *95*, 73−76.

Serrano, M.; Martínez-Madrid, M. C.; Martínez, G.; Riquelme, F.; Pretel, M. T.; Romojaro, F. Review: Role of polyamines in clilling injury of fruit and vegetables. *Food Sci. Technol. Int.* **1996**, *2*, 195−199.

Serrano, M.; Martínez-Madrid, M. C.; Pretel, M. T.; Riquelme, F.; Romojaro, F. Modified atmosphere packaging minimizes increases in putrescine and abscisic acid levels caused by chilling injury in pepper fruit. *J. Agric. Food Chem.* **1997**, *45*, 1668−1672.

Songstad, D. D.; Duncan, D. R.; Widbolm, J. M. Proline and polyamine involvement in chilling tolerance of maize suspension cultures. *J. Exp. Bot.* **1990**, *41*, 289−294.

Wang, C. Y. Involvement of abscisic acid in reducing chilling injury of zucchini squash. *HortScience* **1990**, *25* (9), 1096.

Wang, C. Y. Approaches to reduction chilling injury of fruits and vegetables. *Hortic. Rev.* **1993**, *15*, 63−95.

Wang, C. Y. Combined treatment of heat shock and low-temperature conditioning reduces chilling injury in zucchini squash. *Postharvest Biol. Technol.* **1994**, *4*, 65−73.

Wang, C. Y.; Ji, Z. L. Effect of low-oxygen storage on chilling injury and polyamines in zucchini squash. *Sci. Hortic.* **1989**, *39*, 1−7.

Xin, Z.; Li, P. H. Abscisic acid-induced chilling tolerance in maize suspension-cultured cells. *Plant Physiol.* **1992**, *99*, 707−711.

Received for review October 6, 1997. Revised manuscript received February 25, 1998. Accepted May 7, 1998. This work has been funded by the Comisión Interministerial de Ciencia y Tecnología (Project ALI 95/134).

JF970864C