Ripening Affects High-Temperature-Induced Polyamines and Their Changes during Cold Storage of Hybrid Fortune Mandarins

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Polyamine levels were measured in the flavedo of three ripening stages of Fortune mandarins exposed to high-temperature conditioning before storage at a chilling (2 °C) and a nonchilling temperature (12 °C). Increasing periods of conditioning at 37 °C delayed the development of chilling injury (CI) and produced an increase in the content of the polyamines in the following proportion: spermine > spermidine > putrescine. Storage at 2 °C of conditioned fruits reduced the polyamine titer to levels similar to those of nonconditioned fruits. Changes in polyamines during storage of conditioned and nonconditioned fruits were dependent on the ripening stage of the fruit. In general, the levels of polyamines in nonconditioned fruits stored at 2 and 12 °C were similar. No change in spermine was induced in the most mature fruits at 2 °C or in response to high-temperature conditioning despite conditioning-reduced CI. With this exception, the data indicate that conditioning fruits at 37 °C elevated the content of polyamines and reduced CI in Fortune mandarins.

Keywords: *Citrus; chilling injury; high-temperature conditioning; maturity; polyamines*

INTRODUCTION

Cold stress-induced injury, like senescence, is thought to involve alteration of membrane structure (Wang, 1982; Raison and Orr, 1990), and it has been related to toxic oxygen forms (Purvish and Shewfelt, 1993). Polyamines are inhibitors of many senescence-related processes (Galston and Kaur-Sawhney, 1990) and have been associated with the response of plant tissue to different biotic and abiotic stresses (Faust and Wang, 1992). Their antisenescent activity is thought to be membrane related since polyamines may retard membrane deterioration, possibly by interacting with anionic components of the membrane and stabilizing the bilayer surface (Roberts et al., 1986). The radical-scavenging properties of polyamines have been also suggested to be involved in protecting membranes from oxidative stresses (Kramer and Wang, 1989). Therefore, the involvement of polyamines in reducing chilling injury (CI) has been of great interest. Several pieces of evidence suggest a relationship between polyamines and CI, but controversial results have been found. Storage of grapefruit, pepper fruits, and zucchini squash at low temperature increased the putrescine content (McDonald and Kushad, 1986), but in zucchini squash it decreased also the levels of spermidine and spermine (Kramer and Wang, 1989). In peaches, it has been suggested that an increase of spermidine, but not of putrescine, may be a consequence of chilling stress (Valero et al., 1997). Yuen et al. (1995) found, however, that the cold sensitivity of different citrus cultivars was not related to the chilling-induced levels of putrescine.

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Different postharvest conditioning treatments have been developed to increase the tolerance of horticultural crops to cold stress, but it is still unclear if the synthesis and accumulation of polyamines are linked to the thermal tolerance provided by these treatments. The effect of temperature conditioning and low-oxygen storage, which reduced the susceptibility to CI of zucchini squash, was associated with changes in spermidine and spermine (Kramer and Wang, 1989; Wang and Ji, 1989). In lemon fruits, however, no relationship between the adaptive process induced by temperature-conditioning treatments against CI and the content of putrescine and spermidine was found (McDonald, 1989).

Fortune mandarin (*Citrus reticulata* Blanco) is a hybrid of Dancy mandarin (*Citrus tangerina* Hort. Ex Tan.) × Clementina Fino (*Citrus clementina* Hort. Ex Tan.). Fruits of this cultivar are very sensitive to chilling, which induces peel "pitting", as depressed necrotic spots on the fruit surface. Different heat treatments before cold storage have been useful in increasing its chilling tolerance. The effectiveness of hot water dips at high temperature (>47 °C) for short terms (3−6 min) was lower than that of a conditioning treatment at 37 °C and 90−95% relative humidity (RH) for 3 days, or curing. In addition, dips may originate heat damage in the fruits (Mulas et al., 1995). We have previously shown that the polyamine content increased in the flavedo of Fortune mandarins with the temperature of the hot water dips, but polyamine accumulation appeared to be merely an incidental heat stress metabolite instead of the cause of the heat-induced tolerance to chilling (Gonzalez-Aguilar et al., 1997; Mulas et al., 1997). The tolerance induced to low temperature by hot water dips is variable and changes with the picking date (Mulas et al., 1995; Gonzalez-Aguilar et al., 1997) as occurs in Tarocco oranges (Schirra et al., 1997). The efficacy of curing, however, did not depend

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on the stage of maturity despite the change in the sensitivity of mandarins to CI during the season (Lafuente et al., 1997).

This paper reports on the changes in the levels of polyamines in the flavedo of Fortune mandarins (a) stored at a temperature that induces chilling symptoms (2 °C) as compared with those of fruits held at a nonchilling temperature (12 °C), (b) conditioned at 37 °C for 3 days, and (c) stored at 2 and 12 °C after conditioning. Fruits differing in their maturity stage and in their susceptibility to CI were used to establish if these seasonal changes in CI may be related to a different pattern of polyamine accumulation upon storage.

MATERIALS AND METHODS

Plant Material and Treatments. Fruits of Fortune mandarin (*Citrus reticulata* Blanco) were harvested at random from adult trees growing in a commercial orchard at Sagunto (Valencia, Spain). Fruits were hand-harvested, immediately delivered to the laboratory, and selected by size and color uniformity. To study the effect of duration of heat conditioning on CI and polyamine content, fruits harvested in January, the stage of maximum susceptibility to CI (Lafuente et al., 1997), were used. The sample of fruits was divided into three replicates of 20 fruits for each time of heat conditioning to estimate CI damage and three replicates of 10 fruits to analyze polyamine content. Fruits were subjected to 1, 2, or 3 days at 37 °C and 90−95% RH and then stored for up to 90 days at 2 °C and 80−90% RH. After each heat-conditioning period, flavedo tissue was peeled from 10 fruits and stored at −20 °C for polyamine analysis.

The effect of 3 days of heat conditioning on chilling susceptibility and polyamine changes was evaluated in fruits harvested at three different stages of development and maturation (December 2, $h° = 55.8$; January 13, $h° = 44.7$, and April 7, $h° = 42.3$) and showing different response to chilling temperature (Lafuente et al., 1997). Fruits were randomly divided into two lots. The first lot was subdivided into two subgroups, which were stored for up to 28 days at 80−90% RH at either 2 °C (chilled control) or 12 °C (nonchilled control). The second lot was conditioned for 3 days at 37 °C and 90−95% RH, subdivided into two subgroups, and then stored at the same temperature and RH as the nonconditioned fruits. At 7-day intervals, three replicates of 20 fruits for each temperature regime were scored for CI symptoms and in three replicates of 10 fruits flavedo tissue were randomly sampled for polyamine analysis.

Fruit Color. Color of the fruit was determined using a HunterLab meter as previously described by Lafuente et al. (1997) and expressed as the hue angle ($h°$).

CI Index. CI symptoms in Fortune mandarins are brown pit-like depressions along the fruit. CI was rated visually on the following scale: 0 = none; 1 = slight; 2 = moderate; and 3 = severe, depending on the extent of damage. A CI index was determined by summing the product of the number of fruit in each category by the score of each category and then dividing this sum by the total number of fruit evaluated (Lafuente et al., 1997).

Polyamine Analysis. Free polyamines were analyzed according to the method of Carbonell and Navarro (1989). One gram of frozen flavedo was homogenized in a chilled mortar with 3 mL of 0.2 N $HClO_4$ and 1 mL of 0.6 mM 1,6-hexanodiamine as an internal standard. The homogenate was then centrifuged at 12000*g* for 20 min at 4 °C. Free polyamines left in the supernatant were dansylated as previously described (Gonzalez-Aguilar et al., 1997) and then extracted with 500 μL of toluene. The organic phase was dried under a stream of N_2 at 70 °C, and the residue was resuspended in 200 μL of acetonitrile (HPLC grade) and filtered through an HV-4 filter (Millipore, pore size = 0.45 μm) for HPLC analysis. Polyamines were eluted through a 200 × 4.6 mm reverse-phase C18 column packed with a 5 μm Hypersil ODS resin. Elution was done at a flow rate of 1.5 mL min^{-1} with a gradient of 60−90% acetonitrile in 25 min at 35 °C. Dansylated polyamines in the extracts were detected by fluorescence at an extinction wavelength of 365 nm and an emission wavelength of 447 nm and quantified by peak area comparison using 1,6-hexanodiamine as the internal standard and standard curves for putrescine, spermidine, and spermine.

Statistical Design. Experimental data are the mean ± SE of three replicates of the determinations for each sample. A variance analysis using the Tukey test at the 5% level was performed to determine if the polyamine content induced by different times of conditioning showed significant differences ($p < 0.05$).

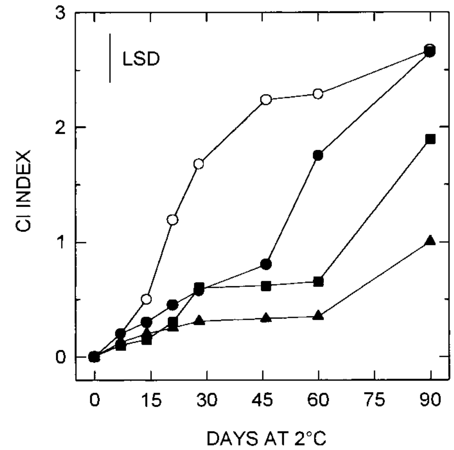

Figure 1. CI index of Fortune mandarin fruits conditioned for 0 (○), 1 (●), 2 (■), and 3 days (▲) at 37 °C and 90−95% RH and stored for up to 90 days at 2 °C. Fruits were harvested in January, and each value is the mean of three replicate samples containing 20 fruits.

RESULTS

Conditioning of Fortune mandarins at 37 °C and 90−95% RH reduced the susceptibility of fruits to CI. The effectiveness of the treatment depended on time of exposure at 37 °C (Figure 1). The longer the conditioning treatment, the higher was the induced tolerance to low temperature. No significant difference in the CI severity among 1, 2, or 3 days preheated fruits was found for up to 21 days of storage at 2 °C. Fruits conditioned for 1 or 2 days showed slightly higher peel damage than the fruit conditioned for 3 days after 28 days of storage. However, the CI indices of the pretreated fruits were always considerably lower (CI index < 0.6) than that of the nonconditioned fruit (CI index = 1.7). After 90 days of storage at 2 °C, CI was not alleviated in fruits pretreated for 1 day, whereas conditioning for 2 or 3 days reduced the CI index by 35 and 60%, respectively. Therefore, a 3 day conditioning treatment at 37 °C was clearly the most effective.

Conditioning Fortune mandarin fruit at 37 °C increased putrescine levels in a time-dependent manner (Figure 2). Putrescine levels changed significantly from 220 to 305, 340, and 430 μg g^{-1} after 1, 2, and 3 days at 37 °C, respectively (Figure 2). No significant differences were found in the concentrations of spermidine or spermine in the flavedo of fruits conditioned for 1 and 2 days at 37 °C, which were about twice those of freshly harvested (Figure 2). After 3 days of conditioning, spermidine and spermine levels increased 2.5- and 6-fold, respectively, and were significantly higher than those of mandarins conditioned for 1 or 2 days (Figure 2).

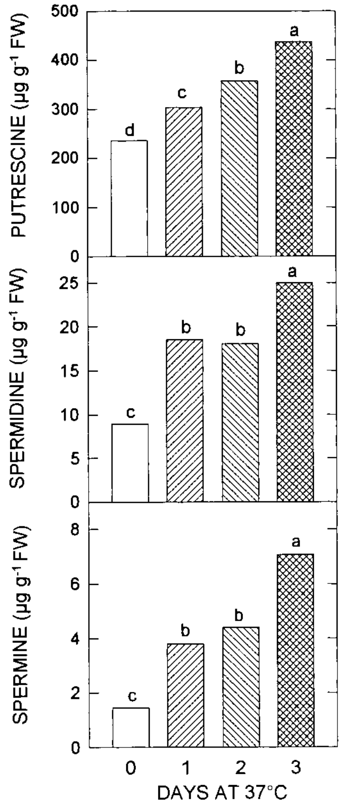

Figure 2. Putrescine, spermidine, and spermine contents in the flavedo of Fortune mandarin fruits conditioned for 1, 2, and 3 days at 37 °C and 90−95% RH. Fruits were harvested in January. Values labeled with the same letter are not different at the 5% significance level.

The changes in putrescine, spermidine, and spermine in the flavedo of nonconditioned and 3-day-conditioned mandarins harvested at different maturity stages and held at 2 or 12 °C for up to 28 days are shown in Figures 3, 4, and 5, respectively. Conditioning increased, in general, polyamine levels, although this effect varied from one stage of maturity to another. Changes in polyamines during storage at 2 and 12 °C of freshly harvested or conditioned fruits were also dependent on the stage of maturity.

The content of putrescine in the flavedo of nonconditioned fruits harvested in December (Figure 3A) and January (Figure 3C) slightly increased during storage at 2 °C for up to 28 days. A similar increase in putrescine was observed after 2 weeks in fruits harvested in April, which declined thereafter (Figure 3E). However, the contents of putrescine detected in fruits chilled or exposed to 12 °C at the three stages of ripening were comparable (Figure 3). A 2-fold increase in putrescine occurred after the fruits were exposed for 3 days at 37 °C at all maturity stages (Figure 3). Putrescine decreased after 7 and 14 days of storage at 2 °C in conditioned fruits from December and January, respectively, until reaching levels similar to those of nonconditioned fruits (Figure 3A,C). However, putrescine levels induced by conditioning treatment on fruits harvested in April were not altered during storage at the chilling temperature (2 °C) (Figure 3E). Putrescine decreased after fruits were transferred from 37 to 12 °C at all maturity stages. The decline in this polyamine was always faster at 12 °C (Figure 3B,D,F) than at 2 °C (Figure 3A,C,E), but putrescine levels in the flavedo of mandarins kept at 2 and 12 °C were similar after 21 days of storage.

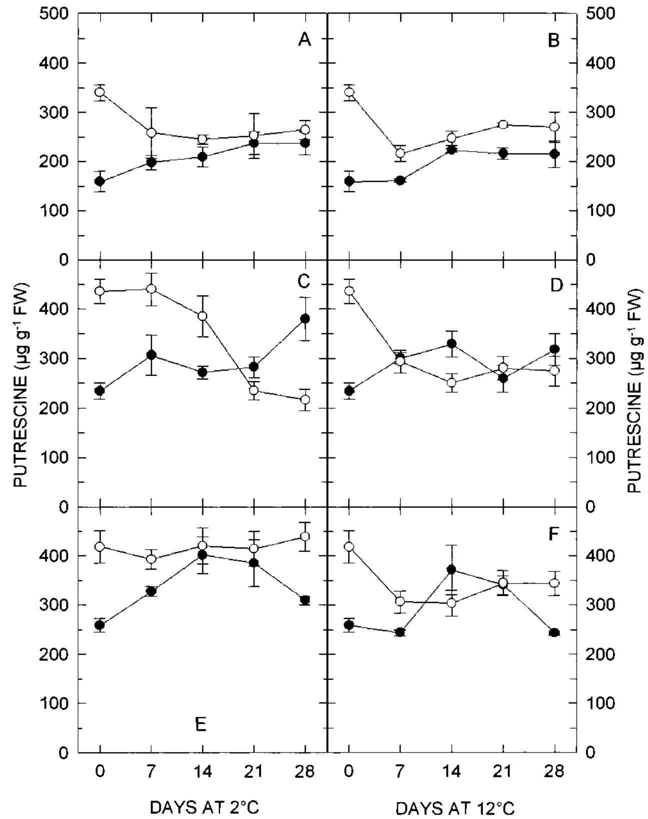

Figure 3. Effect of harvest date on putrescine content in the flavedo of Fortune mandarin fruits stored at 2 °C (left panel) or 12 °C (right panel) immediately after harvest (●) or after 3 days of conditioning at 37 °C and 90−95% RH (○). Fruits were harvested in December (A and B), January (C and D), and April (E and F). Fruits colors ($h°$) were 55.8, 44.7, and 42.3, respectively. Values are the mean of three replicate samples containing 10 fruits ± SE.

Spermidine levels in nonconditioned fruits harvested in December increased slightly for up to 21 days at 2 or 12 °C (Figure 4A,B). Thereafter, spermidine decreased, more at 2 °C than at 12 °C. In fruits harvested in later stages of maturity, spermidine concentration declined during the first week of storage at 2 °C and remained nearly constant afterward (Figure 4C,E). Spermidine levels of fruits kept at 12 °C were, in general, similar to or slightly higher than those of chilled fruits (Figure 4). Heat conditioning for 3 days increased about twice the spermidine content in the flavedo at all stages of maturity (Figure 4). The heat-induced spermidine levels were significantly reduced by 7 days of holding at either 2 or 12 °C but thereafter showed only slight changes (Figure 4). The rate of decrease was greater in fruits harvested in April (Figure 4E,F). No relevant differences in spermidine levels were, however, observed in conditioned or nonconditioned fruits for any stage of maturity between fruits held at 2 or 12 °C after 7 days of storage.

Spermine levels in nonconditioned fruits harvested in December and April remained relatively constant during the 28 days of holding at 2 or 12 °C (Figure 5 A,B,E,F), whereas spermine increased with time of holding in fruits from January (Figure 5C,D). At this stage of maturity, the rate of increase in spermine content in the flavedo of fruits stored at 12 °C (Figure 5D) was considerably greater than at 2 °C (Figure 5C) for up to 14 days of storage. Spermine content in the flavedo of 3-day-heated fruits from December and Janu-

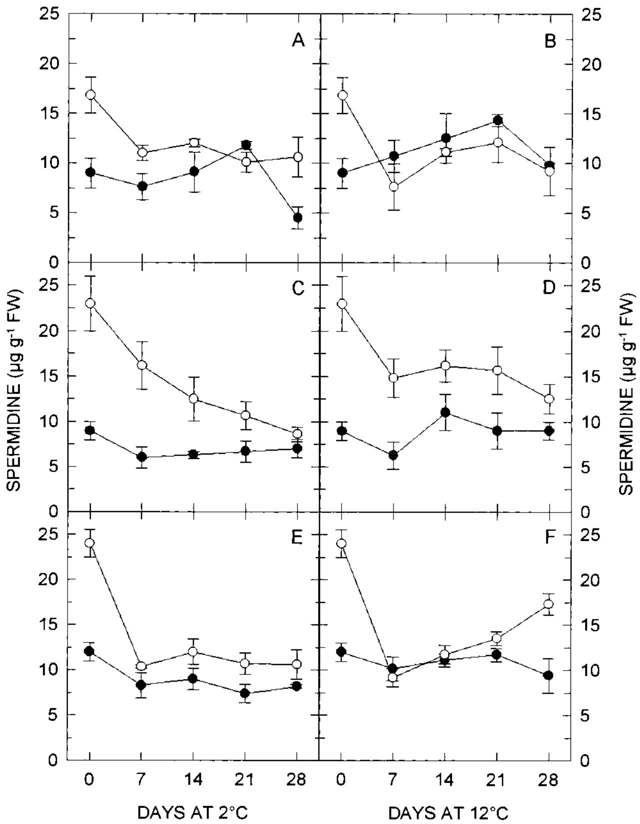

Figure 4. Effect of harvest date on spermidine content in the flavedo of Fortune mandarin fruits stored at 2 °C (left panel) or 12 °C (right panel) immediately after harvest (●) or after 3 days of conditioning at 37 °C and 90−95% RH (○). Fruits were harvested in December (A and B), January (C and D), and April (E and F). Fruits colors (*h*°) were 55.8, 44.7, and 42.3, respectively. Values are the mean of three replicate samples containing 10 fruits ± SE.

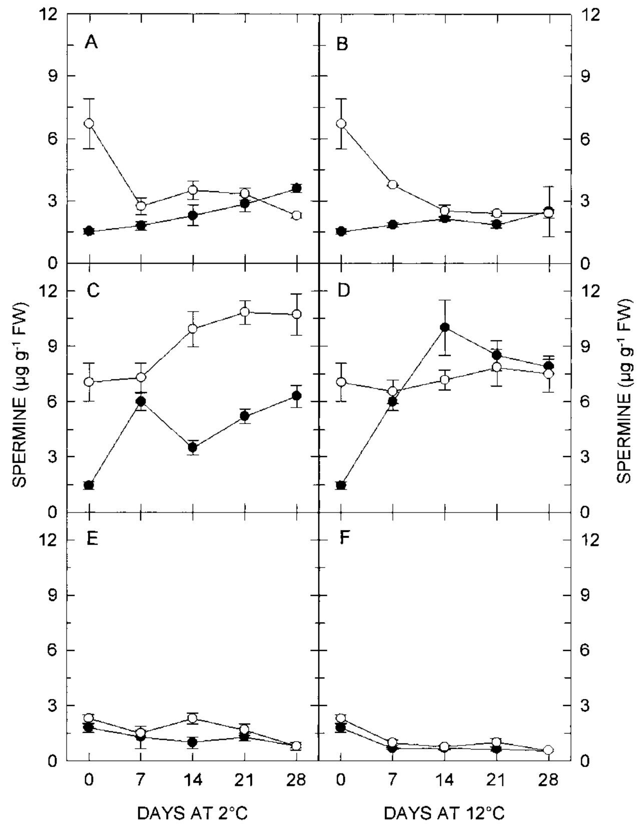

Figure 5. Effect of harvest date on spermine content in the flavedo of Fortune mandarin fruits stored at 2 °C (left panel) or 12 °C (right panel) immediately after harvest (●) or after 3 days of conditioning at 37 °C and 90−95% RH (○). Fruits were harvested in December (A and B), January (C and D), and April (E and F). Fruits colors (*h*°) were 55.8, 44.7, and 42.3, respectively. Values are the mean of three replicate samples containing 10 fruits ± SE.

ary was ∼5-fold greater than in freshly harvested fruits. However, heating the fruits harvested in April barely affected spermine levels. After conditioned fruits were held at 2 °C, spermine levels declined in fruits from December, kept increasing in fruits from January, and remained constant in fruits from April. A similar pattern of changes was observed when fruits were transferred at 12 °C except in fruits from January, in which spermine showed little change during holding.

DISCUSSION

Fortune mandarins are prone to develop CI symptoms upon storage at low temperature. We have previously shown that the sensitivity to CI varied substantially with maturity and that middle-season fruits (January−February) exhibited maximum susceptibility to CI. A conditioning treatment of 3 days at 37 °C has been proved to be highly effective in reducing CI, irrespective of the sensitivity of the fruit at harvest (Lafuente et al., 1997). Stressful temperatures, by themselves, have been shown to induce changes in endogenous polyamine levels (Faust and Wang, 1992). In this study we have compared the effect of increasing time of conditioning at 37 °C on CI susceptibility and polyamine content and evaluated the involvement of these compounds in the heat-induced tolerance to CI in fruits of Fortune mandarin harvested during the season.

The results demonstrate that heat conditioning influenced polyamine content in the flavedo of Fortune mandarin more than chilling. Conditioning fruits for increasing periods at 37 °C delayed the development of CI symptoms and, although the titer of each polyamine was differently affected, a gradual increase was observed with the time of conditioning. These results are consistent with observations in other plant tissues that postharvest treatments that elevate the content of polyamines before cold storage are effective in reducing CI (Wang, 1993; Serrano et al., 1996). It is interesting to note that at the time that Fortune mandarin shows the maximum sensitivity to CI (January), heat treatment resulted in a greater induction in spermine than in spermidine and putrescine. After 3 days at 37 °C, a 5-fold increase in spermine, a 2.5-fold increase in spermidine, and a 1.9-fold increase in putrescine were induced relative to the nonconditioned fruits. Similar results have been also found in zucchini squash subjected to a preconditioning treatment that alleviates CI (Kramer and Wang, 1989) and are consistent with the proposed effectiveness of polyamines as antioxidant and membrane stabilizing in relation to the number of positive charges per molecule (Drolet et al., 1986). In mandarins harvested earlier or later in the season, however, the heat-induced increase in polyamines was not proportional to their antisenescent activity.

The effect of 3 days of conditioning at 37 °C on fruits harvested at different maturity stages did not show a uniform response in polyamine content despite its always reducing CI. The major differences between the levels of the three polyamines in conditioning and nonconditioned fruits held at 2 °C were detected in the fruits more susceptible to developing CI. However, a comparison of the changes in polyamines, especially those in spermine (Figure 5), induced by heat treatment in Fortune mandarin harvested earlier or later in the season reveals that the response to the treatment appears to be more likely related to the stage of fruit maturity rather than to their susceptibility to CI or to the induced resistance. In Tarocco oranges Schirra et al. (1997) have reported a major resistance of middle-season fruits to develop heat-induced peel damage and reinforce the idea that the response of *Citrus* fruits to heat treatments may be dependent on the stage of maturity.

In different plant tissue it has been reported that the increase in polyamine is a common response to heat treatment (Flores, 1991). The involvement of polyamines, however, in the heat-induced tolerance to CI is still unclear. In lemon, McDonald (1989) did not find a relation between the reduction of CI induced by different temperature conditioning treatments and the increase in putrescine and spermidine. In Fortune mandarin we have recently shown that increasing the time or temperature of hot water dip treatment increased the content of the three polyamines, but this effect could not be related to the tolerance induced to subsequent cold storage (Gonzalez-Aguilar et al., 1997; Mulas et al., 1997). In the present study, we show that heating Fortune mandarin, even at a temperature less extreme than those used in hot water dips, enhanced polyamine content in the flavedo tissue and that the induced levels may quickly be reduced after the fruits are transferred to low temperature. It is further shown that spermine content in the flavedo of fruits harvested in April was not affected by 3 days of heat conditioning (Figure 5E), despite the polyamine being more sensitive to heat in fruits harvested early in the season (Figure 5A,C). From these pieces of evidence, the high variability induced in the titer of each polyamine and the similar levels of polyamines induced by hot water dip treatments that did not induce resistance to CI (Gonzalez-Aguilar et al., 1997), we cannot conclude that the increase in polyamines by heat treatment is directly linked to the induced tolerance to CI.

The changes in polyamines during storage of nonconditioned fruits were also different depending on the ripening stage of the fruit but, in general, their changes could not be associated with the different behaviors of the fruits upon chilling. In fruits harvested in December or April, which have similar susceptibilities to CI, the changes in the contents of the three polyamines during storage at 2 °C were also different. In the flavedo of lemon and grapefruit McDonald and Kushad (1986) found an increase in the content of putrescine when fruits were stored at chilling temperature. In zucchini squash the development of CI was correlated with an increase in putrescine and decreases in spermidine and spermine (Kramer and Wang, 1989; Wang, 1994). It has been also suggested that only plant tissues susceptible to CI are able to increase putrescine and spermidine under low-temperature storage (McDonald and Kushad, 1986). In flavedo of Fortune mandarin, however, similar patterns of changes in the three polyamines were observed in fruits stored at the chilling and nonchilling temperatures. This observation may reflect the fact that 12 °C is a temperature low enough to induce polyamine changes even though it did not cause peel damage. This pattern of changes in polyamines appears not to be associated with the development of CI symptoms, being more likely a response to the alteration in the temperature regime imposed on the fruits. These results are in agreement with observations in other *Citrus* cultivars, in which the enhancement of CI by ethylene was not associated with changes in the content of putrescine (Yuen et al., 1995). Collectively, the results indicate that in Fortune mandarin the ripening stage of the fruit has a greater influence on the changes in polyamines under storage than the sensitivity to CI.

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