Postharvest Calcium Infiltration Delays Membrane Lipid Catabolism in Apple Fruit

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Postharvest changes in membrane lipids of Ca^{2+}-infiltrated apples (*Malus domestica* Borkh. cv. Golden Delicious) have been evaluated. Calcium infiltration (2% or 4% w/v $CaCl_2$) improved fruit firmness retention over control (water-infiltrated) fruit following 6 months of 0 °C storage and after 7 subsequent days at 20 °C. During cold storage, total phospholipid (primarily phosphatidylcholine and phosphatidylethanolamine) and acylated steryl glycoside concentrations increased in Ca^{2+}-infiltrated fruit but decreased in control fruit. Seven days following transfer from cold storage to 20 °C, total phospholipid concentration remained highest in fruit infiltrated with 4% $CaCl_2$. Free sitosterol and steryl glycoside concentrations were generally increased with increasing infiltrated Ca^{2+} concentration throughout the postharvest evaluation period. Greater conservation of specific membrane lipid components in the Ca^{2+}-infiltrated fruit, both during and after low-temperature storage, may contribute to the well-known beneficial effects of Ca^{2+} infiltration in maintaining apple quality.

Keywords: *Plant senescence; ripening; glycerolipids; steryl lipids; Malus domestica Borkh.*

INTRODUCTION

Postharvest Ca^{2+} infiltration represents a safe and potentially effective method for increasing the storage life and quality of apple fruit (Conway, 1989). Although Ca^{2+} infiltration of harvested apples is not used commercially (Conway et al., 1994), its effectiveness and adoption by the fruit industry could be advanced by a better understanding of how Ca^{2+} affects the tissue at the cellular level.

The significance of membrane stability in the storage life of apples is based on growing evidence that cell turgor reduction, through increased permeability during senescence, partially accounts for loss of fruit firmness (Shackel et al., 1991; Stow, 1993). Other senescence-driven, membrane deteriorative processes affect quality changes through the extensive loss of ultrastructural organization within the plant cell (Stanley, 1991). An involvement of Ca^{2+} in cell membrane structure and function has long been recognized (Marinos, 1962). Thus, the beneficial effects of Ca^{2+} infiltration on postharvest quality of apples are probably mediated, in part, through the stabilizing influence of Ca^{2+} on cell membranes. Limited data on whole apples infiltrated with Ca^{2+} solutions (based on fruit hydraulic conductivity assessments) support this likelihood (Glenn et al., 1988). *In vitro* study with tissue slices or isolated membrane fractions has shown that cell membranes of apple fruit are more stable in Ca^{2+}-containing solutions than in the absence of Ca^{2+} (Legge et al., 1982; Lieberman and Wang, 1982), but data on the effects of postharvest Ca^{2+} infiltration on membrane stability in whole apple fruit are very limited.

In plant tissue, phospholipids and galactolipids are primarily membrane-associated (Galliard, 1978), and relatively recent evidence indicates that glycosylated as well as free sterols are also membrane structural lipids (Moreau and Preisig, 1993; Goad, 1991). Analysis of these lipids thus provides a means of assessing membrane stability.

In a previous study, we evaluated the effects of postharvest Ca^{2+} infiltration on firmness and on membrane lipid components of apple fruit (*Malus domestica* Borkh. cv. Golden Delicious) following 6 months of 0 °C storage (Picchioni et al., 1995). We found that Ca^{2+} infiltration maintained greater levels of conjugated steryl lipids and galactolipids, but had no effect on phospholipids, in fruit transferred to 20 °C for up to 2 weeks. However, the effect of Ca^{2+} infiltration on membrane lipid metabolism during the cold storage period was not evaluated in the study. An earlier investigation involving noninfiltrated apples stored in both air and low-O_2 environments showed marked changes in the pattern of membrane lipid concentrations, namely phospholipids and free sterols (Bartley, 1986).

The objectives of the current study were to evaluate the effects of postharvest Ca^{2+} infiltration on firmness and membrane lipid changes of Golden Delicious fruit during and after 0 °C storage.

MATERIALS AND METHODS

Postharvest Handling. Preclimacteric Golden Delicious apples were harvested from a commercial orchard and randomized into nine, 20-fruit lots (one lot for each of three infiltration treatments and three postharvest stages). One day following harvest, whole fruit were pressure infiltrated for 3 min at 103 kPa with deionized water (0% $CaCl_2$ control) or with 2% or 4% (w/v) $CaCl_2$ (99% $CaCl_2{\cdot}2H_2O$, Sigma Chemical

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Co.). Fruit were allowed to air-dry overnight at 20 °C, and one of three lots per treatment (0 days storage) was evaluated for firmness, Ca^{2+} concentration, and lipid class concentrations (see below). The remaining lots were stored in air at 0 °C and 85 ± 5% RH for 6 months. These fruit were then evaluated for firmness and lipid classes immediately after 0 °C storage and following an additional 7 days at 20 °C.

Evaluation of Fruit Firmness, Ca^{2+} Concentration, and Lipid Class Concentrations. Fruit firmness was determined on 20 individual fruit per treatment at each postharvest stage. An electronic pressure tester (EPT-1, Salt Lake City Technical Products) was set in the Magness−Taylor mode and interfaced with a personal computer. The instrument had an 11-mm tip which penetrated to a depth of 8 mm. Two measurements were obtained per fruit on opposite, equatorial sides after removing the peel. A 2−3 mm layer of outer cortical tissue was then excised using a mechanical peeler (peel and outermost 1−2 mm of cortical tissue first discarded). This tissue was lyophilized, ground into a fine powder, and stored under N_2 gas at −70 °C prior to Ca^{2+} and lipid class analyses. For Ca^{2+} and lipid evaluations, the pooled outer cortical tissue from five apples made up one sample replicate; four replicates per treatment were analyzed.

Calcium analyses were made only for nonstored fruit (0 days storage). Subsamples of 1 g were ashed in a muffle furnace overnight, dissolved in 2 N HCl, and filtered. Calcium concentrations were then determined using a plasma emission spectrometer (model 61, Jarrel Ash Corp.).

Total lipid extracts were obtained by homogenizing and then filtering 500-mg subsamples of lyophilized tissue in $CHCl_3$−MeOH (2:1). The homogenates were filtered, and then the tissue residue was resuspended in 2:1 $CHCl_3$−MeOH. The combined filtrates were washed with 0.85% (w/v) NaCl and then MeOH−H_2O (1:1). The $CHCl_3$ phase was evaporated under N_2, and the lipid residue was redissolved in 1 mL of $CHCl_3$. Components of the total lipid extracts (neutral lipids, glycolipids, and phospholipids) were separated and collected using silica sep-pak cartridges (Waters). The lipid fractions were prepared for gas and liquid chromatographic analyses as described earlier (Picchioni et al., 1995).

Free sterols were precipitated from the neutral lipid fraction with digitonin and analyzed by capillary GLC−FID using lathosterol as an internal standard. The flow rate of the carrier gas (He) was maintained constant at 1 $mL \cdot min^{-1}$, and the oven temperature was isothermal at 260 °C. The injector and detector temperatures were 300 and 350 °C, respectively.

Glycolipid and phospholipid classes were separated by HPLC using a Waters 600E solvent delivery system and a 10 cm × 3 mm Chromsep LiChrosorb Si (5-μm) silica cartridge (Chrompak). Quantification was achieved using a Varex IIA evaporative light scattering detector (Varex Corp.) with the N_2 flow rate constant at 2.5 $L \cdot min^{-1}$ and the drift tube temperature at 90 °C. For calibration, authentic glycolipid classes were purchased from Matreya, Inc. (Pleasant Gap, PA), and authentic phospholipid classes, from Sigma.

The solvent flow rate for glycolipid and phospholipid HPLC separation was kept constant at 0.5 mL min^{-1}, and the composition was selected from earlier reports by Moreau et al. (1990) and Letter (1992) with slight modifications (Picchioni et al., 1995).

Statistical Analyses. The experiment was completely randomized as a 3 × 3 factorial (3 infiltration treatments and 3 postharvest stages). The analysis of variance was performed using MSTAT-C, version 2.11 (Michigan State University, East Lansing, MI). The main effects for *F*-tests are noted in the presentation of the data, and where appropriate, *t*-tests of significance were performed for individual mean comparisons. Each observation is reported as the mean ± se of four, 5-fruit replicates. The average firmness of five individual fruit (2 measurements per fruit) and the Ca^{2+} and lipid analysis of pooled samples from five fruits represented a single replicate.

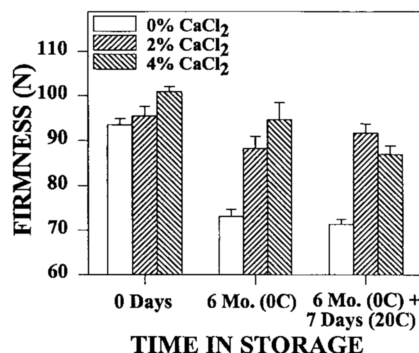

Figure 1. Firmness of Golden Delicious fruit at three stages following Ca^{2+} infiltration. Fruit were infiltrated with deionized water (0% $CaCl_2$) or $CaCl_2$ solutions 1 day after harvest and then stored for 6 months in air at 0 °C and then for 7 days in air at 20 °C. Each value represents the mean ± se of four, 5-fruit replicates.

Table 1. Main Effects for *F*-Tests Involving Fruit Firmness, Membrane Lipid Concentrations, and Relevant Membrane Lipid Ratios over Time (Postharvest Stage) in Golden Delicious Fruit Cortical Tissue following Ca^{2+} Infiltration

response variable	effects[a] $CaCl_2$	Stage	$CaCl_2$ × stage
fruit firmness	***	***	***
TPL	**	NS	*
PC	**	***	**
PE	*	*	NS
PC:PE mass ratio	NS	*	NS
PI	NS	***	NS
PA	NS	***	*
LPC	NS	**	NS
sitosterol	*	*	*
campesterol	***	NS	NS
TFS	*	*	*
TFS:TPL mole ratio	NS	***	NS
SG	***	***	*
ASG	NS	***	**
MGDG	NS	***	NS
DGDG	NS	**	NS

[a] NS, *, **, ***: nonsignificant by *F*-test at $P \leq$ 0,05 or significant at $P \leq$ 0.05, 0.01, or 0.001, respectively.

RESULTS AND DISCUSSION

The $CaCl_2$ infiltration treatments (2% and 4%) increased Ca^{2+} concentration of the outer fruit cortical tissue by an average of 10 times that of the control (0%) treatment (266 ± 22, 2474 ± 132, and 2852 ± 108 mg of $Ca \cdot kg^{-1}$ dry wt for the 0%, 2%, and 4% $CaCl_2$ treatments, respectively). The tissue Ca^{2+} concentration did not increase in proportion to the Ca^{2+} treatment concentration, indicating a finite capacity for the fruit to absorb Ca^{2+} from the infiltration solution.

After 6 months storage at 0 °C, firmness of control fruit (0% $CaCl_2$) decreased by 22%, whereas firmness of Ca^{2+}-infiltrated fruit (2% and 4% treatments) decreased by only 6−8% (Figure 1). The contrasting changes caused an interaction between infiltration treatment and postharvest stage (Table 1).

Overall, firmness changed little following transfer from 0 °C to 20 °C for 7 days, by which time Ca^{2+}-infiltrated fruit were 25% firmer than control fruit. Firmness did not differ between 2% and 4% $CaCl_2$ (*t*-test, $P \leq$ 0.05).

The main effect of postharvest stage on total phospholipid (TPL) concentration (Table 1) was not significant (TPL averages of 258, 270, and 255 $mg \cdot 100\ g^{-1}$ dry wt at 0 days, 6 months, and 6 months plus 7 days, respectively). However, inverse changes in TPL concentration in control fruit and Ca^{2+}-infiltrated fruit during 0 °C storage were observed (Figure 2). Following 6 months of storage at 0 °C, TPL concentrations of Ca^{2+}-

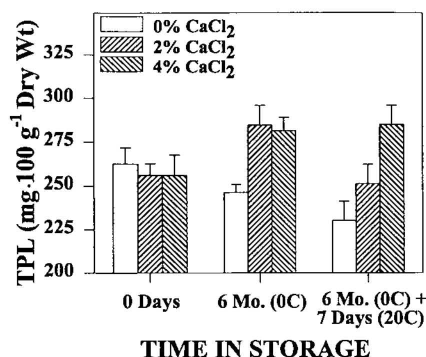

Figure 2. Total phospholipid concentrations (TPL) in Golden Delicious fruit cortical tissue at three stages following Ca^{2+} infiltration. Each value represents the mean ± se of four, 5-fruit replicates (treatment and handling described in Figure 1).

infiltrated fruit (2% and 4% treatments) had increased to a similar level and averaged 15% greater than in control fruit. After 7 additional days at 20 °C, TPL concentration of fruit infiltrated with 2% $CaCl_2$ decreased by 12%, but TPL concentration of fruit infiltrated with 4% $CaCl_2$ remained stable and was the highest of all treatments (*t*-tests, $P \leq 0.05$). In marked contrast to Ca^{2+}-infiltrated fruit, TPL concentrations of control fruit declined with each postharvest stage.

Mature apple fruit are known to accumulate phospholipids in low-temperature environments. In non-Ca^{2+}-treated apples, Bartley (1986) showed that total phospholipid concentration increased by about 25% in cortical tissue following 3 months storage in air at 3.5 °C. We have observed similar changes in similar conditions (e.g., 21% increases in TPL concentration of control, non-Ca^{2+}- treated Golden Delicious fruit during 3−4 months of 0 °C storage; unpublished data). However, results in the present study suggest that unless the fruit Ca^{2+} concentration is significantly increased (through Ca^{2+} infiltration), longer cold storage periods (6 months) may deplete the capacity of the fruit to synthesize membrane phospholipids (compare 0% $CaCl_2$ to 2% and 4% $CaCl_2$ treatments from 0 days to 6 months in Figure 2). This hypothesis is supported by an earlier time course study involving non-Ca^{2+}-infiltrated apples stored for 6 months at 0 °C (Lurie et al., 1987). Using microsomal membranes, these investigators measured overt cell permeability changes coincident with net phospholipid breakdown.

In higher plants, phospholipid accumulation is believed to play an adaptive role in low-temperature acclimation which favors the maintenance of membrane fluidity and membrane function (Graham and Patterson, 1982). Thus, the present results suggest that Ca^{2+} induced membrane restructuring in response to low temperature.

Changes in TPL concentration mainly reflected changes in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which comprised over 80% of the TPL mass (Figure 3). The proportions of individual phospholipid classes (wt %) were unaffected by infiltration treatment (data not shown), but during 0 °C storage (6 months), the ratio of PC:PE increased from an average of 1.47 at 0 days to an average of 1.96 at 6 months (Figure 3). The relatively high PC:PE ratios persisted following 7 days of 20 °C storage (average of 1.85).

Following 6 months at 0 °C plus 7 days at 20 °C, PE concentrations were Ca^{2+}-dependent, and the increased PC:PE ratio in control fruit was mainly the result of net PE degradation. Conversely, in fruit infiltrated with 4% $CaCl_2$, PE concentrations were relatively stable during storage, and a sizeable net increase in PC concentration during 0 °C storage led to the increased PC:PE ratio. Fruit infiltrated with 2% $CaCl_2$ were intermediate in their relative PE and PC changes.

Infiltration treatment did not affect phosphatidylinositol (PI), phosphatidic acid (PA), or lysophosphatidylcholine (LPC) concentrations, but these lipid classes changed during storage (Table 1 and Figure 3). Among the infiltration treatments, average PI concentration remained relatively unchanged during 0 °C storage (23.9 and 24.1 mg·100 g^{-1} dry wt at 0 days and 6 months, respectively), but PI declined sharply to an average of 15.6 mg·100 g^{-1} dry wt following 7 days storage at 20 °C. This again reflects membrane degradation.

The average concentrations of PA and LPC increased by 29−35% during 0 °C storage (from 16.8 to 21.7 mg·100 g^{-1} dry wt and from 2.0 to 2.7 mg·100^{-1} g dry wt for PA and LPC, respectively). During the subsequent 7-day exposure of fruit to 20 °C, the average concentrations of PA and LPC decreased by 4% and 9%, respectively. The increases in LPC and PA during 0 °C storage may have been attributed to membrane catabolism resulting from the action of phospholipase A_2 (André and Scherer, 1991) and phospholipase-D (Chéour et al., 1992), respectively. Alternatively, PA is known to accumulate in disrupted (e.g., homogenized) plant tissue (Galliard, 1978); thus, part of the increase in PA we observed also may be an artifact of the extraction procedure. Using our methods, we were unable to determine the percentage of the PA accumulation which may have arisen from natural senescence.

On average, sitosterol represented over 95 wt % of the total free sterols (Figure 4). Thus, changes in total free sterols (TFS) were largely a function of this predominant component. In control fruit, sitosterol concentration had decreased both after 6 months of 0 °C storage and after 7 subsequent days at 20 °C. In Ca^{2+}-infiltrated fruit, however, average sitosterol concentration either remained unchanged (2% $CaCl_2$ treatment) or increased (4% $CaCl_2$ treatment) during 6 months of 0 °C storage, resulting in an interaction between infiltration treatment and postharvest stage (Table 1). In all treatments, sitosterol and TFS concentrations decreased following 7 days exposure to 20 °C.

Campesterol represented a minor fraction of the TFS pool, and its concentration was unaffected by the postharvest stage (Table 1 and Figure 4). Campesterol concentrations were greater with increasing Ca^{2+} concentrations in infiltration solutions following 6 months of 0 °C storage, although this trend was less apparent following 7 days at 20 °C. Free stigmasterol, free cholesterol, and steryl esters were below detection limits.

Similar patterns of change in the TFS:TPL mole ratio during storage occurred with all infiltration treatments (Table 1 and Figure 4). During 6 months of 0 °C storage, the TFS:TPL ratio decreased from an average of 0.62 to 0.51 and then increased to 0.56 following 7 days of 20 °C storage.

The increase in PC relative to PE during 0 °C storage is significant for low-temperature membrane adaptation in that PC is known to impose less rigidity to plant membranes than PE (Leshem et al., 1992). The de-

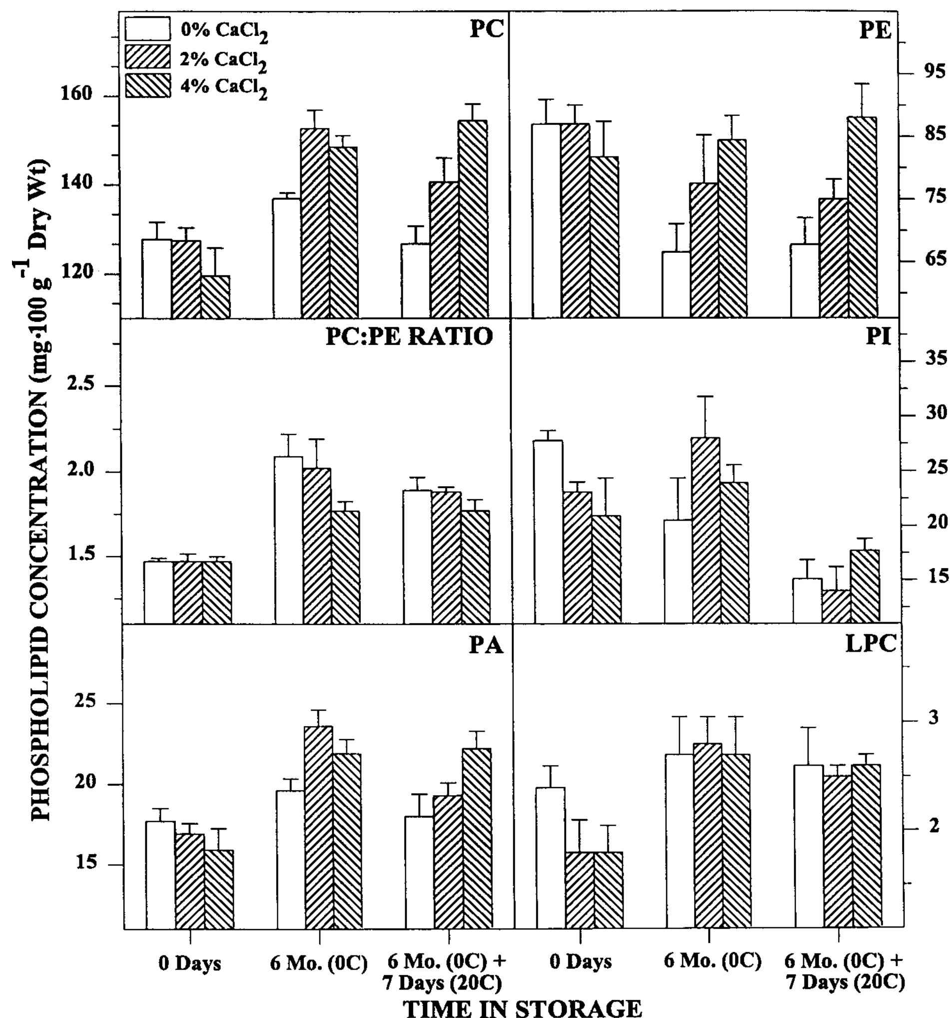

Figure 3. Phospholipid concentrations and the mass ratio of phosphatidylcholine:phosphatidylethanolamine in Golden Delicious fruit cortical tissue at three stages following Ca^{2+} infiltration. Each value represents the mean ± se of four, 5-fruit replicates (treatment and handling described in Figure 1).

crease in TFS relative to TPL during 0 °C storage would presumably further contribute to membrane fluidity, since an increase in this ratio is thought to add rigidity to cell membranes, particularly those in apple fruit (Lurie and Ben-Arie, 1983). Although Ca^{2+} infiltration had no effect on the PC:PE or TFS:TPL ratios during 0 °C storage, it prevented the net loss of phospholipids (PE) and free sterols (sitosterol) that accompanied the changes in these ratios in water-infiltrated fruit.

Storage affected the concentrations of all glycolipid classes that we evaluated (Table 1 and Figure 5). Moreover, steryl glycoside (SG) concentrations were greater with increasing Ca^{2+} concentration following 6 months of 0 °C storage and after 7 days at 20 °C. After 6 months of 0 °C storage, SG concentrations decreased by 16% in control fruit, whereas Ca^{2+}-infiltration maintained SG concentrations closer to the day 0 value. After 7 days at 20 °C, SG concentrations declined by a similar amount in each infiltration treatment but nonetheless remained greater in Ca^{2+}-infiltrated fruit.

During 0 °C storage, a net reduction in acylated steryl glycoside (ASG) concentration in control fruit and a net increase in ASG concentration in Ca^{2+}-infiltrated fruit caused an interaction between infiltration treatment and postharvest stage (Table 1 and Figure 5). Concentrations of ASG subsequently decreased appreciably in all treatments after 7 days at 20 °C.

Previously, we observed substantial increases in ASG concentrations in Ca^{2+}-infiltrated Golden Delicious fruit during a 7-day period of storage at 20 °C (after an initial 6-month period at 0 °C; Picchioni et al., 1995). This did not occur in the present study, and the basis for the difference between the studies is unclear. Nonetheless, ASG comprised less than 5 mol % of the total steryl lipids in the present study; thus, the significance of changes in ASG may not be as important as changes in the more dominant steryl lipids (sitosterol and SG).

Calcium infiltration seems to alter sterol conjugation during postharvest handling of Golden Delicious fruit. Both sterol conjugates and the processes of sterol conjugation (glycosylation and acylation) are thought to occur on the plasma membrane (Goad, 1991; Moreau and Preisig, 1993; Larsson et al., 1990; Hartman and Benveniste, 1987). In addition, sterol conjugation may be an important determinant of membrane function (Benveniste, 1978; Wojciechowski, 1980). The mechanism by which Ca^{2+} regulates these processes in Golden Delicious fruit needs to be elucidated.

Only the main effects of postharvest stage affected galactolipid concentrations (Table 1 and Figure 5). The major overall reduction in monogalactosyldiacylglycerol (MGDG) concentration occurred during 6 months of 0 °C storage (average net reduction of 8.4 mg·100 g^{-1} dry wt), with a smaller reduction occurring following 7 days

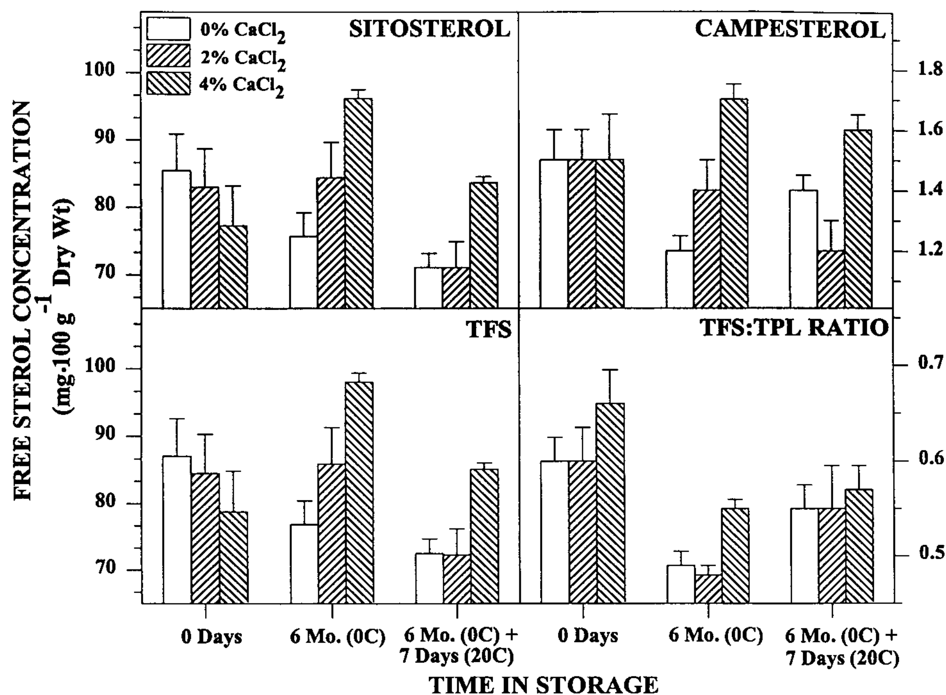

Figure 4. Free sterol concentrations and the mole ratio of total free sterols (TFS):total phospholipid (TPL) in Golden Delicious fruit cortical tissue at three stages following Ca^{2+} infiltration. Each value represents the mean ± se of four, 5-fruit replicates (treatment and handling described in Figure 1).

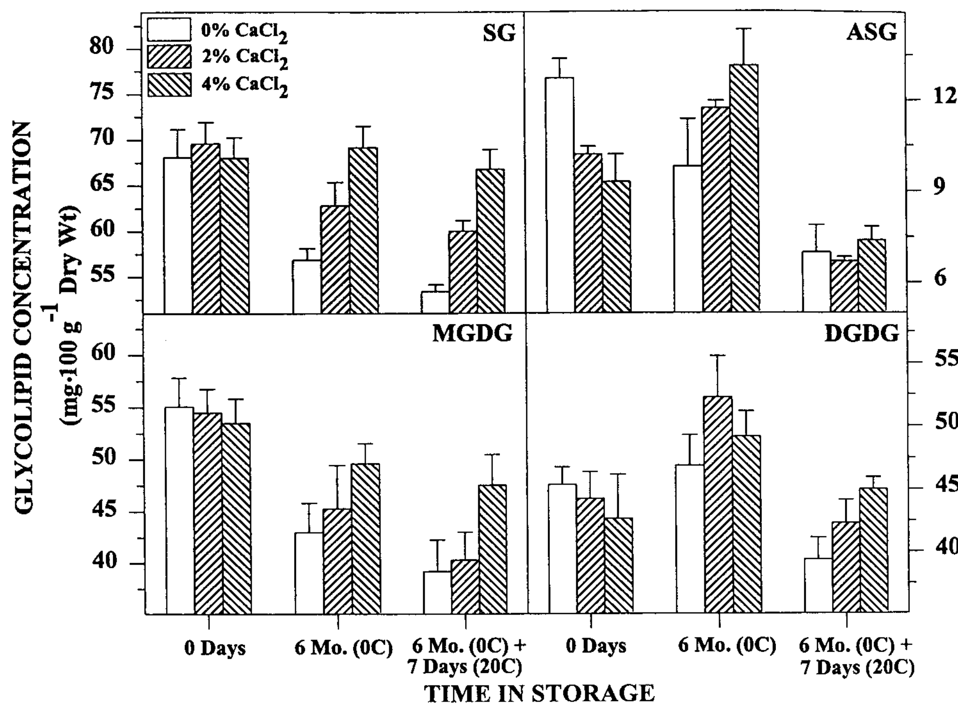

Figure 5. Glycolipid concentrations in Golden Delicious fruit cortical tissue at three stages following Ca^{2+} infiltration. Each value represents the mean ± se of four, 5-fruit replicates (treatment and handling described in Figure 1).

at 20 °C (average net reduction of 3.6 mg·100 g^{-1} dry wt). In contrast, concentrations of digalactosyldiacylglycerol (DGDG) increased during 0 °C storage by an average of 5.4 mg·100 g^{-1} dry wt and then decreased by an average of 7.3 mg·100 g^{-1} dry wt after 7 days at 20 °C.

Infiltration treatments did not alter galactolipid concentrations during storage (Table 1), but there was a trend toward higher galactolipid concentrations in Ca^{2+}-infiltrated fruit following 0 °C storage, as previously noted by Picchioni et al. (1995). Also, results of *t*-tests showed that MGDG concentrations did not decrease in fruit infiltrated with 4% $CaCl_2$ following 6 months of 0 °C storage plus 7 days at 20 °C as compared to the 6-month value ($P \leq 0.05$). In contrast, reductions in MGDG occurred in fruit infiltrated with water or 2% $CaCl_2$ during this same period ($P \leq 0.05$). During 6 months of 0 °C storage, DGDG concentrations increased in both 2% and 4% Ca^{2+} infiltration treatments but remained unchanged in the water-infiltrated fruit ($P \leq 0.05$). Thus, Ca^{2+} infiltration may have a relatively small but important counteractive influence on

galactolipid catabolism in Golden Delicious fruit. This would likely preserve plastid membranes which are known to be enriched with galactoliplids (Miernyk, 1985; Mudd, 1967).

In summary, we have shown that Ca^{2+} infiltration improves firmness retention and delays the net loss of membrane phospholipids, free sterols, and sterol conjugates in Golden Delicious fruit. The firmness and membrane lipid changes reported in the present study are similar to earlier findings with this apple cultivar, where fruit were evaluated only during a 2-week period at 20 °C (following a 6-month cold storage treatment; Picchioni et al., 1995). However, as shown in the present study, major changes in membrane lipid components are associated with low-temperature storage. Water-infiltrated fruit had lower concentrations of TFS, TPL, SG, and ASG, particularly at the end of 0 °C storage, compared with Ca^{2+}-infiltrated fruit. This suggests smaller losses in total membrane lipid components and increased membrane integrity in Ca^{2+}-infiltrated as compared to control fruit.

Because membrane lipid alterations are viewed as a central factor in the senescence of plant tissues, we conclude that Ca^{2+} may serve a key role in delaying apple fruit quality losses after Ca^{2+} infiltration, especially by delaying membrane lipid catabolic processes.

ABBREVIATIONS USED

TPL (total phospholipids); PE (phosphatidylethanolamine); PI (phosphatidylinositol); PA (phosphatidic acid); PC (phosphatidylcholine); LPC (lysophosphatidylcholine); TFS (total free sterols); SG (sterol glycoside); ASG (acylated sterol glycoside); MGDG (monogalactosyldiacylglycerol); DGDG (digalactosyldiacylglycerol).

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