Effects of Calcium Compounds on Fruit Puffing and the Ultrastructural Characteristics of the Subepidermal Cell Walls of Puffy and Calcium-induced Non-puffy Satsuma Mandarin Fruits

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Summary

The alleviating effects of calcium compounds on fruit puffing were investigated in satsuma mandarin. The ultrastructural changes in the subepidermal cell walls of puffy fruits and the inhibiting strategies of calcium against these changes were also observed under transmission electron microscope. Although puffing occurred in both treated and non-treated fruits under certain environmental conditions, pre-harvest applications of CS−2H (CaSO₄ · 2H₂O, CaCl₂) and CS−1B (CaCO₃ microcrystal suspension) significantly reduced it and with the exception of peel puffing these chemicals did not affect other fruit characteristics. The subepidermal cells of non-treated puffy fruits had bigger intercellular spaces in the cell corners and schizogenous intercellular spaces between adjacent walls. Occasionally, cell corners and abutting walls did not possess these spaces, but the disintegration of middle lamella therein was distinct. The disintegration caused loosening of the intercellular connection and swelling of walls. The sporadic destruction of the cell wall was also found near the plasma membrane. The fibrillar materials of loose cell walls separated distinctly in the longitudinal direction; this separation was crosswise near the plasma membrane. In a few cases, the middle lamella and its adjoining walls had disintegrated. The application of calcium compound (CS−2H) prevented these ultrastructural disorders so that intercellular spaces remained small, schizogenous intercellular spaces did not develop, and the dissolution of middle lamella was checked considerably. The cell corners retained their middle lamella or sometimes developed small intercellular spaces. The fibrillar materials in the cell walls were compact and their destruction was not evident. Cell wall swelling was rarely observed, and cellular separation was not found.

Key Words: satsuma mandarin, calcium compound, dissolution of middle lamella, electron microscopy, peel puffing.

Introduction

Satsuma mandarin belongs to the loose-skin group of citrus fruits whose fruits are characterized by easy separation of rind from flesh at maturity (Kawase et al., 1981). Fruits, especially those cultivated in southwestern Japan, usually develop into puffy fruit in late autumn as harvest approaches. The puffy fruits are easily damaged during harvesting, sorting, packing and transporting. Furthermore, they can not be stored for a long period, because their taste deteriorates early, and they are susceptible to post-harvest decay. Serious post-harvest losses occur so that preventive methods are important to producers and buyers (Kawase et al., 1981). Thus practices which would reduce this disorder were of economic consideration during the last decades (Kuraoka et al., 1975a, 1975b, 1976, 1977; Kawase et al., 1981; Kawase and Hirai, 1983).

Application of GA₃ and sprays of corpuscular calcium carbonate (CaCO₃ micro crystal suspension) are known to reduce peel puffiness to some extent (Kuraoka et al., 1977; Kawase et al., 1981). However, GA₃ delays peel coloring and causes rind injury, making the treatment impractical and useless to citrus growers (Kawase and Hirai, 1983). The effect of corpuscular calcium carbonate is also sometimes nullified by rainfall. Moreover, at harvest the fruit surface is coated with a thin layer of calcium residues which makes the fruit unappealing to consumers and thereby yielding low prices. Thus, peel puffing is a serious problem and a burning issue in the citrus industry.

From histological studies, Kuraoka (1962) suggested that the disintegration of albedo cells causes the affected
fruits to become puffy followed by swelling on account of rain. During this process, the albedo is transformed to somewhat spongy structures having large schizogenous intercellular spaces (Kuraoka et al., 1975b). In other studies, loss in firmness of citrus fruits has been associated with the activity of cell wall-degrading enzymes (Rouse, 1977; Williamson, 1991). Orange peel contains these cell wall modifying enzymes which increase in activity during fruit ripening (Monselise et al., 1976; Rouse, 1977). That anatomical changes of albedo cells in puffy citrus fruits occur and that the subepidermal cells are under continuous expansion during the later growing period of fruits are known (Kuraoka et al., 1975b). As the subepidermal cells contain cell wall modifying enzymes, it is a reasonable hypothesis that the enzymatic activities could lead also to their structural alterations in puffy fruits. On the other hand, the calcium compound is reported to alleviate peel puffing through its penetration into the rind cells (Kuraoka et al., 1975a) which would indicate that the subepidermal cells receive calcium immediately after penetrating the epidermis. Hence, calcium may inhibit some enzyme activities resulting in non-puffy fruits.

This investigation was intended to assess the potential of CS-2H (CaSO₄·2H₂O-57%, CaCl₂-27%) to inhibit peel puffing of satsuma mandarin fruit in contrast to CS-1B (CaCO₃ microcrystal suspension) and control. Simultaneously, ultrastructural deformities of the subepidermal cell walls of puffy fruits and the inhibiting strategies of calcium against these deformities were investigated.

Materials and Methods

The experiment on fruit puffing and other qualitative characters was conducted in the Shizuoka Prefectural Citrus Experiment Station and the ultrastructural studies on the same material were made in the Citiculture Laboratory, Faculty of Agriculture, Ehime University, Japan from August to December of 1995, 1996, and 1997. Sixteen-year-old satsuma mandarin (Citrus unshiu Marc. cv. Aoshima) trees, allotted 6.7m² per tree, in an experimental orchard of the station were provided with horticultural care for normal growth throughout the year. Fifteen trees per year were administered three treatments of CS-2H, CS-1B and a water control and replicated 5 times. The active ingredients of CS-2H were CaSO₄·2H₂O (0.25 g/100 ml)-57%, CaCl₂ (4.25 g/100 ml)-27% and others (0.09 g/100 ml)-16%; the solubility of this compound was 0.37g/100ml. CS-1B consisted of CaCO₃-97% and organic polymer-3%; the solubility was 0.00014 g/100 ml. A Randomized Complete Block Design was used for the application of treatments. Trees were sprayed with CS-2H (300 times diluted) and CS-1B (100 times diluted) on August 21 and September 27 in 1995 and on September 2 and September 27 in 1996 and 1997; whereas the control plants were sprayed with distilled water. Twenty fruits per tree were collected at random on December 10, yielding 100 fruits per treatment which were investigated immediately.

The degree of peel puffing was rated as 0, 1, 2, and 3, from firm to touch (0) to marked puffing (3) by feel. Fruits in Grades 2 and 3 are easily injured and generally called rind-puffed fruit. The samples were rated by the number of puffy fruit and degree of rind puffing according to Tsuji et al. (1987). This degree of rind puffing was termed as puffing index and expressed as a percentage. Specific gravity was determined by weighing fruits in air and water. Coloration was assessed from 1 to 10, namely from entirely green to orange. The percent of fruit flesh was calculated by using the ratio of flesh to whole fruit weight multiplied by 100. Brix was measured with a refractometer while titratable acidity content was obtained by the titration of the juice with 0.1N NaOH. The brix-acid ratio was calculated. The average values on the qualitative parameters taken each year were analyzed; means were separated by the Duncans New Multiple Range Test (DNMRT).

For transmission electron microscopy, the samples of the rind, including subepidermal cells of puffy and CS-2H treated non-puffy fruits, were cut into 2mm cube, pre-fixed in the Karnovsky's solution (Karnovsky, 1965), post-fixed in 2% osmium tetroxide, dehydrated in a graded ethanol series and embedded in epoxy resin. The ultrathin sections were cut, and double stained with uranyl acetate and lead citrate. Ten samples per treatment were observed, using about 30 grids (with 5-7 sections each) each year. The sections were viewed under a Hitachi H-7100 transmission electron microscope at 100 kV and photographed. The width of cell walls was measured by at least ten photographs from each treatment.

Results

1. Peel puffing and other qualitative characters of fruits under different treatments

The peel puffing (puffing index) and other qualitative characters of Ca-treated and control fruits are summarized in Table 1. Although puffing occurred in both treated and non-treated fruits, its magnitude was significantly less in CS-2H and CS-1B treatments than in that of the control each year. The average fruit weight and specific gravity showed insignificant differences among treatments but the coloring of fruit rind and the percentage of fruit flesh were unaffected. Brix and titratable acidity differed slightly among the treatments but not significantly. Brix/titratable acid ratio similarly exhibited insignificant variations among the treatments each year. The alleviating effects of CS-2H and CS-1B against peel puffing were statistically identical but the extent of peel puffing varied considerably from year to year in both treated and non-treated fruits (Table 1).
Table 1. Effects of calcium compounds spray on the peel puffing and fruit quality in satsuma mandarin (Citrus unshiu Marc. cv. Aoshima).

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment</th>
<th>Peel puffing index (%)</th>
<th>Avg. fruit wt. (g)</th>
<th>Specific gravity</th>
<th>Degree of rind color</th>
<th>Percentage of flesh</th>
<th>Brix (%)</th>
<th>Titrable acidity (%)</th>
<th>Ratio of brix to acidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995</td>
<td>CS - 2H</td>
<td>30.4b&lt;sup&gt;5&lt;/sup&gt;</td>
<td>121&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>-&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>8.1&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>76.6&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>11.4&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>0.82&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>13.9&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CS - 1B</td>
<td>26.1b</td>
<td>128</td>
<td></td>
<td>7.4</td>
<td>77.2</td>
<td>11.8</td>
<td>0.81</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>68.0a</td>
<td>123</td>
<td></td>
<td>7.6</td>
<td>77.1</td>
<td>11.5</td>
<td>0.87</td>
<td>13.2</td>
</tr>
<tr>
<td>1996</td>
<td>CS - 2H</td>
<td>7.9b</td>
<td>131</td>
<td>0.84</td>
<td>8.2</td>
<td>74.2</td>
<td>11.1</td>
<td>0.93</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>CS - 1B</td>
<td>14.6b</td>
<td>135</td>
<td>0.84</td>
<td>7.8</td>
<td>73.0</td>
<td>11.1</td>
<td>0.92</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>27.2a</td>
<td>131</td>
<td>0.84</td>
<td>7.9</td>
<td>73.9</td>
<td>11.3</td>
<td>0.83</td>
<td>12.5</td>
</tr>
<tr>
<td>1997</td>
<td>CS - 2H</td>
<td>36.0b</td>
<td>128</td>
<td>0.86</td>
<td>9.4</td>
<td>78.1</td>
<td>10.8</td>
<td>0.87</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>CS - 1B</td>
<td>48.0b</td>
<td>131</td>
<td>0.84</td>
<td>9.3</td>
<td>78.3</td>
<td>11.3</td>
<td>0.92</td>
<td>12.7</td>
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<tr>
<td></td>
<td>Control</td>
<td>70.0a</td>
<td>130</td>
<td>0.83</td>
<td>9.3</td>
<td>77.6</td>
<td>11.8</td>
<td>1.00</td>
<td>12.1</td>
</tr>
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</table>

<sup>5</sup> Mean separation within column by Duncan's new multiple range test at 1% level.
<sup>NS</sup> Non significant at 1% level. CS - 2H means CaSO₄ · 2H₂O, CaCl₂ and CS - 1B represents CaCO₃ suspension.

2. Transmission electron microscopic observations on the subepidermal cell walls of puffy and non-puffy fruits

The subepidermal cell walls of puffy fruits under went different ultrastructural disorders and destruction (Figs. 1 A - F), i.e. almost all cells possessed bigger intercellular spaces in the cell corners and the schizogenous intercellular spaces between adjacent walls (Fig. 1 A). The shape and size of the spaces in different locations varied but they were omnipresent. Rarely cell corners did not possess intercellular spaces but the absence of the middle lamella near those corners was distinctly evident (Fig. 1 B). The distortion of middle lamella along with the recurrence of intact and disintegrated portions in the cell corners were evident in the photomicrograph, indicating a distinct disorganization. A few common walls between cells seemed normal or semi-swollen and devoid of schizogenous intercellular spaces but they seemed to be separating or when seen under comparatively high magnification (Fig. 1 C). In these semi-swollen walls which were 4-6 μm width, the middle lamella had hydrolyzed, causing the connection between the neighboring cells to loosen (Fig. 1 C). The most evident dissolution of the middle lamella occurred near the intercellular space in the cell corners as revealed by the presence of a narrower middle lamella and its absence in other zones of the tissue (Fig. 1 D). The cell walls also appeared to be disintegrated (Fig. 1 D), i.e. the apparently attached portions of cell walls between two intercellular spaces also displayed considerable abnormality and swelling (Fig. 1 E). These swollen cell walls, 5-8 μm wide, had a middle lamella which was intact in a few places but the adhesion between neighboring cells caused them to swell and expose distinct cellulose microfibrils (Fig. 1 E). This stretching revealed the distinct longitudinal arrangements of cellulose micro fibrils throughout the cell walls. Contrarily, the fibrillar arrangement near the middle lamella was crosswise (Fig. 1 E). The separation of the cell wall components was also accompanied primarily by their sporadic destruction near the plasma membranes. As the middle lamella disappears, large intercellular spaces appear (Fig. 1 E). In advance stages of puffing, the cell wall exposed to the intercellular space seems to rupture, exposing the fibrillar materials (Fig. 1 F).

In contrast, considerable ultrastructural preservation was observed in the subepidermal cell walls of CS - 2H - treated non-puffy fruits (Figs. 2 A - H). The subepidermal cells of these non-puffy fruits have minimal, small intercellular spaces in the cell corners but schizogenous intercellular spaces were not present (Fig. 2 A). The shape and size of spaces varied in different locations but cell corners having no intercellular spaces were also seen. The extremely large spaces were not found in any location as they were in the puffy fruits. The cell corners without intercellular spaces maintained their structural integrity (Fig. 2 B), and where the dissolution process was initiated, the middle lamella was comparatively intact (Fig. 2 C). This characteristic continuously prevailed even after the formation of small intercellular spaces (Fig. 2 D). The destruction or irregular arrangements of fibrillar materials were not found in most of the cell walls (Fig. 2 E). The cell walls rather than the position of intercellular spaces were completely intact with distinct middle lamella; the compact cellulose microfibrils were always extended longitudinally along the cell wall (Fig. 2 E). These regularly organized normal cell walls were 2-3 μm wide. The hydrolysis and separation along the middle lamella were slightly visible and not extensive (Fig. 2 F). The adjoining cell walls between two intercellular spaces were comparatively longer and also tightly connected by the middle lamella; the width of this semi-swollen cell wall was 3-4 μm (Fig. 2 F). The highly visible middle lamella
remained intact even near the compact intercellular space (Fig. 2 G). The fibrillar materials in the cell walls were compact and the bridging substances among the fibers appeared dense (Fig. 2 H).

**Discussion**

This study showed that pre-harvest applications of CS-2H decreased peel puffing to a considerable level, statistically comparable to CS-1B. The qualitative characters of fruits were unaffected by CS-2H. Peel
Fig. 2. Transmission electron micrographs of the subepidermal cells of CS–2H (CaSO₄ ⋅ 2H₂O, CaCl₂) treated non-puffy satsuma mandarin fruits. A: Comparatively lesser and smaller intercellular spaces (IS) in the cell corners. B: Intact cell corners (IC). C: Cell corners having the initial stage of intercellular space (arrowheads). D: Cell corners with small intercellular space (arrowhead). E: The fibrillar arrangement in longitudinal direction with the cell wall (CW) and the presence of distinct middle lamella (ML). F: Longer connection of middle lamella (ML) between two intercellular spaces (IS). G: Existence of compact middle lamella (ML) near the intercellular space. H: Compact layered cellulose microfibrils in the cell walls (arrowheads); middle lamella (ML) near an intercellular space. (A, X1000; G, X3000; B, C, D, E, F, H, X6000.)

Puffing usually decreases the specific gravity of early maturing fruits, such as Miyagawa Wase and Okitsu Wase even at harvest. In ‘Aoshima’, a late maturing cultivar, the specific gravity is influenced minimally by puffiness during harvest because the puffing index is low. As the magnitude of puffiness increases with storage, its specific gravity decreases. Thus our data collected immediately after harvest of fruit, reveal a low puffing index.

During 1996, peel puffing was comparatively less, whereas the specific gravity remained the same in all treatments. In 1997, specific gravity decreased along with higher rates of peel puffing but even these decreases were not statistically significant. The specific
gravity of satsuma mandarin fruit often depends not only on peel puffing but also on other factors, such as thickness and hardness of peel. The insignificant differences of specific gravity among treatments were presumed to be due to the low magnitude of peel puffing index and the other unknown factors.

CS-1B is known to alleviate peel puffing (Kawase et al., 1981) but its use was discontinued for other demerits, especially the discoloration of the fruit surface by calcium residues while CS-2H has not been studied as an agent against peel puffing. Our data on the efficacy, merits, and demerits of CS-2H indicate that it reduces peel puffing in satsuma mandarin fruit because it is more soluble than CS-1B. This resolved the problem of calcium residues on the fruit surface. Another calcium compound, calcium nitrate effectively controls physiological disorders of the peel in citrus (Zaragoza et al., 1996a, 1996b; Agusti et al., 1997). Many environmental factors, such as elevated temperature and high humidity during fruit ripening enhance puffing (Kawase and Hirai, 1983). This experiment on the efficacy of calcium-containing chemicals on the alleviation of rind puffing was carried out under field conditions. The uncontrolled environmental factors undeniably varied in different years so that the extent of peel puffing differed from year to year in all treatments.

Kuraoka et al. (1975b) reported that the flavedo tissue continues cell division with the enlargement of fruit, whereas the albedo cells go under the cessation of cell division earlier. The difference in the rates of cell division in the albedo and flavedo stresses the junctions between the two tissues. In addition, enzymatic activities in the peel region i.e., the cellulase and pectinase activities remain high during fruit development. These enzymatic activities decrease at different rates in the two tissues with the ripening of fruit; their differences become negligible near harvest. The asynchrony in the developmental speed between the albedo and flavedo produces peel puffing (Kuraoka et al., 1975b). Separation of the subepidermal cells, which decreases the contact area among cells to form large intercellular spaces in the cell corners and schizogenous intercellular spaces between adjacent walls was accompanied by the disorientation of cell wall components. This anomaly is attributed to the tendency of cells to become spherical during the cell enlargement stage.

During fruit softening in pear the changes in the middle lamella and the walls were anticipated as the combined actions of pectolytic enzymes and cellulase (Ben-Arie et al., 1979). Kuraoka et al. (1975b) stated that the differences in the developmental speed of albedo and flavedo cells produce peel puffing. Therefore, the mechanism of peel puffing may be the consequence of a decrease in enzymatic activities and the osmotic pressure gradient brought on by cell enlargement. Cassab and Varner (1988) reported that cell walls are modified by enzymatic actions during growth, development, and environmental stress. However, we found the major cause of cellular detachment to be dissolution of the middle lamella, the ultrastructural and histological bases for puffing. Kuraoka et al. (1975a) found maximum pectinaceous fractions during the early developmental stage of fruit and a subsequent gradual decrease towards harvest. These pectic substances have been reported to be the main component of the middle lamella (Albersheim et al., 1960). Changes in the cell wall structure during normal ripening have also been observed under the electron microscope in many fruit, including avocado (Pesis et al., 1978), pear (Ben-Arie et al., 1979), and tomato (Crookes and Grierson, 1983). These changes were associated with the normal ripening process of fruits. However, changes in this study were associated with the physical disorder known as peel or rind puffing. Different trends of destruction in the middle lamella, cell walls, and distinct variations in their fibrillar arrangement implicate a ultrastructural modification in the cell walls caused by fruit puffing. Thus, the present study reveal that peel puffing is not only associated with the changes in the albedo cells but also with considerable changes in the subepidermal cells.

The loosening and swelling of cell walls, distinct separation of fibrillar materials and dissolution of the middle lamella in the subepidermal cells were reduced by the application of calcium compounds in our study. The cell walls of the albedo in non-puffy fruit usually contain more calcium than those in puffy fruit peel (Kuraoka et al., 1975a). Chamel (1989) found that exogenously applied calcium penetrates into fruit directly through the cuticle. Kawase et al. (1981) postulated that the controlling mechanism of calcium is physical rather than physiological by keeping the stoma open to facilitate transpiration. Our data reveal that CS-2H and CS-1B are inhibitors of peel puffing but that they offer no explanation on their mode of actions. Thus, the question as to whether or not Ca enters the peels and its action is physical still remains unanswered. To derive this information, the peel can be studied under the electron microscope utilizing the specific fixation method as performed by Peng and Iwahori (1995).

In conclusion, the application of different growth regulators and even the other calcium compounds used earlier to control peel puffing had some drawbacks in economic constraints. Whereas, peel puffing was not be completely controlled by CS-2H, the reagent might be useful to control peel puffing to an economical level without causing any deleterious effects on fruit characteristics. This alleviating mechanism is associated with the inhibition in the separation of the middle lamella, and disorganization and separation of cell walls.

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**Literature Cited**


ウンシュウミカンの浮皮発生に及ぼすカルシウム混合剤の効果と同剤処理果実の表皮下細胞壁における微細構造の特性

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摘 要

浮皮発生に及ぼすカルシウム混合剤の軽減効果について、ウンシュウミカンを供試して調査した。浮皮発生果における表皮下細胞壁の微細構造領域における変性とカルシウム剤による浮皮発生抑制について透過型電子顕微鏡を使用して詳細に観察した。

浮皮果は処理や無処理区においても発生したが、CS-2H(CaSO4・2H2O, CaCl2 混合剤)やCS-1B(CaCO3, その他混合剤)の収穫前散布は浮皮発生を明らかに減少させ、両剤ともに果実品質には影響しなかった。無処理区において発生した浮皮果の表皮下組織では、かなり大型の細胞間隙が細胞間隔に、そして離生細胞間隙が隣接細胞壁に発生した。ときおりこれらの間隙のみられない細胞間隔や細胞壁が存在するが、これらの部位では細胞中層の崩壊が明確に認められた。細胞壁の崩壊は細胞間結合の緩みや細胞壁の膨潤を引き起こした。さらに細胞壁内の局所的な破壊が原形質膜に近い壁内でも観察された。膨潤した細胞壁内の繊維状の物質は原形質膜に近い部位では細胞壁に沿って引き離され、細胞中層に近い部位では突出するように引き離されていた。ごくわずかではあるが、細胞中層や中層には近い細胞壁内の繊維状物質は消失していた。

対照として、CS-2H カルシウム混合剤を葉面散布した区では浮皮果において認められたような微細構造の変性は全く見られなかった。とくに大型の細胞間隔は観察されず、小型であった。さらに離生細胞間隔は発生せず、細胞中層の溶解もかなり抑制されていた。細胞角隅は細胞中層で観察されており、時として小型の細胞間隔が認められた。細胞壁内の繊維状物質は整然と配列し、壁崩壊は観察されなかった。ときどき細胞壁の膨潤が見られたが、浮皮果にくらべると少なく、細胞分離も認められなかった。