Relationship of Bound Calcium and Inoculum Concentration to the Effect of Postharvest Calcium Treatment on Decay of Apples by *Penicillium expansum*

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ABSTRACT

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Golden Delicious apples were pressure-infiltrated (68.95 kPa) at harvest with 0, 1, 2, or 4% solutions of $CaCl_2$ and stored at 0 C. After 6 mo, the fruits were removed from storage and wound-inoculated with a conidial suspension of 10^4, 10^5, or 10^6 spores of *Penicillium expansum* per milliliter. After 7 days at 20 C, the fruits were rated for decay severity. The cell walls of similarly treated but uninoculated fruits were extracted and analyzed for Ca concentration. As the Ca concentration of the solutions with which the fruits were infiltrated increased, the amount of cell wall-bound Ca also increased. As the cell wall Ca content increased and the inoculum concentration decreased, the amount of decay decreased. These results indicate that as the inoculum concentration decreases the relative effectiveness of increased cell wall-bound Ca in reducing decay increases.

Although most efforts in treating apples with calcium (Ca) salts have been designed to reduce losses resulting from physiological disorders, postharvest treatments with Ca solutions also have been found to reduce postharvest losses attributed to decay (15). More recent investigations have shown that when the Ca content of apples was increased by postharvest infiltration of Ca solutions, postharvest decay caused by *Penicillium expansum* Link ex Thom was reduced (5,6). The Ca concentrations in that work (5,6) represented total Ca taken up by the fruit and did not differentiate between Ca bound in the cell walls and that found free in the intercellular spaces and less involved in decay reduction. There is disagreement on the fate of Ca exogenously applied to apples. Results of one investigation indicate that localization of native Ca and that originating from postharvest treatment were the same (17), whereas another study using apple tissue disks concluded that externally applied Ca probably was located in the cellular free space and not involved with maintaining cell structure and function (9).

Inoculum concentration, too, may affect the amount of decay reduction in apples resulting from increased tissue Ca through postharvest treatment. The use of dump tanks to unload bulk boxes of apples provides a major source of inoculum causing storage decay (3). The concentration of *Penicillium* spores in dump tanks was determined by several studies to be 10^4 or fewer spores per milliliter of water (3,16). The inoculum concentration used in previous studies (5,6) was 10^6 spores per milliliter or 100 to 1,000 times greater than the fruits would encounter during the handling process. Because of the inconsistencies noted, the objectives of this investigation were to determine whether exogenously applied Ca became bound to the cell wall and if inoculum concentration influenced the affect of Ca on decay.

MATERIALS AND METHODS

Cultivar Golden Delicious apples (*Malus domestica* Borkh.) were harvested from a commercial orchard and randomized. They were then infiltrated under 68.95 kPa of pressure for 2 min with laboratory-grade USP $CaCl_2$ (76%) in either 0, 1, 2, or 4% solutions in distilled water. After treatment, the fruits were placed on Kraft paper and allowed to drain for 2 hr before storage (0 C). After 6 mo, the length of time this cultivar might typically be kept in cold storage, the fruits were removed from storage and wound-inoculated with *P. expansum* as previously described (5), with one modification. Rather than using a single-spore concentration, fruits were inoculated with either 10^4, 10^5, or 10^6 spores per milliliter. They were then rated for decay as previously described (5). Twenty fruits were used for each treatment. Percent reduction in decay was calculated by comparing the area of decay of the 0% $CaCl_2$-treated fruits with that of the 1, 2, or 4% $CaCl_2$-treated apples within each inoculum concentration, then determining how much less decay occurred as a result of the various $CaCl_2$ treatments.

Ca content of the apple cell walls from similarly treated but uninoculated fruit was determined after removing the peel and outer flesh of the entire fruit to a depth of 2 mm with a mechanical peeler. The next 3 mm of flesh was then removed similarly, immediately frozen in liquid nitrogen, and lyophilized. This layer was used for Ca analysis because this was the depth to which the apples were punctured for inoculation. Cell walls were extracted according to a modification of a procedure previously described (11). Lyophilized apple tissue was homogenized in 80% ethanol for 1 min with a polytron homogenizer (Brinkmann Instruments). After filtering the homogenate through Miracloth (Calbiochem), the residue was suspended in 20 mM HEPES-NaOH (pH 6.9), mixed well, and filtered twice through Miracloth to remove the

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ethanol. The resulting residue was then suspended in a solution of 20 mM HEPES-NaOH (pH 6.9) plus α-amylase (1 mg/ml of solution), one drop of toluene was added, and the mixture was held at 37 C for 18–24 hr with constant shaking. The solution was once again filtered through Miracloth and the residue washed with 20 mM HEPES-NaOH (pH 6.9). The residue was suspended in phenol/acetic acid/H_2O (2:1:1, w/v/v) for 10 min with occasional stirring to inactivate endogenous wall-associated enzymes (12,14). This suspension was filtered through a sintered glass filter and the residue suspended in chloroform-methanol (1:1, v/v) for at least 10 min. During this period, the suspension was homogenized gently for 30 sec with a polytron homogenizer and then filtered through a sintered glass filter. The residue then was washed with chloroform-methanol and suspended in 400 ml of acetone for 10 min, filtered, and washed with excess acetone. Cell wall material was dried over P_2O_5 in vacuo at 25–37 C to a constant dry weight. Dried cell walls (0.125 ± 0.005 g) were ashed at 500 C overnight and the residue dissolved in 5 ml of 2 N HCl and analyzed for Ca content with an Instrumentation Laboratory spectrophotometer. All Ca values are reported on a dry-weight basis. Three samples, each consisting of five fruits, were analyzed for each treatment. Both pathogenicity and cell wall Ca experiments were repeated a second time.

RESULTS

The area of decay resulting from inoculations of the various spore concentrations and its relationship to cell wall Ca concentration is shown in Figure 1. As cell wall Ca content increased and inoculum concentration decreased, the resulting percent reduction in area of decay increased (Fig. 2). Fruits treated with a 1% solution of $CaCl_2$ and inoculated with 10^6, 10^5, or 10^4 spores per milliliter had 10, 12, and 12% less decay than the respective untreated fruits. However, those fruits infiltrated with a 4% solution of $CaCl_2$ and inoculated with the same spore concentrations had 28, 37, and 52% less decay than the respective untreated fruits. Initially, a spore concentration of 10^2/ml was used for inoculation but resulted in a much lower percentage of successful infections at the wound sites. To successfully compare the effects of the $CaCl_2$ treatments, it was necessary to determine the area of decay at the inoculation site. If infection does not occur, the area of decay cannot be calculated and comparisons cannot be made. The concentration of 10^4 spores per milliliter was the lowest one used that resulted in 100% successful infection.

The Ca content of the extracted cell wall preparations (Table 1) is assumed to be mainly that bound to the cell wall because the extensive extraction procedure would remove intercellular and extracellular Ca. The Ca concentration of the untreated control, representing the endogenous cell wall Ca, was about 550 μg/g and increased to 1,900 μg/g in fruits treated with a 4% $CaCl_2$ solution, showing an increase in Ca associated with the cell wall. As the amount of Ca in the solution increased, the percent increase in the cell wall decreased.

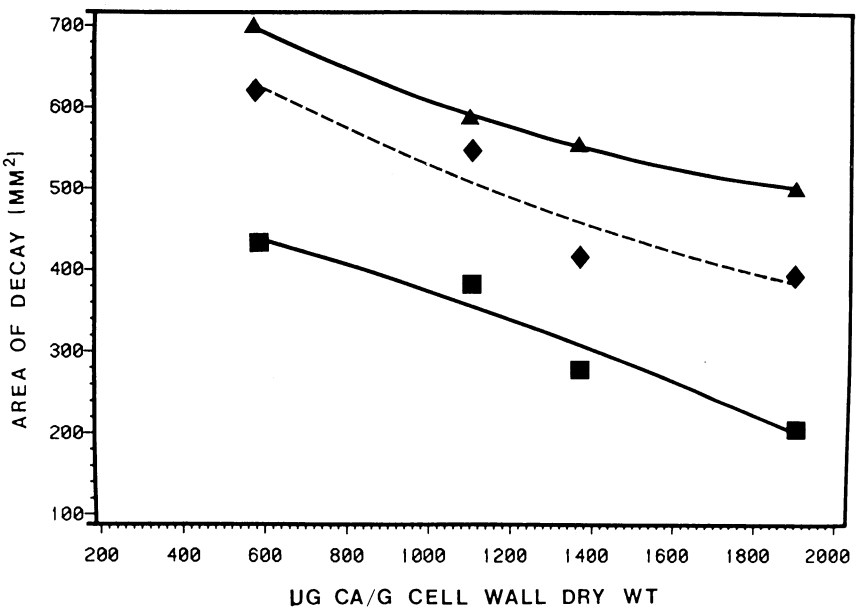

Fig. 1. Relationship between area of decay and calcium concentration of apple fruit cell walls at different inoculum concentration. Regression curves indicate fruit inoculated with a conidial suspension of 1×10^4 spores per milliliter (■) ($y = 511.8100 - 0.1100X - 0.000028X^2$, $r^2 = 0.96$), 1×10^5 spores per milliliter (◆) ($y = 795.2900 - 0.32X + 0.000055X^2$, $r^2 = 0.92$), or 1×10^6 spores per milliliter (▲) ($y = 854.6100 - 0.31X + 0.0000667X^2$, $r^2 = 0.99$). Regressions for all three equations were significant at $P = 0.05$. Data points indicate the concentration of $CaCl_2$ solutions (0, 1, 2, or 4%) from left to right, respectively.

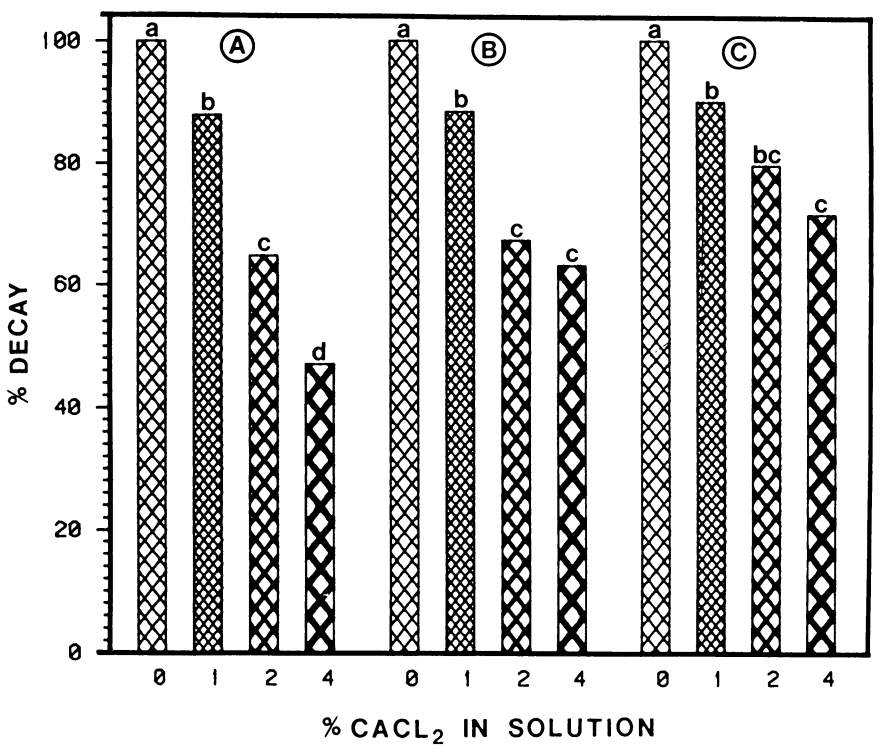

Fig. 2. Relationship between area of decay and concentration of calcium chloride ($CaCl_2$) solutions with which apples were treated when inoculum concentrations differed. Inoculum concentrations included **(A)** 1×10^4, **(B)** 1×10^5, or **(C)** 1×10^6 spores per milliliter. Mean separation by Duncan's multiple range test ($P = 0.05$).

Table 1. Cell wall calcium concentration of Golden Delicious apples pressure-infiltrated (68.95 kPa) with calcium chloride ($CaCl_2$) solutions at harvest[a]

Treatment (% $CaCl_2$)	Cell wall calcium content (μg Ca/g dry wt)
0	554 ± 37[b]
1	1,108 ± 42
2	1,375 ± 91
4	1,909 ± 95

[a]Samples were taken after 6 mo of storage at 0 C.
[b]Numbers represent the mean ± SE.

DISCUSSION

This study showed that as the spore concentration of the inoculum decreases, the relative effectiveness of increased Ca in the cell wall in reducing decay is increased. Earlier work (1) had shown that polygalacturonase (EC 3.2.1.15), produced by many pathogens including *P. expansum* (4), does not readily hydrolyze Ca pectate, so tissue maceration by this enzyme is inhibited by increased Ca content of tissue. Another study (2) suggests that the resistance conferred by Ca pectate can be altered by markedly increasing the inoculum concentration of the pathogen, indicating that tissue resistant to maceration by a low level of polygalacturonase may be macerated by increased concentration of the enzyme. The results of our study support this conclusion. As the concentration of the inoculum increased, resulting in increased production of polygalacturonase, resistance to maceration decreased. Because studies have shown that the spore concentration of apple dump tanks does not usually exceed about 10^4/ml (3,16), more realistic results may have been obtained in earlier studies (5,6) had a lower concentration of inoculum been used. The decrease in area of decay related to an increase in Ca concentration of apple tissue would have been even more pronounced in the earlier studies had inoculum concentration been decreased to a more realistic level.

The relationship between Ca ions and the cell wall has been shown to play a key role in disease resistance. The importance of Ca in retarding decay is thought to be the result of the tight binding of Ca ions in the cell wall (8). Few pectins, if any, are free of neutral sugars, notably rhamnose, and are composed of polygalacturonic acid residues in a chain with rhamnose interspersed in the chain (13). The rhamnose insertion causes a marked kink in the chain. The resulting bunched configuration of the polygalacturonic chain allows spaces for the insertion of a series of cations, all of which may be filled because the binding of one ion causes chain alignment that facilitates binding of the next (10). When ion exchanges are performed in cell walls, either between Ca and monovalent cations or between Ca and Mg, the walls have always shown a marked preference for Ca (7,8). The formation of cation cross-bridges between uronic acids may make the cell wall less accessible to enzymes occurring in the fruit that cause softening or to cell wall-degrading enzymes produced by fungal pathogens. The exact location of Ca binding sites in cell walls has not been determined definitely and would require investigations using an electron microprobe or radioautography; however, Ca is assumed to be bound to the polygalacturonate in the cell wall. This study showed that as the Ca concentration of the solution with which the fruits are infiltrated is increased, there is not a proportional increase in cell wall-associated Ca, probably because there are only a finite number of Ca binding sites in the polygalacturonate. Increasing the $CaCl_2$ concentration over 4% would not increase the amount of Ca bound to the cell wall proportionately and may result in injury to the fruit surface (6).

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