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Botrytis cinerea Decay in Apples Is Inhibited by Postharvest Heat and Calcium Treatments

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Abstract. 'Golden Delicious' apples (*Malus domestica* Borkh.) were treated after harvest with heat (air at 38 °C for 4 days or 42 °C for 1 day) or 2% $CaCl_2$ (w/v; applied as a dip or pressure-infiltrated) or a combination of the two and stored at 0 °C for ≤6 months. Decay caused by *Botrytis cinerea* Pers.:Fr. after inoculation to a depth of 2 mm with a conidial suspension virtually was eliminated in stored fruit heated at 38 °C, regardless of Ca treatment. Apples punctured to a depth of 0.5 mm (but not 2 mm) and inoculated with *B. cinerea* on removal from storage were almost completely protected from poststorage decay if they had previously been pressure-infiltrated with 2% $CaCl_2$, regardless of the heat regime. Heating fruit at 42 °C and dipping in 2% $CaCl_2$ were only partially effective in preventing decay from either pre- or poststorage inoculations. Fruit firmness was not related to resistance to decay.

Consumers increasingly demand that high-quality agricultural commodities be produced by means other than using techniques or chemicals regarded as unsafe. Holding apple or tomato fruit at 38 °C for 4 d or at 42 °C for 1 d after harvest maintains fruit firmness and promotes resistance to physiological and pathological disorders during storage (Conway et al., 1994; Fallik et al., 1993a, 1993b, 1995; Klein and Lurie, 1994). These benefits are enhanced when heated apples subsequently are dipped in $CaCl_2$ solutions (Klein and Lurie, 1994; Lurie and Klein, 1992). However, pressure infiltration with 2% to 4% $CaCl_2$ protects apples from pathogens regardless of heat treatment (Conway et al., 1994; Sams et al., 1993).

Previous research on the prophylactic effect of treatments that use heat, calcium, or both against pathogenic infection in apples involved inoculating fruit after treatment and subsequent storage (Conway and Sams, 1983; Conway et al., 1992). However, most postharvest pathogens are present on the fruit surface at harvest but cause decay only when the tissue is wounded or softens sufficiently in storage to permit pathogen penetration and infection. Using heat (dry, wet, or vapor) for nonchemical disinfestation has been reviewed by Couey (1989). In our study, we compared combinations of extended (4 d at 38 °C) and brief (1 d at 42 °C) exposures to heat and two Ca application techniques (infiltration and dipping) as protective means against storage decay by *Botrytis cinerea* in apples. Some fruit were inoculated before treatment to determine the direct effect of treatments on the pathogen. Other fruit were treated before storage but were inoculated only on removal to determine residual effects of the treatments in promoting resistance to decay-causing organisms.

Materials and methods

Fruit. 'Golden Delicious' apples were harvested in the preclimacteric stage (ethylene content was <0.1 $\mu l \cdot g^{-1}$ and the climacteric rise in CO_2 production had not yet begun) from a commercial orchard in southern Pennsylvania that previously was determined to have a very low incidence of natural infections (data not shown). The apples were divided randomly into eight lots of 270 fruit each.

Disease resistance. Apples in two of the lots were wounded on two opposite sides to a depth of 2 mm by pressing them down on the head of a nail 2 mm in diameter and subsequently were inoculated, treated as described, and stored at 0 °C for 2 or 4 months. Of the remaining lots, three were treated, stored for 2, 4, or 6 months at 0 °C, and inoculated on removal. Fruit in these lots were wounded on opposite sides to a depth of 2 mm by pressing them down on the head of a nail 2 mm in diameter and on the two remaining orthogonal sides to a depth of 0.5 mm by pressing them on the blunted head of a pushpin 1 mm in diameter. All fruit were inoculated by immersion for 15 s in a conidial suspension (10^5 spores/mL) of a quite virulent isolate of *B. cinerea* (ATCC 90870; The American Type Culture Collection, Rockville, Md.) that we have used previously (Conway et al., 1994). The area of decay was calculated from the means of the width and length of lesions after an additional 5 to 7 d at 20 °C. Whether the heat treatment inhibited fungal growth or killed the spores outright was determined by removing 2-mm-thick samples of peel and flesh from the wound inoculation sites and placing them on 15% potato dextrose agar (PDA, Difco, Detroit) at 20 °C for 12 d.

Fruit treatments. Apples (≈120) were placed in tray-packed boxes with perforated polyethylene bags as liners before being heated in thermostatically controlled (±1 °C) walk-in chambers equipped with air circulation. Relative humidity was >95% in the

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boxes, and fruit attained the desired temperature within 3 h. A 20-L, jacketed, stainless steel pressure vessel was used to infiltrate fruit with 2% $CaCl_2$. The treatments were 1) control (no treatment), 2) hold at 38 °C for 4 d, 3) hold at 42 °C for 1 d, 4) dip in 2% $CaCl_2$ at 20 °C for 2 min, 5) hold at 38 °C for 4 d before a dip in 2% $CaCl_2$, 6) hold at 42 °C for 1 d before a dip in 2% $CaCl_2$, 7) pressure infiltration (3 min, 103 kPa, 20 °C) with a 2% solution of $CaCl_2$, 8) hold at 38 °C for 4 d before pressure infiltration with 2% $CaCl_2$, 9) hold at 42 °C for 1 d before pressure infiltration with 2% $CaCl_2$. Heated fruit were treated with Ca within 2 h of removal from heating chambers. Inoculated apples were treated and stored separately from uninoculated apples. All fruit were stored at 0 °C within 2 h of treatment.

FIRMNESS MEASUREMENTS. Three lots of 270 apples each were treated as previously described without being inoculated on removal from storage. Fruit were held at 20 °C for 7 d before firmness measurement with an EPT-1 electronic pressure tester (Lake City Technical Products, Kelowna, B.C., Canada) set in the Magness-Taylor (MT) mode and interfaced to a personal computer. Firmness (bioyield force) was measured at two opposite points on the equator of each fruit after removal of a 2-mm slice with a fixed blade slicer.

TOTAL CALCIUM CONTENT. Fruit from the firmness measurements were used for this part of the study. A 2-mm layer of the peel and outer flesh was removed from the apples with a mechanical peeler, immediately frozen in liquid N, freeze dried, and ground. The next 2 mm of cortical tissue was cut with the peeler and treated similarly to the peel. Dried material (1 g) was ashed, dissolved in 5 mL of 2N hydrochloric acid, and filtered. The samples then were analyzed for Ca concentration by inductively coupled plasma emission spectrometry.

CELL WALL BOUND CALCIUM. A 2-mm layer of cortical tissue was removed as for total Ca analysis. About 50 g of tissue was homogenized in 100 mL of cold 80% ethanol in a Waring blender and filtered through two layers of Miracloth (Calbiochem, La Jolla, Calif.). The residue was washed with 50 mL of 80% ethanol and was used for cell wall extraction following a modification of the procedure of Tong and Gross (1990). The residue then was extracted in 100 mL of 80% ethanol at 100 °C for 5 min, rinsed in water, suspended in 200 mL 20 mM Hepes, pH 7.0, homogenized (Polytron; Brinkmann Instruments, Westbury, N.Y.) for 1 min, and filtered through Miracloth at each stage. The residue was suspended in 100 mL of Hepes buffer in a pressure bomb (Cell Disruption Bomb, Parr Instrument Co., Moline, Ill.) at 13.8 MPa for 10 min and then was stirred for 60 min with 100 mL phenol/Tris buffer at pH 7.5 (Huber, 1991), 10 min with 200 mL chloroform : methanol (1:1, v/v), and 10 min with acetone with filtration through sintered glass at each stage. The wall material was air-dried overnight and dried in vacuo over P_2O_5 at 40 °C for 48 h. Cell wall Ca then was determined using the same procedures as for total Ca content.

STATISTICS. Fruit firmness and disease severity and incidence were measured in each of three replicate samples of 10 apples for each treatment, at each removal time. Calcium analyses were performed on three replicate samples per treatment, each consisting of tissue from five fruit. Mean separations were derived from an analysis of variance of the data using a completely randomized design.

Table 1. Area and incidence of decay caused by *Botrytis cinerea* on 'Golden Delicious' apples inoculated to 2-mm depth before indicated treatment. Fruit were stored for 2 months at 0 °C after treatment and were removed to 20 °C for 5 d before rating for decay.

Treatment	Decay area (mm^2)	Incidence of decay (%)
No Ca		
Unheated	>2200	100
38 °C/4 d	0.1	1
42 °C/1 d	>2200	100
Dip in 2% $CaCl_2$		
Unheated	>2200	100
38 °C/4 d	0.2	3
42 °C/1 d	>2200	100
Infiltrate with 2% $CaCl_2$		
Unheated	1440	91
38 °C/4 d	19	48
42 °C/1 d	1763	96
$LSD_{0.05}$	1.3	5.2

Results

After 2 months at 0 °C plus 5 d at 20 °C, decay lesions on apples that had been inoculated and then held at 38 °C for 4 d before storage were extremely small (Table 1). In comparison, decay was so extensive in nontreated fruit and in fruit held at 42 °C for 1 d that it was not possible to measure accurately the extent of individual lesions. The maximum decay area that could be measured accurately was ≈2200 mm^2. Dipping in 2% $CaCl_2$ neither decreased the extent or incidence of decay of unheated or 42 °C fruit nor further reduced the very small amount of decay found in apples heated 4 d at 38 °C. Fruit that had been pressure-infiltrated with Ca but not exposed to 38 °C after inoculation had a moderately broad (≈4 cm in diameter) area of decay. Heated apples that subsequently were infiltrated with Ca had a high incidence of lenticel burn (data not shown), but this burn was not related to severity of decay. Incidence of decay (development of distinct lesions) was <90% only in fruit exposed to 38 °C for 4 d. In these fruit, decay incidence was significantly greater in pressure-infiltrated apples, although still lower than that in fruit not exposed to 38 °C. After 4 months of storage, the area of decay in apples inoculated before treatment was >2200 mm^2 in all fruit except that no decay occurred in fruit that subsequently had been exposed to 38 °C for 4 d (data not shown). There was no regrowth of *B. cinerea* in plugs taken from inoculation sites on these heat-treated apples and incubated on PDA.

The area of decay in apples inoculated on removal from storage was similar within each treatment at different inspection times. Similar results were obtained with fruit firmness, with the exception of controls. Therefore, data from apples examined after 4 months of storage are presented as representative of results from each of the removal times. Decay severity depended on the depth of inoculation and the prestorage treatment. The area of decay resulting from a shallow (0.5 mm below fruit surface) inoculation was reduced 75% to 90% by heat or Ca dips, alone or in combination (Table 2). However, incidence of decay was reduced only an average of 48% by these treatments. In contrast, infiltrating with Ca, regardless of heating regime, reduced decay area by >99% and reduced decay incidence by ≈90%. Plugs of apple tissue from inoculation sites, when placed on PDA, produced similar amounts of fungal growth, regardless of treatment (data not shown).

Decay area was >5-fold greater in controls inoculated to a 2-mm depth compared with those receiving the shallower 0.5-mm inoculation. Calcium dips and heating fruit at 42 °C for 1 d, alone or in combination, did not significantly reduce the decay area on deeply inoculated fruit. On apples heated at 38 °C for 4 d, decay area was

≈15% smaller compared to controls, but by far the greatest reduction in decay (80%, on average) occurred in Ca-infiltrated fruit, regardless of the heat regime. The deeper poststorage inoculation almost always resulted in 100% decay incidence, with the only reduction, albeit minor, coming from the combination of Ca infiltration and heat treatments.

The firmest apples were those held at 38 °C or infiltrated with Ca (Table 2). Fruit held at 42 °C were less firm than those held at 38 °C before storage, although they were firmer than nontreated controls. Synergistic effects of heating and Ca treatments in firmness maintenance in storage were evident only in the case of fruit infiltrated with Ca after heating at 38 °C.

Table 2. Flesh firmness, area of decay, and incidence of decay in 'Golden Delicious' apples wound-inoculated with *Botrytis cinerea* after 4 months storage at 0 °C and 7 d at 20 °C.

		Decay area (mm^2)		Decay incidence (%)	
		Inoculation depth (mm)		Inoculation depth (mm)	
Treatment	Firmness (N)	0.5	2	0.5	2
No Ca					
Unheated	50	129	703	65	100
38 °C/4 d	73	12	580	30	100
42 °C/1 d	58	33	684	38	100
Dip in 2% $CaCl_2$					
Unheated	55	35	616	38	100
38 °C/4 d	74	21	595	38	100
42 °C/1 d	58	13	626	32	100
Infiltrate with 2 %$CaCl_2$					
Unheated	77	0.5	117	8	98
38 °C/4 d	82	0.6	149	5	88
42 °C/1 d	70	1.4	184	8	95
$LSD_{0.05}$	5.7	1.2	94	16.5	3.7

Calcium concentration in the peel, flesh, or cell wall did not change as a result of heat treatment alone or 2% $CaCl_2$ dips (Table 3). Infiltrating with 2% $CaCl_2$ increased the Ca concentration up to 5-fold in the tissues analyzed, although the increase was significantly less in fruit heated at 38 °C before Ca infiltration.

Discussion

Although Ca and heat treatments in combination previously were shown to have synergistic effects in maintaining apple quality during storage, the added benefits did not include improved phytosanitation (Lurie and Klein, 1992). Exposure of fruit to temperatures >38 °C for <4 d also maintained firmness and prevented scald (Klein and Lurie, 1994) but did not significantly limit decay. This study also demonstrates that infections resulting from prestorage inoculations of apples with *B. cinerea* were controlled only by a subsequent 4-d exposure to 38 °C and that neither holding apples at 42 °C for 1 d nor Ca treatment were effective in preventing decay during storage. Similar heat treatments were effective against *Penicillium expansum* in apples (Fallik et al., 1995).

Combining Ca infiltration with the 38 °C heat treatment greatly increased the area and the incidence of decay compared to 4 d at 38 °C alone or in combination with a 2% $CaCl_2$ dip (which was ineffective without heating). Holding apples at 38 °C for 4 d evidently did not kill all the spores in the inoculum outright, and pressure-infiltration with $CaCl_2$ solution apparently carried some *B. cinerea* spores further into the wound beyond the "zone of protection" afforded by the heat treatment. An increase in decay as a result of pressure-infiltration of pome fruit with $CaCl_2$ solutions has been noted earlier, particularly if the infiltration solution is contaminated with fungal spores that normally infest the fruit surface (Sholberg et al., 1989).

The minimal decay noted in non-infiltrated heated fruit may have resulted from transferring the fruit directly to 0 °C storage. Any surviving heat-weakened spores then would be subjected to a cold shock, and the combined exposure to temperature extremes ultimately would kill all the spores. This may be why *B. cinerea* could not be reisolated from tissues inoculated before heating, even when the inoculation was deep (2 mm) and the excised tissues were placed on an optimal medium for growth. The shallow (0.5 mm) inoculation with *B. cinerea* administered to fruit in the poststorage decay portion of this study typified the stem puncture or scratch wound that we frequently have observed in packinghouses. All of the prestorage treatments tried were at least moderately effective in reducing decay at these shallow infection sites. Calcium infiltration, regardless of heating, provided the best protection and also was the only treatment that provided some protection against decay at the deeper (2 mm) poststorage inoculation sites. The residual added resistance to decay conferred by postharvest Ca treatment, heating, or both would be practically advantageous in cases where not all spores present from the field inoculation were inhibited by heat treatment.

Residual resistance to decay was at best loosely linked with the maintenance of fruit firmness. Although heating fruit at 38 °C or infiltrating with 2% $CaCl_2$ resulted in similar firmness readings, decay area was much less in shallow and deep wounds, and decay incidence was markedly decreased in shallow wounds of Ca-infiltrated apples compared with heat-treated (38 °C) fruit. Similarly, apples stored in low (1%) O_2 or infiltrated with $CaCl_2$ at harvest were equally firm after 6 months in 0 °C storage, but Ca-treated fruit resisted decay better than those held in low O_2 (Conway and Sams, 1984).

Although firmness, per se, may play a role in resistance to fungal decay, the chemical composition of the cell wall, which may also determine firmness, is likely of greater importance (Stermer and Hammerschmidt, 1987). The primary zone of enhanced resistance appeared to be within a few cell layers of the epidermis, since the residual effect of heating was much more profound in inoculation sites that were 0.5 mm rather than 2 mm deep, although cells at both levels surely received the same amount of heating. Hypodermal cells may produce antifungal compounds in response to heating or other stresses (Fallik et al., 1995), and these materials may interact with Ca.

Retention of fruit firmness resulting from heat treatment was not influenced by Ca concentration, either overall or specifically in the cortical cell wall, as Lurie and Klein (1992) have shown. Ca concentration did influence resistance to decay from poststorage inoculation, however, as shown by the residual effect of Ca infiltration, which greatly increased the amount of Ca in all tissues examined. Holding apples at 38 °C before infiltration substantially decreased the amount of Ca taken up, as Sams et al. (1993) also

Table 3. Calcium concentration in epidermal and cortical tissues and in cortical cell walls from 'Golden Delicious' apples treated as indicated.

Treatment	Calcium concn ($\mu g \cdot g^{-1}$, dry mass basis)		
	Epidermis	Cortex	Cell wall
No Ca			
Unheated	610	272	1848
38 °C/4 d	608	244	1857
42 °C/1 d	742	287	1887
Dip in 2% $CaCl_2$			
Unheated	792	286	2017
38 °C/4 d	829	298	1939
42 °C/1 d	871	386	1907
Infiltrate with 2% $CaCl_2$			
Unheated	3365	1554	8492
38 °C/4 d	2626	1119	6132
42 °C/1 d	3146	1509	5762
$LSD_{0.05}$	391	238	558

demonstrated. The decreased uptake of Ca may have been the result of epicuticular wax flowing during heating and sealing surface cracks in the wax through which $CaCl_2$ solutions might otherwise enter the fruit (Roy et al., 1994). Fruit receiving the 38 °C heat treatment before Ca infiltration were firmer and more resistant to decay than apples with higher tissue Ca concentrations, but they also had reddened damaged lenticels typical of Ca injury. Further research on combined applications of heat and Ca should focus on decreasing the Ca concentration of the infiltrating solution so that the synergistic benefits of heating and Ca infiltration can be realized without damage to the treated apples.

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