

## Development of Resistance to Infection by *Botrytis cinerea* and *Penicillium expansum* in Wounds of Mature Apple Fruits

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### ABSTRACT

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Wounds in harvested, mature, preclimacteric Golden Delicious and Granny Smith apples exhibited healing by formation of wall thickenings. Microscopic examination revealed a barrier of cells with wall thickenings extending four to six cell layers or more from the wound. Histochemical tests of cell walls near healed wounds were positive for phenolic substances,

tannins, lignins, and callose after 38 days at 5 C and 14 days at 20 C. Healed wounds were significantly more resistant to conidia of *Botrytis cinerea* and *Penicillium expansum* than freshly inflicted wounds. Wounds became resistant within 4 days at 5 C.

Mechanical injury, caused during harvesting and postharvest handling, is an important locus for infection by many postharvest pathogens of fruits and vegetables. Avoidance of injuries during harvesting and handling is usually considered an important disease-reduction measure. Nevertheless, one commonly observes fruit wounds that do not become infected, although the opportunity for infection appears great. Such a condition would be expected if a healing process intervened, but we are unaware of reports of wound healing in apples or other climacteric fruits at harvest maturity.

Because wound healing in developing apples has largely been associated with wound periderm formation, the lack of wound periderm in fruits wounded after harvest was thought to indicate an absence of wound healing (32,36,37). However, Kahl (23) indicated that three types of healing exist in plant tissues. In one case, the wound reaction is not linked to meristematic activities of cells near the wound; instead, the walls of cells beneath the wound surface become lignified. In another case, potato tubers and many immature fruits develop a wound periderm (3,20). In an intermediate type of wound healing, observed in *Raphanus sativus*, a few cell divisions and the development of callus occur along with lignification. Lignification has been shown to be hastened by various elicitors of ligninlike compound formation such as higher plant cell-wall fragments, fungal spore or mycelial fragments, pectin, polygalacturonic acid, and chitosans (28,33,40).

Regardless of the type of healing in plant tissues, immediately after the fruit is wounded, nuclei of nearby cells usually migrate to the cell wall nearest the wound (6). Usually within 24 hr after injury the volume of the nucleus and nucleolus enlarges considerably. Cells that directly border the wound suberize and soon die (3).

In oranges, a nonclimacteric fruit, increased resistance to infection of injured orange peel has been related to lignification of cells in the injured area (8). The accumulation of phenolic and ligninlike materials in exocarp cell wall thickenings and increased phenylalanine ammonia lyase activity evidently are major factors involved in the resistance of wounds to infection (11-13, 18, 19, 25). The progressive resistance of the peel of lemon to infection by *Geotrichum candidum* Link after injury is associated with the formation of ligninlike compounds in the zone near wounds (4,5).

Lignin accumulation in the peel of tangerines is believed to be a response to the penetration of germinating conidia of *Colletotrichum gloeosporioides* (Penzig) Sacc.; the resistant tangerine peel also contains phenolic and tannin compounds,

particularly in cells as many as four cell layers beneath the appressorium (8-10). Stages of maturity, extent of color changes, and ambient conditions play an important role in wound healing and resistance to infection in citrus fruits (8).

Citrus fruits can be stored on the tree for considerable periods after reaching maturity. Oranges may become highly colored but then regreen under certain climatic conditions. These differences in behavior between citrus and deciduous fruits caused us to doubt that citrus-type wound healing would be found in apples.

The objectives of this study were to determine if wounds in mature apple fruits incite the formation of wall thickenings that may provide a barrier against infection, and if so, whether the wounds become resistant to invasion by *Botrytis cinerea* Pers. ex Fr. and *Penicillium expansum* (Lk.) Thom.

### MATERIALS AND METHODS

**Fruits.** Golden Delicious apples were obtained from the coastal production area of California, and Granny Smith apples were grown in the San Joaquin Valley near Bakersfield. Fruits were brought to the Department of Pomology Postharvest Laboratory without delay and were placed at 0 C for one to several days until the experiments were initiated.

Fruits were washed with a sodium hypochlorite solution (0.5%) and air dried. Penetrometer readings were done, with skin removed at point of penetration, using a 1.1-cm tip. Maturity was further tested by soluble solids analysis using a refractometer and by analysis of starch using an iodine-potassium iodide solution. From the results of these tests, fruits were judged to be preclimacteric when wounded.

Fruits were wounded with an apparatus consisting of a 2-mm-diameter stainless-steel nail, the blunt end of which protruded 2 mm from a wood block. The apparatus was sterilized by dipping it in 80% ethanol and then submersing it in 0.5% sodium hypochlorite. A puncture wound 2 mm deep was produced by pressing the protruding nail against the surface of the fruit at the equatorial region.

An initial test on wound healing was done in 1984 to determine if resistance to *B. cinerea* and *P. expansum* could be demonstrated. Groups of 10 Golden Delicious apples each were left unwounded, were wounded but not inoculated, or were wounded and then inoculated with conidia of *B. cinerea* either immediately or after being held at 5 C for 8 or 16 days following wounding. Fruits were held for 38 days at 5 C after inoculation. Little disease had developed at that time, so the temperature was raised to 20 C for disease development. After 14 days, the number of fruits with

actively developing lesions was recorded. A second test was identical, except that Granny Smith apples were wounded and inoculated with *P. expansum*.

Wounds were inoculated with a syringe that delivered 0.01 ml of spore suspension. Approximately five conidia were provided per wound, numbers were verified by plating 0.01 ml of spore suspension in a series of petri dishes, and developing colonies were counted.

Confirmatory tests were done on fruits from the 1985 harvest season. Wounds were made in groups of 10 fruits each, five replicates for each inoculation time. Fruits were wounded as described above and inoculated. Wounds were inoculated 0, 4, 8, 16, or 24 days after wounding. After inoculations, fruits were stored in apple cartons (49 × 31 × 30 cm) for 56 days at 5 C. During that time, fruits were examined weekly, and fruits with a young active fungal lesion were removed.

**Pathogens.** The fungal pathogens *B. cinerea* and *P. expansum* both had been isolated from apples stored at 0 C in commercial storage. Cultures were maintained on potato-dextrose agar (PDA) slants at about 4 C and transferred to fresh medium at least once per year. Cultures were grown on PDA in 300-ml cotton-stoppered Erlenmeyer flasks. Conidia were harvested from 3-wk-old cultures by aseptically adding sterile distilled water containing one drop of Tween 80 per 100 ml, adding a few sterile glass beads, and gently swirling the flask to dislodge spores. The resulting spore suspension was filtered through sterile cheesecloth into sterile screw-capped centrifuge tubes, which were shaken vigorously to ensure thorough spore wetting. Wetted spores were pelleted by centrifugation at 2,000 rpm in a clinical centrifuge. The liquid, along with conidia that failed to be wetted and were at the surface, was decanted and replaced with fresh Tween 80 solution. Conidia were washed twice by resuspension, centrifugation, and replacement of decanted liquid with fresh Tween solution. The concentrations of the conidial suspensions were determined with the aid of a Bausch and Lomb Spectronic-20 spectrophotometer. Absorbancy at 490 nm was related to a curve previously established with a hemacytometer. Conidial concentrations were confirmed by placing the same volume of suspension as used to inoculate fruits onto PDA in 20 petri dishes and counting developing colonies.

**Histochemistry.** Anatomical and histochemical studies were carried out on fruit from the 1984 harvest. Treatments (10 fruits each) included unwounded control, wounded but uninoculated control, and wounded fruits inoculated immediately or after 8 or 16 days at 5 C. All fruits were stored for 38 days at 5 C followed by 14 days at 20 C. Tissues were killed, fixed in FAA (Formalin 10:50% ethanol 85:acetic acid 5), and vacuum-infiltrated (17,21,22). The material was later subjected to a tertiary butyl alcohol dehydration schedule (21), embedded in Paraplast Plus, and sectioned at a thickness of 10–15 μm with a rotary microtome. Histochemical tests were made only with sections cut from embedded material. Tests for phenolic compounds were made with Gibb's (2,6-dichloro-*p*-benzoquinone-4-chlorimine) reagent (26,32), fast red salt B (diazotized 5-nitro-2-aminoanisole) reagent (32,39), and ferric ferricyanide reaction (24). The nitroso test (30) and the ferric sulfate reaction (31) were employed for detecting tannins. Lignin was detected by phloroglucinol-HCl (21), chlorine water-sodium sulfite (35), toluidine blue O (27,34), and the periodic acid-Schiff (PAS) reaction (21). PAS reaction also served as a test for total carbohydrates. The lacmoid (35) and aniline blue fluorescence tests (14,21,29) were employed to detect callose. A simple iodine test (21) was used to detect starch. Safranin and fast green and Haidenhain's haematoxylin and fast green (22) were used as general stains to observe cellular details.

## RESULTS

**Susceptibility of wounds to fungal infection.** In exploratory tests following the 1984 harvest, four of 10 Golden Delicious apples developed lesions after *B. cinerea* had been inoculated into freshly inflicted wounds, and the fruits were stored for 38 days at 5 C. After those fruits were stored an additional 14 days at 20 C, nine of 10

were diseased, whereas only two of 10 fruits that had been provided 8 days and one of 10 provided 16 days at 5 C for wound healing developed disease. Thus, when wounds were inoculated immediately after they were inflicted, they were significantly more likely to develop lesions ( $P = 0.001$ ; chi-square analysis) than if they were allowed to heal 8 or 16 days at 5 C before inoculation. Before the experiment was terminated, 19 fruits with healed wounds were reinoculated with 50 conidia per wound. No infections occurred after an additional 10 days at 20 C.

Granny Smith apples were treated similarly to the Golden Delicious, but *P. expansum* conidia were used as inoculum. After 38 days at 5 C and 14 at 20 C, seven of 10 apples that had been inoculated immediately after wounding became diseased. Of fruits allowed to heal for 8 or 16 days before inoculation, four and zero of 10 fruits had active disease lesions, respectively. Fruits not provided with a healing period after wounding were significantly more likely ( $P = 0.01$ ; chi-square analysis) to become diseased.

Confirming tests conducted with Golden Delicious and Granny Smith apples following the 1985 harvest showed that the incidence of active lesions in wounds, regardless of the pathogen, was significantly less when fruits were allowed to heal for 4 days or more at 5 C before inoculation (Table 1). Longer healing periods (8, 16, and 24 days) did not result in the development of significantly fewer lesions.

**Appearance of healed wounds.** Sectioned tissues of Golden Delicious or Granny Smith apples adjacent to wounds showed that cell walls were thickened in a zone near the wound (Fig. 1).

TABLE 1. Suppression of rot in mature apples by delays between wounding and inoculation with conidia of *Botrytis cinerea* or *Penicillium expansum*<sup>x</sup>

Test pathogen No.	Fruits with lesions				
	Days at 5 C between wounding and inoculation				
	0	4	8	16	24
Golden Delicious <sup>y</sup>					
<i>Botrytis cinerea</i>	5.2 a <sup>z</sup>	1.8 b	2.6 b	1.0 b	1.6 b
Granny Smith					
<i>Botrytis cinerea</i>	2.4 a	0.4 b	0.0 b	0.0 b	0.0 b
<i>Penicillium expansum</i>	7.2 a	0.8 b	0.8 b	0.8 b	2.2 b

<sup>x</sup>Data indicate percent of fruit with active fungal lesions, average of five replicates of 10 fruits. Fruits were stored at 5 C for 56 days for disease development following inoculations.

<sup>y</sup>Golden Delicious and Granny Smith apples were stored in air at 0 C for 29 and 31 days, respectively, before the beginning of tests.

<sup>z</sup>Numbers within a row followed by the same letter are not significantly different by Duncan's multiple-range test.  $P = 0.01$  (tests 1 and 3) and 0.05 (test 2).

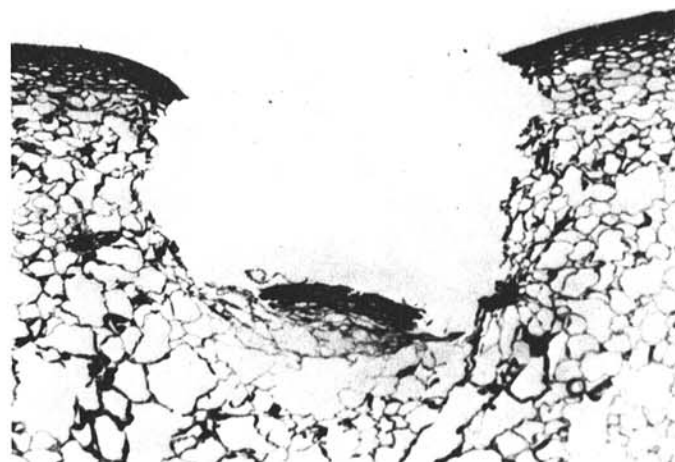


Fig. 1. Section through wound in Granny Smith apple fruit showing the heavily stained (Haidenhain's haematoxylin and fast green) thickened cell walls (×10).

Thickenings were frequently more pronounced in walls on the side of the cell nearest the wound. The zone extended four or five cells, or more, from the wound and extended out from the wound a distance of 10 or more cells, and the zone enclosed the wound from the epidermis extending to below the wound and up to join the epidermis on the far side of the wound.

Mycelia were frequently observed microscopically in wounds that had been inoculated after healing without resulting in lesion development. Invasion of cells ruptured during wounding was observed, but mycelia did not extend into the zone of cells with thickened walls.

**Histochemistry.** Wall thickenings were evident in the cells forming a zone between the wound and unmodified tissues. Histochemical tests used for lignin (phloroglucinol-HCl, Cl<sub>2</sub>-sodium sulfite, toluidine blue O, and PAS) were positive (Table 2).

Tests for phenolic compounds were strongly positive, with ferric ferricyanide staining tissues near wounds a deep Prussian blue. Also providing intense reactions in tissues near healed wounds were Gibb's reagent, which detects phenolic compounds by the formation of gray-blue indophenols, and fast red salt B, which gives a coupling reaction with phenolic compounds to form yellowish-brown products. Only faint or no color was associated with similar tissues near freshly inflicted wounds. Positive reactions for phenolics were found throughout the cytoplasm of

TABLE 2. Histochemical tests of tissues near wounds in Golden Delicious and Granny Smith apples, with and without inoculation with *Botrytis cinerea* conidia, compared with unwounded tissues

Stain	Test <sup>w</sup>	Treatments					
		Golden Delicious			Granny Smith		
		C <sup>x</sup>	WC <sup>y</sup>	INOC <sup>z</sup>	C <sup>x</sup>	WC <sup>y</sup>	INOC <sup>z</sup>
Gibb's reagent	Phenolics	-	+	++	-	+	++
Fast red salt B	Phenolics	-	+	++	-	+	++
Ferric ferricyanide	Phenolics	-	++	++	-	++	++
Nitroso test	Tannins	-	+	++	-	+	++
Ferric sulfate	Tannins	-	+	+	-	+	++
Phloroglucinol + HCl	Lignins	-	+	++	-	+	++
Cl <sub>2</sub> -Sodium sulfite	Lignins	-	+	++	-	+	++
Toluidine blue O	Lignins	-	+	++	-	+	++
Periodic acid-Schiff	Lignins	-	+	++	-	+	++
Lacmoid	Callose	-	++	++	-	++	++
Aniline blue	Callose	-	++	++	-	++	++
I <sub>2</sub> + KI	Starch	-	+	++	-	+	++

<sup>w</sup> Fruits were stored 38 days at 5 C and 14 days at 20 C between wounding and preparation of tissues. Intensity of color reactions: negative -; positive +; intense ++.

<sup>x</sup> Unwounded control.

<sup>y</sup> Wounded but not inoculated.

<sup>z</sup> Inoculated 8 days after wounding.

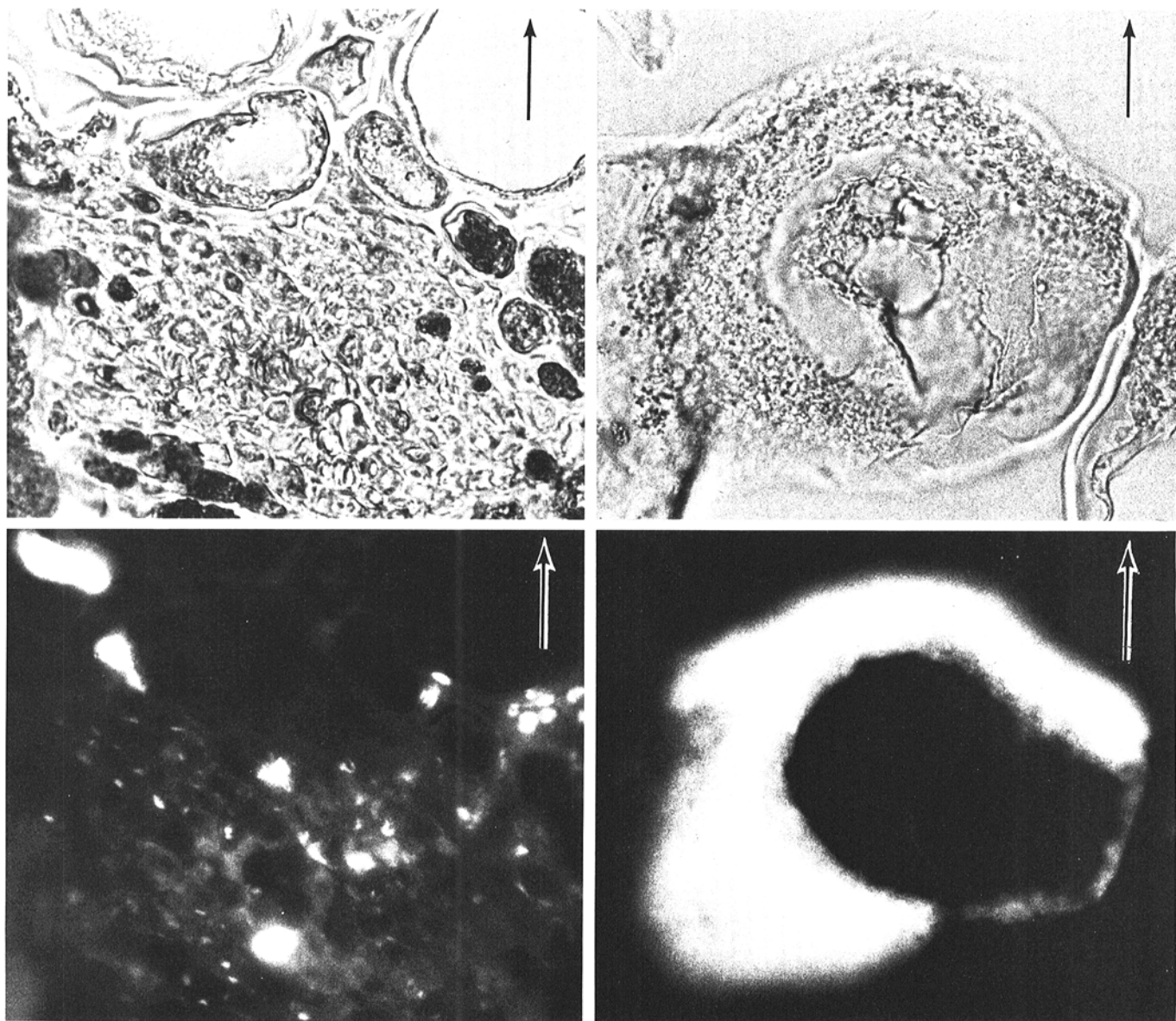


Fig. 2. Dense lignin and callose in individual cells (left,  $\times 160$ ) and in vascular elements (right,  $\times 240$ ) stained with aniline blue and photographed in visible light (above) and ultraviolet (below), the latter showing white areas of callose deposition. Arrows indicate direction of the wound.



cells in the zone between four or five and eight to 10 cells from the limits of the wound.

Aniline blue gave intense fluorescence under ultraviolet light in tissues near well-healed wounds, whereas tissues near wounds freshly inflicted in previously unwounded controls did not fluoresce. Entire groups of parenchyma cells beneath wounds showed the presence of callose (Fig. 2), which appeared to contribute, along with lignin, to a structural barrier surrounding the wound. With lacmoid stain, the callose and thickened cell walls appeared bluish green.

The nitroso test gave a positive cherry-red color, indicative of catechol tannins in the affected zone near healed wounds, and ferric sulfate gave a blue precipitate characteristic of tannins. No staining occurred in similar tissues near freshly inflicted wounds.

When fruits were inoculated with *B. cinerea* or *P. expansum* 8 days after wounding, tissues associated with wounds became more intensely stained by indicators of ligninlike compounds and in certain tests for phenolics and tannins (Table 2), compared with wounded uninoculated controls.

## DISCUSSION

These studies demonstrated that, after a period of 38 days at 5 C and 14 days at 20 C, wound healing had occurred in mature Granny Smith and Golden Delicious apples. Evidence for that statement is the following: 1) fruit cell walls in a boundary zone bordering the wound were thickened, and histochemical tests were positive for ligninlike substances and callose as well as for high levels of phenolics and tannins; 2) healed wounds were highly resistant to infection compared with nonhealed wounds; and 3) mycelia were frequently observed in the wound cavity and in the surrounding layer of ruptured cells but appeared to be contained by the barrier of thickened cell walls.

Wounds also demonstrated a striking resistance to infection by *B. cinerea* and *P. expansum* within 4 days at 5 C following wounding. We are less certain that modifications of cell walls were responsible for resistance at that early time. Factors other than wound healing may be involved. Enhanced production of phenolic substances near wounded tissues, for example, is known to be a common response. These phenolics may be incorporated into lignified cell walls during wound healing. Alternatively, the phenolics may be directly toxic to the pathogen (1,15).

Callose in thickenings, as shown by aniline-blue fluorescence, may play an important role in the wound's resistance to disease. On the other hand, the presence of callose in association with ligninlike materials in wall thickenings does not necessarily indicate that it contributes to resistance to penetration by pathogens. In fact, Smart et al (38) cast doubt on its importance in resistance when callose was removed from barley papillae by laminarinase digestion without affecting resistance to *Erisiphe graminis* DC.

The development of resistance in wounds, as demonstrated in this study, may have contributed to the results of Baker and Heald (2), who spread a paste of rotting tissue over Delicious and Winesap apples immediately after harvest and after 60, 120, and 180 days of storage at 0 C; stored fruits developed fewer blue mold lesions. English et al (16) reported that in Delicious apples, a delay of 3 days in a nonrefrigerated warehouse before storage at 0 C reduced the susceptibility of lenticels to infections by *P. expansum*. Bompeix (7) showed that lenticel infections in apple fruits resulted largely from the partial separation of the lenticel from the surrounding epidermis in some cultivars. Such separations created wounds that likely healed with time.

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