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Effect of Chemical Applications to Peach Bark Wounds on Accumulation of Lignin and Suberin and Susceptibility to *Leucostoma persoonii*

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ABSTRACT

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Fourteen extracts and chemical treatments were tested in the field on orchard trees in May and June of 1987 for their effects on wound responses in the bark. Pruning wounds on 1-yr-old branches were treated and the wound sites were excised up to 14 days later for histological study and chemical analysis of lignin. Fungal cell wall extracts and cellobiose stimulated lignin synthesis and/or enhanced the wound response. In contrast, the commonly used sealants, Lac Balsam and white paint, retarded the wound response. In a second set of experiments, five treatments were tested in the laboratory for effects on wound response and susceptibility to *Leucostoma persoonii*. Branch segments were wounded and exposed to the treatment solutions for 72 hr before fungal inoculation. All treatments except calcium were associated with increased lignin formation before inoculation. Percentage of infection of twigs treated with GA_3 + auxin was greater than the control, whereas twigs treated with cell wall extract from *L. persoonii*, cellobiose, and calcium exhibited fewer infections than the control. Canker length was reduced by all treatments. Lignin production following inoculation was related to the amount of disease present on the twigs, with increased lignin accompanying increased size of cankers.

Peach canker, caused by the fungi *Leucostoma cincta* (Pers. & Fr.) Hohn. (anamorph = *Cytospora cincta* (Pers.) Fr.) and *L. persoonii* (Nits.) Hohn. (anamorph = *C. leucostoma* (Pers.) Fr.), continues to be a major limiting factor in peach production in the northern areas of North America. The pathogens initiate disease in wounds created by pruning, leaf abscission, winter injury, and insect damage (29). The disease appears as perennial cankers on trunks, scaffold limbs, and branches, and causes crop losses mainly through reduction in bearing surface and premature tree death. All the currently grown peach cultivars are susceptible to these pathogens (23), and there is no known treatment that will prevent infection over the long term. A common practice to decrease infection in orchard trees is to seal pruning wounds with white latex paint or Lac Balsam. Preliminary experiments in which wounds in peach twigs were sealed and then exposed to *L. persoonii*, indicated that both treatments prevented infection for a period of 2 wk. However, treatment with sealants often fails to provide long-term protection, because with age the sealants tend to split open and the underlying tissues are once more susceptible to infection. Acceleration of the plant's own wound response so that the tree would continue to be protected as it grows, would be preferable.

Recent histological studies of wounds and fungal infections of peach bark have demonstrated the importance of lignin and suberin in cell walls of new tissues (3,6,7,20). Similar processes have been described for other stone fruit pathosystems (8,12,13). Doster and Bostock (12) illustrated the role of lignin in the resistance of almond to infections by *Phytophthora syringae* (Kleb.) Kleb.; Biggs and Miles (7) recently demonstrated the correlation of suberin in the wound response of peach trees with the past field performance of several cultivars.

With the understanding that postwounding changes in the infection court exert a significant influence on the subsequent frequency and severity of wound-related fungal infections (6,7), it seemed reasonable to investigate methods that could enhance the plant's

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natural defense mechanisms. The objectives of the experiments described herein were to determine the effects of exogenous applications of materials on lignin and suberin accumulation in the infection court, and the subsequent effect of these treatments on infection. Materials were tested first in field experiments and then laboratory experiments were conducted with the most promising materials over a range of concentrations.

MATERIALS AND METHODS

Field experiments. Two-year-old nursery-grown peach trees (*Prunus persica* Batsch.) cultivar Vivid on Brompton rootstock were established in May 1985. The orchard was designed with nine trees in each of 11 rows planted at 3.5- × 4.0-m spacing and was maintained with standard commercial procedures.

The orchard was divided into three plots each consisting of seven trees in each of three rows. These plots served as separate experiments initiated on 12 May, 27 May, and 16 June 1987, and were treated statistically as blocks over time. The entire three-plot arrangement was surrounded by a single row of guard trees. For each of the three experiments, the design was randomized to assign two treatments and one control to each of the seven trees in a single row for a total of 14 treatments and seven controls. A series of wounded but nontreated controls were set up on seven adjacent trees. The three single rows in each plot served as replicates, and the treatments were rerandomized twice more so that, for each experiment, each treatment was repeated three times on single tree plots.

Treatments consisted of 100 μM abscisic acid (ABA), 100 μM gibberellic acid (GA_3) + 100 μM indole-3 acetic acid (IAA), chitosan at 200 mg/ml, 0.3 M cellobiose, 15 μM $CaCl_2$, 150 μM $CaCl_2$, chitosan at 20 mg/ml + 15 μM $CaCl_2$, cell wall extract of *L. persoonii*, peach leaf cell wall extract, fungal cell wall extract + leaf cell wall extract, 3.25 μM glutathione, 7.0 mM ethephon, Lac Balsam artificial bark, white outdoor latex paint, sterile distilled water control (wrapped control), and wounded bark with no treatment (nonwrapped control). All treatment solutions were prepared in distilled water, except for ABA, which was dissolved in 0.1 M sodium bicarbonate, and were sterilized by passage through 0.22-μm Millipore filters into sterile flasks.

Plant cell walls were isolated by the procedure of Hahn et al (14), with the following modifications. In early May, approximately 340 g fresh weight of young peach leaves (1–3 cm long) were collected and frozen at −70 C. Frozen material was powdered with a hammer and passed through an 850-μm mesh screen. The frozen leaf powder was suspended in distilled water (4 C) in portions of about 35 g of powder in 250 ml of water and homogenized with a Polytron (Brinkman Instruments) at full speed for 2 min. The suspensions were centrifuged at 16,000 *g* for 15 min at 4 C, and the pellets washed twice by resuspension and recentrifugation as described above. The final individual pellets were resuspended in 1–2 L of boiling ethanol for 1 hr. After cooling, the walls were collected and washed in ethanol by suction through a coarse sintered-glass funnel. The walls then were washed with methylene chloride/methanol (1:1, v/v) by repeated suspension and removal of solvent by suction. The walls were washed similarly with acetone and the final wall preparation was air dried. The dried residue was passed twice through a 250-μm mesh screen, and then 21 g of material was recovered and frozen at −70 C. Crude cell wall hydrolysate was prepared following the procedure of Nothnagel et al (21).

Fungal cell wall extracts from *L. persoonii* were prepared as described by Anderson-Prouty and Albersheim (1). Fungal material, consisting of 7-day-old germinated spores that had been collected from pycnidia growing on 2% malt agar, were incubated in 0.5% malt broth on a rotary shaker at 30 RPM. A crude cell wall hydrolysate from the fungal walls was prepared by autoclaving for 20 min at 121 C. After cooling, the suspension was passed through a coarse sintered-glass filter, followed by passage through GF/A, 0.45-, and 0.22-μm Millipore filters. Chitosan was purified with the procedure described by Young et al (31).

Treatment, sampling, and analysis. Six pruning cuts were made on each of three branches per tree. The pruning cuts were made flush to the larger branch with either a razor blade or hand-held pruning shears and were arranged to leave at least one viable node between each cut. One half of a sterile rayon ball was placed on five of the six cuts and wrapped with Parafilm. Treatment solutions (about 1 ml) were injected into the rayon with a hypodermic needle until saturation. Water (wrapped) controls were prepared similarly. The rayon balls were removed on the following day.

Pruning cuts were sampled for lignin analysis after 0, 7, and 14 days, and for histological study after 0, 3, 7, 10, and 14 days. A portion of the stem supporting the pruning cut was removed with pruning shears and was trimmed to about 5 mm above and below the wounded area. The trimmed stem portion then was bisected through the wound site with a longitudinal razor blade cut, and one half was placed in formalin/acetic acid/alcohol (FAA) fixative. Bark from the other half was placed in methanol for lignin analysis. Both halves were placed in FAA on days 3 and 10.

Lignin was determined by the lignin-thioglycolic acid procedure of Doster and Bostock (12) from the regression equation of a standard curve. Tissues fixed in FAA were dehydrated and embedded in paraffin (3). Sections were affixed to glass slides, the paraffin was removed with xylene, and the sections were either mounted in glycerine or stained with toluidine blue O and mounted in a nonfluorescent medium (4). Sections mounted in glycerine were examined with fluorescence microscopy and the total autofluorescence intensity (in millivolts), a measure of combined lignin and suberin accumulation, was determined in the outer bark cortex with an MPV compact microscope photometer (5). Sections stained with toluidine blue O were examined similarly and the remaining autofluorescence intensity, which was due solely to suberin, was quantified.

Laboratory experiments. Twigs from the previous year's growth were collected from the same orchard used in the field experiments. Segments were prepared by removing 160-mm lengths from the apical and basal ends of the branches; the remaining branch was cut into 140-mm-long segments with any leaves and flowers removed. The segments were placed in test tubes containing 3–5 ml of distilled water; 15 replicates were prepared for each of 24 treatments, 10 to be inoculated with the pathogen, and five to remain uninoculated. This experiment was performed twice.

The treatments, which consisted of the most promising materials from the field experiments included: 1) fungal cell wall extract from *L. cincta* or *L. persoonii* applied in a 10-fold dilution series; 2) combinations of GA_3 and IAA, i.e., 100 μM/100 μM, 1.0 μM/1.0 μM, 1.0 μM/100 μM, and 100 μM/1.0 μM, respectively; 3) $CaCl_2$ at 1.5 μM, 15 μM, 150 μM, 1.5 mM, and 15 mM; and 4) cellobiose at 0.3 and 0.6 μM, and 1, 3, and 10 mM.

Each segment was wounded through the bark to the xylem in the internode region, midway along the segment with a 4-mm-diameter cork borer. The wounded bark disk was discarded. Wounds were covered with rayon balls and Parafilm and injected with treatment solutions as described above. The tubes were capped and placed in a 21 C incubator in the dark for 72 hr. The Parafilm and rayon were removed and 10 segments per treatment were inoculated with mycelium of *L. persoonii* prepared as described previously (3).

Five uninoculated segments per treatment were harvested and prepared for lignin determination and histological study as described above. The 10 inoculated segments were rated for the frequency and length of cankers 9 and 14 days postinoculation. On day 14, samples were taken from the inoculated plants for lignin analysis and histological study.

Data analysis. Data were analyzed with the general linear models procedure and a randomized complete block design with Type III sums of squares (SAS Institute, Cary, NC). Means were separated by making single degree of freedom orthogonal comparisons (25). Simple correlations were used to examine the relationships between preinoculation lignin, postinoculation lignin, disease frequency, and canker length.

RESULTS

Field experiments. Cell wall extracts from *L. persoonii* and cellobiose stimulated lignin production in peach bark adjacent to pruning wounds by 81 and 71%, respectively, relative to the distilled water (wrapped) control. Wounds treated with white latex paint and Lac Balsam exhibited reduced lignin levels (50 and 36%, respectively) relative to the wrapped control (Table 1). Wounds treated with ethephon produced visible quantities of polysaccharide gum, which exuded from the vascular cambial region approximately 10 days after the treatment was applied; however, lignin production was similar to that of the wrapped control. The nonwrapped control had 81% less lignin than the wrapped control. The amount of lignin in wounds treated with the fungal cell wall extract was approximately 10 times the amount detected in the nonwrapped control. All other treatments were not significantly different from the wrapped control.

Total autofluorescence in the region of wound periderm formation in the outer bark cortex was highest in pruning wounds treated with the combinations of chitosan and calcium, GA_3 and IAA, and 150 μM calcium alone (Table 2). Treatments with Lac Balsam and white latex paint had the lowest total tissue autofluorescence. Only the former was significantly different from the wrapped control with a reduction in total autofluorescence of 12%. Fungal cell wall extract and cellobiose, the two treatments with the highest levels of extractable lignin, exhibited tissue autofluorescence levels similar to both control treatments. Both control treatments had virtually the same levels of total autofluorescence.

Suberin autofluorescence was highest in the wounded bark with no treatment, followed by the chitosan + calcium and the fungal cell wall extract treatments, with increases above the wrapped control of 35, 35, and 33%, respectively (Table 2). However, due to great variation in the data, these values were not significantly different from the wrapped control. Wounds treated with Lac Balsam possessed the least amount of suberin showing a significant reduction of 64% relative to the wrapped control. The amount of suberin in the nonwrapped control was approximately 3.7 times the amount measured in wounds treated with Lac Balsam.

Laboratory experiments. Within some treatment groups, the concentration of applied phytohormones influenced the production of lignin 3 days after treatment. Wounds treated with GA_3 at 10^{-3} M were more than twice as lignified than wounds treated with 10^{-4} M GA_3 (data not shown). On the other hand, the degree of lignification did not vary with IAA concentration. No other treatment group exhibited a dose-response relationship for preinoculation lignin and, therefore, all concentrations were pooled into treatment groups for further analyses. All treatment groups except calcium were associated ($P < 0.05$) with increased levels of lignin (Table 3).

Percentage of infection differed significantly within the cellobiose treatment group following inoculations with *L. persoonii*. Wounds pretreated with 0.6 and 0.3 M cellobiose exhibited 69 and 62% infection, respectively, compared with 35, 32, and 25% infection for wounds pretreated with 0.001, 0.01, and 0.003 M cellobiose, respectively. Because there were no other within treatment group differences related to percentage of infection, the data from the concentration series of each treatment were pooled for further analyses. Percentage of infection was greatest for peach twigs treated with phytohormones (Table 4), where values were greater than 80% for all four treatment combinations. Infection also tended to be high in wounds treated with cell extract from *L. cincta* and were greater than 70% at all five dilution levels (data not shown). However, only the phytohormone treatments consistently exhibited infection levels significantly higher than the distilled water control. Branch segments treated with phytohormones often appeared water soaked and necrotic and also were more likely to become infected with secondary organisms. The other treatment groups, i. e., cell wall extract from *L. persoonii*, cellobiose, and calcium, all exhibited infection levels significantly lower than the control, 1.5 mM calcium showing the least amount of infection, only 10%. Twigs receiving any of the five calcium treatments were less infected than the control.

The lowest dilution of cell wall extract from *L. persoonii* was associated with increased canker length (13.9 mm) relative to the other dilutions (8.0 mm or less, $P < 0.01$, data not shown). When concentrations were combined within treatment groups, canker length was greatest in the distilled water control, whereas all other treatments were associated with significantly smaller cankers (Table 4). Wounds pretreated for 72 hr with various calcium concentrations generally had the smallest cankers, although the calcium treatment group could not be separated statistically from the cellobiose and wall extract from *L. cincta* groups.

Generally, when wounds were inoculated with *L. persoonii* following the pretreatment, the wounds with greater amounts of induced lignin exhibited smaller cankers ($r = -0.63$, $P < 0.10$), although, amounts of induced lignin were not associated with percentage of infection. Lignin levels increased in all treatments and the control following inoculation (Table 3).

Wounds pretreated with cellobiose and cell wall extract from *L. persoonii* exhibited significant ($P < 0.01$) dose response curves

TABLE 1. Thioglycolic acid lignin extracted from bark adjacent to peach pruning wounds 14 days after treatment with various substances

Treatment	Lignin (mg/g)[y]
Fungal cell wall extract	31.0 a[z]
Cellobiose	29.2 a
Plant + fungal cell wall extract	23.2 ab
Ca^{2+} (15 μM)	22.8 ab
Glutathione	20.6 ab
Plant cell wall extract	20.3 ab
Chitosan + Ca^{2+} (15 μM)	20.3 ab
Ca^{2+} (150 μM)	18.8 ab
Control (wrapped)	17.1 bc
Ethephon	15.1 bcd
Chitosan	15.1 bcd
GA_3 + IAA	14.9 bcd
ABA	14.8 bcd
Lac Balsam	11.0 cd
White latex paint	8.6 d
Control (nonwrapped)	3.2 d

[y] Each value is the mean of nine observations, except for control (wrapped) ($n = 48$) and control (nonwrapped) ($n = 15$).
[z] Letters denote significant differences determined with single degree of freedom orthogonal comparisons ($P < 0.05$).

TABLE 2. Total and suberin autofluorescence from wound-induced tissues in regenerated peach bark adjacent to pruning wounds 14 days after treatment with various substances

Treatment	Autofluorescence (mV)	
	Total[x]	Suberin[y]
Chitosan + Ca^{2+} (15 μM)	38.9 a[z]	7.4 a
GA_3 + IAA	38.5 a	4.2 bcd
Ca^{2+} (150 μM)	37.8 a	4.2 bcd
Chitosan	37.0 a	5.3 abc
Plant cell wall extract	36.9 a	5.6 abc
Fungal + plant cell wall extract	36.6 a	3.9 cd
ABA	36.4 a	6.8 ab
Cellobiose	35.9 a	5.0 abc
Ca^{2+} (15 μM)	35.7 a	3.7 cd
Glutathione	35.6 a	5.8 abc
Control (wrapped)	35.3 ab	5.5 abc
Control (nonwrapped)	35.2 ab	7.4 a
Ethephon	35.1 abc	6.9 a
Fungal cell wall extract	34.8 abc	7.3 a
White latex paint	31.3 bc	4.0 cd
Lac Balsam	31.0 c	2.0 d

[x] Each value is the mean of nine observations, except for control (wrapped) ($n = 60$) and control (nonwrapped) ($n = 10$).
[y] Each value is the mean of six observations, except for control (wrapped) ($n = 38$) and control nonwrapped) ($n = 37$).
[z] Letters denote significance of differences determined with single degree of freedom orthogonal comparisons ($P < 0.05$).

with respect to lignin produced following inoculation. With cellobiose, postinoculation lignin increased with cellobiose concentration according to the relationship $Y = 122.74 + 100.98 (X)$ where Y = mg/g lignin and X = molar concentration of cellobiose ($R^2 = 0.91$). With cell wall extracts from *L. persoonii*, lignin increased as the concentration of the extract decreased according to the relationship $Y = 93.14 - 7.26 (LN(X))$ where Y = mg/g lignin and LN(X) is the natural log of the extract dilution. The greatest amounts of lignin were detected in twig wounds that had received phytohormone treatments or *L. cincta* cell wall extracts before inoculation. Lower lignin levels following inoculation were observed in the calcium and *L. persoonii* cell wall extract treatments. The amount of lignin recorded following inoculation was correlated significantly with infection frequency ($r = 0.87$, $P < 0.05$) with greater amounts of lignin detected in treatments exhibiting higher levels of infection.

DISCUSSION

Peach canker disease is managed in Ontario with a combination of horticultural and pest control practices that seek to delay disease onset until the fourth or fifth year after orchard establishment. Procedures for controlling pruning wound infections range from no wound treatments at all to painting every wound with a mixture of thiram and white latex paint. In addition, some fungicides for the control of brown rot blossom infections may protect pruning wounds from infection by *Leucostoma* spp.

The wound reaction in peach bark consists of deposition of suberin and lignin in the walls of the cells near the injury, followed by the differentiation of a cork cambium from underlying parenchyma cells. This cork cambium, which fuses with the uninjured cambium of the stem, produces a layer of suberized cork cells that forms a boundary around the wound (3). There is a positive correlation between the rate of suberin accumulation in peach bark wounds and the ability of the wounds to resist infection by *L. persoonii* (6).

In the present study, we found that application of the commonly used physical sealants, Lac Balsam or white latex paint actually inhibited the deposition of suberin and/or lignin. These observations may help explain the lack of long-term protection afforded by such treatments. They are able to control disease in the short term by forming a physical barrier over the surface of the wound and preventing fungal spores from entering, but once the seals are broken, the underlying tissue is very susceptible to fungal attack.

Several chemicals were tested for their potential to stimulate the plant's own wound response. Most of these were selected based on their reported enhancement of the wound reaction itself, or of one of the reaction's components, e.g., the production of lignin, suberin, periderm, callus, callose, or phytoalexins (1,2,9-11,14-18,22,24,26,28,30). Not all the above chemicals were effective in significantly stimulating the wound response in peach bark or in reducing the susceptibility of the wounds to the pathogen. In the field experiment, in which the greatest range of different chemicals was tested, only the fungal wall extract and cellobiose treatments resulted in increased lignin deposition and none were effective in increasing suberin deposition. Chitosan in combination with 15 μM Ca^{2+} was associated with the highest total autofluorescence and the highest suberin autofluorescence, although neither was significantly different from those of either control. In the laboratory experiments, the mixture of GA_3 and IAA stimulated lignin production at both times tested but also increased susceptibility to infection by *L. persoonii* and secondary pathogens. Ethephon had no effect on the production of lignin or suberin but did induce gum duct formation in the bark (see 19).

There has been much recent interest in the stimulation of defense reactions in plants by polysaccharides of animal, plant, and fungal origin. Although treatment with wall extracts from both *L. persoonii* and *L. cincta* stimulated lignin production and resulted in smaller canker size after infection, only treatment with the extract from *L. persoonii* resulted in decreased percent infection rates. These results suggest that a mechanism(s) in addition to lignification is involved in the resistance of peach to these two fungi. Recent studies have shown that suberin may be more important than lignin in the resistance of this system (6,7) but a correlation with suberization could not be established in the present study. The involvement of other factors, such as the production of phytoalexins, has not been investigated for this system.

One of the two most promising chemical treatments in terms of reducing both infection frequency and canker size was cellobiose. It stimulated lignin production in both the field and laboratory experiments. To the best of our knowledge, this is the first report of lignin stimulation by this chemical. The most promising chemical treatment included in our experiments was calcium. Although this ion did not stimulate the production of lignin or suberin, it was the most effective chemical for reducing both infection frequency and size of cankers. The mode of action of calcium in this system is unknown at present but some possibilities could be mentioned. One is that the ion could stimulate the synthesis of phytoalexins and/or phenols as suggested by Kohle et al (17). A second possibility is that it may reduce fungal polygalacturonase activity by forming cation cross bridges between pectic acids in the plant, thus making its cell walls more resistant to digestion. The latter possibility is supported by the observation that breakdown of the pectins present in peach bark cell walls (3) and the sequestration of calcium by fungal oxalic acid (27) have been shown to occur during pathogenesis by *L. persoonii*.

Even the best chemical treatments used in the present study failed to provide total protection to peach bark against infection by *L. persoonii*, the causal agent of peach canker disease. Therefore, treatment with cellobiose or calcium in an orchard could be expected to slow but not prevent colonization by the fungus. However, since our inoculation method was very favorable for infection, it is possible that under orchard conditions these chemicals would perform better than in the laboratory because

TABLE 3. Thioglycolic acid-lignin from peach bark wounds 3 days after treatment with various substances and 7 days after treated wounds were inoculated with *Leucostoma persoonii*[y]

	Thioglycolic acid-lignin (mg/g)		
Treatment group	Posttreatment	Postinoculation	Change (%)
Cellobiose	77.6 a[z]	142.0 abc	83.0 b
LPWE	75.7 a	127.8 c	68.8 b
GA_3 + IAA	73.7 a	178.7 a	144.4 ab
LCWE	72.6 a	172.7 ab	137.9 b
Calcium	62.2 ab	117.4 c	88.7 b
Control (wrapped)	41.6 b	134.6 bc	223.6 a

[y] Values in columns are combined data from two experiments and are the means of 100 observations except for control (n = 20) and GA_3 + IAA (n = 80). LPWE and LCWE are cell wall extracts from *L. persoonii* and *L. cincta*, respectively.

[z] Letters denote significant differences determined with Duncan's multiple range test ($P < 0.05$).

TABLE 4. Percentage of infection and canker length 7 days postinoculation from twigs pretreated with various substances[y]

Treatment group	Canker length (mm)	Percentage infection
Control	23.4 a[z]	66.7 b
GA_3 + IAA	16.4 b	82.5 a
LPWE	12.9 bc	48.5 c
LCWE	11.2 cd	74.7 ab
Cellobiose	10.8 cd	47.4 c
Calcium	6.6 d	21.0 d

[y] Values in columns are combined data from two experiments and are the means of 50 observations, except for control (n = 10) and GA_3 + IAA (n = 40). LPWE and LCWE are cell wall extracts from *Leucostoma persoonii* and *L. cincta*, respectively.

[z] Letters denote significant differences determined with Duncan's multiple range test ($P < 0.05$).

of lower inoculum levels in the field. Further work on times and methods of application, and amending the treatment solution with other chemicals will be required to capitalize fully on the promising results obtained to date.

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