Effects of Low Temperature on Resistance of Almond Trees to Phytophthora Pruning Wound Cankers in Relation to Lignin and Suberin Formation in Wounded Bark Tissue

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ABSTRACT

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As the temperature was lowered at which bark wounds were aged, the development of resistance to bark cankers caused by *Phytophthora syringae* was slowed. All fresh wounds inoculated with *P. syringae* resulted in cankers. In stem wounds aged for 2 wk at 25 C before inoculation, only 38% of the inoculated wounds developed cankers, while when aged at 6 C, all of the inoculated wounds developed cankers. In orchard trees throughout the fall and winter, inoculation with *P. syringae* resulted in cankers in all of the fresh wounds, most of the 2-wk-old wounds, and many of the 4-wk-old wounds, but almost all inoculated 6-wk-old wounds were immune to infection. As temperature was decreased, less lignin and suberin were observed histochemically and less ligninthioglycolic acid (LTGA) was detected in almond bark wounds using the thioglycolic acid assay. There was almost a threefold increase in LTGA in 2-wk-old wounds when aged at 25 C than when aged at 6 C. During winter, the resistance to infection by *P. syringae* of 2- and 4-wk-old wounds did not consistently correspond to the mean temperature for the period of wound aging, suggesting that other factors in addition to temperature are involved in resistance development.

Phytophthora pruning wound canker, a disease of almond trees (*Prunus dulcis* (Mill.) Webb) in California, occurs when *Phytophthora syringae* (Kleb.) Kleb enters pruning wounds and attacks the inner bark (5). Cankers are observed soon after pruning in the fall and winter when temperatures are relatively low (mean daily temperature usually 5–15 C) and within a range where *P. syringae* grows very well in culture media and in almond bark (5). It was observed that pruning wounds were very susceptible initially to infection, but eventually became immune to infection, suggesting that wound-induced responses in the bark were responsible for the immunity to infection of older wounds (*unpublished*).

A suberized periderm similar to the normal periderm of the outer bark is produced around wounds in almost all dicotyledonous plants (9). Within 2 wk after wounding, a strongly lignified zone and a suberized periderm were observed histologically in the inner bark of almond trees (16) and other tree species (3,13). Increases in phenolic polymers such as lignin as almond bark wounds aged (8) corresponded to increases in resistance to the canker-forming pathogen *Ceratocystis fimbriata* (6,16), suggesting that the ligno-suberized tissue contributes to wound resistance. A negative correlation has been observed between suberization around bark wounds in peach trees and incidence of cankers caused by *Leucostoma* species (4). The lignification and suberization around bark wounds appear to be involved with resistance to canker-forming pathogens.

Lignin and suberin formation around wounds in apple trees was strongly inhibited at low temperatures (13). Because infection by *P. syringae* of pruning wounds in almond trees occurs during fall and winter when temperatures are low (5), it seemed likely that wound periderm formation and resistance development of the wound would be compromised by the low temperature. The objective of this study was to determine the extent that low temperatures inhibit wound-induced lignin and suberin formation and affect resistance development of almond bark wounds to *P. syringae*.

MATERIALS AND METHODS

General. The almond cultivar Nonpareil, the most common cultivar in commercial almond production in California, was used in all experiments. Potted trees were maintained in a lathhouse until needed for experiments. The isolate (F-79) of *P. syringae* was maintained in amended lima bean agar (ALBA) (7) or V-8 agar and grown in cornmeal agar (CMA) or ALBA at 15–20 C for use in inoculations. For inoculations mycelial agar plugs of approximately the same size as the wound were placed on the wound and kept in place by wrapping with Parafilm. Experiments using potted trees were performed in growth chambers with a 12-hr light period (10 Wm^{-2}). In all experiments after wounds had aged, whole branches with aged wounds were cut off, kept in an ice chest during transport to the laboratory, and the wounded tissues were prepared for analysis the same day.

Development of resistance. The bark of 2-yr-old potted trees was wounded by cutting to the xylem with a 5-mm-diameter cork borer and the trees were kept at 6 and 25 C. The wounds were allowed to age so that each of eight trees had 0-, 2- and 4-wk-old wounds, then inoculated with mycelial CMA plugs containing *P. syringae*, and all trees were placed in a 12 C growth chamber. For controls, aged and fresh wounds were left uninoculated. One month later the inner bark was examined for cankers and the length of inner bark discoloration measured. The methods for the studies where the resistance of the aged wounds to *P. syringae* was investigated are given below.

Histological studies. Radial sections were made for all histological studies as follows: 5- × 5-mm pieces of the wounded bark were stripped from the xylem, sectioned fresh with a microtome at a thickness of approximately 0.12 mm, and placed in glycerol. The stains used were phloroglucinol-HCl for lignin and Sudan black B for suberin (10). The percentage of the width of the inner bark tissue from the cambium to the outer bark staining positive was measured with an ocular micrometer using a microscope. In the sections of inner bark tissue, the only suberin observed was associated with wounds, but there was a slight staining for lignin associated with fiber bundles, which was not measured. For the analysis of the data, analysis of variance was performed on the arcsine transformed (14) percentage of bark width staining positive.

Studies were performed using stems of 1-yr-old potted trees, excised branches, and branches on mature trees in an orchard. Potted trees were wounded by making cross-sectional cuts with

pruning shears and were placed in growth chambers kept at 6, 13, 15, 20, and 25 C. Five replications of 10- and 20-day-old wounds were examined.

Excised twigs of approximately 15 cm length and 2 cm diameter were obtained from orchard trees during winter. One end of the twig was sealed in warm paraffin wax to prevent excess loss of moisture. The twigs were wounded by making a cross-sectional cut with pruning shears, and the twigs were placed in incubators kept at 5, 9, 12, 15, 20, and 27 C. Every week for 6 wk new twigs were wounded and placed in the incubators and then the six replications were examined for lignin and suberin formation as above (twigs were not aged for the longer time periods at 15–27 C because of the problem of maintaining twigs at high temperatures for long periods). Fresh and aged wounds were inoculated with *P. syringae* and kept for 4 wk at 12 C. Uninoculated wounds served as controls.

Trees selected at random in an almond orchard in Colusa County, CA, were wounded by making cross-sectional cuts with pruning shears of branches (approximately 2 cm diameter) every 2 wk from October 1984 to April 1985. Wounds were allowed to age 2, 4, and 6 wk in the orchard before inoculation or histological examination. For each wound age and wound date combination there were 10 replications. For controls, aged and fresh wounds were not inoculated. Six weeks after inoculation with *P. syringae*, the length of inner bark discoloration from the wound was measured.

Biochemical assay for lignin. The thioglycolic acid assay (8) was used to quantify the amount of lignin in the wounded inner bark. In the procedure, ligninthioglycolic acid (LTGA) was extracted from the tissue and then quantified by measuring the absorbance at 280 nm using a spectrophotometer. The absorptivity used for LTGA in wounded almond bark was 11.9 g^{-1} L cm (8).

In an experiment investigating the effect of temperature on lignification, the bark in the stem of 2-yr-old potted trees was wounded with a 6-mm-diameter cork borer to the xylem and the trees kept in 6, 12, 19, and 25 C growth chambers. Each week for 4 wk wounds were made on each of five trees. Wounded and nonwounded bark tissues were removed with an 11-mm-diameter cork borer and the amount of lignin determined.

In an experiment investigating lignification in orchard trees during fall, winter, and spring, branches (approximately 2 cm diameter) of five orchard trees in Yolo County, CA, were wounded every 4 wk from September 1985 through April 1986 by making cross-sectional cuts with pruning shears. After 4 wk, 2- × 15-mm pieces of wounded and nonwounded inner bark were removed for measurements of lignin.

RESULTS

Effect of temperature on development of resistance in wounds. Resistance to infection by *P. syringae* developed more rapidly in wounds in potted trees kept at 25 C than for wounds in trees kept at 6 C (Fig. 1). In trees kept at 25 C, all 4-wk-old wounds were immune to infection, while at 6 C there were half as many cankers in inoculated 4-wk-old wounds as in fresh wounds. For both 6 and 25 C, lengths of cankers in 2-wk-old wounds were less than those in fresh wounds by paired Student's *t*-tests ($P < 0.05$). No cankers were observed around the uninoculated controls. Cankers in inoculated fresh wounds in trees that were kept at 6 C before inoculation were smaller ($P = 0.04$) than cankers in fresh wounds in trees that were kept at 25 C before inoculation.

Although in excised twigs inoculation of aged wounds with *P. syringae* always resulted in cankers, the inoculated aged wounds had smaller cankers than inoculated fresh wounds (Fig. 2). Furthermore, in wounds aged at 12 C and above, the difference in canker expansion rate in inoculated 1–3-wk-old wounds decreased as temperature increased (Fig. 2). There was little difference between expansion rates of cankers resulting from inoculation of 1–3-wk-old wounds and fresh wounds when wounds were aged at temperatures less than 12 C before inoculation. However, when wounds were aged at 5 C for 4–6 wk, there was slower ($P = 0.0001$) canker expansion in aged wounds than in fresh wounds. Although no cankers were observed in the uninoculated wounds, there was a slight (3.6 mm) necrosis around the wounds.

Effect of temperature on wound-induced lignin and suberin formation. The extent of the lignified zone and suberized wound periderm observed in the histological study in potted trees was substantially less at low temperatures (Fig. 3). In 20-day-old wounds, no lignin was detected at 13 C or below, while at 25 C in all wounds a lignified zone was observed reaching from the outer periderm to the xylem. In 20-day-old wounds, no suberin was detected at 15 C or below, while at 20 and 25 C there was abundant suberin (Fig. 3). Although there were significant ($P < 0.05$) differences in the percent bark width staining positive for both lignin and suberin between the various temperatures in 20- and 10-day-old wounds, there was a significant ($P < 0.001$) temperature and wound age interaction. Lignin was detected before suberin in all experiments.

The amount of lignin detected in the histological study using excised twigs was substantially less at low than at high temperatures (Fig. 4). No suberin was detected in 1-wk-old wounds

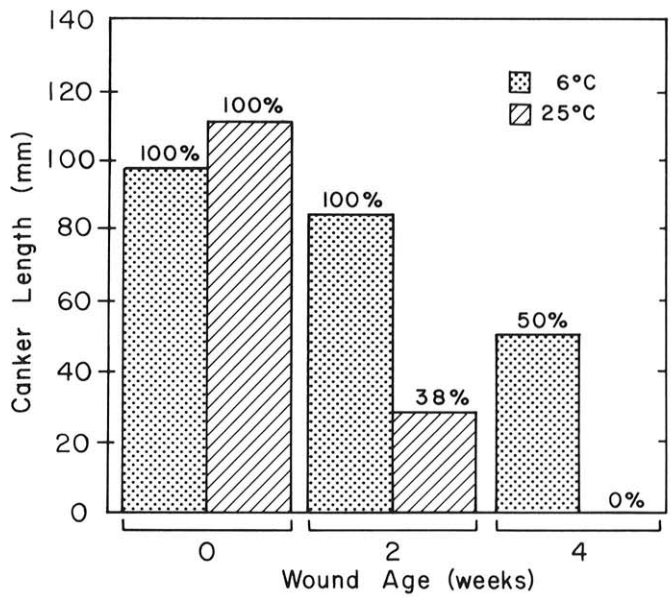

Fig. 1. The effect of preinoculation temperature on development of cankers in aged inner bark wounds in potted almond trees inoculated with *Phytophthora syringae*. After inoculation trees were kept at 12 C for 1 mo. The percentages presented on top of the bars represent the percentage of inoculations resulting in cankers. For the calculation of mean canker expansion rate only inoculations resulting in cankers were included.

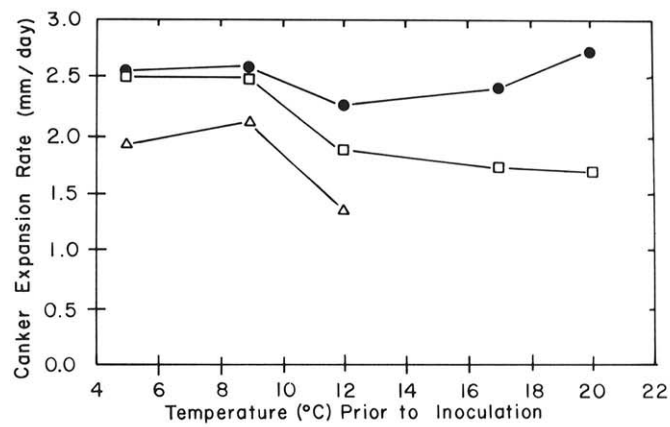

Fig. 2. The development of cankers caused by *Phytophthora syringae* in excised almond branches after inner bark wounds were aged at various temperatures. After inoculation branches were kept at 12 C for 4 wk. Each point represents data from at least nine branches. Fresh wounds were made in branches that had been aged at various temperatures similar to the aged wounds. • = fresh wounds; □ = young wounds (1–3 wk old); Δ = old wounds (4–6 wk old).

at any temperature, but in 2-wk-old wounds, 18% of the bark width was suberized at 20 C although no suberin was detected below 20 C (data not shown in figure). Even after 6 wk no suberin was observed in any wound kept at 12 C or below.

At low temperatures aged wounds of potted trees formed less lignin as determined by the thioglycolic acid assay (Fig. 5). Because it was observed previously that the amount of LTGA increases linearly as wounds age (8), linear regression was performed on the data in Figure 5. For all four temperatures, the lack-of-fit test (18) showed that the linear regression adequately described the data. As the temperature increased, the slopes (rate of LTGA increase) of the regression lines increased (Fig. 5). In the comparison of slopes of the regression lines (11), the 6 C slope was not significantly different from the 12 C slope, but in all other comparisons there were significant differences ($P = 0.05$). At 6 C there was more LTGA detected in the 1-wk-old wounds than in the nonwounded tissue ($P = 0.02$).

Orchard experiments. During the period of the histological study in the orchard, the cumulative degree days (developmental threshold was assumed to be 0 C) for 2-wk periods ranged from 188 in early October, to 62 in January, to 181 in February (Fig. 6A). As the temperatures during this period went from relatively high to low to high, the amount of lignin observed in the aged wounds tended to follow the same pattern as temperature with the least amount of lignin occurring in wounds made in late December or early January (Fig. 6B). The most lignification in the aged wounds was observed in the 6-wk-old wounds and the least in the 2-wk-old wounds. As the temperatures during the experiment went from relatively high to low to high, the amount of suberin observed in 4- and 6-wk-old wounds followed the same pattern with the least occurring in wounds made in late December or early January (Fig. 6C). Only rarely was suberin observed in 2-wk-old wounds during this period. Although the amount of lignin (Fig. 6B) and suberin (Fig. 6C) in wounds increased with the temperature during the aging period (Fig. 6A), there was no clear relationship between temperature and development of resistance to infection by *P. syringae* or inoculated 2- and 4-wk-old wounds (Fig. 6D). All inoculated fresh wounds resulted in cankers. Almost all of the 6-wk-old wounds were immune to infection by *P. syringae*. Substantially smaller cankers occurred in inoculated 2- and 4-wk-old wounds than in inoculated fresh wounds (Fig. 7). Although no cankers were observed in the uninoculated controls, there was a slight dieback (3.5 mm). In the period from October through February, the expansion rates of cankers resulting from inoculations of fresh wounds with *P. syringae* ranged from 1.2 to 1.7 mm/day (Fig. 7). However, as the temperatures rose in March and April, the canker expansion rate for inoculated fresh wounds decreased to 0.6 mm/day.

Throughout the fall and winter during a separate orchard study, substantially more ($P < 0.002$) LTGA was detected in 4-wk-old wounds than in nonwounded inner bark tissue (Fig. 8). Substantially more LTGA was detected in wounds made in February and March than in wounds made in December. As the mean temperatures decreased from September to December the amount of LTGA decreased and as the mean temperatures increased from December to March the amount of LTGA increased (Fig. 8).

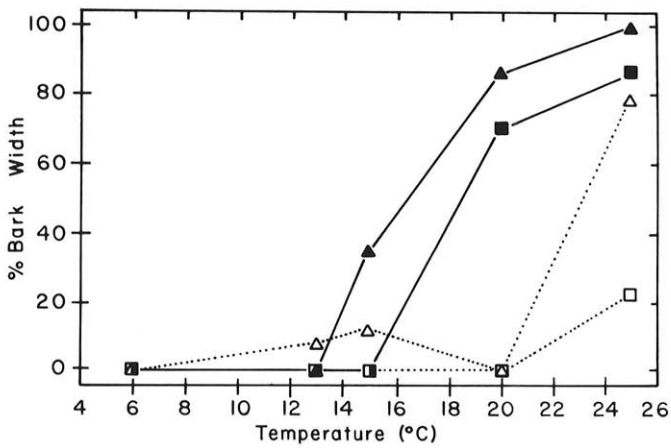

Fig. 3. The effect of temperature on lignification and suberization in wounded inner bark of potted almond trees as determined by histological methods. The percentage bark width was determined by measuring how much of the inner bark from the cambium to the outer periderm stained positive for lignin with phloroglucinol-HCl or stained for suberin with Sudan black B. For the 20-day-old wounds the $LSD_{0.05}$ was 25 and 24 for the arcsine transformed percentage bark width for lignin and suberin, respectively. Δ = lignin, 10-day-old wounds; □ = suberin, 10-day-old wounds; lignin, ▲ = 20-day-old wounds; ■ = suberin, 20-day-old wounds.

DISCUSSION

Low temperatures slowed the development of resistance to infection by *P. syringae* in almond bark wounds (Figs. 1 and 2). Resistance in aged wounds was evident as fewer (Fig. 1) or smaller cankers (Figs. 1 and 2), similar to the resistance to *C. fimbriata* observed with age in almond bark wounds (6,16). Before the present study, very little work had been done on the effect of low temperature on the development of resistance of tree wounds to canker pathogens. Aged pruning wounds in apricot trees developed some resistance to Eutypa dieback at 20 C but not at 3 C (19), and apple fruit scar wounds, which respond similarly to pruning wounds, remained susceptible to *Nectria galligena* longer when kept at 6 C than at 12–24 C (13).

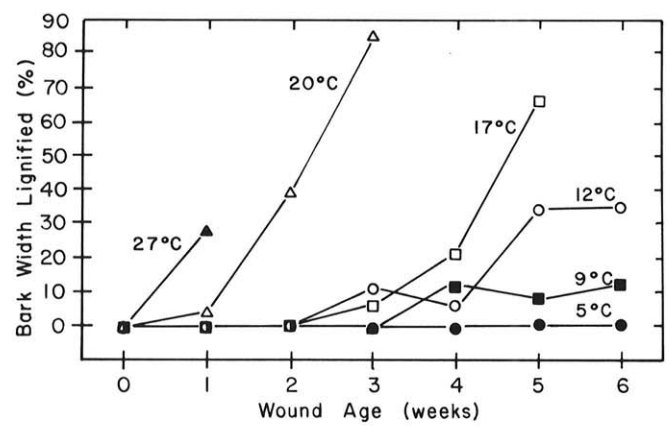

Fig. 4. The effect of temperature on lignification of wounded inner bark of excised almond twigs. The percentage bark width was determined by measuring how much of the inner bark from the cambium to the outer periderm stained positive for lignin with phloroglucinol-HCl.

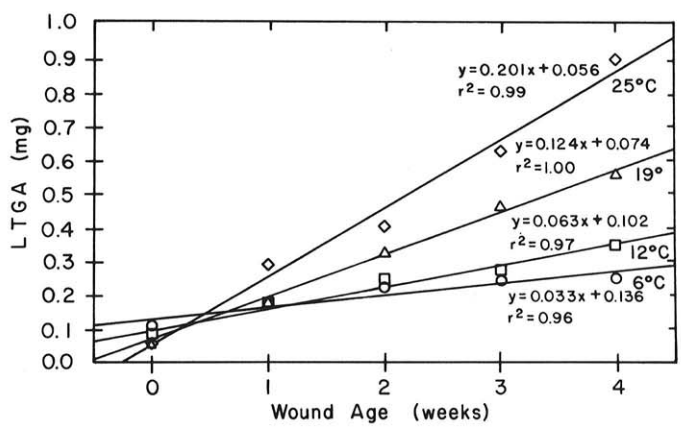

Fig. 5. The effect of temperature on the formation of lignin as measured as ligninthioglycolic acid (LTGA) in wounded inner bark of potted almond trees over a 4-wk period. o = 6 C; □ = 12 C; Δ = 19 C; ◇ = 25 C.

In almond bark wounds low temperature markedly reduced the lignification (Figs. 3 and 4) and suberization (Fig. 3) observed histologically, and less lignin was detected using the thioglycolic acid assay (Fig. 5). Similarly, lignin and suberin formation in wounded apple bark was strongly inhibited at temperatures below 10 C (13) and corresponded to the accumulated degree days with a developmental threshold of 0 C in wounded apple, cherry, and peach bark (2). The rate of wound healing in potato tubers decreased as temperatures decreased from 20 C (26). In the histological studies of bark wounds, the suberin was found in the wound periderm, and the lignin was observed primarily in a separate adjacent zone, both of which eventually extended from the xylem to the outer periderm forming a barrier (3,8,13). The thioglycolic acid (TGA) assay measures the amount of bound phenolic polymers in the inner bark, which would include the lignin and the phenolic component of suberin in and around the wound periderm (8). Suberin consists of two principal components, and it has been suggested that the phenolic component would prevent pathogen entry while the aliphatic component prevents water loss (12). Thioglycolic acid reacts readily with the α-alkoxy function in lignin to give ligninthioglycolic acid (LTGA) derivatives (25), and the LTGA can be extracted from the tissue with base. The TGA assay was sensitive, detecting a substantial increase in LTGA in 1-wk-old wounds kept at 6 C (Fig. 5), whereas even after 20 days no lignin or suberin was detected histochemically (Fig. 3). Even though there was more rapid lignification and suberization in wounds kept at the higher temperatures (Figs. 3–5), increased wound-induced lignification was detected at low temperatures with the TGA assay but not with phloroglucinol-HCl (Figs. 3–5).

The development of resistance to *P. syringae* corresponded to the formation of the lignified zone and of the wound periderm as measured as LTGA in wounded almond bark (8). In the present study this was also true because 2-wk-old wounds aged at 6 C had 13% smaller cankers than inoculated fresh wounds (Fig. 1) and slightly more LTGA (increase of 0.12 mg per wound) (Fig. 5), whereas 2-wk-old wounds aged at 25 C had 74% smaller cankers (when cankers did form) than inoculated fresh wounds (Fig. 1) and substantially more LTGA (increase of 0.34 mg per wound) (Fig. 5). Several possible mechanisms by which lignification could halt fungal development in the host tissues have been proposed (20,24). Because inoculation of almond bark wounds with *P. syringae* greatly increased LTGA levels beyond those induced by the normal wound response (8), the temperature after inoculation may be very important and some of the resistance of wounds aged at low temperatures may have developed after inoculation when all the trees had been moved to a higher temperature.

The results obtained in the histological experiments using excised almond branches (Fig. 4) were similar to those obtained using potted almond trees (Fig. 3). For example, at 20 C the percentage bark width lignified after 20 days was 79% in wounded potted trees and after 21 days was 87% in wounded excised branches. However, there were differences in canker development by *P. syringae* in inoculated wounds in excised branches and in potted trees. Cankers caused by *P. syringae* were found to expand 25% faster in excised branches than in trees (*unpublished*).

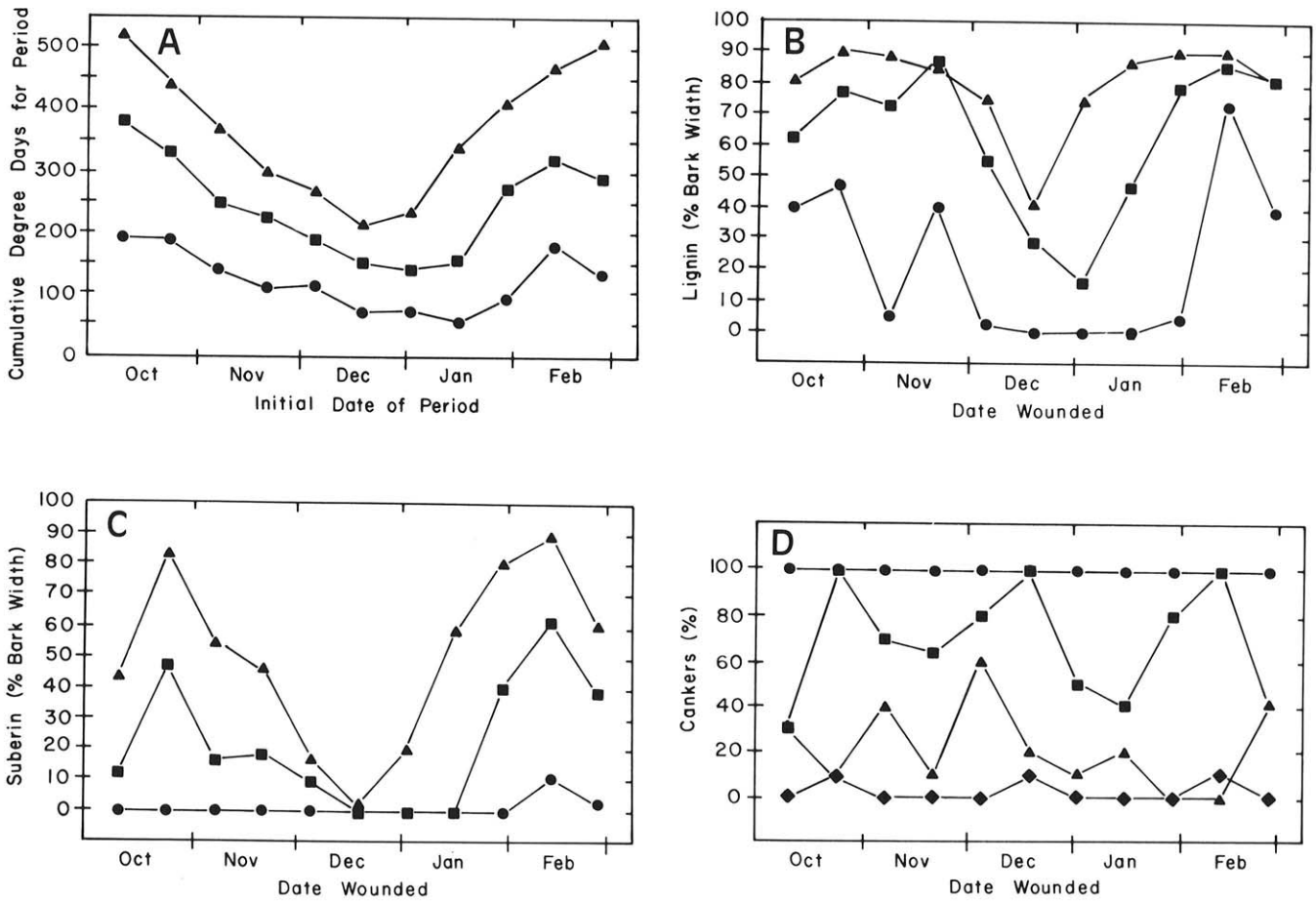

Fig. 6. A, Cumulative degree days for 2-, 4- and 6-wk periods from October 1984 through February 1985 for Colusa, CA. The developmental threshold was assumed to be 0 C. • = 2-wk period; ■ = 4-wk period; ▲ = 6-wk period. **B,** Lignification in wounded inner bark of almond trees in an orchard in Colusa County, CA, in fall 1984 and winter 1985. The percent bark width was determined by measuring the amount of the inner bark from the cambium to the outer periderm staining positive for lignin with phloroglucinol-HCl. • = 2-wk-old wounds; ■ = 4-wk-old wounds; ▲ = 6-wk-old wounds. **C,** Suberization of wounded inner bark of almond trees in an orchard in Colusa County, CA, in fall 1984 and winter 1985. The percent bark width was determined by measuring how much of the inner bark from the cambium to the outer periderm stained positive for suberin with Sudan black B. • = 2-wk-old wounds; ■ = 4-wk-old wounds; ▲ = 6-wk-old wounds. **D,** The percentage of aged wounds inoculated with *Phytophthora syringae* resulting in cankers in almond trees in an orchard in Colusa County, CA, in the fall 1984 and winter 1985. • = fresh wounds; ■ = 2-wk-old wounds; ▲ = 4-wk-old wounds; ◆ = 6-wk-old wounds.

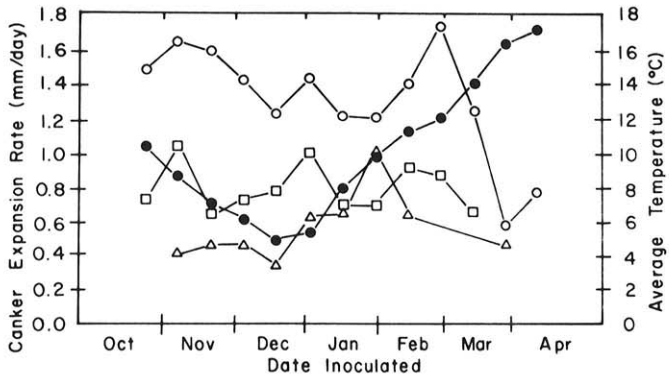

Fig. 7. The mean expansion rate of cankers resulting when aged wounds in almond trees in an orchard in Colusa County, CA, were inoculated with *Phytophthora syringae* in fall 1984 and winter 1985. For the calculation of mean canker expansion rate only inoculations resulting in cankers were included. o = fresh wounds; □ = 2-wk-old wounds; Δ = 4-wk-old wounds; • = mean temperature (C) for 6-wk periods.

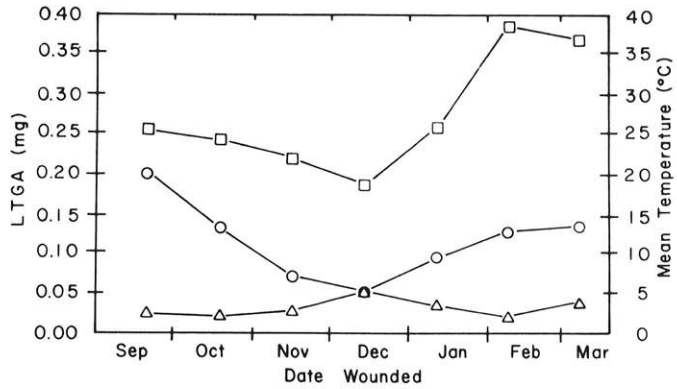

Fig. 8. The formation of lignin as measured as ligninthioglycolic acid (LTGA) in 4-wk-old wounded and nonwounded inner bark of almond trees in an orchard in Yolo County, CA, in fall 1985 and winter 1986. The $LSD_{0.05}$ for LTGA in 4-wk-old wounds was 0.05 mg. □ = 4-wk-old wounds; Δ = nonwounded; o = mean temperature (C) for 4-wk period.

Cankers in walnut trees caused by *Phytophthora citricola* also expanded faster in excised stems than in intact stems (15). All almond bark wounds in excised branches kept at 12 C or below for up to 6 wk and inoculated with *P. syringae* resulted in cankers (Fig. 2), whereas only 50% of the wounds in potted trees kept at 6 C for 4 wk resulted in cankers (Fig. 1). The convenience of using excised branches may make their use in wound response research useful. However, there is a problem in maintaining the excised branches for the duration of experiments lasting several weeks, and the results obtained with excised branches may not accurately predict how orchard trees respond to wounding or infection.

In general, the results from the almond orchard experiments concerning wound-induced lignification and suberization agreed with those obtained in potted trees and excised twigs. Wound periderm formation in apple trees in the orchard (23) and wound response in the barks of several conifer species in the forest (17) have been observed to be much slower in winter than in summer. In our histological study, the formation of lignin (Fig. 6B) and suberin (Fig. 6C) was strongly inhibited in orchard trees when temperatures were low during the winter and corresponded to the accumulated degree days (Fig. 6A). However, the resistance to infection by *P. syringae* of 2- and 4-wk-old wounds did not show any consistent relationship to temperature during winter in orchards (Fig. 6D), even though a significant effect of temperature on resistance development had been observed in wounded potted trees under more controlled conditions (Fig. 1). In the orchard experiments using the TGA assay, substantial amounts of LTGA were detected in 4-wk-old wounds even in the coldest period of winter (Fig. 8) and, although there was less wound periderm formation during the coldest period of winter, perhaps it was sufficient to give some resistance to the wounds. As was observed with potted trees, resistance to *P. syringae* in aged bark wounds of orchard trees was observed as smaller cankers (Fig. 7). Because throughout the fall and winter almost all inoculated 6-wk-old bark wounds had become immune to infection by *P. syringae* (Fig. 6D), any control measure directed at wounds would only have to protect the wounds from infection for about 6 wk.

There is evidence that some trees are susceptible to certain *Phytophthora* species only during certain seasons of the year. When potted walnut trees maintained in a lathhouse were wounded and inoculated monthly with *P. citricola* and then kept in a growth chamber at a constant temperature, there was a significant difference in the susceptibility of the trees during different months of the year, with the longest cankers occurring in May (15). Substantially larger cankers formed during April and May when wounded apple trees were inoculated with *Phytophthora cactorum* (21). Seasonal variations have been observed in the moisture content and in the amounts of starch and other carbohydrates, lipids, proteins, and aromatic constituents in the barks of trees (22). Several of these factors could be important in affecting susceptibility of trees to *Phytophthora* species during different periods in the year. Although during summer *P. syringae* will not infect apple trees (21) or almond trees (5), this is probably due to the sensitivity of the mycelium to high temperatures (5) and not due to changes in the physiology of the tree. In the present study, as temperatures rose in March through May, canker expansion in inoculated fresh wounds slowed (Fig. 7) probably because *P. syringae* does not grow at temperatures above 25 C (5). There was no indication that formation of lignin or suberin in almond bark wounds were inhibited during the spring and although in fact, both increased (Figs. 6B and C and 8), there was not a corresponding increase in resistance as measured by prevention of canker formation (Fig. 6D). Weak points in an established wound periderm through which fungi could penetrate into the healthy bark have been observed in apple (23), peach (1), and almond trees (6, *unpublished*). Even if lignification or suberization is not inhibited, it is possible that wounds increase in susceptibility because of weakening of the wound periderm at certain points. For example, when the cambium is active it is more difficult for the wound periderm to extend to the xylem, allowing the fungus to penetrate near the cambium, even though there may be an otherwise well-formed periderm (1). The changes in the bark of trees in the spring are complex (22), and at this time it is not clear which factors would increase and which would decrease susceptibility of bark wounds to canker pathogens.

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