Project No. 86-U6 - Almond Diseases
Canker Complex in Almonds

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Objectives: Complete studies on Phytophthora pruning wound canker and finalize control recommendations. (2) Evaluate peach-almond hybrid rootstock selections for resistance to Phytophthora crown and root rot caused by Phytophthora cactorum. (3) Complete experiments on Ceratocystis canker clean-up procedures. (4) Identify foamy canker bacterium to species and determine conditions necessary for disease development.

Interpretive Summary:

The following recommendations for control of Phytophthora pruning wound canker, caused by Phytophthora syringae, are derived from several years of laboratory and field studies.

1. Cultural control measures. Pruning at a time to avoid the cool, rainy periods favorable for infection in late fall and early winter is advisable. In mature bearing orchards, pruning in early fall would take advantage of the relatively warm weather and rapid development of natural resistance to infection in wounds (about 2 weeks). In young nonbearing trees, damage to limbs being trained to main scaffolds can be severe and delaying pruning until February or March is an option. If infections do occur, damage is not great since canker development ceases with the onset of warm temperatures of late spring. Wounds on Nonpareil remain susceptible for a longer period of time and greater attention may need to be paid to this cultivar. Most other commercially important cultivars are similar in their susceptibility to Phytophthora syringae.

It is important to be able to discriminate Phytophthora pruning wound canker from Ceratocystis canker. The most striking symptom of Phytophthora pruning wound canker is the association with pruning cuts and the amber colored gum and inner bark dieback surrounding the wounds during the spring. Canker surgery during midwinter to contain Ceratocystis could serve to uncover more tissue susceptible to Phytophthora syringae. Delaying surgery until February or March might be a better choice.

2. Chemical protection. In many orchards, altering pruning schedules is impossible. Since pruning wounds generated during the rainy periods of November and December remain susceptible for up to 6 weeks, chemical protection of large pruning wounds to the trunk and lower main scaffolds is an option. In most situations this would apply to wounds in the lower 5-6 feet of the tree. Aliette, an experimental fungicide from Rhone Poulenc, appears promising and is still being tested. However, it is not yet registered for use in almonds. In the meantime, label rates of Kocide will protect wounds but suffers from the problem of being phytotoxic to wounded tissue.

THE DEVELOPMENT OF PRUNING WOUND CANKERS CAUSED BY *PHYTOPHTHORA SYRINGAE* IN ALMOND ORCHARDS AND STUDIES ON THE PROCESSES RELATED TO EXPRESSION OF DISEASE RESISTANCE IN WOUNDED ALMOND BARK.

SECTIONS OF THE DISSERTATION OF MARK DOSTER

1. INCIDENCE, DISTRIBUTION, AND DEVELOPMENT OF PRUNING WOUND CANKERS CAUSED BY *PHYTOPHTHORA SYRINGAE* IN ALMOND ORCHARDS IN CALIFORNIA.

2. QUANTIFICATION OF LIGNIN FORMATION IN ALMOND BARK IN RESPONSE TO WOUNDING AND INFECTION BY *PHYTOPHTHORA* SPECIES.

3. THE EFFECT OF LOW TEMPERATURE ON RESISTANCE TO *PHYTOPHTHORA* PRUNING WOUND CANKER AND ON LIGNIN AND SUBERIN FORMATION IN WOUNDED ALMOND BARK.

4. SUSCEPTIBILITY OF ALMOND CULTIVARS AND STONE FRUIT SPECIES TO *PHYTOPHTHORA SYRINGAE*.

5. CHEMICAL PROTECTION OF PRUNING WOUNDS FROM INFECTION BY *PHYTOPHTHORA SYRINGAE*.

6. THE EFFECT OF TEMPERATURE AND TYPE OF MEDIUM ON OOSPORE PRODUCTION BY *PHYTOPHTHORA SYRINGAE*.

SECTION 1.

INCIDENCE, DISTRIBUTION, AND DEVELOPMENT OF PRUNING WOUND CANKERS CAUSED BY PHYTOPHTHORA SYRINGAE IN ALMOND ORCHARDS IN CALIFORNIA

ABSTRACT

Almond trees in 3 orchards in California were surveyed for pruning wound cankers caused by Phytophthora syringae. In one orchard 23% of all pruning wounds examined had cankers and some sections of the orchard had over 50% of the wounds infected. The number of infections increased linearly as the diameter of pruning wounds increased (r^2=0.97, orchard A; r^2=0.94, orchard B). Pruning wound cankers were frequently observed high in the trees. In orchard A, the percentage of pruning wounds with cankers increased with height, while in orchard B, there was no clear trend. Cankers resulting from inoculations with P. syringae expanded throughout the fall, winter, and spring, but ceased expansion in summer. In the orchards surveyed, there was no sign of canker expansion during or after summer. P. syringae was isolated from 26% of recently fallen almond leaves on the orchard floor in orchard A and from 18% in orchard B. Abundant oospores were formed in the leaves. In orchard C pruning wound cankers occurred most frequently in the parts of the orchards pruned during periods of heavy rainfall. Rainfall at or near the time of pruning appears to be crucial for disease development. It is recommended that pruning should be avoided during periods of heavy rainfall and that large wounds, especially those on scaffolds, should be protected. Research investigating protection of wounds with fungicides is in progress.

INTRODUCTION

Phytophthora syringae (Kleb.) Kleb. has been known to cause crown and collar rot of several species of stone fruit trees in California (13). Recently P. syringae was found to enter through pruning wounds and cause cankers in almond trees (Prunus dulcis (Mill.) D.A. Webb) (1). P. syringae grows and infects well at low temperatures but the

mycelium is killed above 25 C (1). Pruning in almond orchards in California usually takes place from September through February. Pruning wound cankers were observed in winter and spring when the temperatures were relatively low, but seemed to stop expansion in late spring or early summer as the temperature rose (1).

P. syringae has been observed to cause above ground diseases with several other tree species. In Italy an aerial fruit rot of peaches was caused by the zoospores of P. syringae in the irrigation water being dispersed by sprinklers (2). Apple fruit in England was found to be infected by P. syringae while still on the tree resulting in a fruit rot (16). Brown rot of twigs, leaves, blossoms, and fruit on citrus trees in California is caused by several Phytophthora species including P. syringae (6).

Although pruning wound cankers were present in many almond orchards in California, little was known about the disease since it has only been observed recently. Surveys were conducted in several bearing almond orchards in order to better understand disease incidence, distribution, and development. A preliminary report of this work has been published (5).

MATERIALS AND METHODS

Three bearing almond orchards were surveyed (Table 1). Orchards A and B are in Colusa Co. and orchard C in Yolo Co., CA. During the summer, drip irrigation was used in orchard A and sprinkler irrigation in orchards B and C. Orchards A and B were pruned in December 1983 and orchard C in October 1984 through January 1985. The orchards were surveyed the following springs. Using PVP medium (1), P. syringae was isolated from pruning wound cankers in all three orchards. In orchards A and B, for all trees examined, the diameter and height from the ground for all pruning wounds were measured. In orchard C only the number of pruning wound cankers were counted for each tree. In orchard C the progress of the pruners through the orchard was noted

throughout the fall. The cultivars in each orchard and the number of trees and pruning wounds surveyed are presented in Table 1.

Eighty-three leaves on the orchard floor were collected throughout orchards A and B in the falls of 1983 and 1984. The leaves were kept in an ice chest until isolations were performed the same day. Some of the leaves were surface sterilized with 95% ethanol. Isolations were made in PVP medium (1) using ten 5 x 5 mm pieces from each leaf including the petiole.

In another orchard in Colusa Co., CA, fresh wounds were inoculated with P. syringae from November, 1983 to April, 1984. Branches (1-2 cm diamteter) were wounded by making cross-sectional cuts with pruning shears and inoculated by placing one 9 mm diameter corn meal agar plug containing mycelium of P. syringae (isolate F-79) from the colony margin on each wound which was then wrapped with parafilm. At various times throughout the winter and spring the length of discoloration in the inner bark from the point of inoculation was measured.

RESULTS

All of the pruning wound cankers observed in all orchards showed the symptoms typical of cankers caused by Phytophthora syringae (Fig. 1). Of all three orchards, orchard B had the highest disease incidence with about 23% of the pruning wounds surveyed having cankers and for some parts of the orchard the disease incidence was over 60% (Table 1). For many of the trees in orchard B, all or most of the pruning wounds developed cankers (Fig. 2). In orchard A the cultivar Nonpareil had a substantially higher percentage of cankers than Ne Plus Ultra, whereas in orchard B both cultivars had about the same disease levels (Table 1). Although in orchard A the percentage of pruning wounds with cankers in Nonpareil ranged from 0 to 40% for groups of 10 trees (Fig. 3A), the runs test on the distribution of cankers did not indicate nonrandomness (Table 2). However, for orchard B the runs tests indicated that cankers were distributed nonrandomly or clustered for all three diameters (2, 3, and 4 cm) for

the whole orchard. In orchard B there was less disease in the southernmost third of the orchard (Fig. 3) and when the orchard was divided into thirds the runs tests no longer indicated a strongly nonrandom distribution (Table 2).

As the diameter of the pruning wound increased, the percentage with cankers increased also. The number of infections was estimated using the multiple infection transformation (8). The estimated number of infections increased linearly as the diameter increased in both orchard A and B (Fig. 4). Only the data from the northern third of orchard B are presented in Figure 4, because of the clustering of cankers in the orchard.

In orchard A the percentage of wounds with cankers increased as height increased (Fig. 5). However, in orchard B there was no consistent trend. For example, the percentage cankers for wounds of 4 cm diameter increased substantially with height, but decreased substantially for wounds of 5 cm diameter (Fig. 5). In orchard B, cankers were observed at heights greater than 5 m.

The year following the surveys in orchards A and B no new pruning wound cankers were observed and there were no signs, such as abundant gumming, of the old cankers being active. In orchard A the distance from pruning wound to the canker margin for 9 cankers was measured as 109 mm in the fall 1984 and was still 109 mm in late spring 1985, indicating that the cankers had ceased expansion. An inactive pruning wound canker in a young tree is presented in Figure 6.

P. syringae was commonly isolated from fallen almond leaves on the orchard floor. P. syringae was isolated from 26% of the leaves gathered from orchard A in the fall of 1983 and 1984, and from 18% of the leaves from orchard B in the fall of 1984. The leaves when gathered were in various conditions of decay. P. syringae was isolated from 17% of the leaves which were completely green as though they had just fallen off the tree, from 10% of the leaves showing some yellowing, and from 50% of the leaves showing some browning. Pythium spp. were frequently isolated from leaves with some

yellowing or browning. P. syringae was never isolated from leaves still attached to the tree. Oospores resembling those of P. syringae (1) were observed in 78% of the leaves from which P. syringae was isolated but in only 4% of the other leaves.

The disease incidence in orchard C was correlated to rainfall (Fig. 8). The part of the orchard with the highest disease incidence (0.62 cankers per tree) was pruned during November, the period of the highest mean daily rainfall (9.6 mm/day). The part of the orchard that was pruned during a period of little rain (0.9 mm/day) had very few pruning wound cankers (0.04 cankers per tree).

Cankers resulting from wound inoculations with P. syringae expanded well throughout the fall, winter, and spring with the mean canker expansion rate ranging from 1.0 to 1.9 mm/day (Table 3). The slowest canker expansion rate was during late spring and no canker expansion was observed during summer.

DISCUSSION

In the orchards surveyed the incidence of pruning wound cankers caused by P. syringae was high (Table 1), especially in some sections of the orchards (Fig. 3). Although these almond orchards were chosen for their high disease incidence, there were many other orchards observed during the course of this study with similar disease incidences. In media P. syringae grew well at low temperatures but the mycelium was killed above 25 C (1). Cankers produced from artificial inoculation expanded throughout the fall, winter, and spring, but ceased expansion during summer (Table 3). In orchards A and B there were no signs of canker expansion during or after summer. It is difficult to isolate P. syringae from pruning wound cankers in early summer (1). So, even though disease incidence can be high and the cankers expand relatively fast, the direct damage to the tree as a result of these cankers was limited since the cankers were annual. However, the damage to developing scaffolds of young almond trees can be serious (Fig. 6).

The results of these surveys indicate the difficulty in comparing the susceptibility to P. syringae of various almond cultivars by observing disease incidence in the orchard. In orchard B considering the percentage of pruning wounds with cankers, Price had the lowest percentage (Table 1). However, by examining the number of cankers per tree, Price had substantially more cankers than Nonpareil or Ne Plus Ultra, because Price had more pruning cuts. In orchard A there was a substantially lower percentage of pruning wounds with cankers for Ne Plus Ultra than for Nonpareil, whereas in orchard B, Ne Plus Ultra had a slightly higher percentage than Nonpareil. Because disease severity is influenced by wound diameter, rainfall, inoculum levels, and amount of pruning, observations of disease levels in orchards could be misleading in discerning relative susceptibility of almond cultivars. In inoculation studies, there was little difference in canker development in fresh wounds among the major almond cultivars, but it was observed that wounds in Nonpareil remained susceptible to P. syringae longer (3).

P. syringae was commonly isolated in this study from recently fallen almond leaves on the orchard floor. Fallen apple leaves from orchards in England were found to contain oospores of P. syringae (9), which remained viable for 32 months in the orchard (10). It was found that P. syringae rapidly infected freshly abscised apple leaves and it was concluded that substantial populations of P. syringae in the apple orchards were maintained by the development of P. syringae in fallen apple leaves (11). Similarly in our study, P. syringae was isolated from recently abscised almond leaves and abundant oospores were observed in almond leaves from which P. syringae was isolated (Fig. 7). These oospores could function as the means of survival for the fungus through the hot summers. The abundance of fallen almond leaves on the orchard floor in autumn during the heavy rainfall in California and the high percentage of these leaves containing P. syringae (18 to 26%) indicate that colonization

of the fallen almond leaves by P. syringae could be important for inoculum maintainence and increase in almond orchards in California.

The estimated number of infections of pruning wounds by P. syringae increased linearly as the diameter of the pruning wound increased in both orchard A and B (Fig. 4). However, the surface area of the pruning wound does not increase linearly but according to the square of the diameter. For pruning wounds in apricot trees attacked by Eutypa armeniacae Hansf. & Carter, which invaded the xylem of the tree through pruning wounds, it was found that when the wound diameter was doubled the estimated infections was not doubled but increased 4.8 times or approximately the square of the increase in diameter (15). However, P. syringae seemed to be restricted to the inner bark and did not penetrate deeply into the xylem (personal observation). If infection only occurred in the exposed inner bark of the pruning wound then the surface area of the pruning wound would not be important but only the circumference, which increases linearly as the diameter increases. In general, the relationship between disease incidence and diameter of wound depends on which of the exposed wounded tissue can be invaded by the pathogen.

Pruning wound cankers were very frequent high in the tree. In orchard A there was actually a higher percentage of cankers higher up in the tree, whereas, in orchard B there was approximately the same percentage (Fig. 5). With apple fruit rot caused by P. syringae infection occurred mainly on fruit within 50 cm of the soil, although infected fruit have been observed at heights over 1 m (16). In citrus orchards in California, brown rot caused by several Phytophthora species was usually found on fruit within 1 m of the ground, although occasionally P. hibernalis was isolated from diseased fruit up to 3 m in the tree (6,12). In these cases where the inoculum was from the soil there was a definite decrease in disease as height increased. It was found that infections in cocoa pods by P. palmivora were mainly near the soil early in the year when inoculum came from the soil, but later, high levels of infection

occurred high in the trees when inoculum came from previously infected pods (14). Although an aerial fruit rot of peaches caused by P. syringae was found to be dispersed by sprinklers (2), irrigation practices seem not to be involved in the development of pruning wound cankers in almond trees. Orchard A used drip irrigation, and although orchards B and C used sprinkler irrigation, no irrigation was done during the fall and winter when infection occurred. Pruning wound cankers caused by P. syringae may be common high in the tree because inoculum was coming from previously infected pruning wounds, there were some special means of dispersal, or wounds high in the tree are more susceptible to infection.

The distribution of cankers in orchard C seemed strongly affected by the amount of rainfall, since only the parts of the orchard pruned during heavy rainfall had many cankers (Fig. 8). In orchards A and B there was almost 200 mm of rainfall during the month the trees surveyed were pruned, which was 2.4 times the normal rainfall (the mean of 3 nearby weather stations, NOAA). Cankers have been observed in pruning wounds made throughout the fall and winter in various almond orchards (personal observation) indicating that the time of year was not as important as the amount of rainfall for disease development. For citrus brown rot, the propagules of Phytophthora species are believed to be dispersed to the fruit on the tree by wind-blown rain (6,7). In orchard A cankers in Nonpareil were distributed randomly (Table 2), although there was a substantial difference between cultivars in percentage of pruning wounds with cankers. In orchard B the cankers in Nonpareil were not distributed randomly (Table 2), but most of the nonrandomness derived from the southern third of the orchard having very few cankers. No cultural practice or other factor seems to explain this distribution except perhaps inoculum distribution.

At this time the mechanism for dispersal of P. syringae to the pruning wound is unknown. Field studies on pruning wound cankers are difficult because although the disease is severe in some orchards, its occurrence is sporadic. The fungus was

abundant in the leaf litter of orchards A and B, which would seem to be an inoculum source. However, most aerial diseases with an inoculum source in the soil have the highest disease incidence closest to the soil, whereas this is not so with pruning wound cankers caused by P. syringae (Fig. 5). Another possibility is the transmission of the inoculum by pruning equipment. Phytophthora canker of cacao was easily transmitted by pruning equipment, but the disease still occurred when pruning equipment was sterilized (17). In preliminary experiments, pruning shears dipped in a zoospore suspension transmitted P. syringae (unpublished results), but the extent that transmission by pruning equipment may be involved in disease development in almond orchards is unknown.

A grower may want to only protect the larger pruning wounds generated prior to or during favorable infection periods because large wounds were more likely to develop P. syringae cankers. Favorable infection periods occurred during the late fall and winter during periods of heavy rainfall. Preliminary work on protecting wounds from infection by P. syringae using fungicides have been done (4). Since pruning wound cankers caused by P. syringae can be very damaging in young almond trees (Fig. 6) causing the loss of developing scaffolds, the grower may wish to avoid the disease by pruning young trees in the spring or summer.

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Table 1. The number of trees and pruning wounds examined and the incidence of pruning wound cankers caused by Phytophthora syringae in the almond orchards A, B, and C.[y]

		Number observed		Pruning wounds	
Orchard	Cultivar	Trees	Pruning wounds	with cankers (%)	Cankers per tree
A	Nonpareil	224	785	12.4	0.43
	Mission	56	119	4.2	0.09
	NePlusUltra	28	105	3.8	0.14
B	Nonpareil	313	1186	23.2	0.88
	NePlusUltra	86	408	25.5	1.21
	Price	86	541	22.4	1.41
C	Nonpareil	634	------[z]	------[z]	0.38
	Butte	319	------	------	0.30
	Price	301	------	------	0.23

[y] Orchards A and B were pruned in December 1983 and orchard C in October 1984 through January 1985. The orchards were surveyed the following springs.

[z] In orchard C trees were examined for number of cankers only and individual pruning wounds were not measured or counted.

Table 2. Results of runs tests for random distribution of cankers caused by Phytophthora syringae for pruning wounds of the same diameter in whole and parts of orchards A and B.[y]

Orchard	Part of orchard	Wound diameter (cm)	Canker (%)	Runs test probability[z]
A	Whole	2	10.0	0.708
		3	11.9	0.319
		4	15.3	0.806
B	Whole	2	19.4	0.000 ***
		3	19.3	0.049 *
		4	27.9	0.000 ***
	Northern third	2	16.2	0.058
		3	27.2	0.374
		4	30.6	0.015 *
	Central third	2	33.3	0.033 *
		3	22.1	0.177
		4	38.8	0.136
	Southern third	2	3.7	0.924
		3	4.7	0.872
		4	5.7	0.478

[y] Runs tests were performed on pruning wounds of a specific diameter in Nonpareil trees down one row and then up the next row, etc.

[z] Significance level for one-tailed runs test for random distribution. * indicates significantly ($P<0.05$) nonrandom or clustered. *** indicates highly signifcantly ($P<0.001$) clustered.

Table 3. Canker expansion rate in Nonpareil almond trees resulting from inoculations with Phytophthora syringae for different inoculation dates in fall 1983 through spring 1984.[w]

	Mean canker expansion rate (mm/day)			
	Date cankers measured			Canker length (mm)
Date inoculated	Feb. 2	March 29	June 15	March 29
Nov. 8	1.3	1.1[x]	-----[y]	161
Dec. 6	1.5	1.2	-----	135
Jan. 5	1.5	1.4	-----	115
Feb. 2	-----	1.5	-----	82
Mar. 1	-----	1.9	1.2	54
Apr. 12	-----	-----	1.0[z]	----

[w] The length was determined by measuring the furthest range of discoloration in the inner bark from the inoculated wound. The average monthly temperatures from November 1983 to June 1984 were 11, 9, 8, 9, 14, 15, 21, and 23 C (mean of four nearby weather stations, NOAA).

[x] The $LSD_{.05}$ was 0.3 mm/day for the cankers measured March 29.

[y] ----- indicates not determined.

[z] Mean canker size for inoculations made on April 12 was 65 mm when measured on June 15 and 65 mm when measured on October 24, confirming that cankers had ceased expansion in June.

Figure 1. Typical pruning wound canker caused by Phytophthora syringae as observed in the spring in almond orchard B. The fungus entered through the pruning wound (arrow). Typically, several large amber gum balls are associated with the canker. The outer bark has been removed from the lower part of the canker to show the typical zonate canker margin and the necrosis in the inner bark.

Figure 2. Two pruning wounds (arrows) in an almond tree in orchard B from which cankers caused by Phytophthora syringae developed. In orchard B disease incidence was high with all of the pruning wounds in many trees developing cankers.

Figure 3. Orchard maps showing the percentage of pruning wounds with cankers for groups of 10 trees (chosen for presentation purposes) in the same row in orchards A and B. Rows run from the top of the page (north) to the bottom (south). Only data for pruning wounds of diameter between 2 and 6 cm, inclusive, are presented. The cultivar for the row is presented at the top of the row by a capital letter. N represents Nonpareil, M Mission, U Ne Plus Ultra, and P Price.

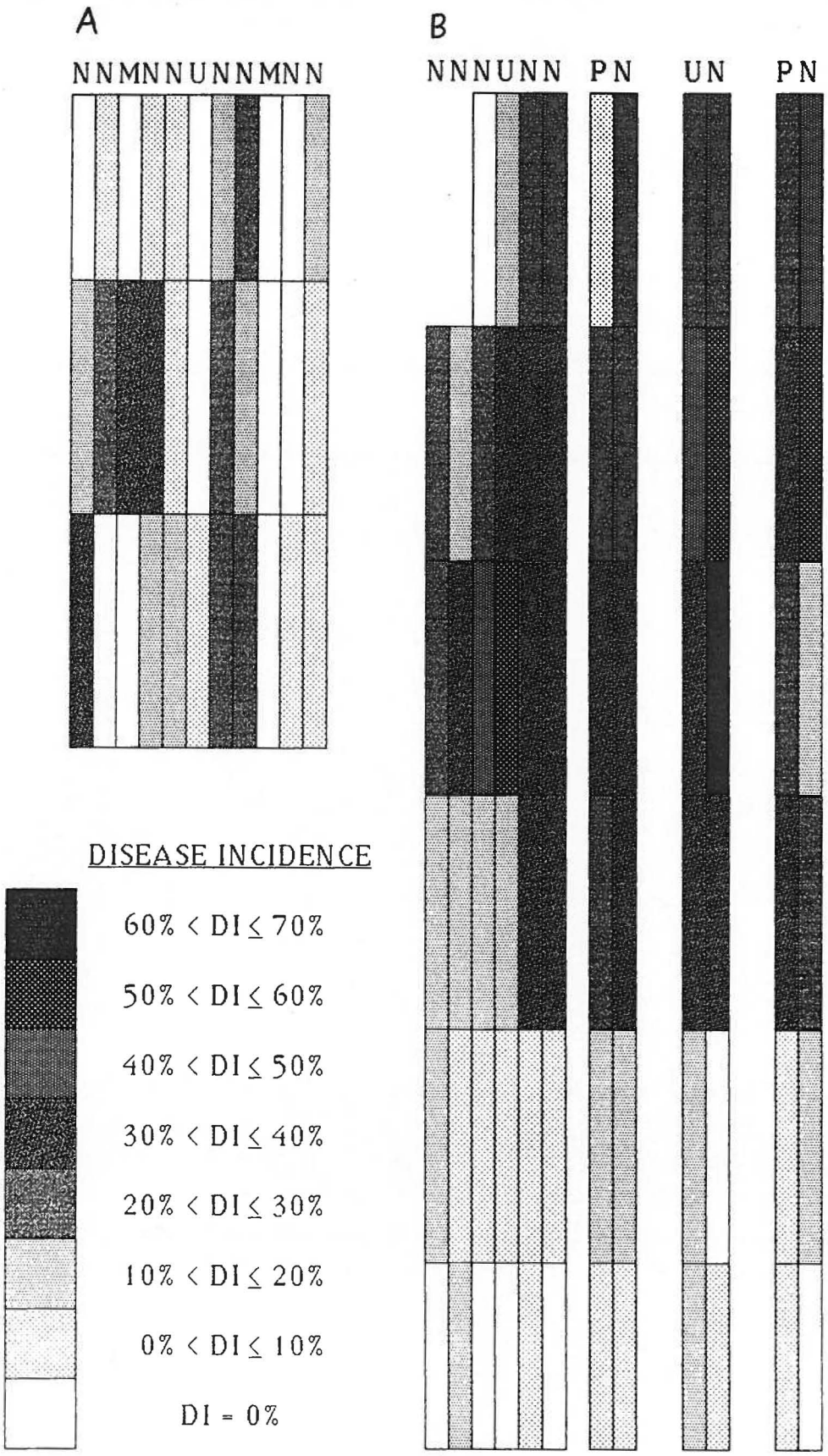

A
NNMNNUNNMNN
B
NNNUNN
PN
UN
PN
DISEASE INCIDENCE
60% < DI ≤ 70%
50% < DI ≤ 60%
40% < DI ≤ 50%
30% < DI ≤ 40%
20% < DI ≤ 30%
10% < DI ≤ 20%
0% < DI ≤ 10%
DI = 0%

Figure 4. Number of infections by Phytophthora syringae per 100 wounds as estimated by the multiple infection transformation for pruning wounds of various diameters in Nonpareil trees in orchard A and the northern third of orchard B. ○ orchard A, □ orchard B.

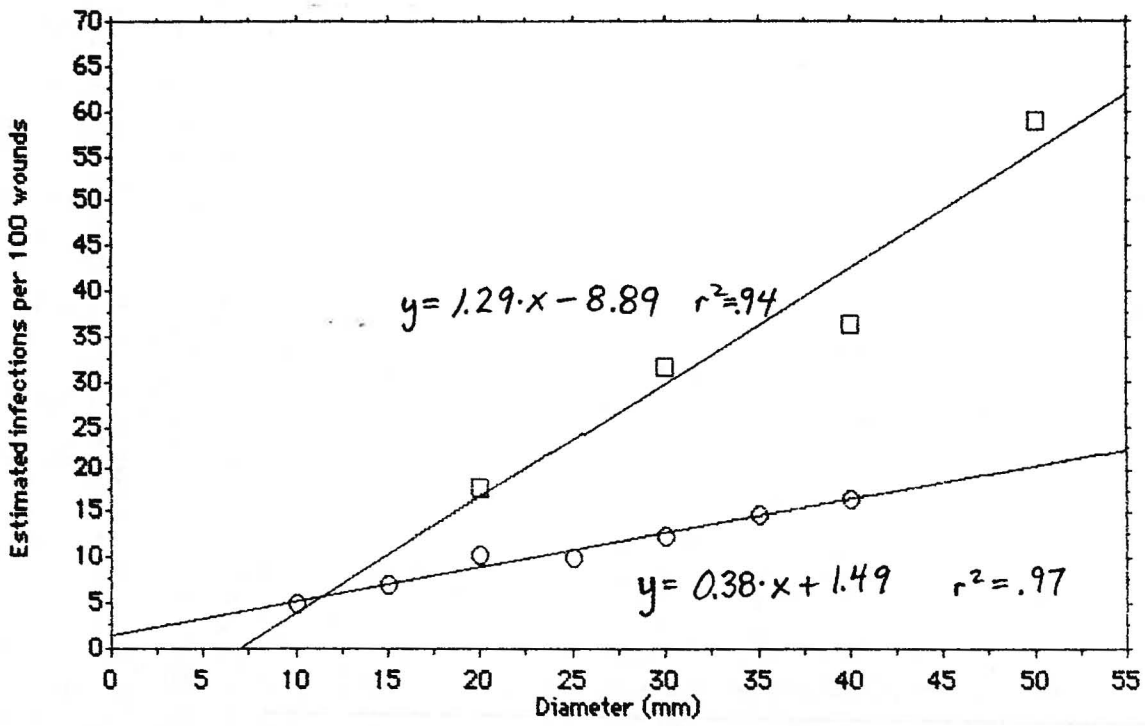

Estimated infections per 100 wounds
Diameter (mm)
y= 1.29·x − 8.89 r²=.94
y= 0.38·x + 1.49 r²=.97
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70

Figure 5. The percentage of pruning wounds with cankers at various heights from the ground for different pruning wound diameters for Nonpareil trees in orchards A and B. Only those data points representing more than 15 pruning wounds are presented. The data points represent on the average 26 pruning wounds for orchard A and 46 for orchard B. Orchard A: ○ 2.5 cm diameter, □ 3 cm; orchard B: ● 2 cm, ■ 3 cm, ▲ 4 cm, and ◆ 5 cm.

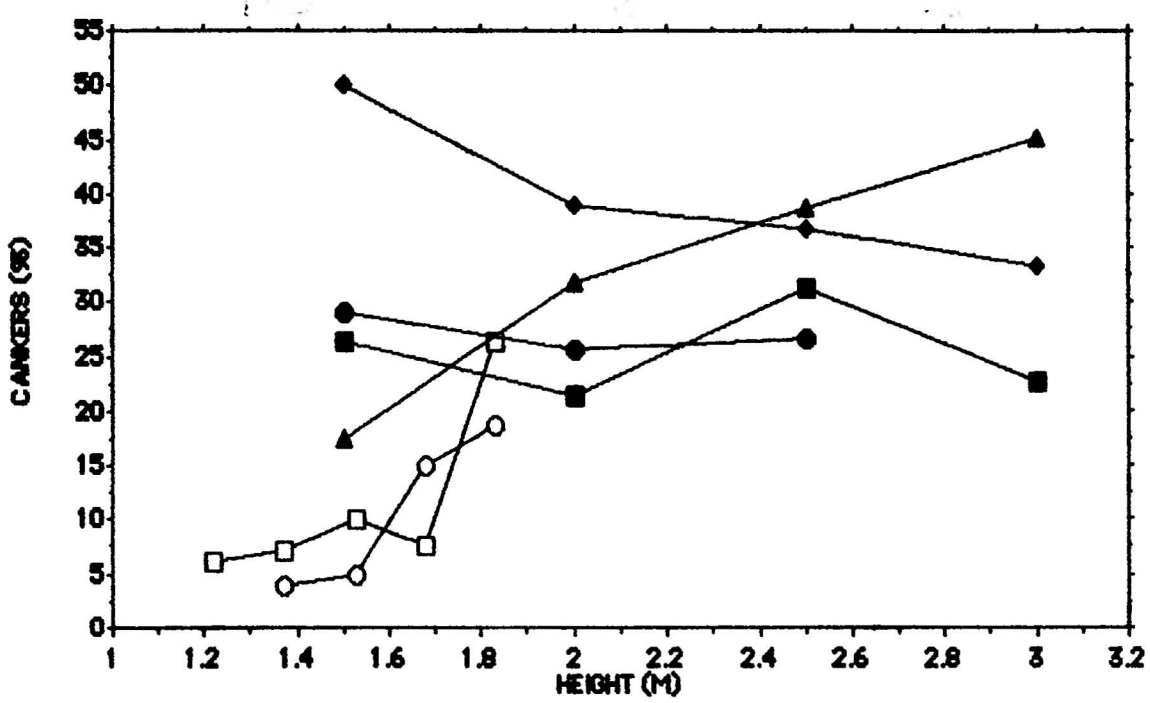

CANKERS (%)
55
50
45
40
35
30
25
20
15
10
5
0
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
3
3.2
HEIGHT (M)

Figure 6. An inactive pruning wound canker caused by Phytophthora syringae in a scaffold of a young almond tree. The canker is about one-year-old and probably ceased expansion in late spring or early summer when the high temperatures killed the mycelium of the fungus.

Figure 7. Abundant oospores (small brown circles) in an abscised almond leaf gathered from the ground of orchard A and from which Phytophthora syringae was isolated. Bar length represents 0.1 mm.

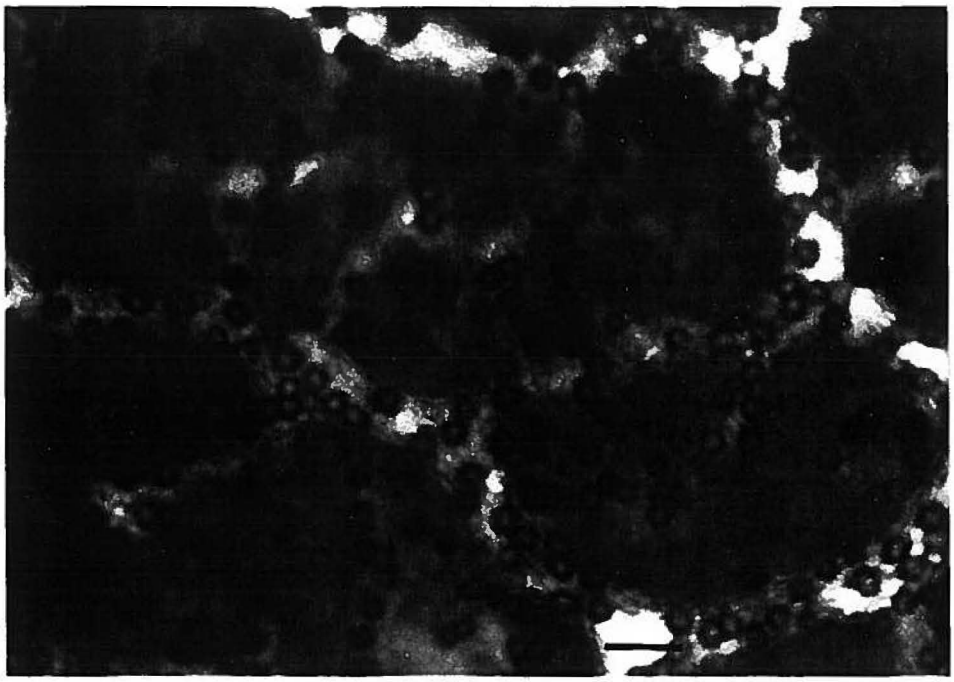
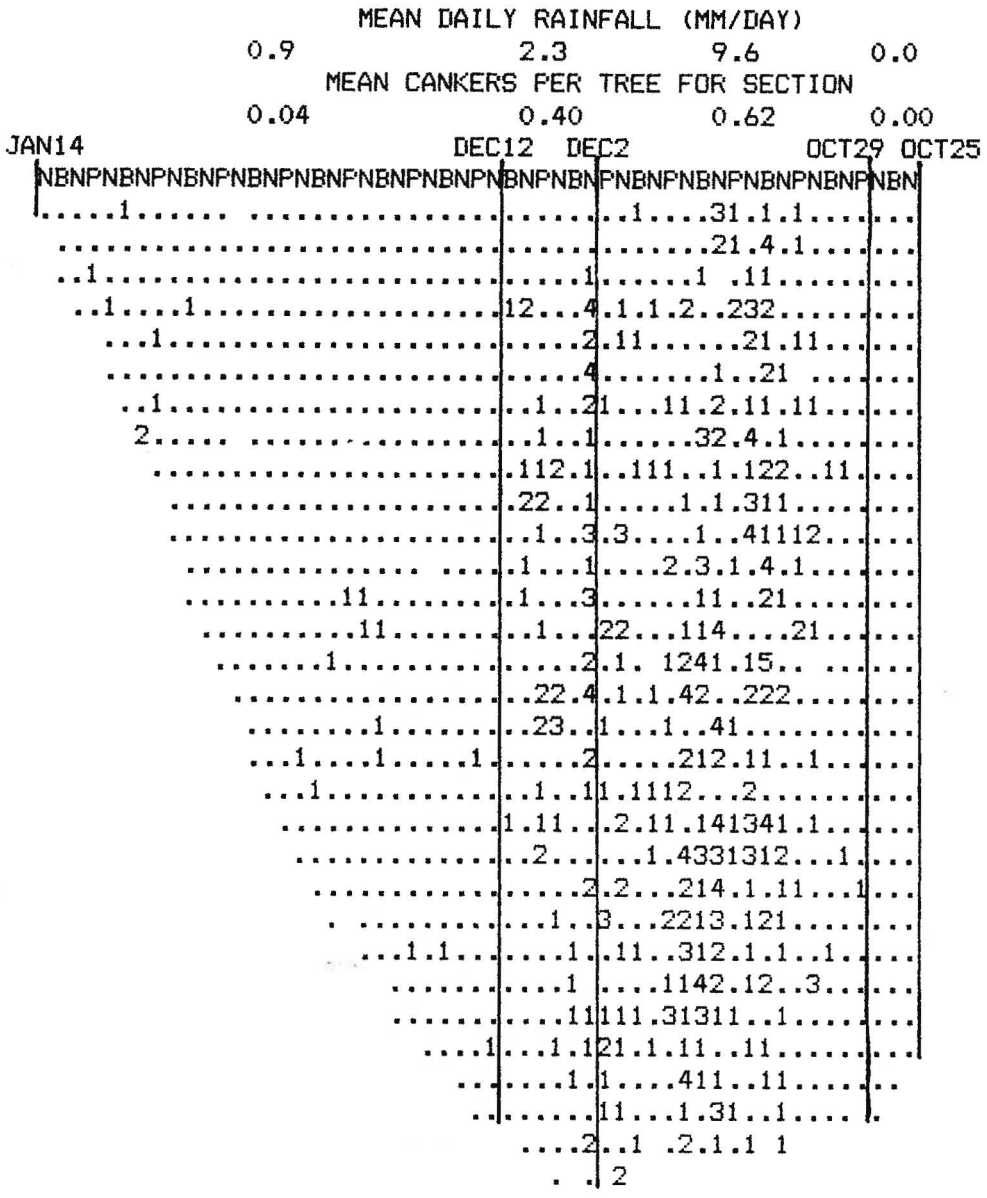


Figure 8. The number of pruning wound cankers per tree in orchard C. The cultivar for each row is represented by N, B, and P which stands for Nonpareil, Butte, and Price, respectively. The '.' represents trees with no cankers. The mean daily rainfall, mean cankers per tree, and inclusive dates for pruing for each of the four sections of the orchard are presented. Pruning was performed on rows which run from top to bottom starting with the rows at the right and moving left.


MEAN DAILY RAINFALL (MM/DAY)
0.9
2.3
9.6
0.0
MEAN CANKERS PER TREE FOR SECTION
0.04
0.40
0.62
0.00
JAN14
DEC12
DEC2
OCT29
OCT25
NBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBNPNBN

SECTION 2.

QUANTIFICATION OF LIGNIN FORMATION IN ALMOND BARK IN RESPONSE TO WOUNDING AND INFECTION BY PHYTOPHTHORA SPECIES

ABSTRACT

A quantitative assay for lignin using thioglycolic acid was adapted for use in investigating lignification in inner bark tissue of almond trees in response to wounding and infection by several Phytophthora species. One-day-old wounds in potted trees kept at 25 C had 71% more ligninthioglycolic acid (LTGA) than fresh wounds. In these trees, LTGA increased linearly (r^2=.98) over the 9 day period observed. Two-day-old bark wounds made in orchard trees during early October 1985 had 194% more LTGA than fresh wounds. In the orchard trees, LTGA of wounded tissue increased linearly (r^2=.95) over the 3 week period observed. Three-day-old wounds treated with chitosan contained 114% more LTGA than nonwounded inner bark tissue, whereas LTGA in water treated wounds increased by 57%. Cupric oxide oxidation of LTGA from 2-wk-old wounds yielded significantly more p-hydroxybenzaldehyde, vanillin, and syringaldehyde than from nonwounded tissue, and confirmed that lignin was produced in response to wounding. The mean lignin detected in variously aged wounds was negatively correlated (r=-1.00) to the mean length of cankers resulting after inoculation of similarly aged wounds with P. syringae. In cankers caused by P. syringae substantially more LTGA was detected in the inner bark than in nearby healthy tissue. Healthy tissue 0.9 cm from the canker margin had 34% more LTGA than healthy tissue 15 cm from the margin. Wounds inoculated with P. syringae, P. hibernalis, and P. cactorum had substantially more LTGA 3 days after inoculation than noninoculated, whereas wounds inoculated with P. infestans, a nonpathogen of almond, had the same amount of lignin as the noninoculated wounds. The deposition of lignin

in almond inner bark appeared to be a response to wounding and infection by pathogens.

INTRODUCTION

In studies of fungal canker diseases of almond trees (Prunus dulcis (Mill.) Webb) it was observed that pruning wounds eventually become immune to infection by Phytophthora syringae (Kleb.) Kleb. (6,9). During preliminary histological studies with almond inner bark, soon after wounding a general dieback of the cells near the wound surface was observed, which was followed by the formation of a lignified zone and later a suberized wound periderm extending from the outer bark to the xylem (Fig. 1) (personal observation). The formation of lignin and of a wound periderm around tree bark wounds have been reported in several histological studies (5,9,15,17). Lignin formation has been suggested as a mechanism of resistance to ingress by plant pathogens (25). The suberized periderm formed around bark injuries appears to provide a barrier to infection by canker pathogens (5,15,22).

During initial investigations of the role of lignification in development of resistance we found it difficult to quantify wound-induced lignin formation using histological techniques and were concerned that other phenolics besides lignin caused the stain reaction. A routine assay was needed which would be quantitative and specific for lignin and permit the analysis of a large number of samples. An important long-established method of isolating and characterizing lignin from wood has involved thioglycolic acid (10). Thioglycolic acid reacts readily with the α-alkoxy function in lignin to give ligninthioglycolic acid derivatives (26) and the ligninthioglycolic acid can be extracted from the tissue with base. This method has been adapted to quantify lignin in tissue cultures (27) and potato tubers (12).

The objectives of our research were to adapt the thioglycolic acid assay for determination of lignin in inner bark tissue of almond trees and then to use the assay to determine the time-course of wound-induced lignification. Additionally, the assay

was used to investigate the formation of lignin in response to fungi in the genus Phytophthora and the effect of chitosan, an elicitor of lignin in wheat (18), on wound lignification in almond bark.

MATERIALS AND METHODS

General. The almond cultivar Nonpareil was used in all experiments. Potted trees were maintained in a lathhouse until needed for experiments. All fungi used were maintained on amended lima bean agar (ALBA) (7) or V-8 agar at 15 C. For inoculations the fungi were grown on cornmeal agar (CMA) or ALBA. All experiments using potted trees were performed in a growth chamber with a 12 hr light period (10 Wm^{-2}) kept at 25 C unless otherwise noted. In all experiments after wounds had aged, whole branches were cut out, kept in an ice chest during transport to the lab, and the wounded tissues were prepared for analysis the same day.

Thioglycolic acid (TGA) assay. Unless indicated otherwise the following procedure was used for the experiments. The outer bark was carefully peeled or cut away from the inner bark. The inner bark tissue was separated from the xylem and extracted in 4 changes of methanol over 24 to 48 hr. The samples were finally dried overnight in a vacuum desiccator and then weighed. To each tissue sample, 5 ml of 2 N HCl containing 0.5 ml thioglycolic acid (Sigma) was added and the capped tubes placed in a 95 C oven for 4 hr. The acid was drained off and the samples rinsed in 5 ml water. The samples were then extracted with 5 ml of 0.5 N NaOH for 18 hr at room temperature. The NaOH extracts were decanted into 12 ml conical centrifuge tubes. The samples were rinsed with 5 ml of water and this water was then added to the NaOH extract. The extracts were acidified with one ml of concentrated HCl and then placed at 5 C for 4 hr. The precipitate was collected by centrifugation for 10 min in a clinical centrifuge at the second highest setting. The acidic supernatant was drained off and the pellet resuspended in 0.5 M NaOH (the amount depending on amount of ligninthioglycolic acid

present). The resuspensions were centrifuged to remove any undissolved matter. The absorbances of the supernatants were then measured at 280 nm with a spectrophotometer. This absorbance was used as a measurement of relative lignification (12,27) or of the concentration of ligninthioglycolic acid (LTGA) by using the absorptivity value of 11.9 $g^{-1}L$ cm obtained below.

Development of TGA assay for use with the inner bark. Branches of about 1 cm diameter on bearing almond trees in an orchard near Arbuckle, CA were wounded by making cross-sectional cuts with pruning shears in March. Six weeks later, 4 x 4 mm areas were removed from around the wounds and from nonwounded tissue with 3 replications. For half of the samples, the outer bark was not removed; otherwise, the standard TGA assay outlined above was followed.

Branches of about 1 cm diameter on bearing trees in the orchard were wounded by making cross-sectional cuts with pruning shears in April. Six weeks later, 2 x 4 mm areas were removed from around the wounds and from nonwounded tissue of the 4 replications. After methanol extraction and drying, half of the samples were immersed in 5 ml of 0.5 N NaOH for 24 hr, then 1.25 ml of 2 N HCl was added. The liquid was then poured off and the samples rinsed twice with water; otherwise, the standard TGA assay outlined above was followed.

The absorptivity of LTGA derived from the xylem, the outer periderm, and wounded inner bark was determined to obtain a standard curve for LTGA. The outer periderm (approximately 800 mg dry weight per replication) and xylem (approximately 200 mg dry weight per replication) were obtained from 2 cm diameter branches from orchard trees. The wounded inner bark was obtained from about 15 corkborer wounds of 10 mm diameter in orchard tree branches of 4 cm diameter per replication. A 14 mm diameter corkborer was used to remove the wounded tissue. The standard TGA assay was followed for the 3 replications except the quantities were adjusted to account for the larger tissue samples. Also, prior to the acid precipitation the solution was filtered to remove

contaminating tissue, and after the acid precipitation the samples were centrifuged (15 min 7000 rpm), washed in deionized water and centrifuged twice, and then the pellet dried for 3 hr in a 60 C oven and stored in a vacuum desiccator. The resulting LTGA was weighed. A weighed amount of LTGA was then dissolved in 0.5 M NaOH and the absorbance (280 nm) read for various dilutions to obtain the standard curve and the absorptivity.

Cupric oxide oxidation of the LTGA. This procedure was partly adapted from methods developed for lignin analysis in potato tuber disks (12). The main stems of four 2-yr-old potted trees were wounded by making cross-sectional cuts with pruning shears. Two weeks later wounded and nonwounded bark was removed from each tree. The standard TGA assay was followed, except instead of adding 0.5 N NaOH and reading the absorbance, 1.5 ml of 3 N NaOH and 265 mg $CuSO_4$ H_2O were added and the mixture placed in a Parr 4749 acid digestion bomb in a 180 C oven for 2.5 hr. When cool, the contents were transferred with the aid of water to conical centrifuge tubes. After centrifuging for 10 min in a clinical centrifuge, the supernatant was transferred to a test tube. The solids were resuspended in 1.5 ml 0.5 N NaOH, centrifuged as before, and the supernatant was added to the previous supernatant. The pH of the supernatant was adjusted to about 3 with 6 N HCl and then extracted with 3 half volumes of diethyl ether. The ether extracts were partitioned against equal volumes of freshly prepared 1% $NaHCO_3$. The ether was evaporated under nitrogen and the residue dissolved in 0.2 ml methanol. The samples and the p-hydroxybenzaldehyde, syringaldehyde, and vanillin standards were spotted on 250 um HLF Silica gel G TLC plates (Analtech) and developed in chloroform-acetone (94:6). The aldehydes were located with UV irradiation and the silica gel with fluorescent spots corresponding to p-hydroxybenzaldehyde, syringaldehyde, and vanillin was scraped off, extracted in one ml chloroform-methanol (2:1), and the extract filtered through glass wool. The silica gel samples were reextracted 3 more times in one ml chloroform-methanol (2:1) each time and filtered.

The filtrates were combined and evaporated to dryness under a stream of nitrogen and the residues were dissolved in 0.02% KOH in 95% ethanol. The amount of the aldehydes and the UV spectra were determined spectrophotometrically (16).

Time course of lignification in wounded potted and orchard trees. Wounds were made through the bark to the xylem of 2-yr-old potted trees (about 1.5 cm diameter) with a 6 mm diameter corkborer. Wounds were made at various times on each of the 10 trees and were distributed over 30 cm of the trunk. The aged (from one to 9 days) wounded tissue was removed with an 11 mm diameter corkborer and the lignin content of these tissues was determined.

Wounds were made through the bark to the xylem with a 6 mm diameter corkborer in branches (about 2 cm diameter) of 6- and 25-yr-old almond trees located in an orchard near Davis, CA in late September and early October. The average temperature during the course of the experiment was 20.4 C and the daily average ranged from 14.7 to 25.3 C. Wounds were made at various times on each of 8 branches distributed randomly over a half-meter section of the branch. The various aged (from 2 to 21 days old) wounded tissues were removed with an 11 mm diameter corkborer and assayed for lignin.

Correlation of lignification and resistance. Each week for 3 weeks 2 bark wounds were made with a 5 mm diameter corkborer in each of ten 1-yr-old potted trees kept in an 18 C growth chamber. Half of the wounds were inoculated with 5 mm diameter CMA plugs of P. syringae and the other wounds were analyzed by the TGA assay. After 4 weeks the amount of discoloration due to canker formation was measured and the TGA assay was performed on the wound area. For the assay a 10 mm corkborer was used to remove the inner bark tissue.

Effect of fungi on lignification. In the experiment to investigate the effect of infection by Phytophthora species on wound lignification, 1-yr-old potted trees in an 18 C growth chamber were wounded with a 4 mm diameter corkborer. Agar plugs (4 mm

diameter) from colonies of P. syringae (originally isolated from pruning wound canker in an almond tree), P. hibernalis (from orange), P. cactorum (from almond crown rot), and P. infestans (from potato) were placed in the fresh wound and wrapped loosely with parafilm. Mycelial plugs from two media, ALBA and CMA, were used for inoculation . All of the different treatments were applied to each of 5 trees. After 3 days, the wounded area was removed with a 10 mm diameter corkborer and the TGA assay performed.

In order to investigate whether lignin is produced in the inner bark in response to infection, wounds in seven 1-yr-old potted trees were inoculated with mycelial plugs of P. syringae and kept in an 18 C growth chamber. After 6 weeks various parts of the canker and the healthy tissue were removed with a 6 mm diameter corkborer and the TGA assay performed on the inner bark tissues.

Effect of chitosan on wound lignification. A chitosan solution for treating inner bark wounds was made as follows: 10 mg crabshell chitosan (milled to #40 mesh) was dissolved in 2 ml of 0.1 M HCl, then diluted to 5 ml with water, and adjusted to pH 6 with 0.5 M NaOH. Two wounds on each of 7 potted 2-yr-old trees were made through the bark to the cambium but not into the xylem with a 6 mm diameter corkborer. In one wound on each tree, 50 ul chitosan solution (0.1 mg chitosan) was applied with a pipetter and in the other wound, the same amount of sterile distilled water was applied. After 3 days, the wounded area was removed with an 11 mm diameter corkborer and the standard TGA assay performed.

RESULTS

Development of TGA assay for use with bark. The amount of ligninthioglycolic acid (LTGA) was 0.70 mg around 6-wk-old wounds with outer bark, 0.21 mg for 6-wk-old wounds with outer bark removed, 0.45 mg for fresh wounds with bark and 0.03 mg for fresh wounds with bark removed. In the factorial analysis of variance there were

significant differences in LTGA content between wounds with outer bark (0.58 mg) and wounds with outer bark removed (0.12 mg) ($P<.001$) and between the 6-wk-old wounds (0.46 mg) and fresh wounds (0.23 mg) ($P<.01$).

The pretreatment of inner bark tissue with NaOH did not significantly decrease the LTGA detected. For the 6-wk-old wounds, the mean weight of LTGA was 0.25 mg with base pretreatment and 0.23 mg without base pretreatment.

The standard curve for the wounded inner bark LTGA is shown in Figure 2. The absorptivity was determined to be 11.9, 9.4, and 8.4 $g^{-1}L$ cm for the LTGA derived from wounded inner bark, xylem, and outer bark, respectively. The percentage yield (dry weight LTGA / methanol-extracted dry weight sample x 100) was 4%,15%, and 31% for wounded inner bark, xylem, and outer bark, respectively.

Cupric oxide oxidation yielded more of the three phenolic aldehydes, p-hydroxybenzaldehyde, vanillin, and syringaldehyde from the LTGA from 2-wk-old wounded tissue than from nonwounded tissue (Table 1). All three aldehydes were detected in all nonwounded tissues.

Time course of lignification. In potted trees 1-day-old wounds had 71% more LTGA than fresh wounds ($P<.001$ by paired t-test). The amount of LTGA increased linearly ($r^2=.98$) over the 9 day period (Fig. 3). The methanol extracted dry weight of the wounded tissue increased 34% over a 9 day period and this increase was linear ($r^2=94$) (Fig. 3). The variable LTGA per mg dry weight also increased linearly ($r^2=.99$).

In orchard trees 2-day-old wounds had 191% more LTGA than fresh wounds ($P<.01$ by paired t-test). The amount of LTGA increased linearly ($r^2=.95$) over the 3 week period observed (Fig. 4). The methanol-extracted dry weight of the wounded tissue increased 136% over a 3 week period and this increase was linear ($r^2=.92$) (Fig. 4).

Correlation of lignification and resistance. The mean amount of LTGA in variously aged wounds before inoculation was negatively correlated ($r=-1.00$) to the mean length of cankers resulting from infection by P. syringae of similarly aged wounds (Fig. 5). The

amount of LTGA per wound was 0.77, 1.12, 0.93, and .75 mg for inoculated 0-wk-, 1-wk-, 2-wk-, and 3-wk-old wounds, respectively 4 weeks after inoculation ($LSD_{.05}$ = 0.21 and noninoculated controls had 0.31 mg LTGA). The amount of LTGA of inoculated wounds did not correspond to canker length. For example, there was little difference in LTGA between inoculated 0-wk-old wounds and 3-wk-old wounds, although the former had canker lengths of 42.1 mm while the latter had cankers of only 5.6 mm.

Effect of fungi on lignification. Three Phytophthora species, P. syringae, P. hibernalis, and P. cactorum, increased the amount of LTGA detected in 3-day-old wounds, but there was no increase when wounds were inoculated with P. infestans (Table 2). There was a significant (P=0.0001) difference in the amount of LTGA detected between the two inoculation media, CMA and ALBA. However, there was little difference between the media in the case of P. infestans and the noninoculated controls (Table 2). There was a significant (P=0.002) difference by the paired t-test between the noninoculated ALBA treatment and when no medium and no parafilm wrapping was used with the wounds. There was an almost significant (P=0.052) difference between the noninoculated CMA treatment and the no medium and no parafilm wrapping treatment.

There were significant (P=0.0001) differences in the amount of LTGA detected in the bark tissue around a canker caused by P. syringae (Fig. 6). There were substantially more LTGA within the discolored tissue of the canker than in the healthy appearing tissue. The disks of healthy tissue 0.3 (0.084 mg LTGA) and 0.9 cm (0.071 mg) from the canker margin had significantly ($LSD_{.05}$=0.017) more LTGA than the tissue 15 cm away (0.053 mg).

Effect of chitosan on lignification. There was a significant difference in lignification between wound treatments (P<.0001). Chitosan-treated wounds had a 135% increase in LTGA over the nonwounded while LTGA in the water-treated wounds increased 60% (Table 3).

DISCUSSION

The formation of LTGA has been suggested as a criterion for genuine lignin (10). Cupric oxide oxidation of lignins yields the phenolic aldehydes, p-hydroxybenzaldehyde, vanillin, and syringaldehyde, with the amount of each depending on the amount of the corresponding phenylpropanol precursor for the lignin (8). The detection of these phenolic aldehydes in LTGA in this study indicates that lignin was indeed being measured in the TGA assay. The fact that more of these phenolic aldehydes were detected in wounded tissue (Table 1) further supports the contention that genuine lignin is formed in almond bark in response to wounding.

When the TGA assay was performed on the outer bark of almond trees, substantial amounts of LTGA were detected. Since suberin from many plants contains a lignin-like phenolic component (14), this result suggests that in the TGA assay care should be taken to separate the inner bark from the outer bark which is highly suberized and the xylem which is highly lignified (Fig. 1). However, the base pretreatment of the tissues to remove nonlignin phenolic esters was unnecessary and indicated that these compounds were not contributing to the absorbance at 280 nm obtained with the assay.

The absorptivity of LTGA at 280 nm was different depending on the source of the lignin. The absorptivity ranged from 8.4 $g^{-1}L$ cm for the outer bark to 11.9 for wounded inner bark. Hardwood milled lignin for different species showed a wide range of absorptivities at 280 nm from 7.9 to 14.8 depending on the methoxyl:carbon ratio of the lignin (20). Even though there is variability in the absorptivity, the TGA assay for lignin can be used for determining relative amounts of lignin.

LTGA increased linearly as the bark wounds aged in both potted and orchard trees (Fig. 3 and 4). There are several possible sources of this LTGA in wounded inner bark tissue. Histological observations have found some lignified bast fibers present in nonwounded tissue (personal observation) which may have yielded the LTGA detected in nonwounded tissue in the present study. Additionally, the strongly lignified zone of

cells and suberized wound periderm observed in histological studies (Fig. 1) (personal observation,9,17) probably contributed to the LTGA content of wounded almond bark tissues. Similar histological observations have been made in wounded bark tissue of other species of trees (5,15). The suberized outer periderm of almond trees increased the amount of LTGA detected, thus, it seems likely that the suberized wound periderm also increased the amount of LTGA detected. It is also possible that some lignification occurred near the almond bark wound surface, since a common response to wounding for plant tissues is to lay down lignin near the wound surface (19). The TGA assay seems to measure the amount of bound phenolic polymers in the tissue.

The advantages of the TGA assay for lignin in almond inner bark were that it was quantitative and specific for lignin, sensitive to small amounts of lignin, and easy to use with many replications of small tissue samples. However, the assay did not distinguish between LTGA derived from the bast fibers, the lignified zone, or the suberized wound periderm. Histological techniques allow the localization of the lignin and suberin in the tissue, although they are not quantitiative, sensitive, or convenient. Therefore histological techniques complement the use of the TGA assay in research into the development of resistance of the bark to pathogens. Photometric techniques have been used to quantify the amount of lignin and suberin in wounded tree bark (2), but it is not known how these techniques compare to the TGA assay. The TGA assay could be used to compare the wound responses of various cultivars, the effect of various compounds on the response of treated wounds, and the effect of treatments such as temperature on lignification of wounds.

The dry weights of methanol-extracted wounded tissue increased linearly (Fig. 3 and 4). This increase could be due to deposition of compounds such as lignin in cell walls or to newly formed cells derived from the phellogen of the developing wound periderm. The variable LTGA per dry weight would underestimate the degree of lignification around the wound because the dry weight of a wound also increases with age.

Observations on the development of the resistance of almond bark wounds to Ceratocystis fimbriata Ell. & Halst. (17) and Phytophthora syringae (Fig. 5) showed that fresh wounds are very susceptible, and that soon after wounding susceptibility decreased until wounds became immune to infection. In this study, LTGA increased soon after wounding (1 day in potted trees and 2 days in orchard trees) and continued to increase over the time periods observed. Two-day-old wounds inoculated with C. fimbriata resulted in smaller cankers and 14-day-old wounds were completely resistant (17). It was also observed that material reactive with phloroglucinol-HCl, a reagent commonly used for the histological detection of lignin (13), was not apparent until 4-6 days after wounding (17). Our results indicate that the TGA assay can detect an increase in lignin-like material 1-2 days after wounding and thus may be more sensitive than histochemical reagents. It has been observed that Valsa ceratosperma in apple bark (22) and Leucostoma spp. in peach bark (3) are able to penetrate the wound periderm in several weak places. So, in individual cases even when there is a well established ligno-suberized barrier, a canker can form when the fungus penetrates a weak point in the barrier. However, there was an excellent negative correlation ($r=-1.00$) between mean LTGA detected in aged almond wounds and mean canker length resulting from inoculation with P. syringae of similarly aged wounds (Fig. 5) and increases in LTGA as almond bark wounds aged corresponded to increases in resistance to the wound pathogen C. fimbriata , supporting the hypothesis that lignin or lignin-like compounds contribute to resistance of these wounds to infection by canker pathogens.

In this study lignin was elicited by pathogens in a susceptible almond cultivar in 3 cases. There was substantially more LTGA in almond bark wounds inoculated with Phytophthora spp. than in noninoculated wounds (Table 2), in necrotic almond bark tissue associated with a canker caused by P. syringae than in healthy tissue (Fig. 6), and in inoculated wounds of ages ranging from 0 to 3 weeks than in noninoculated wounds (correlation study). Increased lignification was detected in susceptible potato tubers in

response to infection by P. infestans, although the rate and extent of lignification was greater in resistant potato cultivars (11). These results suggest that the rate of lignin deposition once these aggressive pathogens are established is insufficient to prevent canker expansion. However, in susceptible almond cultivars, lignification elicited by pathogens in the genus Phytophthora may still slow canker expansion in the bark since wound-induced lignification in almond bark corresponded to slower expansion of cankers caused by P. syringae (Fig. 5). As has been observed with poplar trees and Cytospora chrysosperma (4), host responses associated with wounding and pathogen infection were similar. However, with sweet potato tubers, which have been investigated more thoroughly, differences in respiration, phytoalexin, polyphenol and lignin formation, and coumarins synthesis have been noted between the plant's response to wounding and to infection by C. fimbriata (24).

Chitosan substantially increased the lignin detected in 3-day-old almond bark wounds (Table 3). Chitosan, a component of many fungal cell walls (1), has been reported to increase lignification in wounded wheat leaves and may be involved in resistance to fungal pathogens (18). Bark wounds might be treated with chitosan or similar materials to stimulate components of the wound response, although further work is necessary to determine if chemically-induced lignification will enhance disease resistance development.

The role of lignin-like polymers in the development of resistance in bark wounds to canker pathogens is still unclear, even though there is a good correlation between increase in LTGA and development of wound resistance. Since canker pathogens usually need wounds before infection can occur (21), the intact suberized outer periderm is an effective barrier to fungal ingress which would indicate that the similar appearing wound periderm would function likewise as a barrier. Periderms have been observed to restrict the expansion of cankers caused by Phytophthora cinnamomi in trees (23) and by P. syringae in almond trees (personal observation).

Phenolic polymers could inhibit canker expansion in several different ways, but it is believed that lignin increases the resistance of host cell walls to compressive forces and to the enzymes of the pathogen (24). Whether cankers develop in wounded bark may depend on the rate and extent of phenolic polymerization versus the rate of pathogen ingress into the tissue.

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Table 1. Cupric oxide oxidation products of ligninthioglycolic acid extracted from 2-wk-old wounded and nonwounded almond inner bark tissue cell walls.[x]

	Oxidation products[y]		
Treatment	p-Hydroxy-benzaldehyde	Vanillin	Syringaldehyde
wounded	38.8[z]	55.8[z]	20.0[z]
nonwounded	12.5	19.3	3.0

[x] The main stems of 2-yr-old potted trees were wounded by making cross-sectional cuts with pruning shears and then kept at 25 C.

[y] Aldehyde ug/g methanol-extracted dry weight.

[z] Wounded tissue had significantly ($P<.05$) more oxidation product than nonwounded according to a paired t-test.

Table 2. Ligninthioglycolic acid (LTGA) detected per bark wound 3 days after inoculation with various Phytophthora species.[y]

	LTGA (mg)		
	Inoculation medium [z]		
Phytophthora species	CMA	ALBA	Mean
P. syringae	0.62	1.31	0.96
P. hibernalis	0.47	1.02	0.75
P. cactorum	0.38	0.73	0.56
P. infestans	0.27	0.28	0.28
Noninoculated	0.29	0.27	0.28
No medium	----	----	0.21
Nonwounded	----	----	0.16
		$LSD_{.05}$	0.16
Mean	0.41	0.72	

[y] The inner bark of potted almonds was wounded and inoculated and the plants were maintained in a 18 C growth chamber.

[z] CMA and ALBA are cornmeal agar and amended lima bean agar, respectively.

Table 3. Effect of chitosan on lignification of wounded almond inner bark tissue.[y]

Wound treatment	LTGA (mg/wound)
Chitosan[z]	0.110
Water	0.075
Nonwounded	0.047
$LSD_{.05}$	0.022

[y] The amount of ligninthioglycolic acid (LTGA) was determined for each 6 mm diameter bark wound made in 2-yr-old potted trees and the 7 replications were kept at 25 C for 3 days.

[z] To each wound 0.1 mg chitosan in solution was applied.

Figure 1. Conceptualized model developed from histological studies for responses after wounding in the inner bark of almond trees. The various tissues are given in capital letters and the wound responses in small letters.

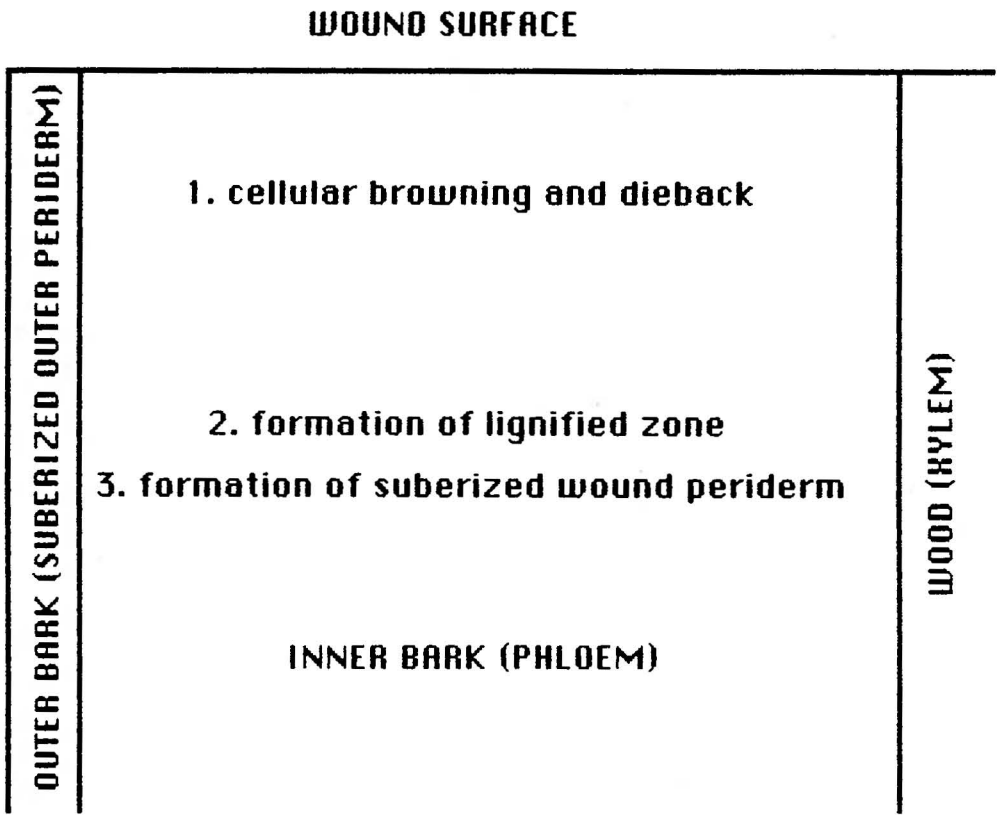

WOUND SURFACE
OUTER BARK (SUBERIZED OUTER PERIDERM)
1. cellular browning and dieback
2. formation of lignified zone
3. formation of suberized wound periderm
INNER BARK (PHLOEM)
WOOD (XYLEM)

Figure 2. Standard curve for ligninthioglycolic acid (LTGA) from 4-wk-old bark wounds in orchard almond trees. The regression formula was derived using only the data from concentrations of .01 to .08 mg/ml and was restricted so that the intercept was zero. Each point represents the mean absorbance from the data from 3 trees.

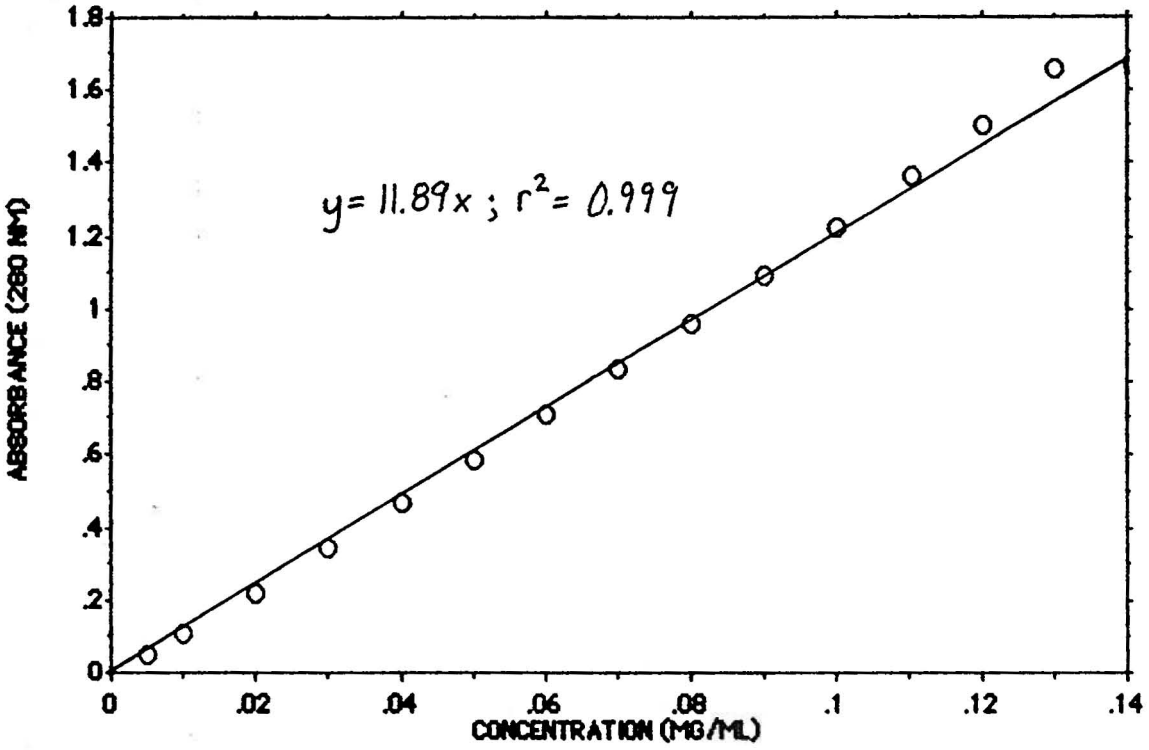

y= 11.89x ; r²= 0.999
ABSORBANCE (280 NM)
CONCENTRATION (MG/ML)
0
.2
.4
.6
.8
1
1.2
1.4
1.6
1.8
.02
.04
.06
.08
.1
.12
.14

Figure 3. Amount of ligninthioglycolic acid (LTGA) and methanol-extracted dry weight of bark wounds of various ages in 10 replications of potted almond trees kept in a 25 C growth chamber.

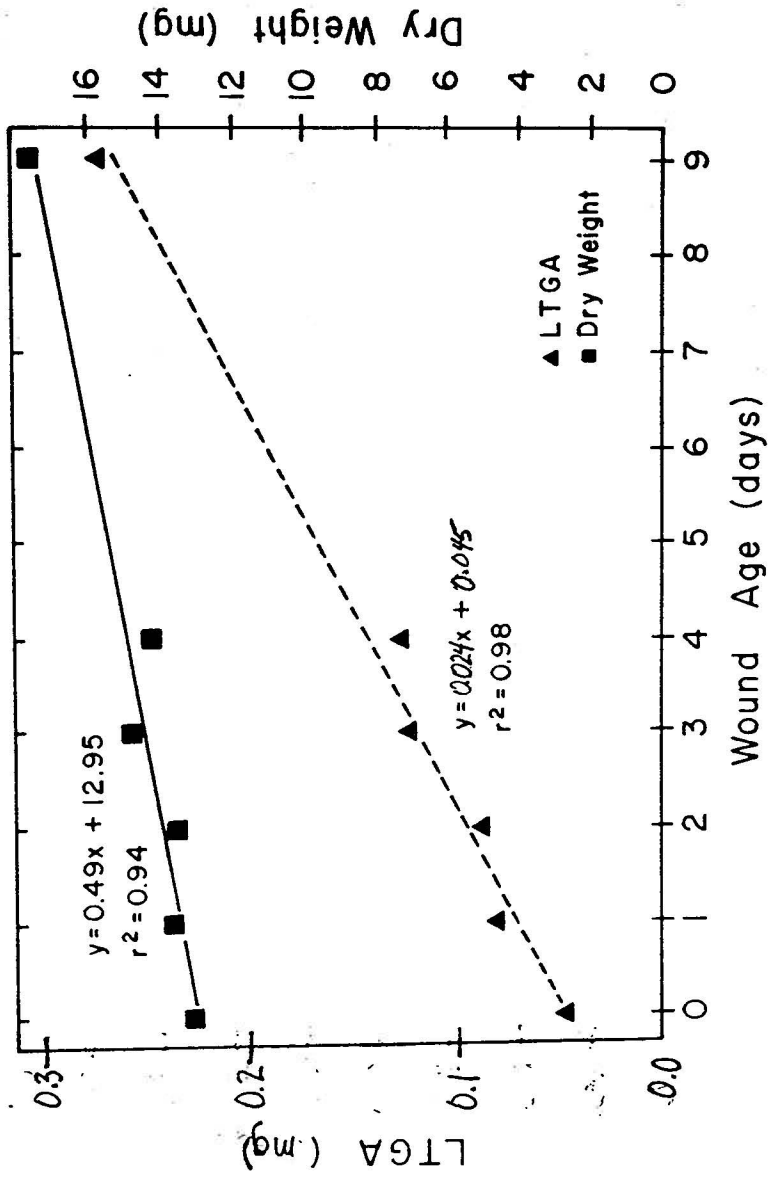

LTGA (mg)
Dry Weight (mg)
Wound Age (days)
▲ LTGA
■ Dry Weight
y = 0.49x + 12.95
r² = 0.94
y = 0.024x + 0.045
r² = 0.98
0.0
0.1
0.2
0.3
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6
7
8
9

Figure 4. Amount of ligninthioglycolic acid (LTGA) and methanol-extracted dry weight of 6 mm diameter bark wounds of various ages in almond orchard trees. The wounds were made in late September and early October, 1985 with 8 replications (average temperature was 20.4 C).

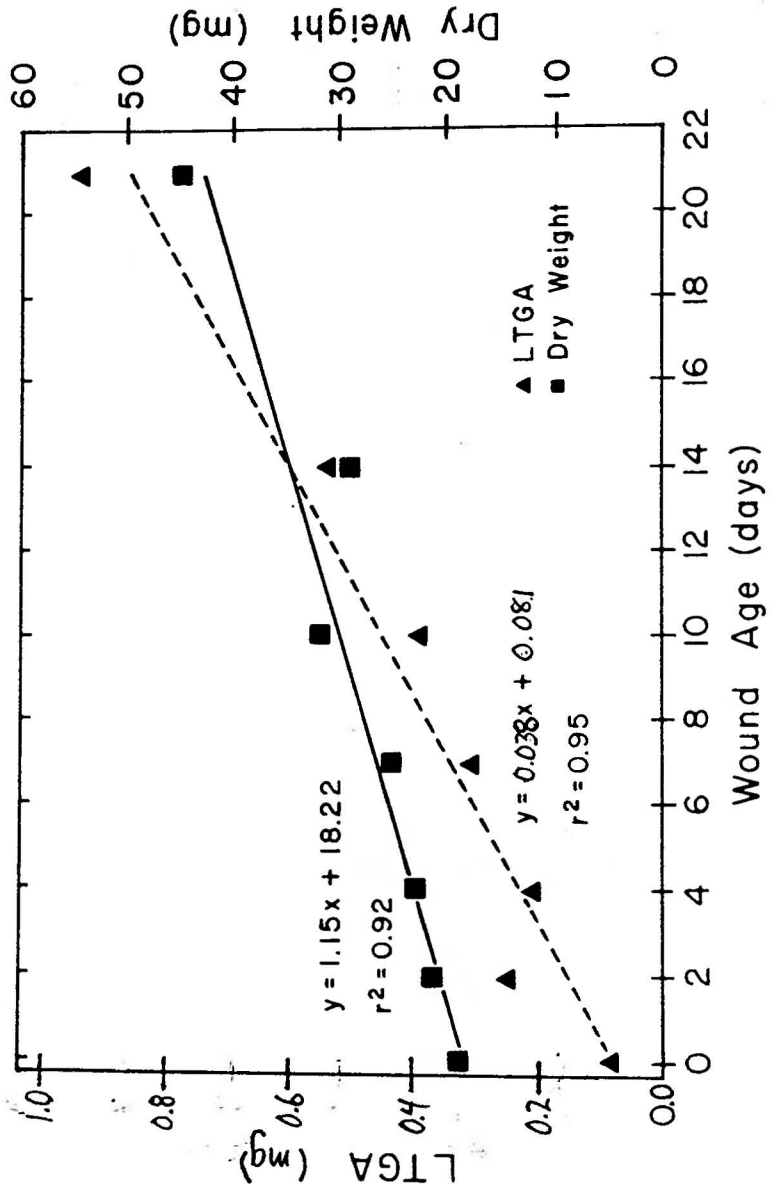

LTGA (mg)
Dry Weight (mg)
Wound Age (days)
▲ LTGA
■ Dry Weight
y = 1.15x + 18.22
r² = 0.92
y = 0.038x + 0.081
r² = 0.95
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20
22

Figure 5. Correlation of ligninthioglycolic acid (LTGA) in wounded almond bark and length of 4-wk-old cankers resulting from inoculation of similarly aged bark wounds with Phytophthora syringae. Potted trees were wounded and kept at 18 C. Each data point represents the mean of 10 replications.

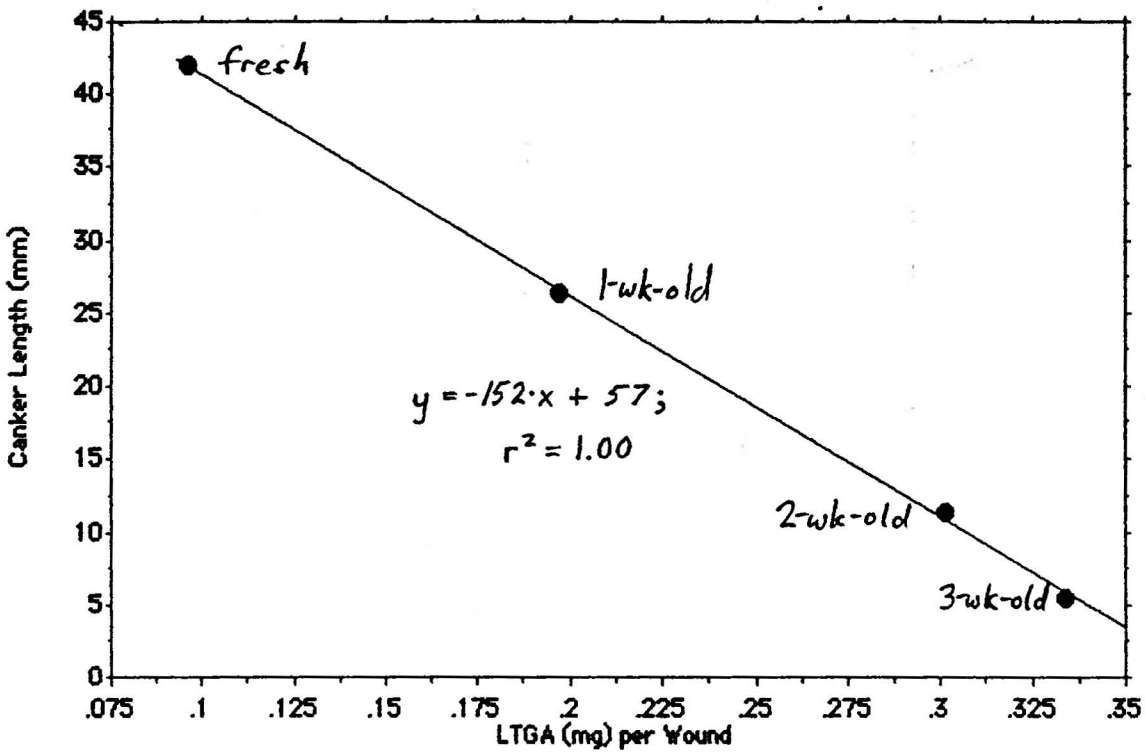
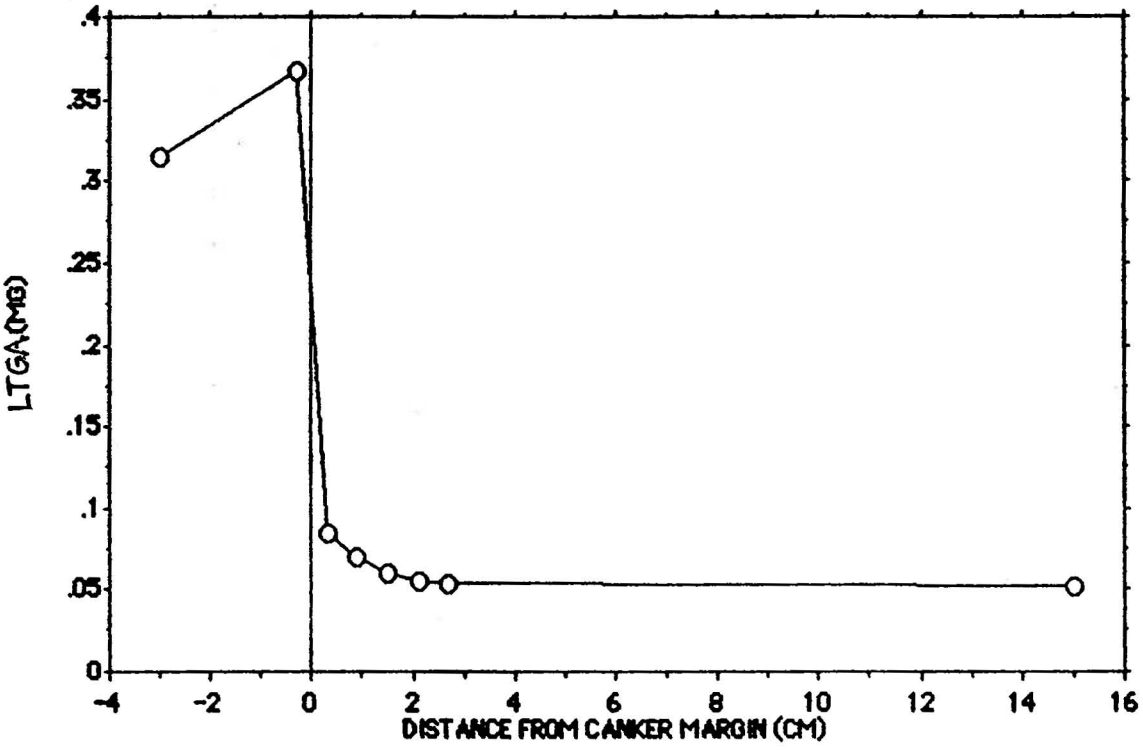

Canker Length (mm)
45
40
35
30
25
20
15
10
5
0
.075
.1
.125
.15
.175
.2
.225
.25
.275
.3
.325
.35
LTGA (mg) per Wound
fresh
1-wk-old
2-wk-old
3-wk-old
y = -152·x + 57;
r² = 1.00

Figure 6. The amount of ligninthioglycolic acid (LTGA) in healthy and Phytophthora syringae infected almond bark near the canker margin in potted trees kept at 18 C. Distances less than zero are part of the canker and positive distances have healthy appearing bark.


LTGA(MG)
.4
.35
.3
.25
.2
.15
.1
.05
0
-4
-2
0
2
4
6
8
10
12
14
16
DISTANCE FROM CANKER MARGIN (CM)

SECTION 3.

THE EFFECT OF LOW TEMPERATURE ON RESISTANCE TO PHYTOPHTHORA PRUNING WOUND CANKER AND ON LIGNIN AND SUBERIN FORMATION IN WOUNDED ALMOND BARK

ABSTRACT

As bark wounds in almond trees aged, inoculation of the wounds with Phytophthora syringae resulted in fewer and smaller cankers. In potted trees and excised branches, as the temperature at which bark wounds were aged was lowered, the development of resistance to P. syringae was slowed. All fresh wounds inoculated with P. syringae resulted in cankers. In potted trees with stem wounds aged for 2 weeks at 25 C prior to inoculation, 38% of the inoculated wounds developed cankers that were 74% smaller than cankers surrounding inoculated fresh wounds. When wounds were aged at 6 C prior to inoculation, all of the inoculated wounds developed cankers, but these were 13% smaller than those surrounding inoculated fresh wounds. 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-week-old wounds, and many of the 4-week-old wounds, but almost all inoculated 6-week-old wounds were immune to infection. At low temperatures in almond bark wounds in potted trees and excised twigs, less lignin and suberin were observed histologically and less ligninthioglycolic acid (LTGA) was detected using the thioglycolic acid assay. A substantial increase in LTGA was detected in 1-week-old wounds kept at 6 C; however, after 20 days no lignin or suberin was detected histologically at this temperature. There was almost a 3-fold increase in LTGA in 2-week-old wounds when aged at 25 C than when aged at 6 C. In the histological study in an almond orchard, both lignification and suberization in wounds were strongly inhibited when temperatures were low during the winter. However, the resistance to infection by P. syringae of 2- and 4-wk-old wounds did not show any consistent effect of the low temperatures during winter. In another orchard

experiment even in the coldest period of winter substantial amounts of LTGA were detected in 4-wk-old wounds, although there were still significant differences for the months throughout the fall and winter in LTGA detected. The inhibition of lignin, suberin, and wound periderm formation by low temperatures would indicate that pruning should be avoided during those periods if possible; however, there was no indication that wounds in orchard trees are actually more susceptible during those periods. The results indicate that treatments applied to pruning wounds for protection against P. syringae should be effective for at least 6 weeks.

INTRODUCTION

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 (4). Cankers are observed soon after pruning in the fall and winter when temperatures are relatively low and P. syringae grows very well in media and in almond bark at these low temperatures (4). 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 resistance of older wounds.

A suberized periderm similar to the normal periderm of the outer bark is produced around wounds in almost all dicotyledonous plants (8). In the inner bark within 2 weeks after wounding, a strongly lignified zone and a suberized periderm (Fig. 1) were observed histologically in almond trees (15) and other tree species (2,12). Increases in phenolic polymers such as lignin as almond bark wounds aged (7) corresponded to increases in resistance to the canker-forming pathogen Ceratocystis fimbriata Ell. & Halst. (5,15) suggesting that the lignified zone or suberized periderm contribute 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 (3). 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 were strongly inhibited at low temperatures (12). Since infection by P. syringae of pruning wounds in almond trees occurs during fall and winter when temperatures are low, 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 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) (6) 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 were cut out, 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 with a 5 mm diameter corkborer and the trees were kept at 6 and 25 C. The wounds were allowed to age so that each of 8 trees had 0, 2 and 4-wk-old wounds. The wounds were inoculated with mycelial CMA plugs containing P. syringae and all trees were placed in a 12 C growth chamber. One month later the inner bark was examined for cankers and the amount of inner bark discoloration measured. The methods for some of the histological

and biochemical studies where the resistance of the aged wounds to P. syringae was also investigated are given below.

Histological studies. Radial sections were made for all histological studies as follows: 5 x 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 (9). The percentage of the width from the cambium to the outer bark of the inner bark tissue staining positive was measured with an ocular micrometer using a microscope.

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

Excised branches of approximately 15 cm length and 2 cm diameter were obtained from orchard trees. One end of the twig was sealed in warm paraffin wax to prevent excess loss of moisture. Wounds were made by cross-sectional cuts with pruning shears and the branches were placed in incubators kept at 5, 9, 12, 15, 20, and 27 C. Every week the branches were rinsed in distilled water to prevent drying out. Every week for 6 weeks new twigs were wounded and placed in the incubators and then the 6 replications were examined as above. Fresh and aged wounds were inoculated with P. syringae and kept for 4 weeks at 12 C.

Trees selected at random in an almond orchard, Colusa County, CA, were wounded by making cross-sectional cuts with pruning shears every 2 weeks from October, 1984 to April, 1985. Wounds were allowed to age 2, 4, and 6 weeks before inoculation or histological examination. For each treatment there were 10 replications. 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 (7) 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 (7).

In an experiment investigating the effect of temperature on lignification, the bark of 2-yr-old potted trees was wounded with a 6 mm diameter corkborer to the cambium and the trees kept in 6, 12, 19, and 25 C growth chambers. Each week for 4 weeks wounds were made on each of five trees. The wounded bark tissue was removed with a 11 mm diameter corkborer and the amount of lignin determined.

In an experiment investigating lignification in orchard trees during fall, winter, and spring, branches of 5 trees in an orchard in Yolo Co., CA, were wounded every 4 weeks from September 1985 through April 1986 by making cross-sectional cuts with pruning shears. After 4 weeks, a 2 x 15 mm piece of wounded inner bark was removed and the amount of lignin in the piece determined.

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. 2). In trees kept at 25 C all 4-wk-old wounds were immune to infection, but even at 6 C there were half as many cankers in inoculated 4-wk-old wounds than in fresh wounds. For both 6 and 25 C, lengths of cankers in 2-wk-old wounds were significantly ($P<.05$) smaller than those in fresh wounds by paired t-tests (Fig. 2). Cankers in inoculated fresh wounds in trees which were kept at 6 C prior to inoculation were significantly smaller ($P=.04$) than cankers in fresh wounds in trees which were kept at 25 C prior to inoculation.

Although inoculation with P. syringae of aged wounds in excised twigs always resulted in cankers, the inoculated aged wounds had smaller cankers than fresh

wounds (Fig. 3). There was little difference between expansion rates of cankers resulting from inoculation of 1- to 3-wk-old wounds and fresh wounds when wounds were aged at temperatures less than 12 C. However, in wounds aged at 12 C and above, the difference in canker expansion rate between inoculated aged wounds and fresh wounds increased with temperature (Fig. 3). Even when wounds were aged at 5 C, there was slower canker expansion in inoculated 4- to 6-wk-old wounds than in fresh wounds.

Effect of temperature on wound-induced lignin and suberin formation. The extent of lignified zone and suberized wound periderm (Fig. 1) in the histological study using potted trees was substantially less at low temperatures (Fig. 4). 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. There were significant ($P=.0001$) differences in the arcsine transformed (13) percentage bark width staining positive between the various temperatures for both lignin and suberin in 20-day-old wounds. Likewise, there were significant ($P<.05$) differences between the various temperatures for both lignin and suberin in 10-day-old wounds. Lignin was detected prior to suberin in all experiments.

The amount of lignin detected in the histological study using excised twigs was substantially less at low than high temperatures (Fig. 5). In 1-wk-old wounds no suberin was detected at any temperature. In 2-wk-old wounds 18% of the bark width was suberized at 20 C but no suberin was detected below 20 C. Even after 6 weeks no suberin was observed in any wound kept at 12 C or below.

At low temperatures less lignin as determined by the thioglycolic acid assay was measured in aged wounds in potted trees (Fig. 6). Since it has been observed that the amount of LTGA increases linearly as wounds age (7), linear regression was performed on the data. For all four temperatures, the lack-of-fit test (17) 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. 6). In the comparison of slopes of the regression lines (10), the 6 C slope was not significantly different from the 12 C slope, but in all other comparisons there were significant differences ($P<.05$). At 6 C there was significantly ($P=.02$) more LTGA detected in the 1-wk-old wounds than in the nonwounded tissue.

Orchard experiments. During the period for the histological study in the orchard, the cumulative degree days for 2 week periods ranged from 188 in early October, to 62 in January, to 181 in February (Fig. 7A). As the temperatures during this period went from high to low to high, the amount of lignin observed in the aged wounds followed the same pattern with the least occurring in wounds made in late December or early January (Fig. 7B). The most lignification in the aged wounds was observed in the 6-wk-old wounds and the least in the 2-wk-old wounds. Examples of sections of bark wounds showing little lignification during January when temperatures were low but a well-formed zone of lignin during March when temperatures were relatively high is shown in Figure 8. As the temperatures during the experiment went from high to low to high, the amount of suberin observed in 4-wk-old and 6-wk-old wounds followed the same pattern with the least occurring in wounds made in late December or early January (Fig. 7C). Only rarely was suberin observed in 2-wk-old wounds during this period. Although the amount of lignification (Fig. 7B) and suberization (Fig. 7C) in wounds corresponded to the temperature (Fig. 7A), there was no clear relationship between temperature and development of resistance to infection by P. syringae for inoculated 2-wk-old and 4-wk-old wounds (Fig. 7D). 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-wk-old and 4-wk-old wounds than in inoculated fresh wounds (Fig. 9). 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. 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 the orchard study, substantially more LTGA was detected in 4-wk-old wounds than in nonwounded inner bark tissue ($P<.002$) (Fig. 10). 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. 10).

DISCUSSION

Low temperatures slowed the development of resistance to infection by P. syringae in almond bark wounds (Fig. 2 and 3). Resistance in aged wounds demonstrated itself as smaller cankers (Fig. 2 and 3) or no canker (Fig. 2), similar to that observed with almond bark wounds inoculated with C. fimbriata (5,15). Aged pruning wounds in apricot trees developed some resistance to Eutypa dieback at 20 C but not at 3 C (18). Apple fruit scar wounds, which respond similarly to pruning wounds, remained susceptible to Nectra galligena longer when kept at 6 C than at 12 to 24 C (12). Prior to 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.

In almond bark wounds low temperature markedly reduced the lignification (Fig. 4, 5, and 8) and suberization (Fig. 4) observed histologically and less lignin was detected using the thioglycolic acid assay (Fig. 6). Similarly, it has been observed histologically that lignin and suberin formation in wounded apple bark were strongly inhibited at temperatures below 10 C (12). The rate of wound healing in potato tubers was decreased as temperatures decreased from 20 C (25). 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 (Fig. 1) (2, 7, 12). The thioglycolic acid (TGA) assay measures the amount of bound phenolic polymers in the inner bark, which would include the lignin in the lignified zone and the phenolic component of suberin in the wound periderm (7). Suberin consists of two components and it has been suggested that the phenolic component would prevent pathogen entry while the aliphatic component prevents water loss (11). Thioglycolic acid reacts readily with the α-alkoxy function in lignin to give ligninthioglycolic acid (LTGA) derivatives (24) 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. 6), whereas even after 20 days no lignin or suberin was detected histologically (Fig. 4). Even though there was more rapid lignification and suberization in wounds kept at the higher temperatures (Fig. 4, 5, and 6), there was substantial wound-induced lignification detected at low temperatures with the TGA assay but not with phloroglucinol-HCl (Fig. 4, 5, and 6).

The development of resistance to P. syringae has been observed to correspond to the formation of the lignified zone and of the wound periderm as measured as LTGA in the wounded almond bark (7). In the present study this was also true since 2-wk-old wounds aged at 6 C had 13% smaller cankers than inoculated fresh wounds (Fig. 2) and slightly more LTGA (increase of 0.12 mg/wound) (Fig. 6), whereas 2-wk-old wounds aged at 25 C had 74% smaller cankers (when cankers did form) than inoculated fresh wounds (Fig. 2) and substantially more LTGA (increase of 0.34 mg/wound) (Fig. 6). Several possible mechanisms by which lignification could halt fungal development in the host tissues have been proposed (19,23). Since inoculation of almond bark wounds with P. syringae greatly increased LTGA detected beyond the normal wound response (7), 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. 5) were similar to those obtained using potted almond trees (Fig. 4). For example, at 20 C the percentage bark width lignified after 20 days was 78.6% in wounded potted trees and after 21 days was 86.6% in wounded excised branches. However, there were differences in P. syringae canker development 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 results). Cankers in walnut trees caused by Phytophthora citricola also expanded faster in excised stems than in intact stems (14). All almond bark wounds in excised branches kept at 12 C or below for up to 6 weeks and inoculated with P. syringae resulted in cankers (Fig. 3), whereas only 50% of the wounds in potted trees kept at 6 C for 4 weeks resulted in cankers (Fig. 2). 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.

In general the results from the almond orchard experiments concerning wound-induced lignification and suberization agreed with the results from experiments using potted trees and excised twigs. Wound periderm formation in apple trees (22) and wound response in the barks of several conifer species (16) have been observed to be much slower in winter than in summer. In our histological study, both lignification (Fig. 7B) and suberization (Fig. 7C) were strongly inhibited when temperatures were low during the winter (Fig. 7D). However, the resistance to infection by P. syringae of 2- and 4-wk-old wounds did not show any consistent effect of the low temperatures during winter (Fig. 7D), even though a definite effect of temperature on resistance development had been observed in wounded potted trees (Fig. 2). In the orchard experiment using the TGA assay, even in the coldest period of winter substantial amounts of LTGA were detected in 4-wk-old wounds (Fig. 10) 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 observed with potted trees, resistance to P. syringae in aged bark wounds of orchard trees was observed as smaller cankers (Fig. 9). Since throughout the fall and winter almost all inoculated 6-wk-old bark wounds were immune to infection by P. syringae (Fig. 7D), any wound treatment would only have to protect the wounds from infection for this period.

There is evidence that some trees are susceptible to certain Phytophthora species only during certain seasons. When potted walnut trees maintained in a lathhouse were wounded and inoculated monthly with Phytophthora citricola and then kept in a growth chamber at a constant temperature, there was a significant difference in the susceptibility of the trees for the different months with the longest cankers occurring in May (14). Substantially larger cankers formed during April and May when wounded apple trees were inoculated with Phytophthora cactorum (20). 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 (21). 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 (20) or almond trees (4), this is probably due to the sensitivity of the mycelium to high temperatures (4) and not due to changes in the physiology of the tree. In the present study, as temperatures rose in March through May, canker expansion slowed (Fig. 9). There was no indication that lignification or suberization in almond bark wounds were inhibited during the spring and although in fact, both increased (Fig. 7B, 7C, and 10), there was not a corresponding increase in resistance (Fig. 7D). Weak points in an established wound periderm through which fungi could penetrate into the healthy bark have been observed in apple (22), peach (1), and almond trees (personal observation). Even if lignification or suberization is not inhibited, it is possible that wounds increase in susceptibility due to weakening of the barrier at certain points. For example, perhaps 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. The changes in the bark of trees in the spring are complex (21) 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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Figure 1. Conceptualized model developed from histological studies for responses of inner bark in almond trees after wounding. The various tissues are given in capital letters and the wound responses in small letters. The chronological order of the initiation of the various wound responses is indicated by the number preceeding the response. The formation of the wound periderm occurs just interior to the lignified zone.

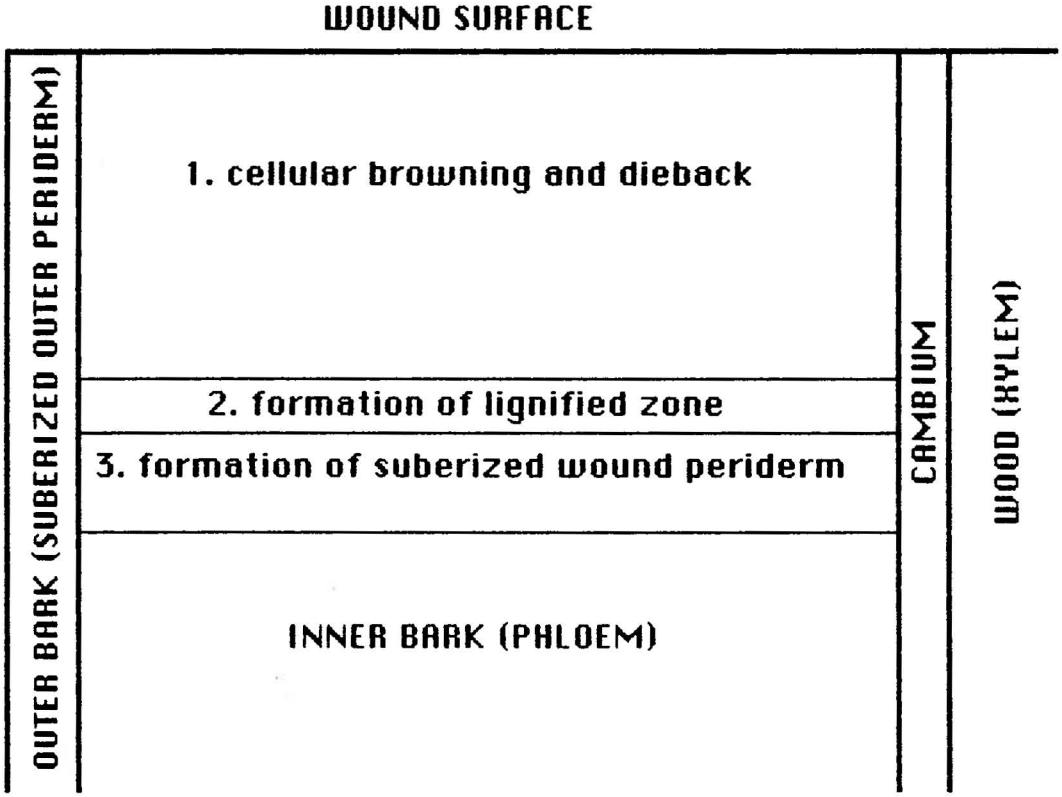

WOUND SURFACE
OUTER BARK (SUBERIZED OUTER PERIDERM)
1. cellular browning and dieback
2. formation of lignified zone
3. formation of suberized wound periderm
INNER BARK (PHLOEM)
CAMBIUM
WOOD (XYLEM)

Figure 2. The effect of temperature on development of cankers in aged inner bark wounds in potted trees inoculated with Phytophthora syringae. After inoculation trees were kept at 12 C for one month. The percentage 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.

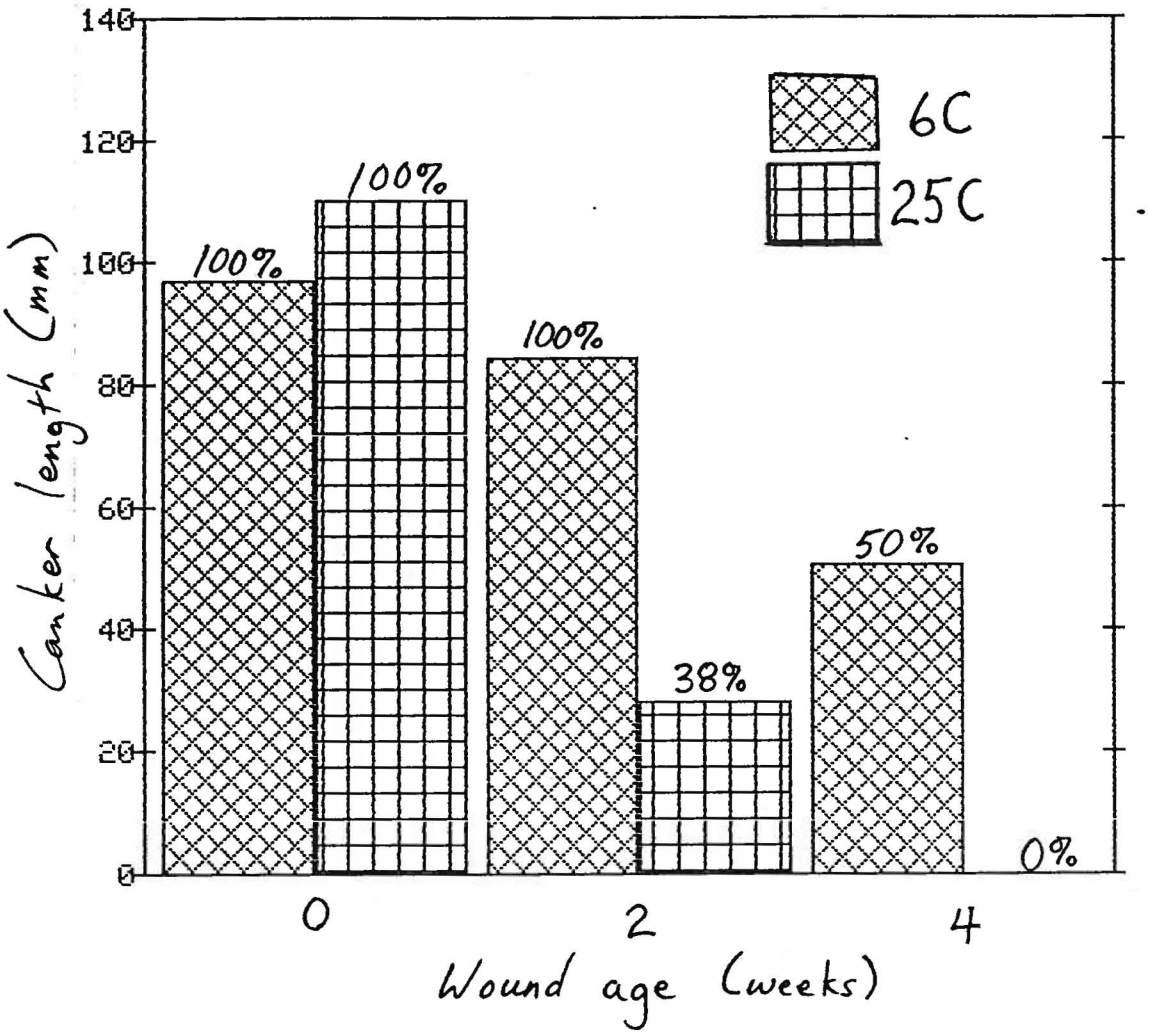

140
120
100
80
60
40
20
0
Canker length (mm)
6C
25C
100%
100%
100%
38%
50%
0%
0
2
4
Wound age (weeks)

Figure 3. The development of Phytophthora syringae cankers in excised branches after inner bark wounds were aged at various temperatures. After inoculation branches were kept at 12 C for 4 weeks. Each point represents data from at least 9 branches. Fresh wounds were made in branches which had been aged at various temperatures similar to the aged wounds. ● fresh wounds; □ young wounds (1-3 weeks old); △ old wounds (4-6 weeks old).

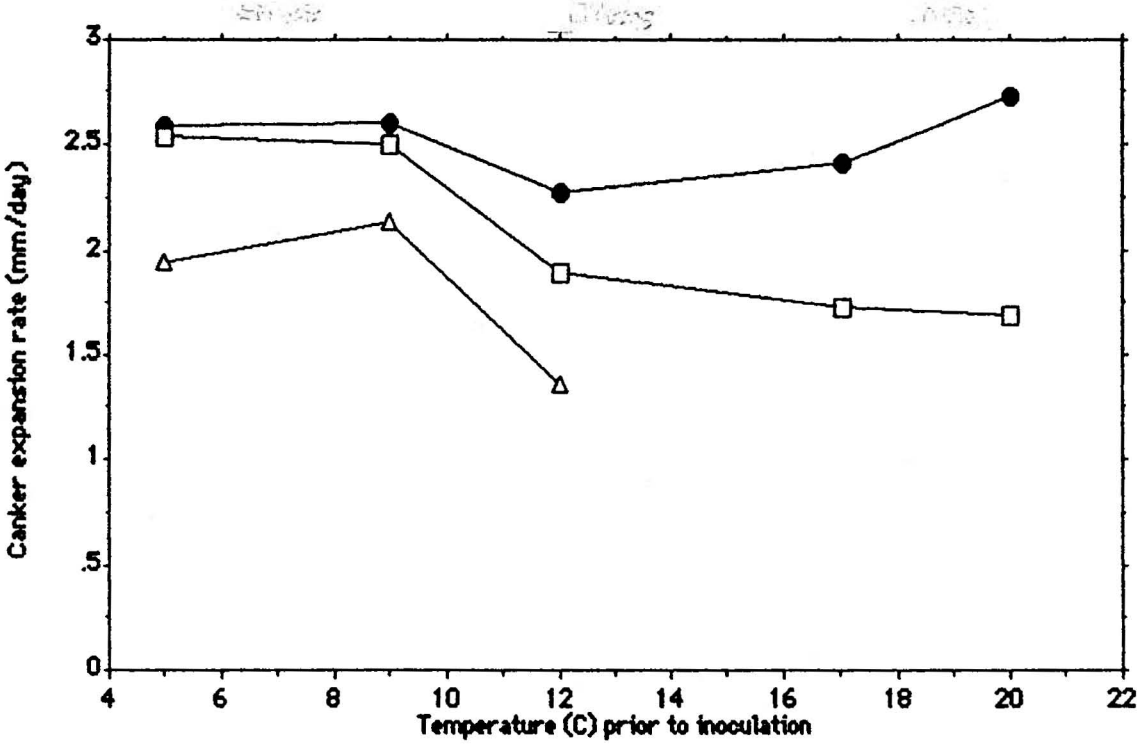

Canker expansion rate (mm/day)
3
2.5
2
1.5
1
.5
0
4
6
8
10
12
14
16
18
20
22
Temperature (C) prior to inoculation

Figure 4. 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 suberin with sudan black B. For the 20-day-old wounds the $LSD_{.05}$ was 25 and 24 for the arcsine transformed percentage bark width for lignin and suberin, respectively. ···△··· lignin, 10-day-old wounds; ···◇··· suberin, 10-day-old wounds; —▲— lignin, 20-day-old wounds; —◆— suberin, 20-day-old wounds.

Fig. 1

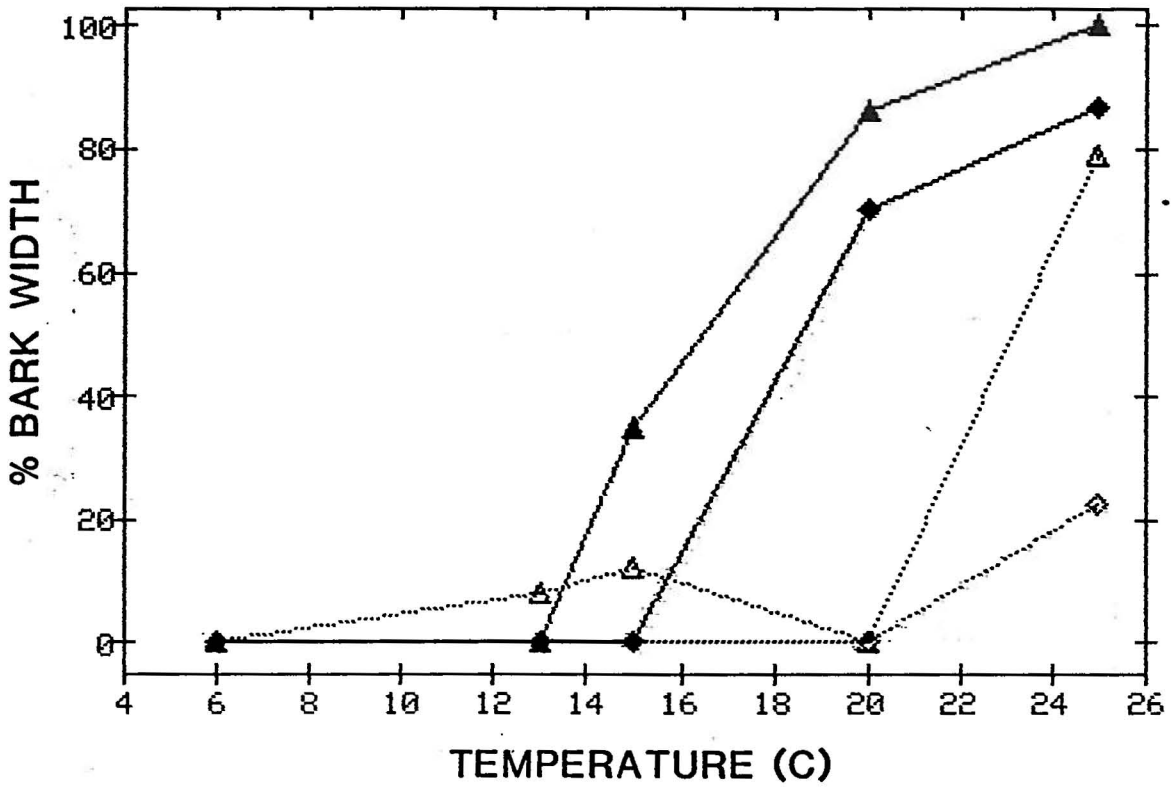

100
80
60
40
20
0
% BARK WIDTH
4
6
8
10
12
14
16
18
20
22
24
26
TEMPERATURE (C)

Figure 5. The effect of temperature on lignifcation 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.

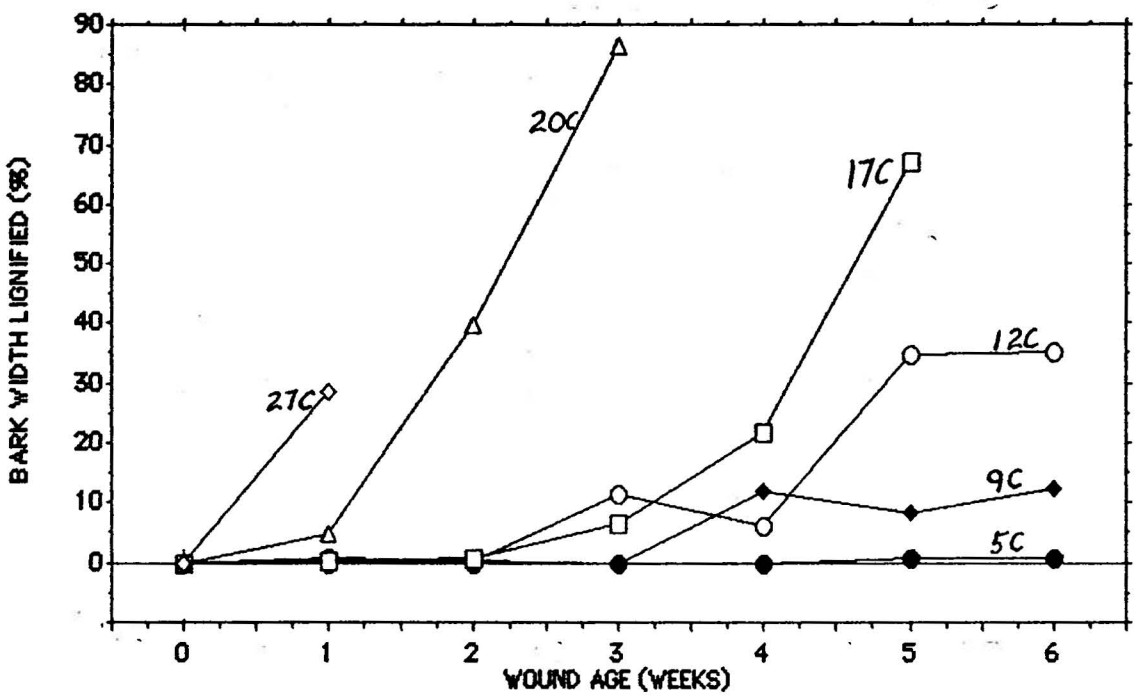

BARK WIDTH LIGNIFIED (%)
WOUND AGE (WEEKS)
0
10
20
30
40
50
60
70
80
90
0
1
2
3
4
5
6
27C
20C
17C
12C
9C
5C

Figure 6. 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 week period. ○ 6 C; □ 12 C; △ 19 C; ◇ 25 C.

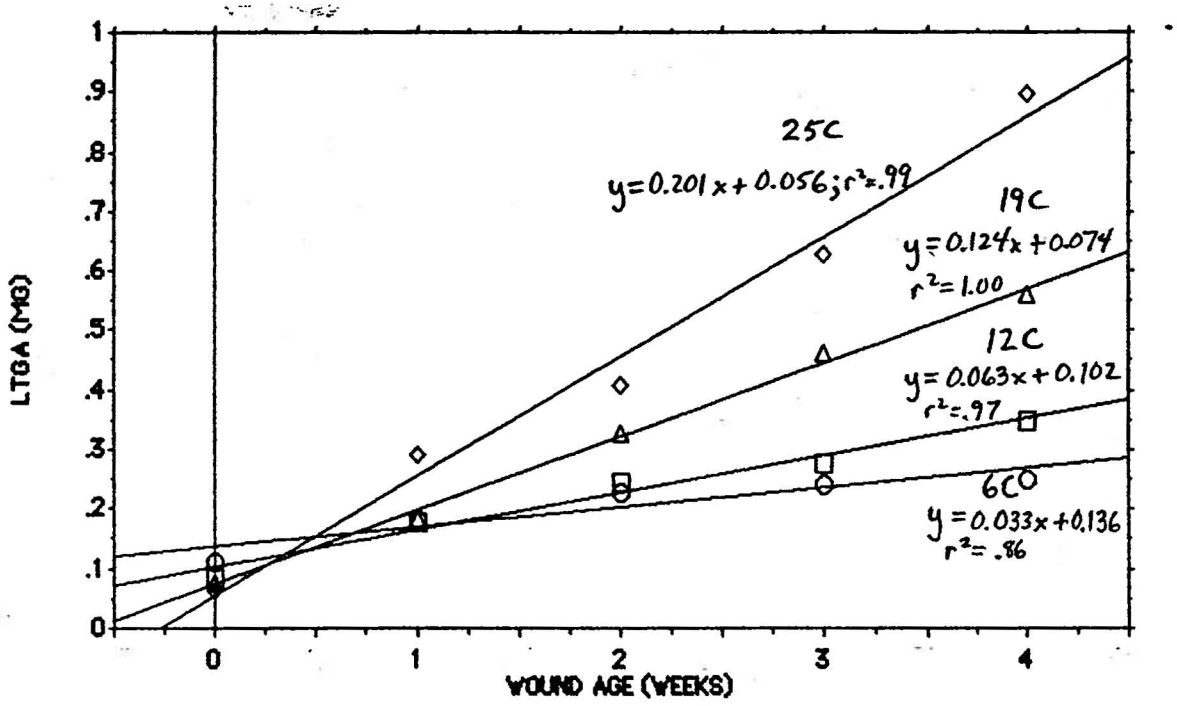

LTBA (MG)
WOUND AGE (WEEKS)
25C
y=0.201x+0.056; r²=.99
19C
y=0.124x+0.074
r²=1.00
12C
y=0.063x+0.102
r²=.97
6C
y=0.033x+0.136
r²=.86

Figure 7A. Cumulative degree days for 2, 4, and 6 week periods beginning from October, 1984 through February, 1985 for Colusa, CA. The developmental threshold was assumed to be 0 C. ● 2 week period; ■ 4 week period; ▲ 6 week period.

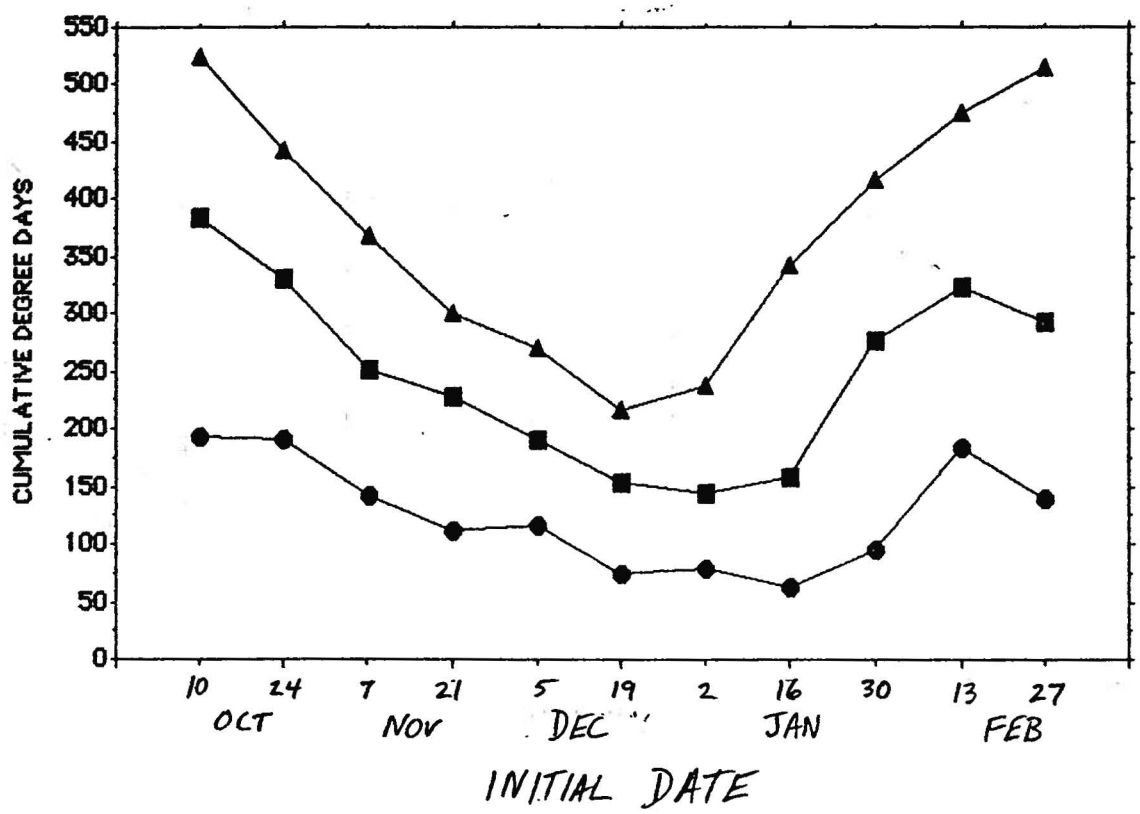

CUMULATIVE DEGREE DAYS
550
500
450
400
350
300
250
200
150
100
50
0
10
24
7
21
5
19
2
16
30
13
27
OCT
NOV
DEC
JAN
FEB
INITIAL DATE

Figure 7B. Lignification in wounded inner bark of almond trees in an orchard in Colusa Co., CA in the fall, 1984 and winter, 1985. 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. ○ 2-week-old wounds; □ 4-week-old wounds; △ 6-week-old wounds.

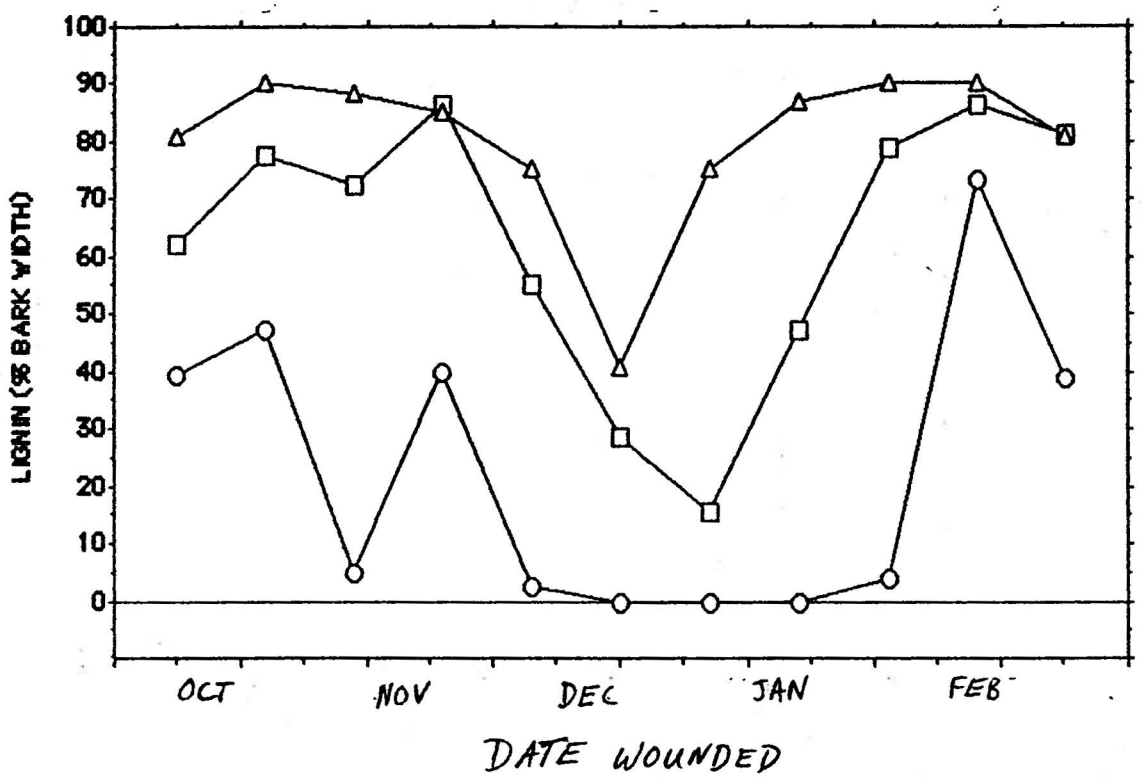

LIGNIN (% BARK WIDTH)
100
90
80
70
60
50
40
30
20
10
0
OCT
NOV
DEC
JAN
FEB
DATE WOUNDED

Figure 7C. Suberization of wounded inner bark of almond trees in an orchard in Colusa Co., CA in the fall, 1984 and winter, 1985. The percentage 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-week-old wounds; □ 4-week-old wounds; △ 6-week-old wounds.

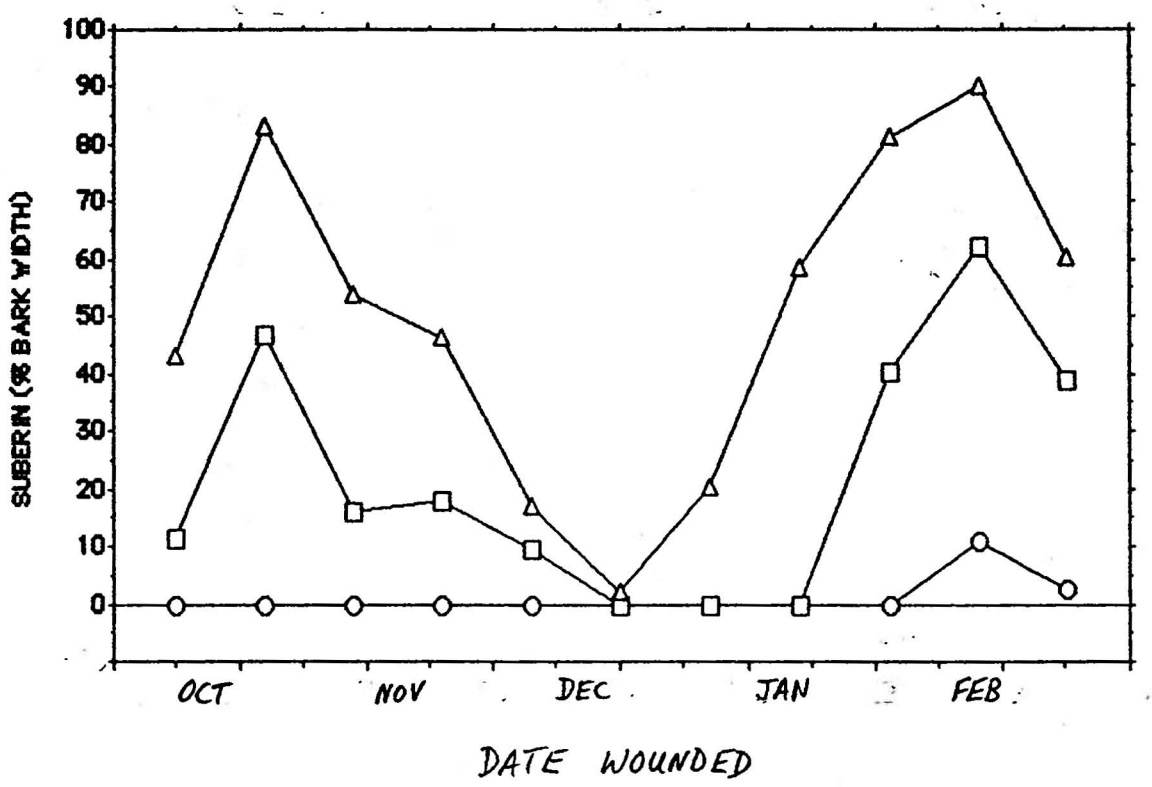

SUBERIN (% BARK WIDTH)
100
90
80
70
60
50
40
30
20
10
0
OCT
NOV
DEC
JAN
FEB
DATE WOUNDED

Figure 7D. The percentage of aged wounds inoculated with Phytophthora syringae resulting in cankers in almond trees in an orchard in Colusa Co., CA in the fall, 1984 and winter, 1985. ○ fresh wounds; □ 2-week-old wounds; △ 4-week-old wounds; ◇ 6-week-old wounds.

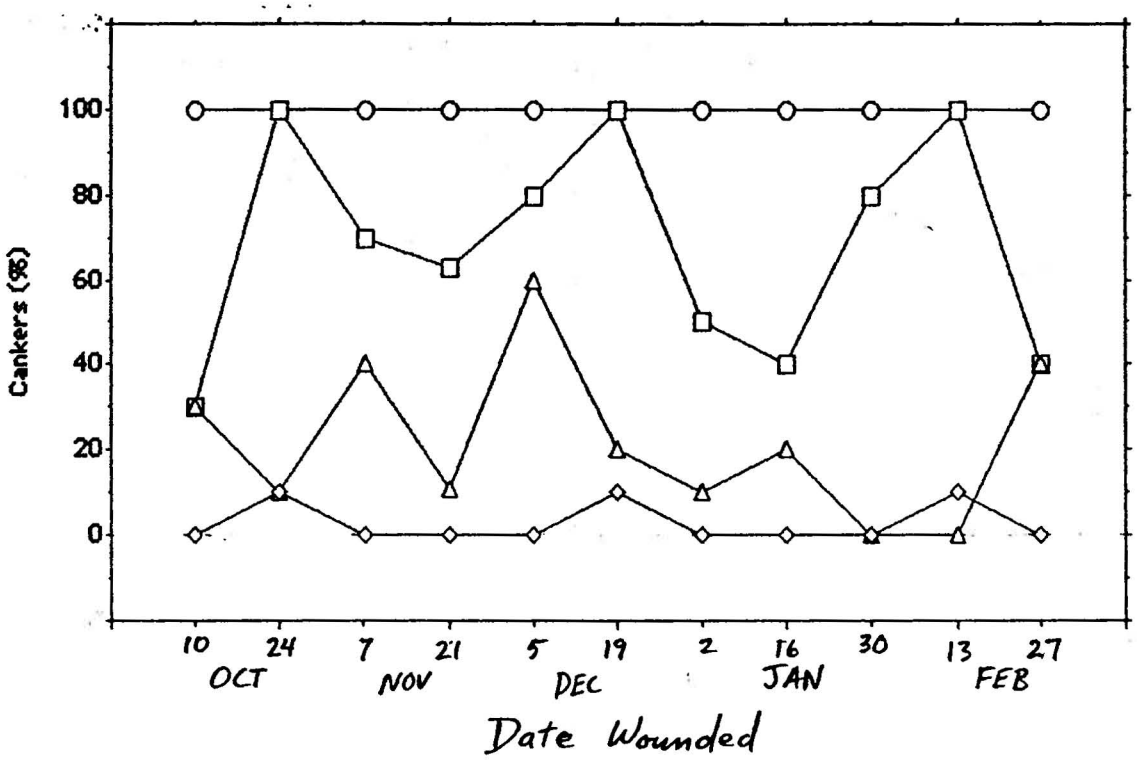

Cankers (%)
100
80
60
40
20
0
10
24
7
21
5
19
2
16
30
13
27
OCT
NOV
DEC
JAN
FEB
Date Wounded

Figure 8. Radial sections of 4-wk-old bark wounds in orchard trees aged in January (A) and March (B) stained for lignin with phloroglucinol-HCl. Lignin is stained red. Only a little lignin is observed in the wound aged in January, but a definite zone of lignin extending from the outer bark to the xylem is seen in the wound aged in March. Bars = 0.1 mm.

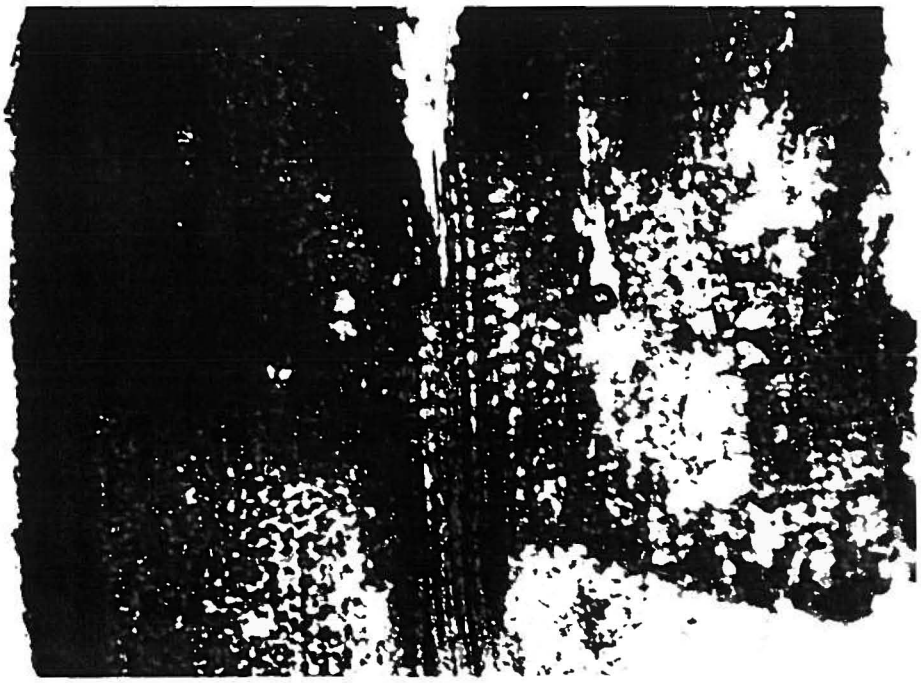


Figure 9. The mean expansion rate of cankers resulting when aged wounds in almond trees in an orchard in Colusa Co., CA were inoculated with Phytophthora syringae in the fall, 1984 and winter, 1985. For the calculation of mean canker expansion rate only inoculations resulting in cankers were included. ○ fresh wounds; □ 2-week-old wounds; Δ 4-week-old wounds; ● mean temperature (C) for 6 week periods.

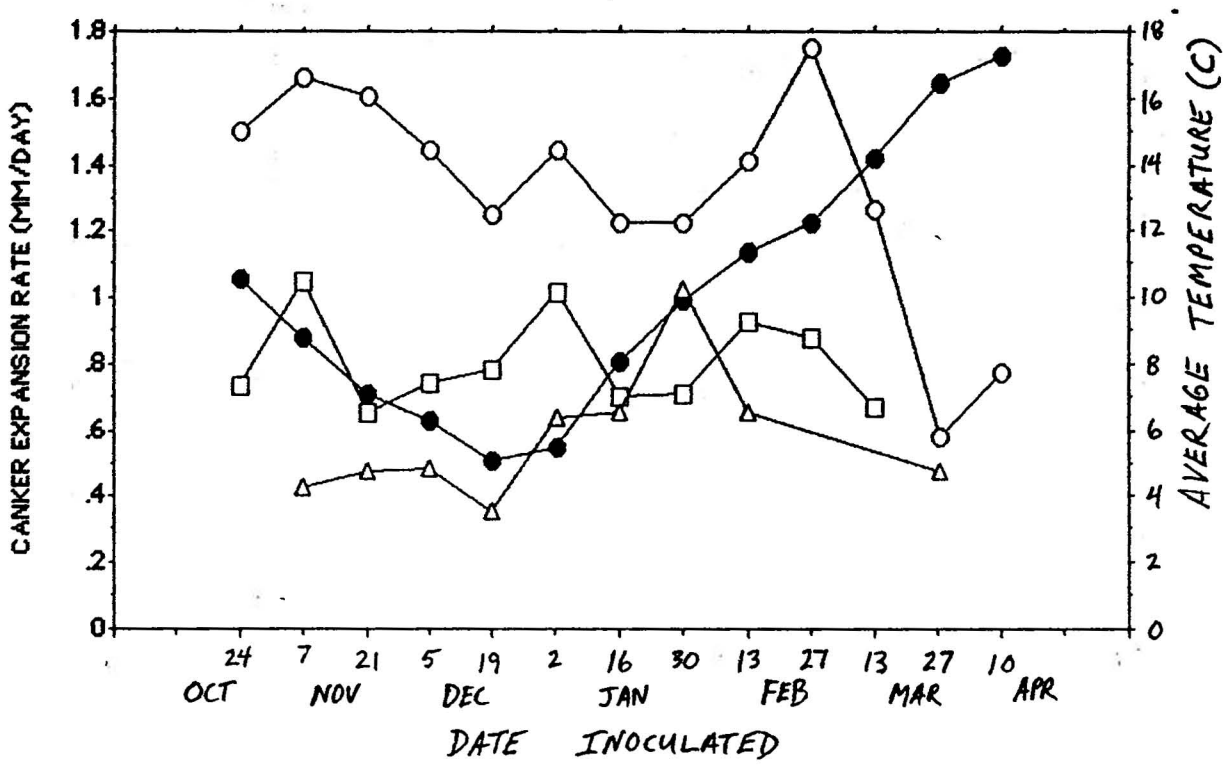
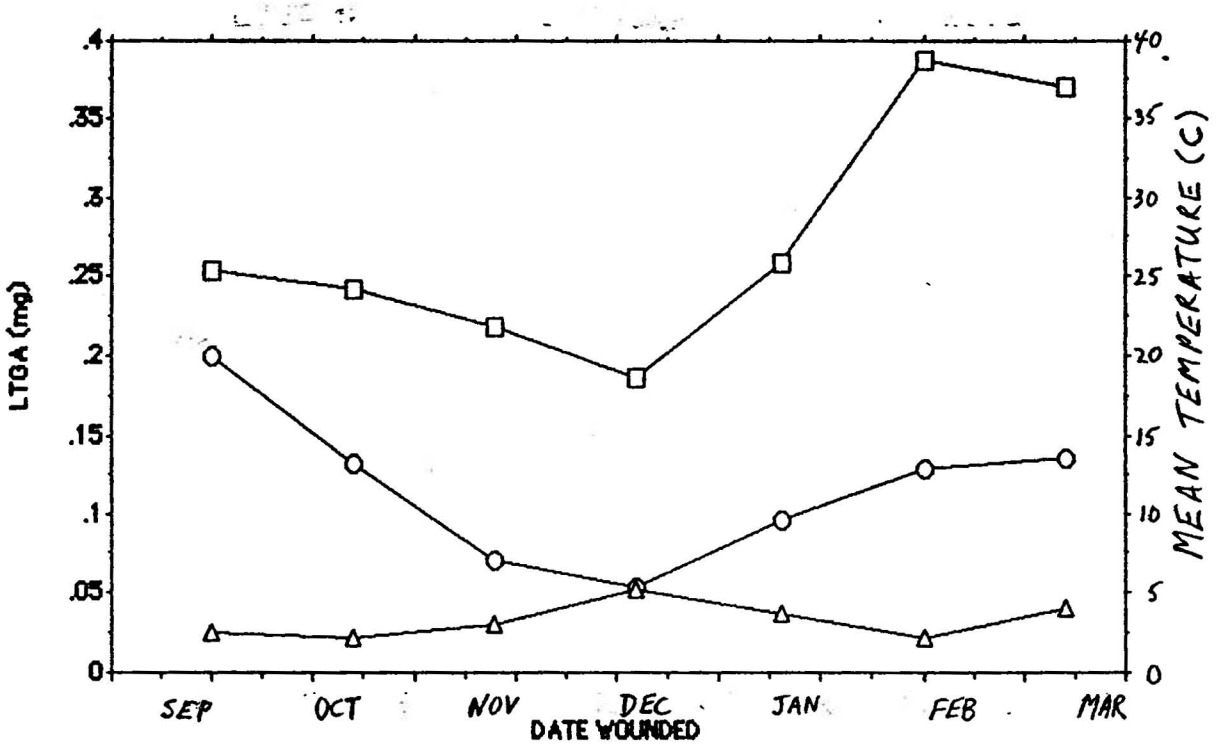

CANKER EXPANSION RATE (MM/DAY)
1.8
1.6
1.4
1.2
1
.8
.6
.4
.2
0
AVERAGE TEMPERATURE (C)
18
16
14
12
10
8
6
4
2
0
24 7 21 5 19 2 16 30 13 27 13 27 10
OCT NOV DEC JAN FEB MAR APR
DATE INOCULATED

Figure 10. The formation of lignin as measured as ligninthioglycolic acid (LTGA) in 4-week-old wounded and nonwounded inner bark of almond trees in an orchard in Yolo Co., CA in the fall, 1985 and winter, 1986. The $LSD_{.05}$ for LTGA in 4-week-old wounds was 0.05 mg. □ 4-week-old wounds; △ nonwounded; ○ mean temperature (C) for 4 week period.


LTGA (mg)
.4
.35
.3
.25
.2
.15
.1
.05
0
40
35
30
25
20
15
10
5
0
MEAN TEMPERATURE (C)
SEP
OCT
NOV
DEC
JAN
FEB
MAR
DATE WOUNDED

SECTION 4.

SUSCEPTIBILITY OF ALMOND CULTIVARS AND STONE FRUIT SPECIES TO PHYTOPHTHORA SYRINGAE

ABSTRACT

The relative susceptibility of almond cultivars and various stone fruit species grown in California to Phytophthora syringae, the causal agent of pruning wound cankers commonly observed in almond orchards in California, was investigated. Pruning cuts were inoculated with mycelium in 1984, 1985, and 1986 and after 9 to 16 weeks canker lengths were measured. All inoculations of fresh wounds in almond trees resulted in cankers. Canker expansion rates varied greatly for most almond cultivars from year to year and Ripon was the only cultivar which consistently had smaller cankers. Inoculation of 3-wk-old wounds in almond cultivars resulted in cankers only in Nonpareil (31.6% cankers). The length of the period when wounds in a cultivar remain susceptible to infection may be important in determining disease severity in the orchard. Both potted and bearing orchard almond, apricot, and peach trees were all very susceptible to P. syringae. Only small cankers formed in plum and French prune trees.

INTRODUCTION

For the last several years profusely gumming cankers were frequently observed associated with pruning wounds in almond trees (Prunus dulcis (Mill.) Webb) in California. Phytophthora syringae (Kleb.) Kleb., which grows well at low temperatures, was found to cause these cankers by infecting pruning wounds in the fall and winter (1). Almond orchards have been observed in which approximately 25% of the pruning wounds developed cankers caused by P. syringae (2). Pruning wound cankers were located throughout the height of the tree to over 5 m high (2).

P. syringae has been found to be involved in crown rots of almond, apricot (Prunus armeniaca L.), cherry (P. avium L.), peach (P. persica (L.) Batsch.), and prune (P. domestica L.) trees in California (5,6,8) and of various species of stone fruit trees besides almonds throughout the world (4,9). Because many hectares of various stone fruit trees are grown in California, there seemed to be a possibility that the fungus could cause pruning wound cankers in other stone fruit trees besides almonds.

Almonds are a major crop in California with over 170,000 ha. The relative susceptibility of the almond cultivars to P. syringae was unknown because the disease has only recently been observed. The objective of this study was to determine the susceptibility of the major almond cultivars and various stone fruit species grown in California to P. syringae.

MATERIALS AND METHODS

An isolate of P. syringae (F-79) obtained from an almond pruning wound canker and maintained on lima bean agar was used for inoculation. This isolate showed typical growth rates in media and almond bark tissue when compared with other P. syringae isolates.

All experiments used the following procedure. During winter, wounds were made in branches 1-3 cm diameter by making cross-sectional cuts with pruning shears. Wounds were inoculated by placing a mycelial plug (9 mm diameter) from a culture growing in lima bean or cornmeal agar on the wound and covering with parafilm. After 9 to 16 weeks, the branches were excised, the outer bark removed, and the extent of inner bark discoloration measured. For controls, agar plugs with no fungal mycelium were placed on some wounds and covered with parafilm.

Almond cultivar trials. Eleven almond cultivars representing large acreages in California were tested in two bearing orchards in both 1984 and 1985. One orchard, in Butte Co., CA, was inoculated January 6, 1984 and January 18, 1985. The resulting

cankers were measured 16 weeks later. The other orchard, in San Joaquin Co., CA, was inoculated January 24, 1984 and February 1, 1985. The resulting cankers were measured about 15 weeks later. For each year, one branch of approximately 2 cm diameter was inoculated on 10 trees per cultivar at each site.

In February, 1985, fresh and 2-wk-old wounds of the cultivars, Nonpareil and Mission, were inoculated in an orchard in Colusa Co., CA. The resulting cankers in the 10 replications were measured 6 weeks later.

In 1986, the resistance of fresh and 3-wk-old wounds in 7 almond cultivars was tested in two bearing orchards in Colusa Co. and San Joaquin Co., CA. The 10 replications per orchard were inoculated in February and cankers measured 12 weeks later.

Stone fruit species trials. Bearing trees of various stone fruit species were inoculated in early March, 1984 in an orchard near Davis, CA. Cankers in 8 branches per species were measured 10 weeks later. Two-yr-old potted trees were inoculated in early March, 1985 and kept in a lathhouse. Cankers in 4 trees for each species were measured 9 weeks later.

RESULTS

The results of the almond cultivar trials for 1984, 1985, and 1986 are presented in Table 1. All inoculations of fresh wounds resulted in cankers. The interaction, cultivar X year, was very significant ($P=0.0001$) for the 1984 and 1985 trials as can be seen by noting that canker expansion rates varied greatly for most cultivars from year to year. For example in Jordanolo, cankers expanded faster than in any other cultivar in 1984, but in 1985 cankers expanded faster in most of the other cultivars. Likewise in the 1986 trial the results for some cultivars (e.g. Price) were quite different than in the previous years. However, in the 1986 trial no cultivars had significantly ($P<0.01$) slower canker expansion and in the 1984 and 1985 trials only Ripon had slower canker expansion. There was a small (7.3%) but significant ($P<0.05$) difference in the canker

expansion rate between the two orchards in 1984 and 1985, but the cultivar X orchard interaction was not significant.

In 1985, 100% of the inoculated 2-wk-old wounds in Nonpareil resulted in cankers, but only 50% of those in Mission. In 1986, 31.6% of the inoculated 3-wk-old wounds in Nonpareil had cankers, whereas, none of those in the other cultivars tested resulted in cankers (Table 1).

In both stone fruit species trials, only small cankers were formed in French prune and plum trees (Table 2). In orchard trees the fastest canker expansion was observed in almond trees (1.86 mm/day), whereas in potted trees, the fastest was observed in apricot (1.66 mm/day). Peach trees also had high canker expansion rates (1.1 mm/day in orchard trees), but they were less than those for almond trees in both the potted and orchard trees.

DISCUSSION

Naturally occurring pruning wound cankers have been observed in all the almond cultivars tested (personal observations). However, since disease distribution in orchards was irregular and clustered and disease incidence was correlated with pruning wound diameter (2), it was difficult to determine by observation the relative susceptibility of the cultivars. Cankers expanded at approximately the same rate in all almond cultivars, except cankers expanded more slowly in Ripon (Table 1). This suggests that there may be no difference in resistance between most cultivars once the canker is initiated.

For all almond cultivars tested, inoculated fresh wounds always resulted in cankers. As almond bark wounds age they gradually develop resistance until by 6 weeks after pruning almost all are immune to infection by P. syringae (3). Wounds in Nonpareil, the cultivar with the most acreage in California, developed this immunity more slowly than wounds in Mission in 1985 and in the other cultivars tested in 1986 (Table 1). This suggests that Nonpareil would be more likely to have pruning wound cankers because

the period that the wounds are susceptible is longer. This would complicate observations of the relative susceptibility of the cultivars in the orchards because if the infection period occurred while the wounds were fresh then all cultivars would be very susceptible. However, if conditions were not favorable for infection until wounds had aged then differences in disease incidence would be observed.

Although no reports in the literature were found of pruning wound cankers in almonds outside of California or in other stone fruit species anywhere, several researchers have examined the relative susceptibility of some of the stone fruit species to collar rot caused by P. syringae (4,7,9). Two of the studies were done in areas distant from California such as New Zealand (9) and Greece (4) and only one included an almond cultivar (4). These studies consisted of inoculating trees and then determining the amount of girdling of 2-yr-old trees (4), the amount of girdling and canker length in 2-yr-old trees (7), and infection severity by subjective observation of trees of unspecified age (9). All studies found that in apricot trees cankers were the most severe, in peaches they were somewhat less severe, and in plums the cankers developed the least and sometimes not at all (4,7,9). In our study, in the 2-yr-old potted trees apricots had the largest cankers, peaches had somewhat smaller, and plums had small cankers. However, in the orchard trees peaches actually had larger cankers than apricots (Table 1). French prune trees had the smallest cankers in both potted trees and orchard trees (Table 1). The only study to include an almond cultivar, found inoculated almond trees to have a girdling index intermediate between apricots and peaches (4). This corresponds to what was observed in 2-yr-old potted trees in our study but in orchard trees the cankers expanded the fastest in almond trees (Table 1). Very young potted trees may not be adequate predictors of mature tree resistance. Although various cultivars and diverse isolates of P. syringae were used in these studies, it is concluded that almond, apricot, and peach trees are very susceptible and plum and prune are resistant. In California, pruning wound cankers caused by P.

syringae have been observed in French prune and apricots, but this is rare (personal observations). At this time it is not known why in California Phytophthora pruning wound cankers have been very common and widespread in almond orchards (1, 2) but not in orchards of other stone fruit trees when the P. syringae isolate grew so well in other stone fruit trees.

The use of mycelial agar plugs to inoculate fresh wounds although very convenient may not be the most appropriate method to determine the relative susceptibility of trees to Phytophthora species. It is possible that some cultivars are resistant to canker initiation by low levels of inoculum, but once the fungus is established in the host the canker expands rapidly. An important component of the resistance of cultivars may be the rate aged wounds become resistant as observed in this study (Table 1).

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Table 1. Development of cankers by Phytophthora syringae in bearing trees of various almond cultivars in three orchards (Butte Co. 1984, 1985; San Joaquin 1984, 1985, 1986; and Colusa Co. 1986).

	Canker expansion rate (mm/day)				Cankers in
Cultivar	1984	1985	Mean[x]	1986	aged wounds (%)[y]
Jordanolo	1.12	0.81	0.97	----	---
Butte	0.91	1.02	0.96	0.97	0.0
Merced	0.85	1.06	0.96	----	---
Mission	0.85	1.01	0.93	1.11	0.0
Fritz	0.87	0.97	0.92	----	---
Nonpareil	1.05	0.78	0.91	1.15	31.6[z]
Carmel	1.09	0.71	0.90	0.94	0.0
Ne Plus Ultra	0.84	0.82	0.83	0.81	0.0
Thompson	0.89	0.77	0.83	0.81	0.0
Price	0.85	0.61	0.73	1.12	0.0
Ripon	0.59	0.48	0.53	----	---
$LSD_{.05}$	0.21	0.32	0.18	0.23	
$LSD_{.01}$	0.30	0.45	0.26	0.34	

[x] Mean of the 1984 and 1985 data.

[y] In 3-wk-old wounds in same orchards as 1986 trials.

[z] Nonpareil had significantly more cankers than any of the other cultivars according to Fisher's Exact Test (P=0.01).

Table 2. The development of Phytophthora syringae cankers in potted and orchard trees of various stone fruit species.

		Canker expansion rate (mm/day)	
Stone fruit tree	Cultivar	Orchard trees	Potted trees
Almond (Prunus dulcis (Mill.) Webb)	Nonpareil	1.86	1.42
Peach (P. persica (L.) Batsch.)	Fay Elberta	1.11	1.20
Apricot (P. armeniaca L.)	Blenheim	0.79	1.66
Cherry (P. avium L.)	Bing	----[z]	0.67
Plum (P. salicina Lindl.)	Santa Rosa	0.32	0.57
Prune (P. domestica L.)	French	0.27	0.17
	$LSD_{.05}$	0.24	0.45
	$LSD_{.01}$	0.32	0.62

[z] not determined.

ADDENDUM

In the following Tables 3 and 4 the complete results of the almond cultivar trials for 1984 and 1985 are presented. Some of the results were summarized in Table 1. But many of the cultivars were present in only one of the orchards, and therefore, were not included in Table 1. The methods used are as described above.

Table 3. Expansion rate of Phytophthora syringae cankers in various almond cultivars at the California State University, Chico cultivar trial, Butte Co. for the winters of 1984 and 1985.

Canker expansion rate (mm/day)

	Year		
Cultivar	1984	1985	Mean
Norman	1.47	1.08	1.28
Milow	1.17	1.34	1.26
Carrion	1.11	1.38	1.25
Padre	1.24	1.16	1.20
Sonora	0.80	1.41	1.10
Jordanolo	1.23	0.94	1.08
Butte	0.97	1.08	1.02
Vesta	0.94	1.08	1.01
Solano	1.00	0.98	0.99
Merced	0.83	1.08	0.96
Robson	1.16	0.74	0.96
Ne Plus Ultra	0.85	1.02	0.94
Mission	0.88	0.95	0.92
Thompson	0.88	0.93	0.91
Fritz	0.83	0.98	0.91
Carmel	0.97	0.84	0.91
Nonpareil	0.94	0.84	0.89
Harvey	0.81	0.88	0.84
Granada	0.78	0.74	0.76
Price	0.79	0.63	0.71
Ripon	0.61	0.55	0.58
Mean	0.97	0.98	0.97
		$LSD_{.05}$	0.17
		$LSD_{.01}$	0.24

Table 4. Expansion rate of Phytophthora syringae cankers in various almond cultivars at Delta College almond cultivar trial, San Joaquin Co. for the winters of 1984 and 1985.

Canker expansion rate (mm/day)

	Year		
Cultivar	1984	1985	Mean
Livingston	1.51	1.25	1.38
Grace	1.30	1.16	1.23
Mono	1.41	1.04	1.22
Sauret #1	1.50	0.82	1.16
Monterey	1.27	0.98	1.12
Ruby	0.72	1.22	0.97
Merced	0.87	1.04	0.96
Mission	0.81	1.07	0.94
Fritz	0.92	0.96	0.94
Nonpareil	1.16	0.71	0.93
Tokyo	0.94	0.87	0.90
Carmel	1.21	0.59	0.90
Butte	0.85	0.96	0.90
Le Grand	0.75	0.98	0.86
Jordanolo	1.00	0.69	0.85
Sauret #2	0.68	0.85	0.76
Yosemite	0.78	0.73	0.76
Price	0.92	0.60	0.76
Thompson	0.89	0.61	0.75
Ne Plus Ultra	0.83	0.62	0.73
Monarch	0.69	0.73	0.71
Planada	0.64	0.77	0.70
Peerless	0.65	0.52	0.59
Ripon	0.57	0.40	0.48
Mean	0.95	0.84	0.90
		$LSD_{.05}$	0.14
		$LSD_{.01}$	0.20

SECTION 5.

CHEMICAL PROTECTION OF PRUNING WOUNDS FROM INFECTION BY PHYTOPHTHORA SYRINGAE

ABSTRACT

Experiments were performed using cupric hydroxide (Kocide 101 77 WP) and fosetyl-Al (Aliette 80 WP) to treat almond tree (Prunus dulcis) pruning wounds for prevention of Phytophthora pruning wound canker caused by Phytophthora syringae. In a trial conducted in 1985, inoculation with P. syringae of wounds treated with cupric hydroxide (740 g active ingredient (ai) per L linseed oil) resulted in no cankers while the nontreated wounds had 40%. However, signs of phytotoxicity were observed. In a trial conducted in 1986, the percentage of cankers resulting from inoculation with P. syringae were 73.5% for nontreated, 20.0% for cupric hydroxide (1.8 g ai / L water) treated, and 0.0% for fosetyl-Al (30 g ai / L water) treated wounds, but again signs of phytotoxicity were observed in wounds treated with cupric hydroxide. Using the thioglycolic acid assay for lignin in the inner bark, substantially more lignin was detected in cupric hydroxide (18.3 g ai / L) treated wounds than in the nontreated wounds, but there was no difference between wounds treated with cupric hydroxide (1.8 g ai / L), fosetyl-Al (30 g ai / L), and the nontreated wounds. The signs of the phytotoxicity caused by cupric hydroxide were formation of clear gum, xylem discoloration near the cambium, and abnormal lignification around wounds.

INTRODUCTION

Phytophthora pruning wound canker of almond trees is caused by Phytophthora syringae (Kleb.) Kleb., which enters wounds made in the fall and winter during pruning (1). Several compounds, including copper fungicides, have been reported to be effective against various Phytophthora species (7). Cupric hydroxide was found to

be effective against Phytophthora parasitica Dast. when applied with a paintbrush to the trunk of citrus trees (10) and fosetyl-Al had high fungitoxic activity against Phytophthora species (8). Some of the almond growers in California have attempted to control Phytophthora pruning wound canker by applying cupric hydroxide in linseed oil to pruning wounds, although the efficacy of this treatment has not been tested.

Wound treatments have been ineffective both as physical and chemical barriers to entry by wood-decay fungi (9). However, P. syringae does not usually attack the xylem but is restricted to the inner bark (personal observation), so different demands are placed on a pruning wound treatment for P. syringae. An example of an effective fungicide treatment of pruning wounds is the protection of apricot trees against Eutypa dieback with benzimidazole fungicides (6).

Wound treatments at times may be detrimental to the tree. One researcher found that after more than 40 wound paints had been tested, only one fungicide was not phytotoxic (5). The objective of this study was to find a chemical which would be effective in protecting wounds against P. syringae but would not be phytotoxic.

MATERIALS AND METHODS

Production of Zoospores. P. syringae (isolate F-79) was grown on amended lima bean agar (2). The agar was cut up into pieces of approximately 2 x 2 cm and placed in sterile distilled water in petri dishes and kept at 15 C. After 7 days the water was drained off, more sterile distilled water was added, and the petri dishes placed at 4 C. Two hours later the water with the zoospores was poured into test tubes and vortexed at medium speed for 90 seconds to encyst the zoospores. The zoospores were then filtered using 0.025 mm mesh filters.

1985 Fungicide Trial. During the winter, pruning wounds were made in bearing almond trees (cv. Nonpareil) in an orchard near Davis, CA by cutting 1 to 2 cm diameter branches off with pruning shears. A mixture of 740 g active ingredient (ai) cupric

hydroxide (Kocide 101 77 WP) in 1 L linseed oil was applied to the entire wound surface with a paintbrush to 20 branches on different trees. One day later half of the wounds were inoculated with 0.3 ml of a zoospore suspension (1.7×10^4 zoospores / ml), while the other half of the wounds were left noninoculated for investigation of phytotoxicity. Seven weeks later the inoculations were examined for formation of cankers and signs of phytotoxicity. Some noninoculated wounds were sectioned and stained using the method of Doster (4) and then examined for wound lignification and periderm formation.

1986 Fungicide Trial. During the winter, a fungicide trial was performed in a Nonpareil almond orchard near Davis, CA. Branches (1 to 2 cm diameter) were cut cross-sectionally about 20 cm from the main branch using pruning shears. The fungicides tested were cupric hydroxide (1.8 g ai / L water) and fosetyl-Al (Aliette 80 WP) (30 g ai / L water). The fungicides were applied to and around the wounds with a paintbrush. A zoospore suspension (1×10^4 zoospores / ml) was produced using the method given above, applied with a paintbrush to 10 wounds for each fungicide treatment and to 49 nontreated wounds, and then plastic bags were tied around the wounds for three days. After 9 weeks the wounds were examined for signs of phytoxicity and canker formation.

Effect of Fungicides on Bark Wound Response. Wounds were made during winter in branches with a 6 mm diameter corkborer through the bark to the cambium but not into the xylem. The three fungicide treatments, cupric hydroxide (1.8 g ai / L), cupric hydroxide (18.3 g ai / L), and fosetyl-Al (30.0 g ai / L), were applied to the wounds with a paintbrush. All treatments were applied to one branch on each of 7 almond trees (cv. Carmel) near Davis, CA. Twenty days later the wounded areas were removed with a 11 mm diameter corkborer and the thioglycolic acid assay for lignin performed on the inner bark (3).

RESULTS

In the 1985 trial, cupric hydroxide completely prevented infection by P. syringae, but the treated wounds usually had clear gumming around the wound. When these wounds were sectioned, stained, and examined with the microscope, they had no lignified zone, which was well developed in the nontreated wounded inner bark (Table 1). In the 1986 trial, cupric hydroxide treated wounds had substantially fewer cankers than the nontreated wounds, but these wounds showed clear gumming and extensive discoloration of the xylem near the cambium (Table 1). Fosetyl-Al treated wounds prevented canker formation completely, while the inoculated nontreated wounds resulted in 73.5% cankers. No signs of phytotoxicity were observed in the fosetyl-Al treated wounds.

Substantially more ligninthioglycolic acid was detected in nontreated 20 day-old-wounded tissue than in the nonwounded tissue (Table 2). Substantially more ligninthioglycolic acid was detected in the wounds treated with the high concentration of cupric hydroxide (18.3 g ai / L) than in the nontreated wounds, but there was no significant difference among the wounds treated with the low rate of cupric hydroxide (1.8 g ai / L), fosetyl-Al, and the nontreated wounds (Table 2).

DISCUSSION

Cupric hydroxide, even at the low rate of 1.8 g ai / L, was observed to be phytotoxic when applied to pruning wounds (Table 1). The low concentration (1.8 g ai / L = 2 lb actual / 100 gal) was less than or equal to the spray rate commonly used in stone fruit orchards. Phytotoxicity observed at such low rates on fresh pruning wounds but not on the foliage may be explained by the absence of a barrier to the fungicide such as the cuticle of leaves or outer bark of branches. Furthermore, the xylem of fresh wounds in winter has a suction which draws in the fungicide mixture (personal observation), and painting wounds may introduce more of the compound than spraying. The signs of phytotoxicity observed in cupric hydroxide treated wounds were clear gumming, xylem discoloration, occasionally additional bark dieback, and abnormal lignification.

Application rates recommended for foliage or branches with intact outer bark may not be appropriate for fresh wounds.

The thioglycolic acid assay quantifies the amount of lignin in wounded inner bark tissue (3), and provides a measure of the progression of wound periderm development. The assay was used in this study as a sensitive test for an abnormal response of the inner bark in treated wounds (Table 2). The wounds treated with cupric hydroxide at the concentration 18.3 g ai / L had substantially more ligninthioglycolic acid (as measured by the absorbance at 280 nm) than the nontreated wounds indicating an alteration of the normal wound response, while the fosetyl-Al and the cupric hydroxide at the concentration 1.8 g ai / L had about the same ligninthioglycolic acid as the nontreated (Table 2). However, in the 1986 trial (Table 1) extensive discoloration of the xylem near the cambium, but not of the inner bark, was observed in wounds treated with the low rate of cupric hydroxide, suggesting that parts of the xylem were more sensitive than the inner bark.

Treating wounds with fosetyl-Al was effective in preventing infection by P. syringae, while treating with cupric hydroxide substantially decreased the number of cankers formed (Table 1). Although wound treatments may not be effective against wood decay (9), it seems likely that fungicidal wound paints could be very effective against canker-forming fungi such as P. syringae. However, more work is needed to determine how well these treatments function in preventing natural infection. During winter, wounds remain susceptible to P. syringae for up to 6 weeks after wounding (4). Studies are in progress to determine fungicide persistence for the period of wound susceptibility.

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Table 1. Percentage of wounds inoculated with P. syringae resulting in cankers and phytotoxicity observed around wounds for trials performed in 1985 and 1986.

Treatment	Rate (g ai / L)	Cankers (%)	Phytotoxicity[v]
---------- 1985 ----------			
Nontreated	none	40	-
Cupric hydroxide	740[w]	0[x]	+
---------- 1986 ----------			
Nontreated	none	73.5	-
Cupric hydroxide	1.8	20.0[y]	+
Fosetyl-Al	30	0.0[z]	-

[v] + means at least one of the following phytotoxicity signs were observed: clear gumming, xylem discoloration, excessive inner bark dieback, and abnormal wound response. - means none of the signs of phytotoxicity were observed.

[w] Mixed in 1 L linseed oil, with no water added. All other fungicides were in water only.

[x] Significantly fewer cankers than nontreated according to Fisher's Exact Test (P = 0.043).

[y] Significantly fewer cankers than nontreated by Fisher's Exact Test (P = 0.002).

[z] Significantly fewer cankers than nontreated by Fisher's Exact Test (P = 0.000).

Table 2. Extent of inner-bark wound response to fungicides as measured by the absorbance (280 nm) after the thioglycolic acid assay.

Wound treatment	Rate (g a.i. / L)	Absorbance (280 nm)[z]
Cupric hydroxide	18.3	0.852
Nontreated	none	0.550
Fosetyl-Al	30.0	0.516
Cupric hydroxide	1.8	0.447
Nonwounded	none	0.125
	$LSD_{.05}$	0.153
	$LSD_{.01}$	0.207

[z] Absorbance (280 nm) measures the amount of ligninthioglycolic acid present in the sample, which is taken to represent the amount of lignin originally present in the tissue.

SECTION 6.

THE EFFECT OF TEMPERATURE AND TYPE OF MEDIUM ON OOSPORE PRODUCTION BY PHYTOPHTHORA SYRINGAE

ABSTRACT

Reliable methods for oospore production by Phytophthora syringae were developed by the use of low temperatures and media amended with vegetable oil or made from the leaves of stone fruit trees. All of the fourteen isolates tested produced oospores in media after 4 weeks at 5 and 9 C but most did not produce oospores at temperatures above 12 C. Similarly, oospores were only observed in almond leaves kept at 12 C and below. Oospore production was greatly increased when corn oil, linseed oil, or wheat germ oil was added to various base media. The more wheat germ oil added to the media (up to 48 ml / L medium), the more oospores that were produced. The addition of β-sitosterol resulted in a slight but significant increase in oospores. Abundant oospores were produced by an almond isolate of P. syringae in almond leaf medium (75 g fresh leaves per liter medium). Oospores were also produced in media made from the leaves of other stone fruit trees. However, the fungus would not grow in apple leaf medium.

INTRODUCTION

Phytophthora syringae (Kleb.) Kleb., which grows well at low temperatures but is strongly inhibited by temperatures above 24 C, causes branch cankers and crown rot in stone fruit trees (1,17), fruit rot and crown rot in apple trees (22,23), and fruit rot in citrus (7). In almond (Prunus dulcis (Mill.) Webb) orchards in California, the fungus infects pruning wounds during the fall and winter when temperatures are relatively low, but the resulting cankers become inactive in early summer or late spring with the onset of high temperature (1). The fungus was commonly isolated from fallen almond leaves on the orchard floor and oospores were observed in these leaves (5). Even

though P. syringae is homothallic (18), during investigations of this disease we had difficulty producing oospores in media.

Several researchers have found media and conditions favorable for oospore production by various Phytophthora species, but in each case P. syringae did not produce any oospores (9,11,16,19). In general, keeping cultures in the dark favored oospore production by Phytophthora species, but P. syringae did not produce any oospores in the light or dark (9). β-Sitosterol was found to enhance oospore formation with other Phytophthora species but not with P. syringae (11). In their investigation of the relationship of the carbon-nitrogen ratio to sexual reproduction in Phytophthora species, Leal et al. (16) did not observe oospores of P. syringae in any of the media used. Savage et al. (21) after many attempts concluded that conditions favorable for production of abundant oospores by P. syringae had not been found.

Chee et al. (4) used an amended V-8 medium to enhance oospore production by Phytophthora palmivora. Vegetable oils have been found to greatly increase oospore formation by several Phytophthora species and other fungi in the Oomycetes, but were not tested with P. syringae (14). These media seemed promising in enhancing oospore production. The objective of our study was to develop a reliable method for oospore production by P. syringae which would aid in species identification, and allow research into oospore genesis, survival, and germination.

MATERIALS AND METHODS

An isolate of P. syringae (F-79), obtained from a pruning wound canker in a California almond orchard, was used in most of these studies. Additional isolates were used for one experiment and are described below. The fungi were maintained in lima bean or V-8 agar at 15-20 C.

General media. The following media were used to determine the effect of various additives on oospore production. The peptone-yeast extract-glucose medium (PYG)

consisted of 1.25 g peptone, 1.25 g yeast extract, 1.25 g glucose, 0.075 g $CaCl_2$ $2H_2O$, and 20 g agar per liter of deionized water (14). The V-8 medium (V8) consisted of 177 ml of V-8 juice (clarified by centrifugation for 20 min at 4000 rpm), 1 g $CaCO_3$, 15 g agar per liter of deionized water. The V-8+$CaCl_2$ medium (V8C) was the same as the V8 medium except 100 mg $CaCl_2$ $2H_2O$ per liter was added instead of the $CaCO_3$. The amended V-8 medium (V8A) consisted of 177 ml V-8 juice (clarified), 30 mg β-sitosterol, 20 mg tryptophan, 100 mg $CaCl_2$ $2H_2O$, 1 mg thiamine, and 15 g agar per 1 liter deionized water (4).

Effect of temperature on oospore formation. Fourteen isolates of P. syringae obtained from 12 different locations in California were used. Eight of the isolates were from pruning wound cankers in almond trees, three isolates were from fallen almond leaves on the orchard floor, two were from apricot trees, and one from lemon fruit. Five media were tested, V8 to which 6 ml wheat germ oil was added, V8C to which 6 ml wheat germ oil was added, PYG to which 1 g linseed oil and 1 g corn oil was added per liter of medium, V8C, and V8A. For the PYG oil, V8C, and V8A media only five isolates were tested. For each medium and isolate, two petri plates were placed in each of the incubators kept at 6 to 21 C at 3 C intervals. After 4 weeks the bottom of the petri plates were examined with the microscope and oospores counted.

An experiment was performed on the effect of temperature for oospore production in almond leaves. Almond leaves were gathered from orchard trees and surface sterilized (2 min in 10% bleach, rinsed 3 times with sterile water). Leaf disks, made with a 9 mm diameter cork borer, were placed on 3-day-old colonies growing in cornmeal agar (CMA) and kept in incubators ranging in temperature from 2 to 24 C. Leaves on noninoculated CMA and inoculated CMA with no leaves were also placed in the incubators. After 4 weeks oospores were counted in leaves and media using the microscope for 6 replications.

Testing of media. Various amounts of corn oil, linseed oil, wheat germ oil, cod liver oil, and β-sitosterol were added to PYG, V8, and V8A. Also, media were made from the leaves

of several fruit trees (almond, apple, apricot, cherry, French prune, peach, and plum) as follows: 75 g fresh leaves were washed in distilled water, ground in deionized water for 1 min in a Waring blender, centrifuged 5 min at 10000 rpm, the supernatant placed in a flask with 20 g agar, deionized water added to make 1 liter, and then autoclaved. After inoculation, 6 petri plates for each medium were kept at 9 and 12 C. After 4 weeks the plates were examined for oospore formation.

Effect of various amounts of wheat germ oil. Wheat germ oil in amounts ranging from 1 to 48 ml and β-sitosterol in amounts of 0 and 180 mg per liter of medium were added to PYG before autoclaving. After inoculation, the 6 petri plates per medium were kept at 9 C. After 4 weeks the oospores were counted by examining the undersurface of the plate with the microscope.

RESULTS

All of the P. syringae isolates tested produced abundant oospores in V8 medium amended with oil at 5 and 9 C (Fig. 1) but most did not produce oospores at temperatures greater than 12 C (Table 1). At most temperatures fewer isolates produced oospores in V8C medium. Although fewer isolates were tested, oospores were produced as frequently in PYG amended with oil as the V8 oil medium, only occasionally in V8A and very rarely in V8, but the relationship of low temperatures resulting in greater oospore production was similar to that with the V8 medium amended with oil. Similarly, oospores were observed inside the almond leaf tissue in all leaf disks kept at 12 C and below, but none at higher temperatures (Table 1). The most oospores were produced at 9 C, although this was not significantly greater than the number of oospores produced at 5 or 12 C. No oospores were observed in inoculated CMA or noninoculated almond leaves.

A summary showing the results for some of the media tested for oospore production is presented in Table 2. Although few or no oospores were observed in PYG, V8, and

V8A media, oospore production was greatly increased when corn oil, linseed oil, or wheat germ oil was added to these media but not when cod liver oil was added. Abundant oospores were produced in some media such as PYG + wheat germ oil (6 ml) where over 50 oospores per mm^2 was commonly observed. However, in the media with oil there was also abundant oil droplets which interfered with rapid quantification of oospores in the media. Abundant oospores (frequently over 50 oospores per mm^2) were produced in the almond leaf medium (Fig. 2), but the fungus was unable to grow at all in the apple leaf medium. P. syringae grew in the media made from leaves of apricot, cherry, French prune, peach, and plum trees, and produced ample number of oospores in all of them.

Increased amounts of wheat germ oil in PYG resulted in greatly increased numbers of oospores (Fig. 3). However, there was only an 11% increase in oospores observed when using 48 ml instead of 24 ml of wheat germ oil. Even at the lowest amount tested of 1 ml per liter of PYG medium, 0.6 oospores per mm^2 were observed which is significantly (P=0.008 Mann-Whitney U test) more than the 0.0 observed with PYG with no wheat germ oil. The addition of β-sitosterol resulted in a mean increase of 6.6 oospores per mm^2 (significant at P=0.001), but this increase was slight in comparison to the effect of the oil.

DISCUSSION

The addition of vegetable oils to several media facilitated abundant oospore production by P. syringae (Table 2, Fig. 1), as has been observed with some other Phytophthora species (14),. Cod liver oil did not substantially enhance oospore production by P. syringae, but all the vegetable oils greatly increased oospore formation when added to the base media PYG, V8A, V8C, and V8 (Table 2). The enhancement of oospore production by P. syringae may be due to more polyunsaturated fatty acids present in the vegetable oils tested (between 55 and 67% by weight) (20,24)

than in cod liver oil (about 26%) (8), although there is only a slight difference in total unsaturated fatty acids since cod liver oil consists of approximately 50% monosaturated fatty acids (8). Also, the carbon chain length of the fatty acids may be important, since about 84% of each of the vegetable oils used consist of fatty acids with an 18-carbon chain length (20,24) while cod liver oil has only about 27% (8). Unsaturated fatty acids were important in enhancing oospore formation by the fungus Lagenidium giganteum, an Oomycete (15). As more wheat germ oil was added to the medium the number of oospores produced increased (Figure 3). It is possible that the presence of sterols in the vegetables oils (24) was responsible for the increased oospore formation. Sterols have been found to be important in sexual reproduction for a number of Phytophthora species (6). Although two other studies (11,16) were not able to demonstrate that β-sitosterol enhanced oospore production by P. syringae, in our studies β-sitosterol increased oospore production by P. syringae. However, it has been suggested that sterols increased oospore production by L. giganteum because the fatty acid uptake by the fungus was enhanced (13,15). Oxidation products of eicosapolyenoic acids may also be involved in regulation of induction and maturation of oospores (12).

Abundant oospores were formed by P. syringae in the almond leaf medium (Table 2, Fig. 2) as well as in almond leaves (Table 1). P. syringae has been frequently isolated from fallen almond leaves in almond orchards and abundant oospores have been observed in these leaves (5). The production of oospores in almond leaves seems to be important for the development of the fungus in the orchard (5). Oospores were likewise produced in all of the media made from the leaves of other stone fruit trees (apricot, cherry, French prune, peach, and plum). However, P. syringae did not grow in the apple leaf medium in our study, even though abundant oospores have been observed in apple leaves (10). The stimulation of oospore formation by host extracts has been observed with P. cinnamomi and avocado roots (25). It is not clear why P.

syringae rarely produces oospores in lima bean or V-8 media, but abundantly in the almond leaf medium.

Most of the isolates of Phytophthora syringae tested only produced oospores at 12 C or below (Table 1), even though the optimum temperature for vegetative growth was 21 C (1). For P. palmivora the optimum temperature for sexual reproduction was almost 10 C less than the optimum for vegetative growth in vitro (2). It is not true in general for Phytophthora species that the optimum for oospore production is less than that for vegetative growth as shown by the case of P. hevea where the optima are the same (2). The medium used can compensate for the inhibition of oospore production above 12 C as shown by more isolates producing oospores in oil-amended V8 medium than in oil-amended V8C medium at 15 and 18 C (Table 1). For P. palmivora, the relationship of temperature to oospore production was the same in the host tissue, Piper nigrum, as in media (3). Similarly with P. syringae, only the temperatures favorable for oospore production in media were also favorable in almond leaf tissure (Table 1). It is possible the reason that many studies failed to demonstrate oospore formation by P. syringae was that the temperature was at 20 C or higher (9, 11, 16, 19), which would be too high for almost all isolates tested in our study. Therefore, it is recommended that experiments investigating oospore formation involving P. syringae should be performed at below 12 C.

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Table 1. Effect of temperature on oospore production by Phytophthora syringae in vegetable oil amended V8 and V8C media and in almond leaves.

	Isolates producing oospores (%)		Oospores /mm^2
Temperature (C)	V8 medium[x]	V8C medium[x]	In almond leaf[y]
2	---	---	1.8[z]
5	100	64	5.6
9	100	100	6.1
12	50	14	3.2
15	21	7	0.0
18	21	7	0.0
21	7	7	0.0
24	---	---	0.0

[x] Percentage of 14 isolates producing oospores in V8 and V8C media amended with 6 ml wheat germ oil. --- indicates not tested.

[y] Only one isolate of P. syringae was used with almond leaf disks. All of the inoculated leaf disks kept at 12 C or below had oospores, while none of the leaf disks kept at temperatures above 12 C. There was a significant difference between any of the temperatures less than or equal to 12 C and any greater than 12 C by Fisher's Exact Test (P=0.001).

[z] For the temperatures less than 15 C, $LSD_{.05} = 3.0$.

Table 2. Production of oospores by Phytophthora syringae in various media after 4 weeks at 9 and 12 C.

Media	Oospore production rating[y]
Almond leaves (75 g fresh weight)[x]	+++
PYG + corn oil (12 ml) + linseed oil (6 ml)	+++
PYG + wheat germ oil (6 ml)	+++
V8 + wheat germ oil (6 ml)	+++
V8A + corn oil (12 ml)	+++
V8C + wheat germ oil (6 ml)	+++
PYG + corn oil (6 ml) + sitosterol (0.18 g)	+++
Cherry leaves (75 g fresh weight)	+++
Apricot leaves (75 g fresh weight)	+++
PYG + corn oil (6 ml)	++
PYG + linseed oil (6 ml)	++
Plum leaves (75 g fresh weight)	++
Peach leaves (75 g fresh weight)	++
PYG + corn oil (2 ml) + linseed oil (1 ml)	++
French prune leaves (75 g fresh weight)	++
V8	+
V8C	+
V8A	+
PYG + corn oil (1 ml)	+
PYG + cod liver oil (6 ml)	+
PYG	-
Apple leaves (75 g fresh weight)	-[z]

[x] The numbers in parentheses represent the quantity used per liter of medium. PYG, V8A, V8C, and V8 stand for the peptone-yeast extract-glucose, amended V-8, V8 with $CaCl_2$ and V-8 media, respectively, described in the text.

[y] The rating was based on the mean number of oospores observed in about thirty microscope fields of about 2 mm^2. - indicates none observed, + indicates > 0 but < 1, ++ indicates > 1 but < 10, and +++ indicates > 10.

[z] No fungal growth occurred in this medium.

Figure 1. At 9 C abundant oospores were produced by Phytophthora syringae in V8 medium amended with 6 ml wheat germ oil per liter of medium. Bar = 0.1 mm.

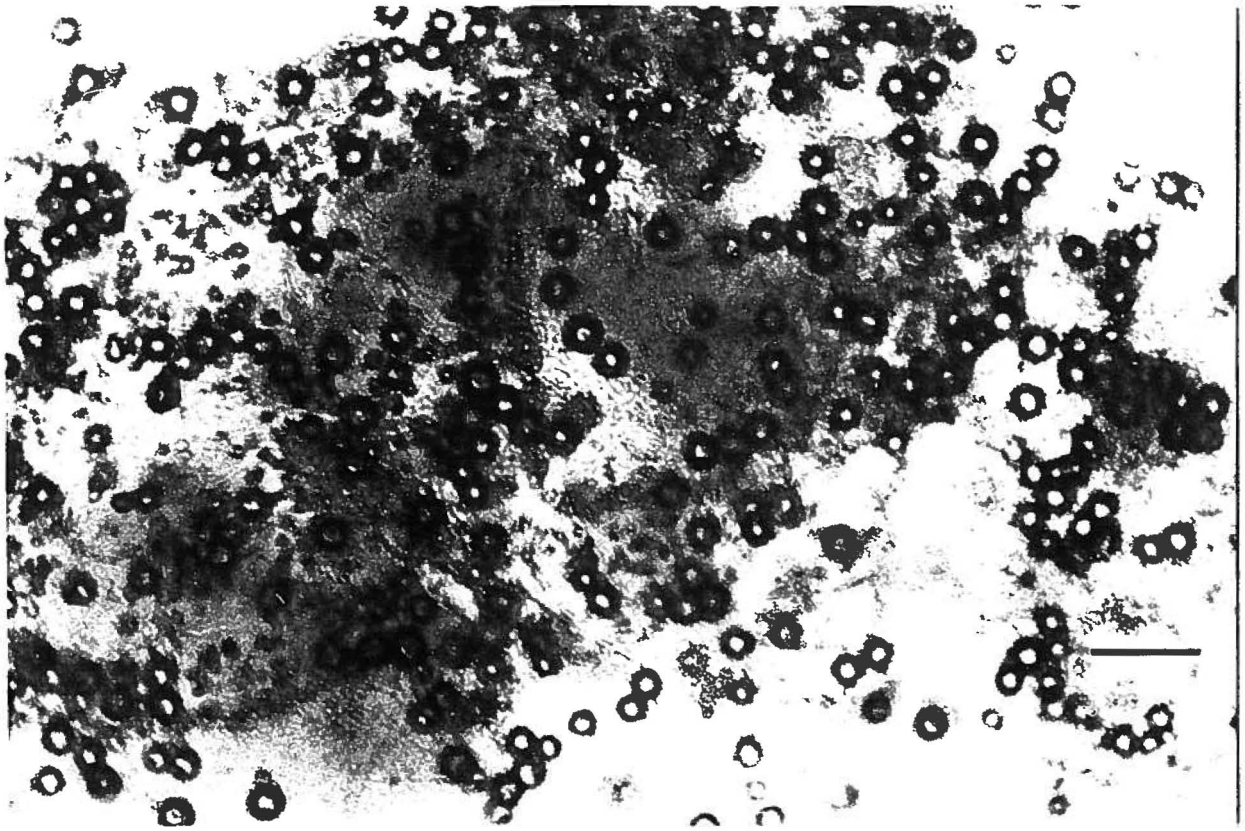


Figure 2. Production of oospores by Phytophthora syringae in almond leaf medium. Bar = 0.1 mm.

Fig. C
15

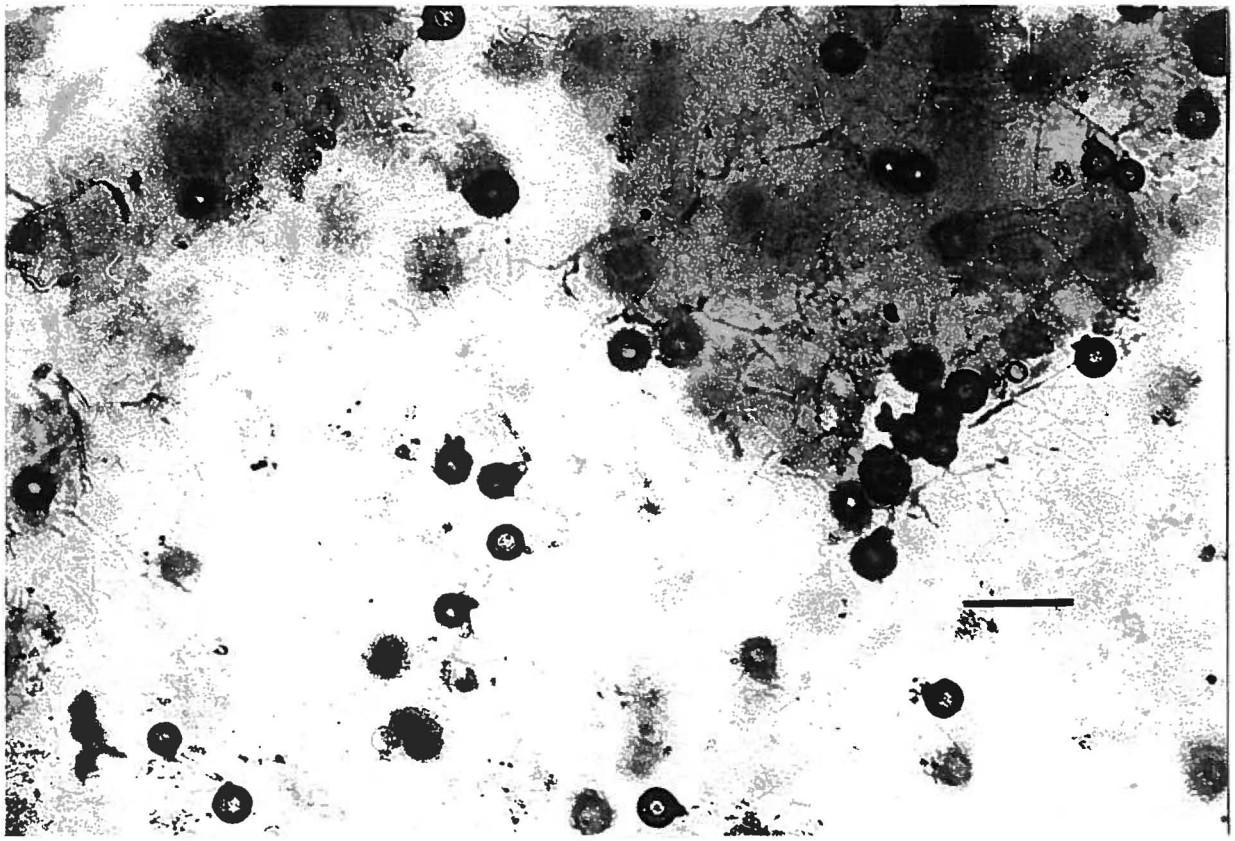
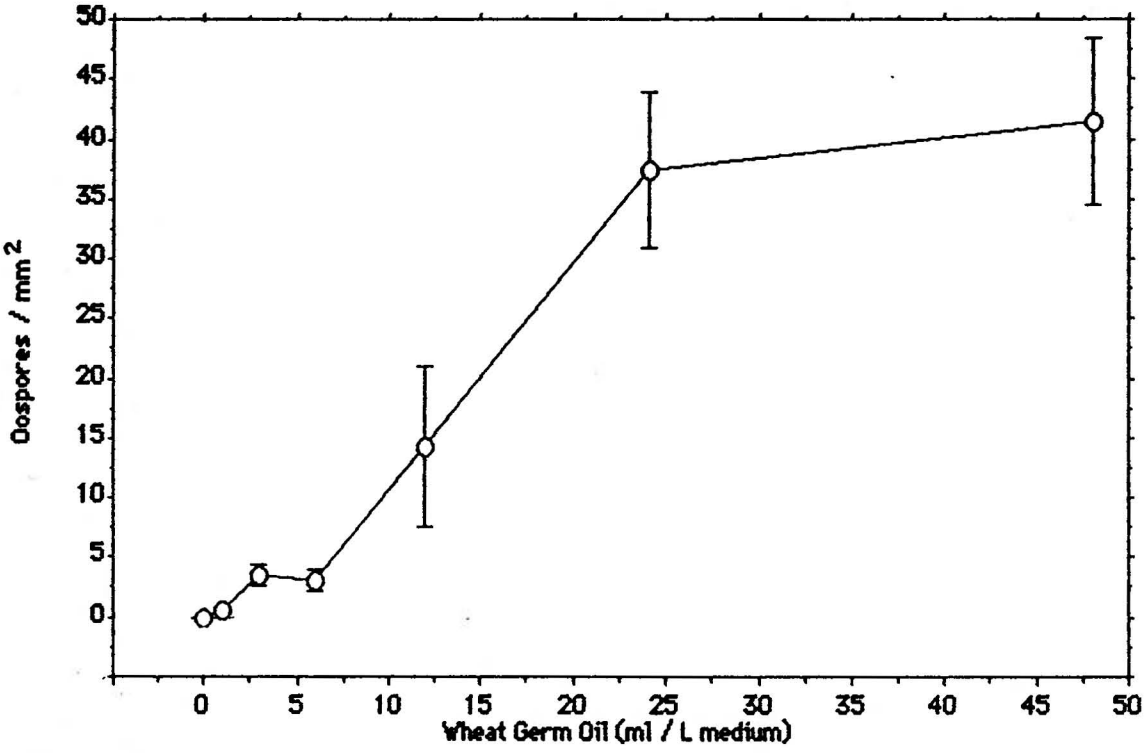


Figure 3. The effect of the addition of various amounts of wheat germ oil to PYG on the production of oospores by Phytophthora syringae at 9 C. The bars equal 1 standard error of the mean greater than and less than the mean.


Oospores / mm 2
0
5
10
15
20
25
30
35
40
45
50
Wheat Germ Oil (ml / L medium)