Factors Influencing Ingress of Xanthomonas pruni through Peach Leaf Scars and Subsequent Development of Spring Cankers

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ABSTRACT

Leaf scars, obtained by the forcible removal of the entire leaf or by delaminating the petioles 7 days prior to their removal, were inoculated immediately and semimonthly from 15 July to 1 November with *Xanthomonas pruni*. The scars were located either at the tip or one third of the way down the twig. Summer cankers were produced following inoculations performed from 15 July to 1 October, whereas spring cankers resulted from inoculations made from 17 September to 1 November. Ingress of *X. pruni* occurred only at fresh scars resulting either from the forcible removal of leaves or from the removal of petioles that had been delaminated 1 and 3 days. Inoculated scars at the tip developed many more spring cankers than those one third of the way down the twigs. Histopathological investigations disclosed that *X. pruni* can enter the twig through the xylem vessel and intercellular spaces in the leaf base. Entrance through the xylem vessels appeared to be of no pathological importance, however, since the bacteria, once inside, could not move out to infect neighboring cells. Bacteria survived the winter in the intercellular spaces of the cortex, phloem, and xylem parenchyma and initiated cankers the following spring. Phytopathology 60:1720-1726.

Additional key words: leaf abscission, ligno-suberization.

In bacterial canker of peach caused by *Xanthomonas pruni,* two types of twig cankers, designated by Thornberry & Anderson (13) as "spring" and "summer" cankers, are recognized. It is well established that the main source of primary inoculum comes from spring cankers occurring in the terminal portions of twigs produced during the past growing season. Goldsworthy & Wilson (6) demonstrated that fall infections of twigs resulted in the development of cankers the following spring; however, they did not identify the site of bacterial ingress that resulted in the development of the cankers. In similar studies using forced stream inoculations, Adam et al. (1) report that August is the opt time in Illinois for the application of *X. pruni* to peach trees for the development of high incidence of spring cankers. Using needle inoculations, these workers found that the highest incidence of spring cankers resulted from inoculation of twig terminals, and that spring canker development was reduced as the distance from the terminal was increased.

Lenticels and leaf scars are suspects as sites for infection resulting in spring canker development, as Smith (12), Rolfs (11), and others report that stoma and lenticel infections result in the development of disease symptoms in leaves and twigs. In addition, leaf scars have been shown to be the site of bacterial ingress into the host by Horne et al. (9), Hewitt (8), Crosse (4, 5), and others.

The purpose of the present investigation was to determine whether leaf scar infections by *Xanthomonas pruni* (E.F.S.) Dows. result in the development of spring cankers. If the leaf scar, as expected, proved to be an important site for bacterial ingress into the peach twig, the intent was to determine (i) the effect of leaf scar age, location on the twig, time of invasion, and development of twig cankers the following spring; (ii) the path taken by the pathogen during invasion.

MATERIALS AND METHODS.—Two-year-old Sunhigh and Rio-Oso-Gem peach trees used in these experiments were grown outdoors in 3-gal porcelain crocks filled with washed sand. They were watered daily with nutrient solution to insure good flushing. The solution contained nutrient elements in the following ppm: $N(NO_3)$, 45; $N(NH_4)$, 22; P, 15.5; K, 78; S, 126; Ca, 80; Mg, 48; Fe^{++}, 1; B, 0.1; Mn^{++}, 0.25; Zn^{++}, 0.01; Cu^{++}, 0.01; and Mo^{++}, 0.01. In addition, Na_2 EDTA was used to prevent precipitation of Fe^{++}.

Inoculum for these experiments was secured from leaves showing the bacterial spot disease previously collected from an orchard and frozen in cubes of ice. Forty-eight-hr-old broth cultures of new isolates from frozen leaves were used in each experiment. The identity of a new isolate was determined by its sensitivity to *X. pruni* bacteriophage.

In all experiments, four trees were used for each treatment, and all twigs were inoculated accordingly. Of these, one tree was used for isolations and histological investigations and three trees were held for observations on canker development.

Inoculation.—Two experiments designed to evaluate the method used in exposing the leaf scar, its location on the twig, and the time of inoculation on the incidence of spring canker development were conducted in 1966 and 1967. The leaf scars inoculated were produced at the tip and one-third of the way down the twig accord-

ing to the method employed by Crosse (5): fresh leaf scars were exposed (Method A) by pulling leaves off forcibly; and (Method B) by removal of delaminated petioles. At the time of petiole detachment (7 days after delamination), abscission development was underway and the exposed leaf scars resembled those resulting from the dropping of mature leaves. A small drop of *X. pruni* suspension was carefully placed on both types of leaf scars immediately after the leaf or petiole was removed. Control plants were either left noninoculated or a drop of the bacterial suspension was placed at the base of uninjured petioles.

Both types of leaf scars located at the tips and one-third of the way down the twigs were inoculated on 15 July, 15 August, 17 September, and 1 and 15 October. On 1 November, only tip inoculations were performed since all but the terminal leaves had normally fallen.

In a second experiment, the Sunhigh cultivar of peach was used to determine the influence of leaf scar age and the effect of varying the time between delamination and petiole removal on infection. Leaf scars produced by the forcible removal of undelaminated petioles (A) were inoculated immediately (0 hour), 1 day, 3 days, and 7 days after exposure of the scar. The effect of abscission development was also determined by detaching the petiole 1 day, 3 days, and 7 days after delamination and then inoculating the scar. These treatments were also made at two locations: the tip and one third of the way down the twig.

Isolation and identification.—All inoculated twigs were observed for canker development and isolations made semimonthly to determine whether infection had occurred and to ascertain the longevity of *X. pruni* in the host tissue under the conditions of the experiment. All yellow bacteria isolated were exposed to *X. pruni* bacteriophage to determine whether the isolate was *X. pruni*. Two cultures of *X. pruni* bacteriophage, one received from Harry L. Keil (USDA) and the other isolated and purified as described below, were used in these studies.

A culture of *X. pruni* bacteriophage was isolated from soils taken from beneath infected peach trees. The phage was purified according to the method described by Anderson (2), and the agar layer method (7) was used in testing isolates from inoculated twigs for plaque formation.

Histological technique.—Histological studies of inoculated twigs were made to determine the path of invasion and the effect on the host tissue. Sections from the check (noninoculated) trees were likewise taken for comparison.

Samples were taken immediately, 1, 3, 5, 7, 9, 11, 15, 30, and 60 days after inoculation. Samples were cut successively into 5-mm segments fixed in formalin-acetic acid-alcohol.

The fixed materials were softened in a solution containing 45 ml of 52% hydrofluoric acid and 7 ml of 60% ethyl alcohol for 9 days, followed by washing in running water for 24 hr.

The segments were dehydrated in a tertiary butyl alcohol series and embedded in Tissuemat with a melting point of 56.5 C (10). The tissues were cut at 8 μ and mounted serially. The sections were stained in 1% solution of thionin in 5% phenol for 1 hr and counterstained in orange G (saturated solution of orange G in cellosolve, pure cellosolve, and 95% alcohol, 1:1:1) for about 10 dips. They were rinsed quickly in a solution of clove oil, cellosolve, and 95% alcohol, 1:1:1, differentiated in a solution of clove oil, absolute alcohol, and xylol, 1:1:1 (10-12 dips), cleared in xylol (3 changes of xylol, at least 10 min each change), and mounted in permount (3).

The presence of suberization or lignification was determined by using standard solution of Sudan IV in 70% alcohol or 0.1 g phloroglucin in 10 cc of 95% alcohol (10), respectively.

RESULTS.—*Type and location of leaf scars and time of inoculation on incidence of spring cankers.*—Observations for canker development were started about 1 week after inoculations were made. Summer and spring cankers were distinguished according to the criteria of Thornberry & Anderson (13); results are given in Table 1.

Only fresh leaf scars obtained by the forcible removal of leaves provided suitable infection courts for the inoculation methods employed. Fresh leaf scars protected by an abscission layer or the base of the petiole of intact leaves were unsuitable for infection. Spring cankers resulted from inoculations made between 17 September and 1 November (17 September, 1 October, $P = < .05$; 15 October and 1 November, $P = < .01$). high incidence of summer cankers resulted from inocu-

TABLE 1. Development of summer and spring cankers in Rio-Oso-Gem peach cultivar following inoculations of fresh leaf scars, 1967-1968[a]

Location	Date of inoculations					
	15 Aug.	1 Sept.	17 Sept.	1 Oct.	15 Oct.	1 Nov.
			Summer canker incidence			
Tip	83	85	83	68	0	0
One-third of the way down	64	65	69	39	0	
			Spring canker incidence			
Tip	0	0	55	56	83	85
One-third of the way down	0	0	0	5	4	

[a] Data represent average percent of twigs observed which showed cankers.

TABLE 2. Effect of leaf scars age at time of inoculation on development of spring cankers in Sunhigh peach cultivar. 15 Oct. 1967[a]

	Period of time between leaf removal or petiole delamination and inoculation of leaf scar						
	0 hr	1 day		3 days		7 days	
Location	B1	B1	B2	B1	B2	B1	B2[b]
Tip	61	0	44	0	38	0	0
One-third of the way down the twig	23	0	30	0	0	0	0

[a] Data represent average percentage of twigs observed which showed cankers.

[b] Treatment B1: fresh leaf scars obtained by forcibly removing leaf. Treatment B2: leaf scars obtained by removal of petioles 1, 3, and 7 days after delamination.

lations made from 15 July to 1 October. Tip inoculations resulted in the development of more cankers than did inoculations one-third of the way down the twig.

Table 2 shows the effect of leaf scar age on the incidence of spring cankers on Sunhigh peach trees. Since the 15 October 1966 inoculations gave a high percentage of spring cankers, and since normal defoliation at nodes one-third of the way down the twig had not occurred at that time, 15 October was picked as the date for inoculations of leaf scars of various ages, types, and locations on twigs in the 1967-1968 experiments. Leaf scars located at the tips of twigs inoculated immediately after the forcible removal of intact leaves showed the highest incidence of spring cankers (Fig. 1). Similarly produced leaf scars inoculated 24 hr or more after leaf removal did not result in the development of spring cankers at either location (tip or one-third of the way down the twig).

Results obtained with leaf scars produced by the removal of the previously delaminated petiole (B2) clearly demonstrate the protective effect of stimulating abscission prior to inoculation. A decrease in disease incidence occurred as time between delamination and removal of the petiole increased. Scars located one-third of the way down the twig became immune to infection earlier than those located at the tip. A significantly higher incidence of canker development was obtained from inoculations of leaf scars at the tip than of those located one-third of the way down the twig.

Leaf abscission in relation to infection.—Leaf scars resulting from the forcible removal of leaves located at the tips of twigs resulted in mechanically damaged fibers, vessels, sieve tubes, and epidermal and parenchyma cells. Except for four to five layers of collapsed and dried cells on the exposed surface a day after leaf removal, no barrier was observed within the tissues of the scars that could provide mechanical protection against invasion by the bacteria. In a few cases in the 1 November inoculations, a trace of ligno-suberization was observed in the area of the protective layer even before the removal of the leaf. Generally, ligno-suberization of the protective layer in such scars is completed in 5-7 days after leaf removal. By the 9th day, the protective layer has, by division, become several cells thick.

In abscission zones of leaves located one-third of the way down the twig, ligno-suberization started as early as 15 October and was completed just after leaf fall.

Fresh leaf scars resulting from the removal of petioles 1 and 3 days after delamination exhibited very little or no ligno-suberization (15 October 1967 inoculation). They apparently did not differ anatomically from scars produced by the forcible removal of the leaf, except that tearing was less when the petiole had been delaminated. Ligno-suberization was found to be almost complete in leaf scars located one-third of the way down the twig when they were produced by the removal of petioles that had been delaminated for 7 days.

Pathological anatomy.—Bacteria were not observed in any of the sections prepared following inoculation of leaf scars resulting from the removal of petioles that had been delaminated for 7 days. Leaf scars resulting from the removal of petioles that had been delaminated for 1 and 3 days and those that were formed by forcible removal of leaves allowed bacterial invasion.

Once the bacteria entered the host, no apparent difference in the course taken in invading the tissues was observed among the different treatments. One day after inoculation, bacteria were observed at the surface of scars and some had penetrated the xylem vessels of the three vascular bundles of the leaf trace. On the 3rd day, the bacteria had penetrated the intercellular spaces 2-3 layers of parenchyma cells of the cortex and also of the xylem and phloem parenchyma. By the 5th day, invasion of the cortex had extended 0.5 to 1.0 mm below the surface of the leaf scar. By the 9th day, some cortical cells had been colonized and the depth of intercellular invasion had increased (Fig. 2). By the 11th day, invaded cortical cells were completely disorganized and contained masses of bacteria.

During the months of July, August, and September, periderm was forming across the leaf scar 4-5 days after forcible defoliation, and was well developed by the 9th day. Obvious formation of periderm subsequent to bacterial invasion was observed by the 30th day after inoculation, with a phellogen and 2-3 layers of phellem surrounding and isolating the invaded area. By this time, the vascular bundles of the leaf trace were completely invaded. Lysigenous cavities formed by the collapse of parenchyma cells and filled with bacteria surrounded the xylem vessels. The latter were frequently filled with bacteria but had intact walls (Fig. 3).

No histological evidence was found to indicate that bacteria can successfully penetrate through the lateral walls of invaded xylem vessels to infect neighboring parenchyma cells, nor can bacteria from infected cells move into the xylem vessels (Fig. 4). Likewise, migration was never observed to occur between vessels of a single vascular bundle. Considerable attention was given to the area where the infected leaf trace joins the healthy vascular cylinder of the main stem. There was no indication that the bacteria moved from infected to healthy vessels even in cases where two vessels lay side by side. In some instances, tyloses were found inside

the infected xylem vessels; however, they probably did not significantly obstruct movement of the bacteria since bacteria were observed above and below the tyloses.

Infection of the phloem occurs first through the mechanical wounding created by leaf removal and later by the invasion of bacteria from the cortical parenchyma. Bacteria are commonly observed in the intercellular spaces of the phloem parenchyma. As bacterial pockets increase in size, adjacent cells are crushed, including the sieve elements and parenchyma cells (Fig. 5). Bacteria from the phloem area readily invade the cambium. In some cases, however, invasion extends only from cortical parenchyma to the phloem fibers, and is checked by periderm formation before it invades the other phloem elements (Fig. 6).

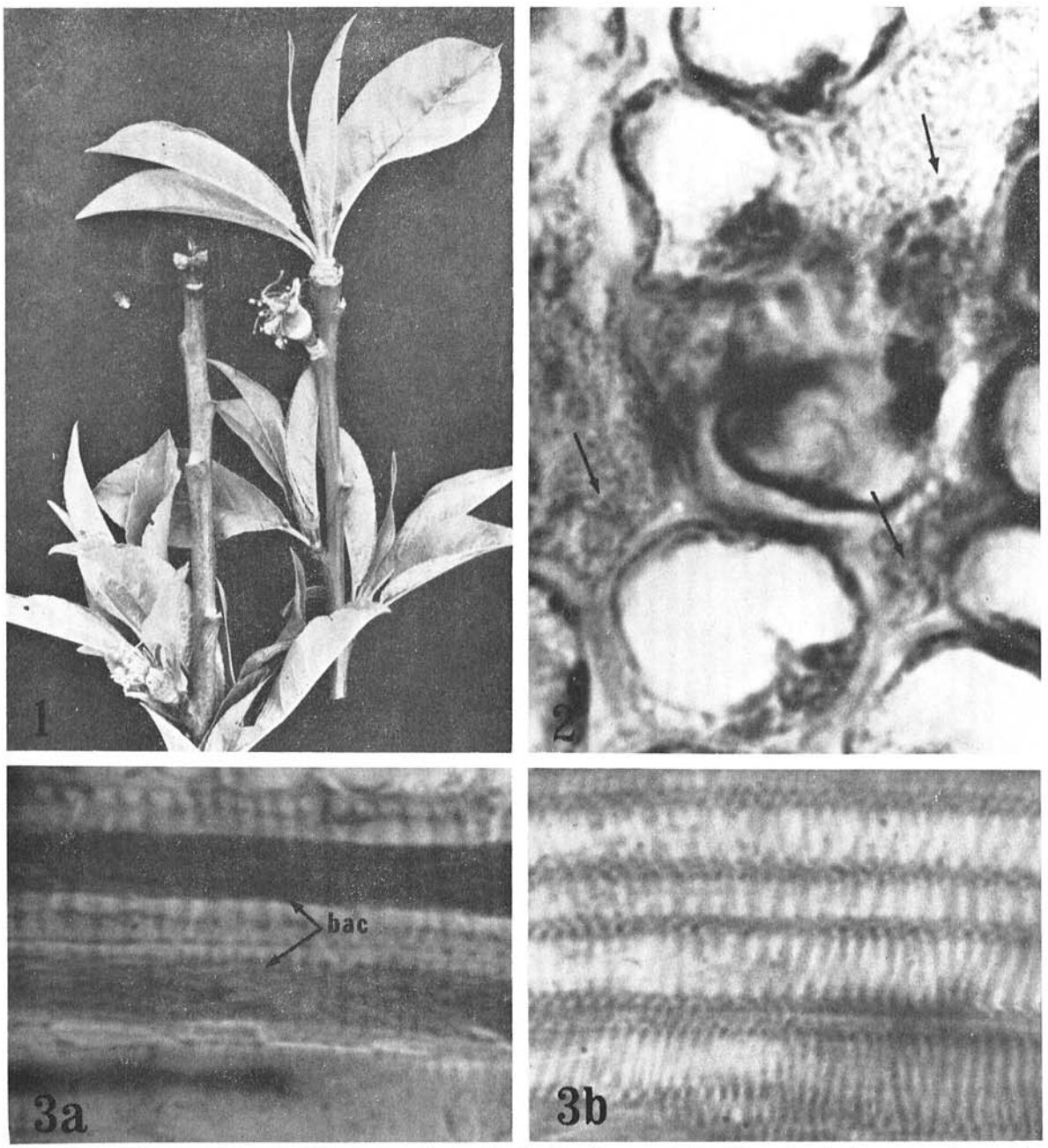
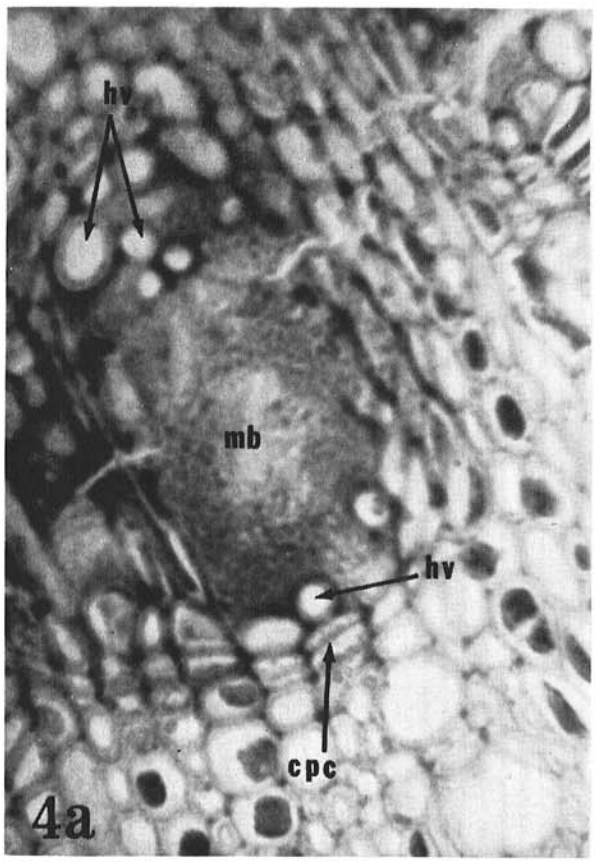


Fig. 1-3. **1)** Diseased and healthy peach twigs at the shuck split stage. Left: Diseased twig shows a spring canker which resulted from the inoculation of a leaf scar located at the tip of the twig on 1 November. **2)** Longitudinal section of inoculated twig showing intercellular invasion (arrows) of the cortical parenchyma (×900). **3)** Longitudinal section through the vascular bundle of the leaf trace showing lumina of the xylem vessels filled with bacteria (bac) (a) in comparison with the healthy xylem vessels (b) (×430).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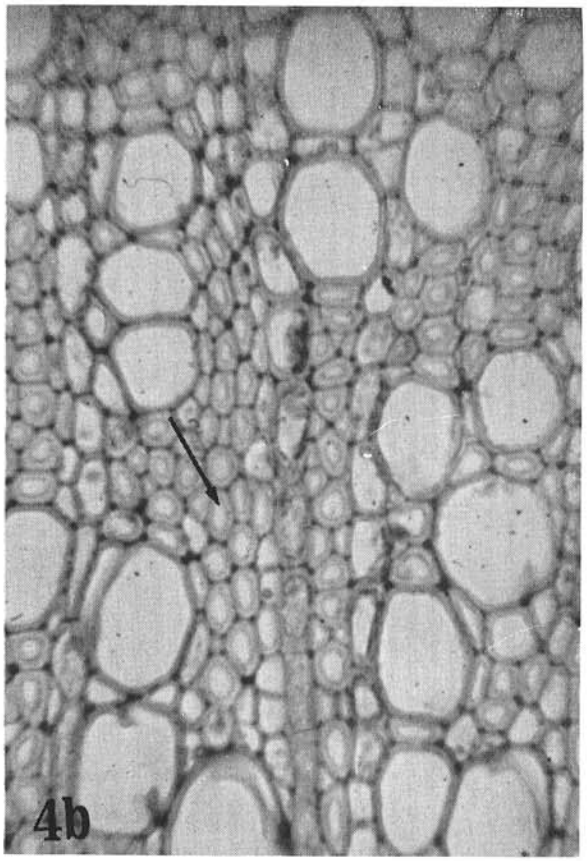
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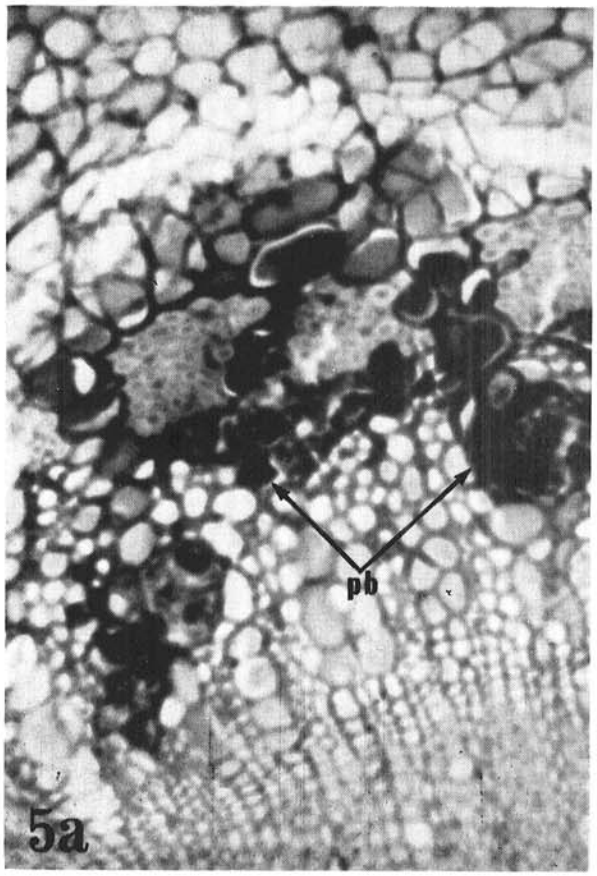
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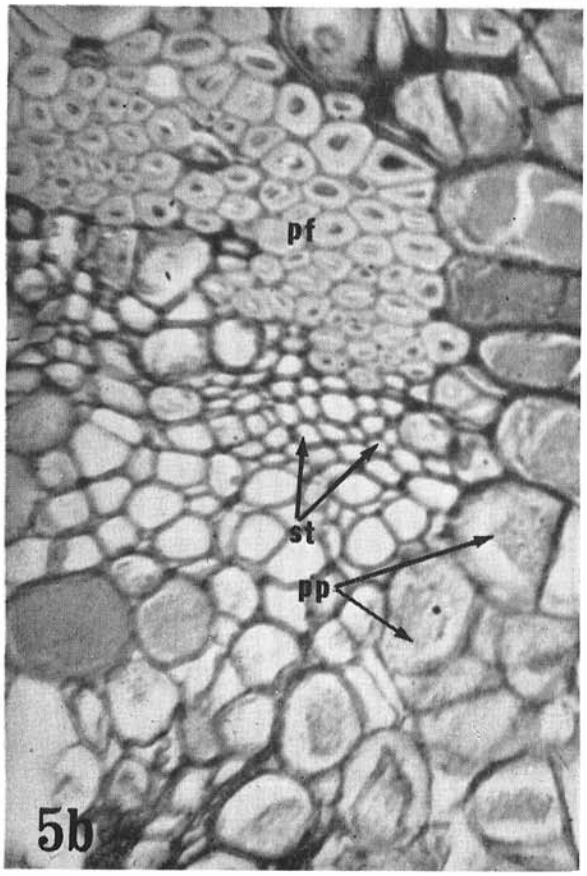
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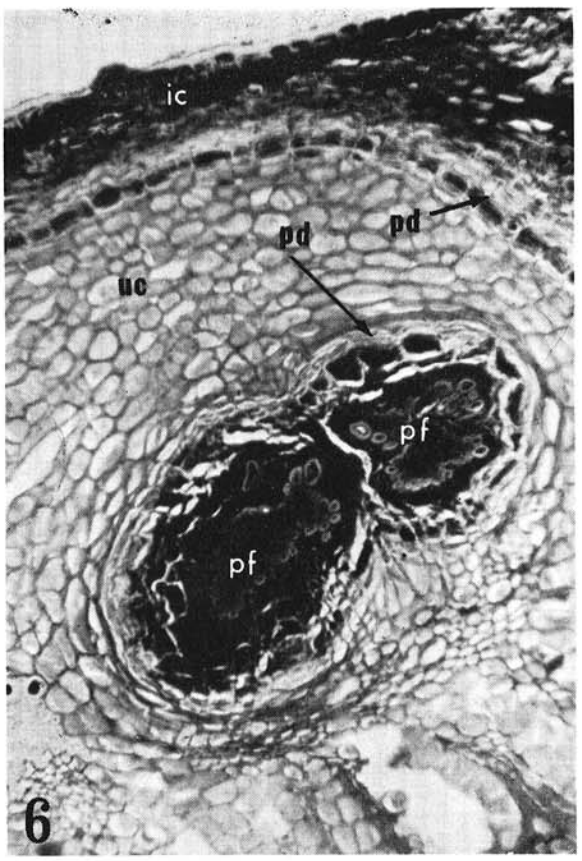

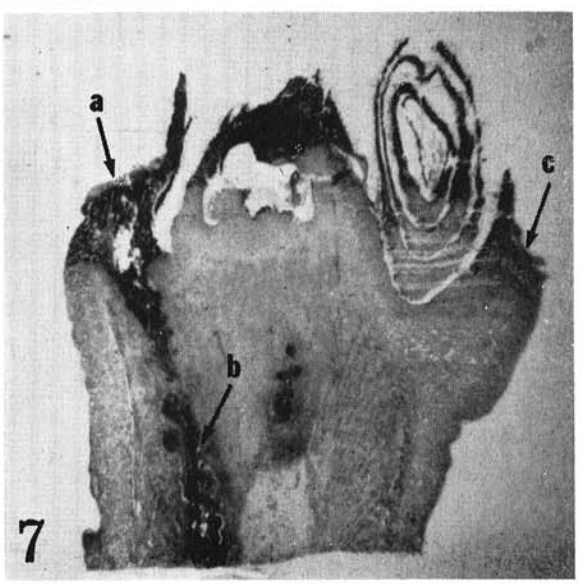

Fig. 6-7. **6)** Transverse section through the stem showing infected phloem fibers (pf) effectively walled off by periderm (pd). Note also the periderm separating the invaded (ic) from the uninvaded cortical (uc) tissues (×200). **7)** Longitudinal section of a diseased twig terminal showing a leaf scar through which infection had occurred. Such infection results in the development of spring cankers. a = point of inoculation; b = path of invasion; c = normal leaf scar (×10).

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Periderm formed around invaded areas during the summer and early fall months (July, August, September, and 1 October inoculations) but not during the late fall (15 October and 1 November inoculations) and winter months. This provides an explanation for the high percentage of spring cankers developing from the late-fall inoculations.

In the spring, overwintering bacteria usually occur in the innermost layer of the cortex, a zone of parenchyma cells rich in starch and abutting the phloem fibers. From here they migrate through intercellular spaces into the rest of the cortex, which is eventually completely destroyed, and into the phloem via groups of parenchyma cells among the fiber strands.

DISCUSSION.—The present investigation serves to increase our understanding of the time and site of ingress of *X. pruni* into the tissue of peach twigs, and some of the factors influencing penetration into the host tissues.

Xanthomonas pruni invades peach twigs in the autumn through fresh leaf scars. In nature, this is accomplished most effectively by wind-driven rain causing the forcible removal of leaves and at the same time depositing inoculum on the peach leaf scars. The incidence of spring cankers, therefore, can vary from year to year depending on the weather conditions during the period when effective infection can occur. In New Jersey, this period occurs from September to early November.

This study emphasizes the importance of fresh wounds produced by tearing for the ingress of bacteria through the leaf scars (Fig. 7). Histological studies revealed that suberized protective layers are formed prior to normal leaf fall (Fig. 8), thus providing defenses against bacterial ingress. Formation of the protective layer also explains the experimentally observed decrease in disease incidence as the interval increased between delamination and detachment of petiole and inoculation.

That the tip of the twig is the part most susceptible to infection and provides the most favorable conditions for the overwintering of the bacteria was earlier demonstrated by Adam et al. (1), and has been confirmed by this study. Under the conditions of the present experiments, formation of the protective layer and its subsequent ligno-suberization starts in the month of October

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Fig. 4-5. **4a)** Transverse section through the stem showing masses of bacteria (mb) in the xylem parenchyma. Note the healthy vessels (hv) besides the compressed parenchyma cells (cpc) (×430). **4b)** Transverse section through the healthy stem showing the normal appearance of the xylem parenchyma cells (arrow) (×430). **5a)** Transverse section through the stem showing pockets of bacteria (pb) in the intercellular spaces of the phloem elements (×200). **5b)** Transverse section of the stem showing healthy phloem elements: phloem fibers (pf), sieve tubes (st), and phloem parenchyma (pp) (×430).

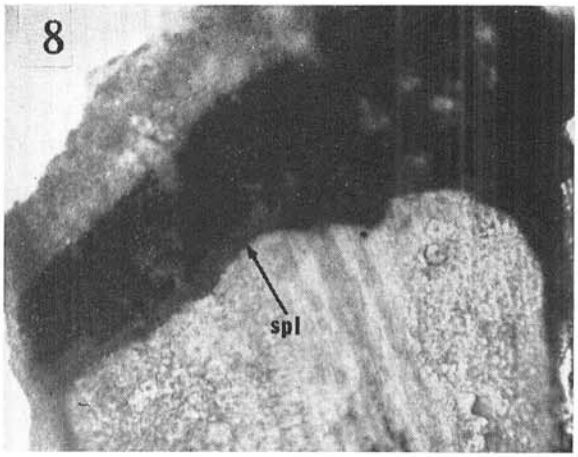

Fig. 8. Free-hand longitudinal section (approximately 100 μ thick) of the leaf scar just after normal leaf fall showing a well-suberized protective layer (spl) already formed (×100).

in petioles located one-third of the way down the twig, and in late October and early November in leaves located at the tip of the twigs. Bacteria were never observed to invade the protective layer in leaf scars of the following types: those located one-third of the way down the twig and resulting from the removal of petioles that had been delaminated 3 and 7 days before inoculation, or those formed by forcible removal of leaves at either the tip or one-third of the way down the twigs 24 hr before inoculation (15 October 1967). There seems to be no anatomical explanation for the resistance to infection shown by the 24-hr-old leaf scar produced by the forcible removal of the leaf. With the inoculation method employed, however, a drying out of the surface cells seems to have been sufficient to preclude infection from occurring. In cases where lignosuberization of the protective layer is not complete at the time of inoculation, whether or not infection occurs depends on whether the bacteria can penetrate into the leaf base before completion of the periderm layer. Since development of the periderm occurs more rapidly in early fall than during October and early November, infections occurring during the latter period have a much greater chance of becoming established than do earlier infections.

Invasion of xylem vessels by *X. pruni* appears to be of little pathological importance, as bacterial cells, once inside, apparently do not move out through the lateral walls. However, *X. pruni* can successfully invade intercellular spaces and parenchymatous cells. Numerous observations made during this investigation suggest that invasion of host tissues by *X. pruni* receives little or no assistance from the dissolving action of pectic or cellulolytic enzymes.

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