HILGARDIA

A Journal of Agricultural Science Published by the California Agricultural Experiment Station

VOLUME 12 OCTOBER, 1938 NUMBER 1

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UNIVERSITY OF CALIFORNIA · BERKELEY, CALIFORNIA

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LEAF-SCAR INFECTION IN RELATION TO THE OLIVE-KNOT DISEASE[1, 2]

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INTRODUCTION

OLIVE KNOT, a serious disease of *Oleo europea* L. in most of the olive-producing districts north of the Tehachapi mountains of California, is characterized by the development of overgrowths, knots on the branches. These knots develop most frequently at the leaf scars except in years when freezing injury occurs. The present study furnishes inoculation data, with histological evidence that the scars, under certain conditions, are portals of entry of the causal agent, *Bacterium savastanoi* E.F.S., into the host. This paper also deals with inoculation experiments used to determine the length of time during which the leaf scars are susceptible to infection. It further describes the microchemical and histological studies of the development of the abscission region just before and after leaf fall, and thereby elucidates the rôle played by leaf scars in infection.

PREVALENCE OF LEAF-SCAR INFECTION

Observations in California olive groves show that a large percentage of the new knots forming each year develop at leaf scars. Horne, Parker, and Daines (*2*)[4] were first to point out this fact: "By far the largest number of knots appear on leaf scars or wound callus." Wilson (*6*) also recognizes the importance of leaf scars as infection courts. He points out that in years other than those in which freezing injury occurs, as high as 90 per cent of the new knots on branches develop at leaf scars.

The distribution of inoculum from active knots necessary to infect leaf scars depends upon the presence of dripping moisture, as from rain. Horne, Parker, and Daines (*2*) state that the bacteria exude in a slimy mass from the fissures of knots during rain. Wilson (*6*), in further and more extensive studies, demonstrates the importance of rain in the exudation and spread of the organism as related to infection. He shows that enough bacteria were present to cause infection within a short period after the knots were moistened. He placed wounded, healthy, potted trees under diseased trees, allowed a fine spray of water to fall over them,

[1] Received for publication March 21, 1938.

[2] A thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, University of California, 1936.

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[4] Italic numbers in parentheses refer to "Literature Cited" at the end of this paper.

and then placed the potted trees in the greenhouse so that the knots might develop. In this experiment he reports numerous knots developing on trees removed from the spray 7 minutes after it was started. Wilson also infected newly formed leaf scars by artificial inoculation and showed

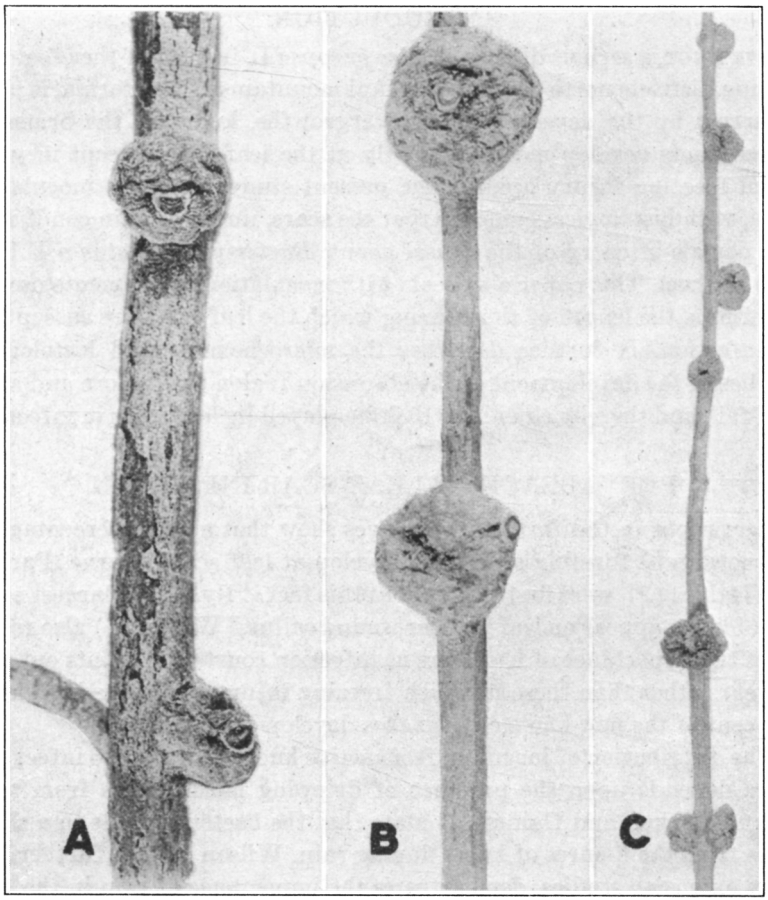

Fig. 1.—*A*, *B*, Olive knots showing leaf scars on the surface. *C*, Young olive knots resulting from natural leaf-scar infections. (*A*, ×2; *B*, ×4; *C*, natural size.)

that the vessels in the leaf scars were open at the time of leaf fall. According to his climatic studies, infection may occur in a range of temperature from about 40° to 100° F.

The origin of the knots can be determined by examination. Those developing at the nodes usually form at the leaf scar, which upon close inspection, can usually be seen on the surface of young knots (fig. 1).

To gain further evidence as to the importance of leaf-scar infection, the following experiments were carried out. On May 5, 1936, in a Mission-olive orchard in Sacramento County, 60 healthy branches were tagged. When these were re-examined in October, 241 knots were discovered, of which 225 (about 93.5 per cent) were located at leaf scars.

On May 14, 1936, in the same olive orchard 339 yellow leaves about ready to fall were removed by bending them slightly backward. This leaf removal was continued until interrupted by a shower. Other light showers occurred on May 28. By August, 229 of the leaf scars (67.5 per cent) had developed knots.

The olive normally drops some of its older leaves each season, and the time of abscission of leaves plays an important part in the infection of leaf scars. Wilson (*6*), studying natural leaf fall, finds that the period of maximum fall varies with the season; and, in unpublished experiments, shows that leaf drop generally starts about the time growth begins in late January or February, rises slowly during the early months, and increases suddenly to a maximum at full bloom around the middle of May. It then decreases through the summer and usually ceases by the latter part of September. Observations by the writer during the seasons of 1935 and 1936 confirmed those of Wilson.

MATERIALS AND METHODS

Choosing Material.—All experiments, unless otherwise stated, were conducted on the Mission variety of olive in an orchard in Sacramento County, California.

Fresh leaf scars were made by removing only the leaves that were three-fourths to entirely yellow. In such leaves the abscission process was well advanced, and the leaves were about to drop. The leaves were removed by placing a pencil or finger at the tip of the leaf and bending them toward the base of the stem. If the leaf fell by the time it was pushed back one half of the distance to the main limb—that is, approximately 45°—the resulting scar was encircled with a red wax pencil mark and retained for use in these studies.

Method of Inoculation.—In artificial inoculations a water suspension of *Bacterium savastanoi* E.F.S. from a 36- to 48-hour culture on potato dextrose agar was used. A drop of this suspension was placed over the leaf scar with a small camel's hair brush.

Collecting Leaf Scars.—Leaf scars were collected by cutting out a small portion of the stem along with the leaf scar (fig. 2, *B*).

Moist Chambers.—Moist conditions were provided by enclosing a

branch in a cylinder of wire gauze (⅛-inch mesh screen) and afterwards wrapping it with three layers of wet cheesecloth. The bottom of the chamber was then placed in a can containing water, and the can was suspended by a wire from a large branch in the tree (fig. 2, *A*).

Method of Holding Fresh or Killed Unimbedded Leaf Scars for Sectioning.—Sliding microtome sections of leaf scars killed, fixed, and stored in formalin-acetic-alcohol solution, together with sections of fresh

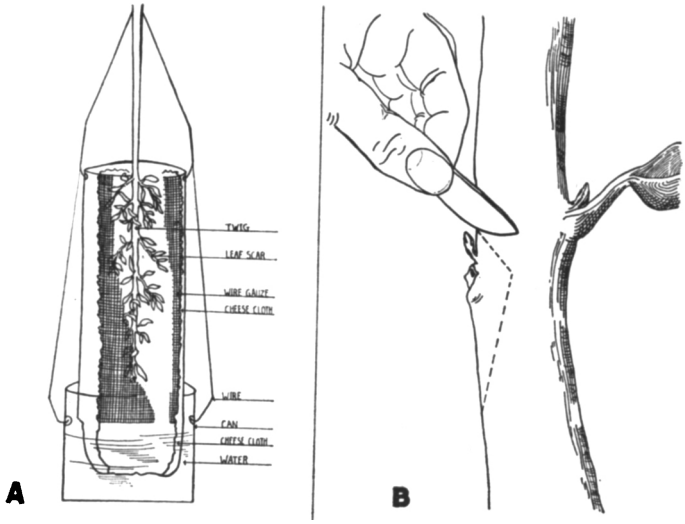

Fig. 2.—*A*, Diagram of the moist chamber used. *B*, Illustrating the method used for cutting leaf scars from the stems before fixation.

leaf scars, were used for these studies. The collected scars were small and in order that they might be sectioned without imbedding, a method was devised to hold the scars. They were mounted directly in paraffin blocks. The tissue was first blotted until the surface appeared dry. A small hole was melted in one end of a paraffin block with a hot iron rod, and the scar was then inserted into the melted paraffin. To loosen air bubbles adhering to the scars, a warm dissecting needle was moved around the material; this also served to orient the scars in the blocks. The paraffin block was then cooled in ice water, trimmed, and sectioned by the sliding microtome. Most of the sections of the leaf scars freed themselves from the surrounding paraffin when placed in water, but sections that adhered to the paraffin were freed easily by prodding with a small brush.

Paraffin Method Used.—The material was killed and fixed in a solution of formalin 10 cc, acetic acid 10 cc, and 50 per cent alcohol 100 cc for 12 hours or longer. It was imbedded in paraffin as follows: (A) The speci-

mens were transferred directly from the formalin-acetic-alcohol into a solution of 10 per cent glycerin in water. The solution was then allowed to evaporate until it became thick. (B) The material was next successively transferred through the following glycerin and normal butyl alcohol mixtures and left in each for 48 to 72 hours:

(a) 75 parts of glycerin and 25 parts of normal butyl alcohol.
(b) 50 parts of glycerin and 50 parts of normal butyl alcohol.
(c) 25 parts of glycerin and 75 parts of normal butyl alcohol.
(d) Pure dehydrated normal butyl alcohol; four changes—the first two 24 hours apart, the next two 48 hours apart—were used. The containers were placed on top of the paraffin oven to warm; this hastened the removal of all the glycerin.

(C) Small amounts of paraffin were added to the last change of butyl alcohol while the specimens were still on top of the oven, and they were allowed to stand for 12 hours. Then the process of infiltration was completed as in standard schedule (*5*).

INOCULATION OF THE ABSCISSION ZONE BEFORE LEAF FALL

If infection of the leaf abscission zone were to precede leaf fall, it might be expected to occur through a break in the epidermis or some other open infection court. This point was tested by inoculating the base of the petiole with a water suspension of *Bacterium savastanoi*. On May 31, 1935, 50 yellow leaves were inoculated at the base of the petiole. When examined on August 31, only 2 out of the 50 leaf scars had developed knots. Again on May 4, 1936, the base of the petioles of 67 yellow leaves were inoculated. The number of leaves that had fallen at intervals after inoculation were as follows: at the end of 1 hour, 29; 3 hours, 42; 24 hours, 55; and 48 hours, 64. By September 1, 2 of the 67 leaf scars had developed knots. The leaves from the leaf scars that developed knots probably fell shortly after inoculation, which allowed the inoculum to spread over the scar and accomplish infection.

The condition of the epidermis of the petiole in the region of the abscission zone was studied in 25 yellow leaves. A break that might serve as an infection court was found at the abscission zone in the axil of only one leaf. Figure 3 shows a photograph of this break. Judging from these results and from the inoculations previously described, infection of leaf scars is not apt to occur before leaf fall.

THE NEWLY FORMED LEAF SCAR

A microchemical and histological study of the abscission zone of 25 yellow Mission-olive leaves was made before and after leaf fall to determine what changes take place in this zone, because the structure and composition of these tissues were suspected to have an important relation to the infection process.

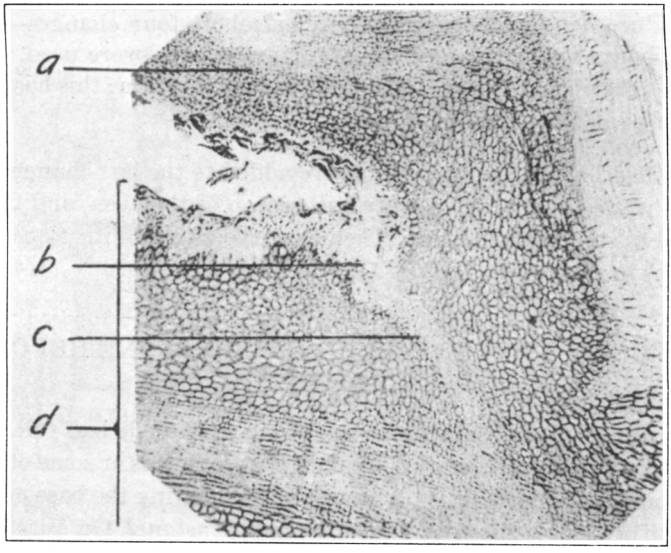

Fig. 3.—A portion of a longitudinal section through the abscission zone of a yellow leaf, showing a break in the epidermis in the axil of the leaf. Details are: *a*, Bud; *b*, a break in the epidermis at the edge of the abscission zone in the axil of the leaf; *c*, abscission zone; *d*, leaf petiole. (×55.)

The mode of abscission in the olive leaf resembled that of *Castanea sativa* Mill., as described by Lee (*4*).

The abscission zone of the olive leaf lies at the base of the leaf petiole very close to the stem and, in longitudinal sections, stains lighter than the surrounding tissues (fig. 3). The number of layers of cells involved in the abscission zone varies from 1 to 7, but is more frequently 3 to 5. The zone extends from the epidermal cells through the entire diameter of the petiole and involves all the living cells. Leaf fall does not occur until the leaf is almost completely yellow. Separation takes place along an irregular plane between the walls of cells in the portion of the zone proximal to the stem.

In no leaves studied were there any cell divisions before separation,

nor any protective layers of lignin, gum, or suberin formed in the tissues above or below the abscission zone. The cuticle, sieve tubes, vessels, and fibers were mechanically broken at leaf fall. The epidermal cells at the separation plane of the scar appeared as though they had been broken at leaf fall. Just after leaf fall, therefore, the scar was an open wound, with exposed unprotected tissue, and open vessels.

SUSCEPTIBILITY OF NEWLY FORMED LEAF SCARS TO INFECTION

In June, 1935, leaf scars made by removing yellow leaves were inoculated immediately after their formation. Some were placed in the moist chambers for incubation; others were left exposed to outside atmospheric conditions. The inoculations were made when the outside air temperature was 28.5° C in the shade and when a gentle north wind was blowing. The temperature in the moist chambers ranged from 20° to 21° C. Of the 196 leaf scars inoculated and placed in moist chambers for 24 hours' incubation, 156 (or 79.6 per cent) developed knots; and of the 224 left outside of moist chambers 184 (or 82.6 per cent) developed knots. The data show that inoculations made immediately after leaf fall produced a high incidence of leaf-scar infection, and indicate that moist chamber conditions are not essential to a high degree of infection.

PERIOD DURING WHICH LEAF SCARS ARE SUSCEPTIBLE TO INFECTION

The period during which leaf scars are susceptible to infection was determined by periodic inoculations after scars were formed.

The leaf scars of one series were left outside of moist chambers until inoculation at which time they were placed in moist chambers for incubation and left 24 hours, after which the moist chambers were removed. The leaves for a second series were removed on the same dates, the scars being placed in moist chambers when formed, to determine the influence of high atmospheric humidity on scar healing and its relation to infection. After the moist chambers had been removed a short time for inoculation, they were replaced and left over the scars for 24 hours after inoculation, then removed.

Groups of leaf scars in each series were inoculated at intervals after they were formed. Series of inoculations were made during June, 1935; made again in May, 1936, because of an early season; and repeated at shorter intervals in June, 1937. The scars were allowed to remain on the

trees until knots were well developed (fig. 1). The results are given in table 1, and presented graphically in figure 4.

As shown in figure 4, the percentage of leaf scars susceptible to infection decreased rapidly within the first day. This drop is much more pro-

TABLE 1

RESULTS OF INOCULATIONS OF LEAF SCARS AT VARIOUS TIME INTERVALS AFTER LEAF REMOVAL

Age of leaf scar at time of inoculation (days)	Inoculations of 1935		Inoculations of 1936		Inoculations of 1937		
	Number of leaf scars inoculated	Per cent of leaf scars forming knots	Number of leaf scars inoculated	Per cent of leaf scars forming knots	Age of leaf scar at time of inoculation (hours)	Number of leaf scars inoculated	Per cent of leaf scars forming knots
Scars left outside moist chambers, except for 24-hour inoculation period							
0	11	81.8	46	97.9	0	51	96.5
1	14	57.2	37	64.8	1	36	100.0
2	12	41.6	45	57.8	3	24	99.2
3	12	25.6	38	39.5	6	24	79.2
4	10	40.0	33	42.4	12	22	68.2
5	12	33.0	44	34.1	24	23	52.2
7	12	8.3	37	13.5	48	23	42.4
8	..	....	40	15.0	72	35	31.7
9	..	....	14	7.0	96	45	28.9
10	..	....	12	0	..	..	....
13	11	0	..	....	..	..	....
15	12	0	..	....	..	..	....
21	10	0	..	....	..	..	....
Uninoculated	15	0	34	14.7	Uninoculated	25	0
Scars kept in moist chambers until 24 hours after inoculation							
0	20	85.0	80	90.0	0	51	96.5
1	24	29.2	74	24.3	1	25	92.0
2	30	3.3	64	17.2	3	20	100.0
3	39	5.1	39	12.9	6	20	85.0
4	16	6.2	98	9.2	12	20	75.0
5	10	0	60	1.7	24	22	18.2
6	..	....	25	0	48	22	13.6
7	16	0	..	....	72	24	8.0
11	11	0	..	....	..	..	....
Uninoculated	10	0	51	....	Uninoculated	15	0

nounced in the scars kept in moist chambers than in the scars left outside. According to these data, leaf scars left outside of moist chambers are susceptible to infection longer than those kept in moist chambers. The latter became immune about the fifth day, whereas the former did not

become immune until they were 7 to 9 days old. The infection of some of the scars used as controls invalidates the curve of 1936 inoculations after the sixth day, since any infection shown after this time may have resulted from earlier natural infection that prevented the curve from dropping to zero. The United States Weather Bureau in Sacramento recorded a

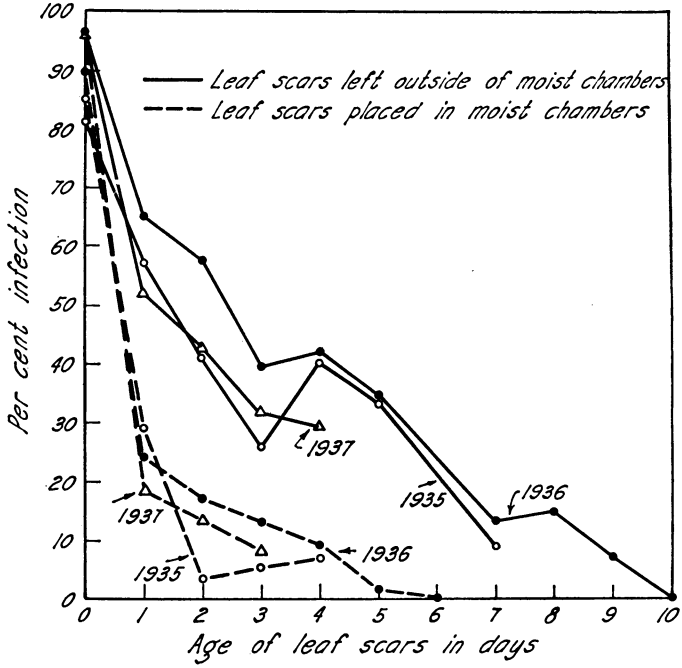

Fig. 4.—Showing the relation between the age of the leaf scars in days at the time of inoculation with *B. savastanoi* and the per cent of scars developing knots for both those kept in moist chambers and those left outside.

trace of rain on May 13 and 0.30 inch on May 14. These rains, occurring on the fifth and sixth days of the series of inoculations, might have initiated some infection in the experimental scars.

In 1937 the inoculations were made at shorter intervals to discover when the drop in infected scars takes place through the first 24 hours (table 1 and fig. 5). During the first 12 hours, the percentage of scars infected at each inoculation period remained about the same, both for those scars kept in moist chambers and for those left outside. In the former, the infected scars dropped from 75 per cent at the 12-hour period to only 18 per cent at the 24-hour period, while in the latter group the percentage of scars infected dropped gradually at each inoculation after 3 hours.

Though the number of scars used in any one year's inoculation is small, the results for the three years consistently show that leaf scars soon become immune to infection (fig. 4).

If the conditions within the moist chambers might simulate a rather long period of high humidity, such as might occur with a rain of a week's

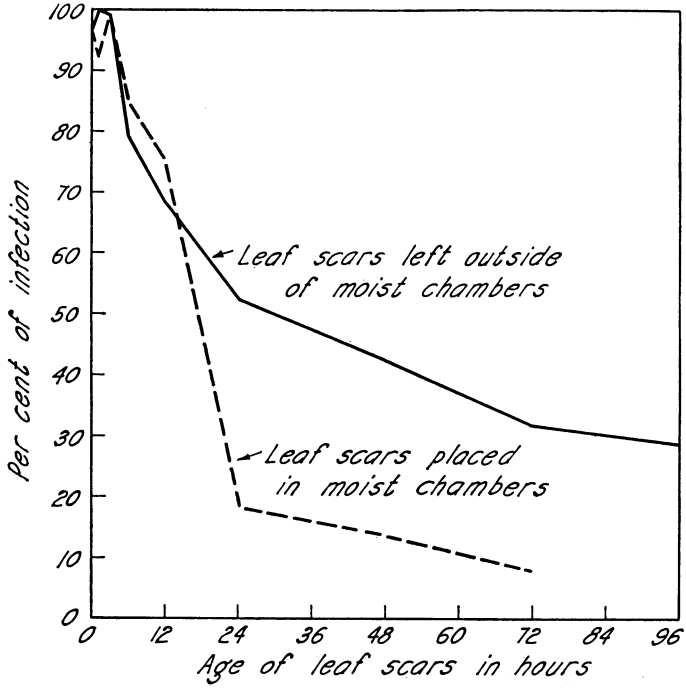

Fig. 5.—Results of the 1937 inoculations of leaf scars at short time intervals after leaf removal, showing the relation between the age of the leaf scars in hours at the time of inoculation and the per cent of scars which developed knots, for both those placed in moist chambers and those left outside.

duration, then, in the early summer the processes that prevent infection take place more rapidly during periods of continuous high humidity than during those of low humidity. Under California conditions, however, the spring rains are usually short, generally lasting only a few hours but seldom continuing more than two days. The leaf scars left outside the moist chambers were subjected, then, to the weather conditions that most frequently occur in the spring. Apparently, therefore, 80 to 95 per cent of the leaf scars are susceptible to infection at the time of leaf fall, the percentage of infectable scars drops to about 40 per cent by the fourth day, and most scars are immune to infection by the end of the ninth day after leaf fall.

MICROCHEMICAL STUDIES OF THE HEALING PROCESSES IN LEAF SCARS

Microchemical studies of the healing processes of uninoculated leaf scars were made to determine the processes which take place in these tissues and which may explain the results obtained in the inoculation experiments. For this purpose leaf scars were formed by removing yellow leaves, but the scars were not inoculated. One group was placed in moist chambers; the other left outside of moist chambers. A collection from each group was made when the leaf scars were formed, and other collections of ten scars each were made on each of the ten days following. The scars were preserved in the alcohol-formalin-acetic acid fixing solution until they were sectioned. The healing processes of the scars used in these studies should correspond to those of the leaf scars used in the inoculation work, for these collections came at intervals corresponding to the inoculation intervals which were made in experiments to determine how long the scars were susceptible to infection.

Longitudinal sections about 15 microns thick were made with a sliding microtome. Microchemical tests consisted of determining the presence of and changes in wound gum, water-soluble gums, lignin, suberin, oil, starch, and tannins. Unless otherwise stated, the microchemical methods used were those described by Rawlins (*5*).

Wound Gum.—Wound gum is defined as a substance often found in vessels of plants adjacent to wounds and in wood invaded by wood-decay fungi. It is insoluble in water and stains red with phloroglucinol in hydrochloric acid (*5*). Haas and Hill (*1*), in a similar description, mention the following properties of wound gum: It does not swell in water; it is insoluble in sulfuric acid and caustic soda; and on oxidation it yields both mucic and oxalic acids. According to Küster (*3*) wound gum is insoluble in alcohol, ether, carbondisulfide, cold nitric acid, and cold aqua regia but soluble in warm nitric acid and in a combination of hydrochloric acid and chlorate of potash.

Table 2 gives the results of microchemical tests in leaf-scar tissues. The formation of wound gum was one of the first and most conspicuous processes observed in the healing of leaf scars. It occurred in the cell walls, intercellular material, intercellular spaces, and lumina of vessels.

The only test that would distinguish wound gum from lignin was the Maule reaction (*5*), which colors lignified tissues light red but does not give a color reaction with wound gum in the leaf scar. Wound gum, therefore, is regarded in this paper as the water-insoluble material that forms

in the leaf-scar tissues, reacts positively to most lignin tests, but does not color with the Maule reaction.

Longitudinal sections of leaf scars killed and fixed immediately after they were formed exhibited no detectable change in the composition of the tissues except in one scar where a trace of wound gum was found in

TABLE 2

MICROCHEMICAL REACTIONS OF THE WOUND-GUM ZONE IN THE LEAF SCAR

Classifications concluded on Opposite Page

Chemical test	Tissues adjacent to the wound-gum zone				
	Reaction of the cell walls of:				Intercellular material of parenchyma
	Cortex parenchyma	Cortex fiber cells	Xylem parenchyma	Vessels	
Iodine and potassium iodide	—*	Yellow	—	Yellow	—
I-KI followed by 72 per cent H_2SO_4	Blue	Yellow, later brown	Blue	Yellow, later brown	—
Zinc chloriodide	Blue	Light clear yellow	Blue	Light clear yellow	—
Ferric chloride and potassium ferricyanide	Green	Light blue	Green	Light blue	Dark green
Dinitro-phenylhydrazine	—	Yellow	—	Yellow	—
Maule reaction	—	Light red	—	Light red	—
Phloroglucinol in HCl	—	Red	—	Red	—
Orcinol followed by HCl	—	Light blue	—	Light blue	—
Ruthenium red‡	—	—	—	—	—
Phloroglucinol in HCl‡	—	—	—	—	—
Zinc chloriodide‡	Blue	Blue	Blue	Blue	—
Ruthenium red¶	Light red	Red	Red	Light red	Red
Phloroglucinol in HCl¶	—	—	—	—	—
Zinc chloriodide¶	Blue	Blue	Blue	Blue	—
Polarized light	Anisotropic	Anisotropic	Anisotropic	Anisotropic	Isotropic

* The dashes indicate negative results.
† The substances were removed by chlorination.
‡ After chlorination and sodium-sulfite treatment.
¶ After chlorination and treatment with 10 per cent ammonium hydroxide.

cortical cells. The first change regularly noted after the scars were formed was in the protoplasm of the cells that were to make up the wound-gum zone. Such protoplasm contained a yellow amorphous material (table 4) which stained a dark brown when treated with phloroglucinol in hydrochloric acid.

Traces of wound gum were found at the end of the first day in the tissues of scars left outside moist chambers (table 3 and plate 1, *A*), but not until the second day in the tissues of the scars placed in moist chambers (table 5 and plate 3, *A*). In the former the wound-gum zone developed in 3 to 6 layers of cells, which were usually from 4 to 11 tiers of cells from the surface of the scar. Photographs of stages in the formation of wound gum under these conditions are shown in plates 1, 2, and 3.

Wound-gum zone of the leaf scar

Reaction of the cell walls of:				Reaction of the material plugging the:		Intercellular material
Cortex parenchyma	Cortex fiber cells	Xylem parenchyma	Vessels	Lumina of vessels	Intercellular spaces	
Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown
Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow
Blue	Blue	Blue	Blue	Blue	Dark blue	Dark blue
Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow
—	Very light red	—	Light red	—	—	—
Red	Red	Red	Red	Red	Red	Red
Blue	Blue	Blue	Blue	Blue	Blue	Blue
—	—	—	—	Removed†	Removed	Removed
—	—	—	—	Removed	Removed	Removed
Blue	Blue	Blue	Blue	Removed	Removed	Removed
Light red	Light red	Red	Light red	Removed	Removed	Red
—	—	—	—	Removed	Removed	—
Blue	Blue	Blue	Blue	Removed	Removed	—
Anisotropic	Anisotropic	Anisotropic	Anisotropic	Isotropic	Isotropic	Isotropic

In those scars outside moist chambers wound gum usually began to stain first in the walls of the cortex cells, though in many sections the material was noted in the walls of living cells of the xylem in the region of the wound-gum zone (plate 1, *A*). With an increase in the age of the scars came a corresponding development of the wound-gum zone (tables 3 and 4). The intercellular spaces generally filled about the second day. The plugging of the vessels, which usually began about the third day,

TABLE 3

RESULTS OF STUDIES OF THE HEALING OF LEAF SCARS* THAT WERE FORMED IN MAY AND KEPT OUTSIDE OF MOIST CHAMBERS

Age of leaf scars (days)	Presence of wound-gum layer	Depth of wound-gum layer (number of cells from scar surface)	Plugging of vessels by wound-gum		Amount of starch†			Number of scars developing periderm	Number of cell divisions in periderm	Suberinlike material in cells of the wound-gum zone	Scars having oil in cells of wound-gum zone	Tannins in leaf-scar tissues
			Vessels counted	Vessels plugged	Exterior to wound-gum zone	In wound-gum zone	Interior to wound-gum zone					
0	−‡	−	−	−	+++	+++	+++	−	−	−	−	−
1	T	5 to 11	−	−	+++	+++	+++	−	−	−	−	−
2	+	4 to 12	−	−	+++	++	+++	−	−	−	−	−
3	+−	4 to 12	243	24	+++	+−	+++	−	−	T	−	−
4	++	2 to 12	254	70	+++	+	++	−	−	+	1	−
5	++−	4 to 12	264	204	+++	T	++	−	−	+	−	−
6	+++	4 to 12	−	−	+++	−	+	2	1 to 2	+−	3	−
7	+++	4 to 12	270	269	+++	−	+	16	1 to 2	++	2	−
8	+++	4 to 13	...	all	+++	−	T	20	1 to 4	++	2	−
9	+++	4 to 10	...	all	+++	−	T	10	2 to 4	+++	−	−

* Twenty scars were studied at each day group except the ninth day in which 10 scars were used.

† The amount of starch is on a comparative basis.

‡ Legend: − = negative; + = positive; +−, ++, and +++ = increasing amounts in their respective order; T = trace.

was well advanced by the fifth, as shown in table 3 and plate 2, *A*. By the sixth to seventh days the wound gum layer appeared completed in a number of scars (plate 1, *C*, 2, *C* and *D*), and the formation of a periderm had started in a few scars (plate 1, *D* and table 3).

TABLE 4

RESULTS OF STUDIES OF THE HEALING OF LEAF SCARS THAT WERE FORMED IN SEPTEMBER

Leaf-scar number	Age of leaf scar (days)	Wound gum by phloroglucinol in HCl	Orcinol test for wound gum	Depth of wound-gum layer (number of cells from scar surface)	Presence of yellow amorphous material	Copper acetate and ferric chloride test for tannins
Scars left outside moist chambers						
1	0	−*	−*	−*	−*	−*
2	0	−	−	−	−	−
3	0	−	−	−	−	−
4	0	−	−	−	−	−
5	0	−	−	−	−	−
6	0	−	−	−	−	−
7	0	−	−	−	−	−
8	0	−	−	−	−	−
9	0	−	−	−	−	−
1	1	T	T	5 to 8	+	−
2	1	T	T	7 to 10	+	−
3	1	T	T	8 to 11	+	−
4	1	−	−	−	+	−
5	1	T	T	7 to 9	+	−
6	1	T	T	5 to 8	+	−
7	1	T	T	6 to 10	+	−
8	1	T	T	4 to 8	+	−
9	1	T	T	5 to 10	+	−
10	1	T	T	6 to 11	+	−
11	1	T	T	6 to 9	+	−
12	1	−	−	−	+	−
13	1	T	T	5 to 8	+	−
14	1	T	T	6 to 11	+	−
15	1	T	T	8 to 12	+	−
16	1	T	T	4 to 9	+	−
17	1	−	−	−	+	−
18	1	−	−	−	+	−
19	1	T	T	7 to 9	+	−
20	1	T	T	9 to 11	+	−
1	2	+	+	4 to 9	+	−
2	2	+	+	5 to 7	+	−
3	2	+	+	6 to 9	−	−
4	2	+	+	7 to 10	−	−
5	2	+	+	4 to 8	−	−
6	2	+	+	6 to 9	+	−
7	2	+	+	6 to 10	+	−
8	2	+	+	7 to 9	+	−
9	2	+	+	6 to 8	+	−
10	2	+	+	4 to 9	+	−

* Legend: − = negative; + = positive; +−, ++, +++ = increasing amounts in their respective order; T = trace.

TABLE 4—(*Concluded*)

Leaf-scar number	Age of leaf scar (days)	Wound gum by phloroglucinol in HCl	Orcinol test for wound gum	Depth of wound-gum layer (number of cells from scar surface)	Presence of yellow amorphous material	Copper acetate and ferric chloride test for tannins
Scars kept in moist chambers						
1	0	−*	−*	−*	+*	−*
2	0	−	−	−	−	−
3	0	−	−	−	+	−
4	0	−	−	−	−	−
5	0	−	−	−	−	−
6	0	−	−	−	−	−
7	0	−	−	−	−	−
8	0	−	−	−	−	−
9	0	−	−	−	−	−
10	0	−	−	−	−	−
1	1	−	−	−	+	−
2	1	T	−	1 to 3	+	−
3	1	−	−	−	+	−
4	1	−	−	−	+	−
5	1	−	−	−	+	−
6	1	−	−	−	+	−
7	1	−	−	−	+	−
8	1	−	−	−	+	−
9	1	−	−	−	+	−
10	1	−	−	−	+	−
11	1	−	−	−	+	−
12	1	−	−	−	+	−
13	1	−	−	−	+	−
14	1	−	−	−	+	−
1	2	T	T	1 to 3	+	−
2	2	+	+	1 to 4	+	−
3	2	+	+	1 to 4	+	−
4	2	+	+	1 to 2	+	−
5	2	+	+	1 to 5	+	−
6	2	+	+	1 to 2	+	−
7	2	T	T	1 to 3	+	−
8	2	+	+	2 to 5	+	−
9	2	+	+	3 to 6	+	−
10	2	T	T	2 to 4	+	−

* Legend: − = negative; + = positive; +−, ++, +++ = increasing amounts in their respective order; T = trace.

Although transverse sections through the wound-gum zone were not very satisfactory for these studies, they show how the wound-gum plugs appear in transverse sections of the vessels (plate 3, *F*).

Two layers of wound gum formed in a number of the scars kept in moist chambers. The first layer usually began to develop in scars collected the second day. Wound gum formed in 1 to 6 rows of cells across the surface of the scar (tables 4 and 5 and plate 3, *A* and *B*) and had generally formed completely by the third to fourth day (plate 3, *C*). About

TABLE 5

RESULTS OF STUDIES OF THE HEALING OF LEAF SCARS* THAT WERE FORMED IN MAY AND PLACED IN MOIST CHAMBERS UNTIL COLLECTED

Age of leaf scar (days)	Presence of wound gum	Number of scars developing two layers of wound gum	Depth of wound-gum layer. (Number of cells from scar surface)		Plugging of vessels by wound gum		Amount of starch†			Number of scars developing periderm	Number of cell divisions	Suberinlike material in cells of the wound-gum zone	Scars having oil in cells of wound-gum zone	Tannins in leaf-scar tissues
			Layer 1	Layer 2	Vessels counted	Vessels plugged	Exterior to wound gum zone	In wound-gum zone	Interior to wound gum zone					
0	−‡	−	−	−	−	−	+++	+++	+++	−	−	−	−	−
1	−	−	−	−	−	−	+++	+++	+++	−	−	−	3	−
2	T	−	1 to 6	−	173	3	+++	+++	+++	−	−	−	−	−
3	+	−	1 to 8	−	203	47	+++	++	+++	1	1	−	1	−
4	+−	3	1 to 5	4 to 10	212	163	+++	+	++	3	1	T	3	−
5	+++	2	1 to 4	4 to 10	...	all	+++	T	+	2	1 to 2	+	1	−
6	+++	13	1 to 4	4 to 8	...	all	+++	T	+	2	1	+−	−	−
7	+++	16	1 to 4	4 to 8	...	all	+++	−	T	3	1 to 2	++	−	−
8	+++	10	1 to 3	4 to 9	...	...	+++	−	T	1	1	+++	−	−

* Twenty scars were studied at each day group except the eighth day in which 10 scars were used.

† The amount of starch is on a comparative basis.

‡ Legend: − = negative; + = positive; +−, ++, +++ = increasing amounts in their respective order; T = trace.

this time a second layer of wound gum began to appear in a layer of cells from 4 to 10 cells from the surface of the scar, below the first layer (plate 3, *D*). Both layers, however, generally converged at the edges of the scar (plate 3, *E*). By the end of the fifth day, wound gum had plugged all the vessels observed in either the first or second zone.

The striking differences in wound-gum formation between the scars left outside moist chambers and those kept in moist chambers are as follows: (1) The scars left outside moist chambers developed only one wound-gum zone, whereas those kept inside developed two layers. (2) The wound gum was usually detectable by the first day in the scars left outside, but not until the second day in those kept in moist chambers. (3) After it once started, however, wound gum formed much more rapidly in the scars kept inside than in those left outside.

Water-Soluble Gum.—The term is used here to designate gumlike materials that are soluble in water as contrasted with wound gum that is insoluble in water. Water-soluble gum, lignin, and wound gum stain blue when treated with orcinol followed by hydrochloric acid. To distinguish, therefore, between wound gum, lignin, and water-soluble gums, a section was first treated with orcinol and HCl. Next, if positive results were obtained, adjacent sections from the same scar were washed in warm water to remove gums, then treated with orcinol. If the material, other than lignified tissues, staining with orcinol was removed by the water, it was considered to be water-soluble gum. Fresh, unfixed leaf scars were used to determine the presence of this material. These studies were made in scars formed September 9. The results for scars kept in moist chambers are recorded under orcinol in table 4, as are also those for scars exposed to outside conditions. Conceivably, water-soluble gums might be deposited in the cell walls before wound gum appeared; but apparently such was not the case, for no positive tests were obtained.

Lignin.—In all cases, phloroglucinol in hydrochloric acid and the Maule reactions were used to distinguish between wound gum and lignin. The former reacts with both substances, whereas the Maule reaction is positive only when the tissues are lignified.

Lee (*4*) described the abscission of leaves and the healing of leaf scars in numerous species of plants. As to the healing processes of *Castanea sativa* Mill., he states: "Directly after leaf fall—the activity of the cells below the surface of separation is at once shown by the change in the chemical composition of the cell wall. Very gradually these become more or less completely lignified." He does not mention wound gum, but the lignification he describes is probably wound gum, which gives the same reactions as lignin with a number of microchemical tests. If he had used

the Maule reaction, possibly he would have failed to get a positive reaction.

According to results of the present studies of olive leaf scars the vessels, fibers, and a few stone cells were the only tissues of the olive leaf scar that were lignified.

Suberin and Oil.—Sudan III was used to determine the presence of suberin and oil.

In describing the healing of leaf scars of *Castanea sativa,* Lee (*4*) states that when the cell walls of the protective layer undergo "lignification" there is deposited on the inside surface of each cell wall of this layer a fine film of suberin. Lee termed the process "lignosuberization." The protective layer he mentions in the leaf-scar tissue of *C. sativa,* is in a position similar to that of the wound-gum zone in the olive leaf scar.

In the cells of the wound-gum zone of the olive leaf scar was found a material deposited as a thin layer or lamella on the inside surface of the cell walls. This material stained red with Sudan III or IV, was insoluble in alcohol, ether, or benzene, but was soluble in 3 per cent KOH and was isotropic when examined with polarized light. As it did not separate from the cell wall with the protoplasm when the cells were plasmolyzed, it is designated as "suberinlike" material because it apparently has the properties of suberin; but its location in the cell differs from that of suberin, which usually impregnates the wall.

The lamella of suberinlike material began to form after some wound gum had been deposited, and showed first in the 3-day-old leaf scars outside moist chambers (table 3) and in the 4-day-old leaf scars kept in moist chambers (table 5). It continued to develop as the age of the scars increased and stopped about the time the periderm began to form.

Oil globules were found, but not consistently, in either those scars kept in moist chambers or those left outside.

Starch.—Iodine in potassium iodide solution was used for starch determination. In all cases the amount of starch in the cells of the wound-gum zone decreased with increase in the quantity of wound gum (tables 3 and 5).

Tannins.—Sections, after treating with a solution of cupric acetate to precipitate tannins, were rinsed in water and treated with ferric chloride. Tannins stain green, blue, or black. By this method, no difference was noted in the quantity of tannin distributed over the leaf scar at any time during healing.

Periderm Formation.—Observations on the formation of a periderm in the leaf-scar tissue were made when the other healing processes were studied. The results appear in table 3 for the scars left outside moist

chambers and in table 5 for those kept in moist chambers. The periderm developed in the tissue just beneath the wound-gum zone. In the scars left outside, the periderm development was regular. The first cell divisions were observed in two 6-day-old scars. The number of scars in which a periderm had begun to develop increased in each day's collection thereafter. By the eighth day all the scars that were sectioned had completed from 1 to 4 cell divisions in the formation of a periderm (table 3).

The formation of a periderm was not regular in the scars kept in moist chambers. One of the 3-day-old scars, and only one, two, or three of the scars collected each day thereafter, through the eighth day, had started to develop a periderm (table 5). This situation differs from the regular increase in the number of scars that formed periderm in those left outside moist chambers.

From the microchemical studies, one cannot easily explain why the number of scars infected dropped so rapidly during the first two days after the leaves were removed (table 1, and figs. 4 and 5). By the end of two days there were apparently no materials deposited within the tissues of the scars that mechanically blocked them to invasion by bacteria. After two days, however, the deposition of wound gum was concurrent with the decrease in the percentage of scars infected. By the time all the vessels of the scars appeared completely plugged with wound gum, the scars had become immune to infection. This held true both for scars kept in moist chambers and for those left outside.

These studies show that during the first two days other factors may be involved in the resistance to infection.

COURSE OF BACTERIA THROUGH LEAF-SCAR TISSUE TO ESTABLISH INFECTION

The processes of healing in the leaf scars previously described do not explain why the susceptibility of scars to infection decreased rapidly during the first two days. They also do not explain why this drop was much more pronounced in the scars kept in moist chambers than in those left outside. A study was made, therefore, of the channels through which the bacteria could enter the tissues to cause infection.

For these studies, a mixture of 1 part of Higgins' American India ink and 5 parts of water proved most satisfactory because it did not diffuse into the living cells and was insoluble in water after it had dried in the scars. This procedure facilitated handling of the scars in water during and after sectioning.

In the experiment several scars were formed as previously described

under methods. One group was placed under moist chambers; another was left outside. When the leaves were being removed and at intervals thereafter, a few scars in each group were covered with the ink mixture, applied with a small brush. The ink mixture usually remained on the surface of the scar about 10 minutes before drying. After the ink dried, the scars were removed and brought into the laboratory for sectioning. The sections were mounted in water, and the depth of ink penetration was measured with a filar micrometer. The greatest depth of penetration

TABLE 6

THE PENETRATION OF INDIA INK INTO THE VESSELS OF LEAF SCARS

Age of leaf scar at time of treatment (hours)	Scars outside moist chambers			Scars inside moist chambers		
	Number of scars treated	Scars in which ink penetrated 5 cells or more (per cent)	Average depth of ink penetration (microns)	Number of scars treated	Scars in which ink penetrated 5 cells or more (per cent)	Average depth of ink penetration (microns)
0	34	86.3	493.3	34	86.3	493.3
½	30	74.2	154.2	20	70.0	139.4
3	25	70.0	65.3	22	63.5	69.5
12	29	48.3	23.9	25	32.0	20.3
24	30	48.2	10.7	22	27.6	8.2
48	25	27.9	8.0	20	15.0	7.3

in each scar was recorded, and the average for each collection was computed from these measurements (table 6, fig. 6). The results show that immediately after the leaves were removed, the ink placed over the scars moves a relatively great distance back into the vessels of the scars. In scars only half an hour old, however, the ink penetrated only a relatively short distance; and this distance decreased thereafter with increasing age of the scar (table 6, fig. 6, *A*). A comparison of these results with those of infection data (fig. 5) suggests that the reason a high percentage of the scars developed knots when inoculated immediately after removal of the leaves was that the inoculum penetrated deeply into the vessels at this time.

In these observations the ink penetrated only through the vessels; in no case was it found to have entered the intercellular spaces or sieve tubes.

If the formation of wound gum in the leaf-scar tissues prevents the entrance of bacteria, the latter, in order to infect the host, must pass beyond the wound-gum zone before it plugs the channels of entrance. The wound gum developed in 4 to 12 cell layers from the surface of the scar. An average thickness of this zone was around 2 to 5 layers of cells.

Apparently, then, if the initial penetration of the bacteria into the scar tissues was 5 cell-layers or more, they were deep enough in the scar tissues to establish infection.

In these studies of ink penetration, therefore, if the India ink placed over the surface of the scar penetrated the tissues 5 cells or more, it was considered deep enough for bacteria to have caused infection. If this

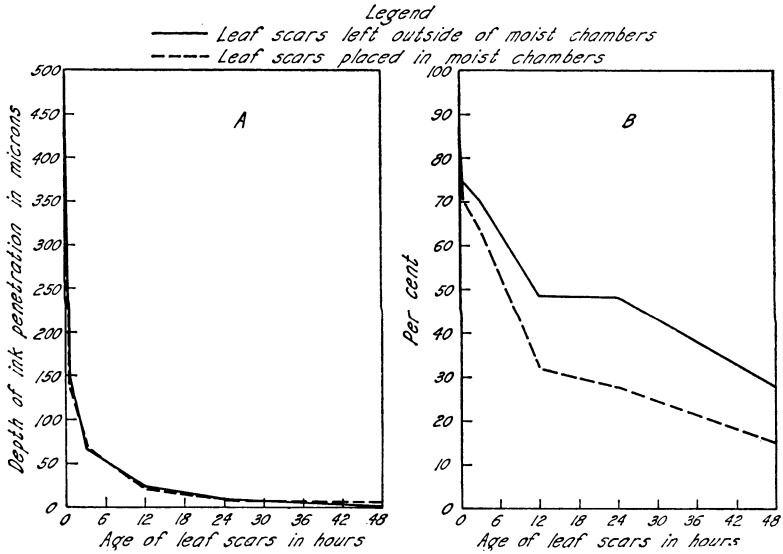

Fig. 6.—The results of studies of India-ink penetration into leaf-scar tissues for scars kept in moist chambers and those left outside. *A*, The relation between the age of the leaf scar in hours when treated with ink and the depth of ink penetration. *B*, The relation between the age of the leaf scars in hours when treated with ink and the per cent of scars in which the ink penetrated to a depth considered sufficient to cause infection if bacteria had been used.

assumption is correct, the percentage of scars in which the ink penetrated 5 cells or more should correspond to the results of inoculation experiments under similar conditions.

The curve (fig. 6, *B*) representing the percentage of scars in which ink penetrated to a depth of 5 cells, or more, compares very closely for the period of the first two days with the results of interval inoculations in the curves of figure 5. For the scars left outside moist chambers the ink penetrated to a depth of 5 cells, or more, in 48 per cent of the scars one day old, and in 28 per cent of those two days old, whereas in the inoculation experiments about 58 per cent of the one-day-old scars developed knots and about 45 per cent of the two-day-old scars developed knots.

For the scars kept in moist chambers the ink penetrated 5 cells or more in about 27 per cent of the one-day-old scars and 15 per cent of the two-

day-old scars. In the inoculation experiments about 23 per cent of the one-day-old scars developed knots, and about 15 per cent of those two days old.

Judging from the ink-penetration studies under the conditions of these experiments, the infection of leaf scars may be influenced by the depth of penetration of the inoculum into the vessels.

THE DEVELOPMENT OF BACTERIA IN THE SCAR TISSUES

The course the bacteria take in establishing infection was studied in artificially inoculated leaf scars. A large number of scars were formed, marked, and inoculated as previously described. Groups of 10 scars each were collected 2, 4, 6, 8, 10, 13, 15, 19, and 26 days after the scars were inoculated. The scar tissues were imbedded in paraffin, as described earlier, and were sectioned with a rotary microtome. The sections were stained with Stoughton's stain for bacteria in tissues (*5*).

Theoretically the bacteria might infect a leaf scar in several ways: (1) by growth on the surface of the scar, (2) by entering the scar by breaking down the tissue, (3) by penetrating the scar tissue through the intercellular spaces, (4) by entering through fissures in the scar, (5) by entering the scar through the sieve tubes, and (6) by entering the scar tissues through vessels. In the scars examined, the only way, with one exception, in which infection had been established was through the vessels. In one case, however, the bacteria had penetrated deeper than the wound-gum zone through a fissure between cortex parenchyma cells. A photograph of this section is shown in plate 4,*A*. In most cases, according to observations, the bacteria established on the surface, in fissures, and in the intercellular spaces of the leaf-scar tissue are later barred from entering more deeply into the tissue by the formation of wound gum and finally of a periderm.

Bacteria pass deeply into the vessels when the leaf scars are inoculated immediately after the leaves were removed. This point is supported by the experiment in which India ink was placed on the surface of scars and passed freely through the vessels of the leaf and, in a few cases, as deep as the vascular system of the stem. Small groups of bacteria were, furthermore, found scattered along in the vessels (plate 4, *B*) and lodged on the rims at the ends of the vessel cells in sections of scars collected 4 days after inoculation. Bacteria were observed between the secondary thickenings, where they may have lodged as the inoculum was drawn back through the vessels. The groups of bacteria shown in these photomicrographs (plate 4, *B*) are stained very heavily in order that the vessel walls

may be shown. They appear, therefore, only as dark masses lodged between the thickening on the sides of the vessels. These small groups of bacteria enlarge rapidly, producing colonies that finally merge and completely fill the vessels (plate 4, *C*).

The bacteria are freed from the vessels into the other tissues of the leaf scar when the forming periderm pulls the vessels apart (plate 4, *D* and *E*). The periderm forms just beneath the wound-gum zone, and the increase in number and size of the cells pulls the vessels apart at this point. This generally occurs from 8 to 10 days after the scars are formed. The bacteria are then released into the newly forming periderm, a region of actively dividing cells (plate 4, *D* and *E*). The bacteria confined in the vessels deeper in the leaf traces remained there and did not break out into the surrounding tissue by the end of the 19 days after inoculation (plate 4, *E*).

The presence of bacteria or their products among the meristematic cells of the periderm apparently stimulates these cells to active division, and it appears that the successive rows of parenchyma cells were derived from the phellogen (plate 5, *A*). The bacteria increase in numbers at the end of the broken vessels and form small pockets, which increase in size and grow with the surrounding tissue. After 19 days the pockets are fairly large (plate 5, *A*). Some of the cells adjacent to them break down, collapse, and remain around the outer portions of the pockets (plate 5, *B*). As the plate shows, more cell divisions occur in the region around the bacterial pockets.

SUMMARY

A study has been made of certain factors involved in the infection of olive leaf scars by *Bacterium savastanoi* E.F.S. from the stage just before leaf fall until a periderm develops in the scar.

It was found that natural infection in the region of the abscission zone rarely occurs before leaf fall.

Evidence obtained confirms the conclusions of previous workers that most of the new knots forming each year develop at leaf scars.

Most leaf scars were susceptible to infection immediately after leaf fall; the susceptibility dropped rapidly during the first day; and the scars became immune by the end of the ninth. The drop in susceptibility was much more rapid for scars kept in moist chambers than for scars left outside.

Microchemical studies of the abscission processes of leaves and the healing of leaf scars showed that (a) no protective layers are formed in the tissues before leaf fall; (b) separation takes place through the inter-

cellular material between two rows of cells in the base of the abscission zone; (c) the sieve tubes, vessels, cuticle, fibers, and apparently the epidermal cells are mechanically broken at leaf fall; (d) during the healing processes of the olive-leaf scar a wound-gum layer is first formed and is followed by the development of a periderm; (e) water-soluble gums, lignin, oil, suberin, starch, and tannins apparently have no influence upon infection.

India ink was used to trace the course of the inoculum from the surface of the leaf scar into the scar tissues. Judging from these studies, infection may depend upon the depth of penetration of the inoculum.

Most infections in the leaf scars were caused by bacteria that entered the tissues through vessels. Bacteria entering the leaf-scar tissue through intercellular spaces progressed slowly and were stopped by wound gum that plugged these spaces. Those entering the vessels were freed into the periderm cells when the vessels were slowly pulled apart by growth of the periderm. Pockets of bacteria were formed in the tissues derived from the phellogen, and the greatest amount of cell proliferation occurred around the pockets of bacteria.

ACKNOWLEDGMENTS

The author is grateful to Dr. T. E. Rawlins, Dr. E. E. Wilson, and Dr. A. S. Foster for their guidance during the research work; to Professor Max W. Gardner for helpful suggestions and criticism and to the owners of the Cross Ranch in Sacramento County for the use of their orchard.

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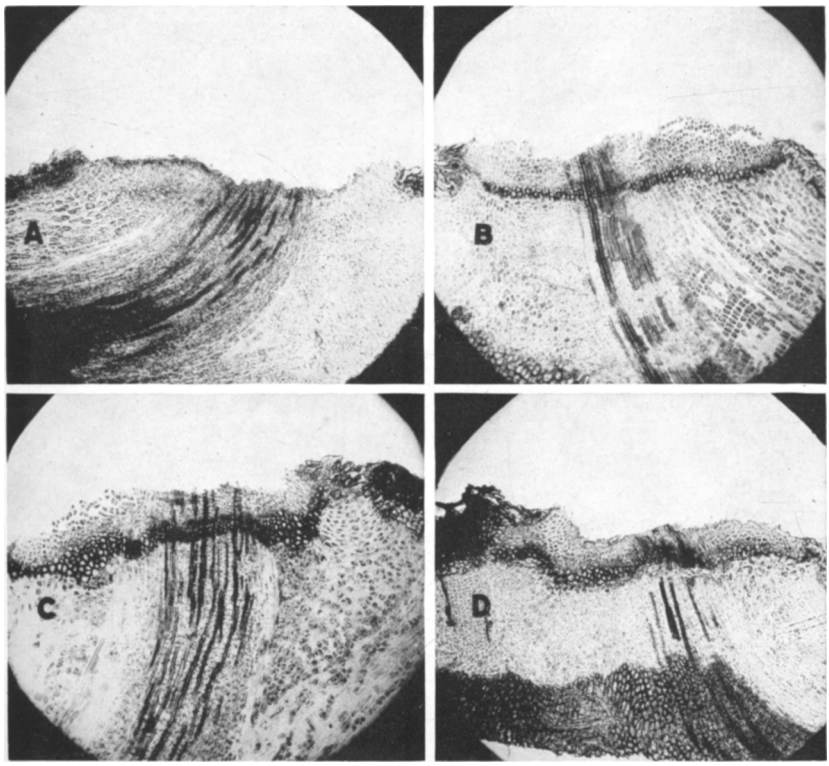

Plate 1.—Sections of leaf scars, approximately 15 microns thick. The sections were treated with phloroglucinol in hydrochloric acid and mounted in the same solution. The lignified walls, a portion of the cell contents, and the wound gum were the only parts of the sections that stained with the phloroglucinol in hydrochloric acid; they appear particularly dark in the photographs. Stages in the development of wound gum in leaf scars left exposed to outside atmospheric conditions: *A*, Longitudinal section of leaf scar 1 day old. The wound-gum layer has begun to form at a distance of 5 to 8 cells from the edge of the leaf scar, as indicated by darker staining in the cortex. *B*, Longitudinal section of a leaf scar 5 days old. The section shows the wound-gum layer being formed and wound-gum plugging some of the vessels. Photographs at higher magnifications showing the plugging of the vessels are in plate 2, *A* and *B*. *C*, Longitudinal section of leaf scar 7 days old. All the vessels of the wound-gum area are completely plugged with gum. Photographs of higher magnifications of the wound-gum zone are shown in plate 2, *C* and *D*. *D*, Longitudinal sections of leaf scars 9 days old. From 1 to 3 cell divisions have occurred beneath the wound-gum zone in an early stage in periderm formation. Also note the vessels that have been pulled apart. (All × 36.)

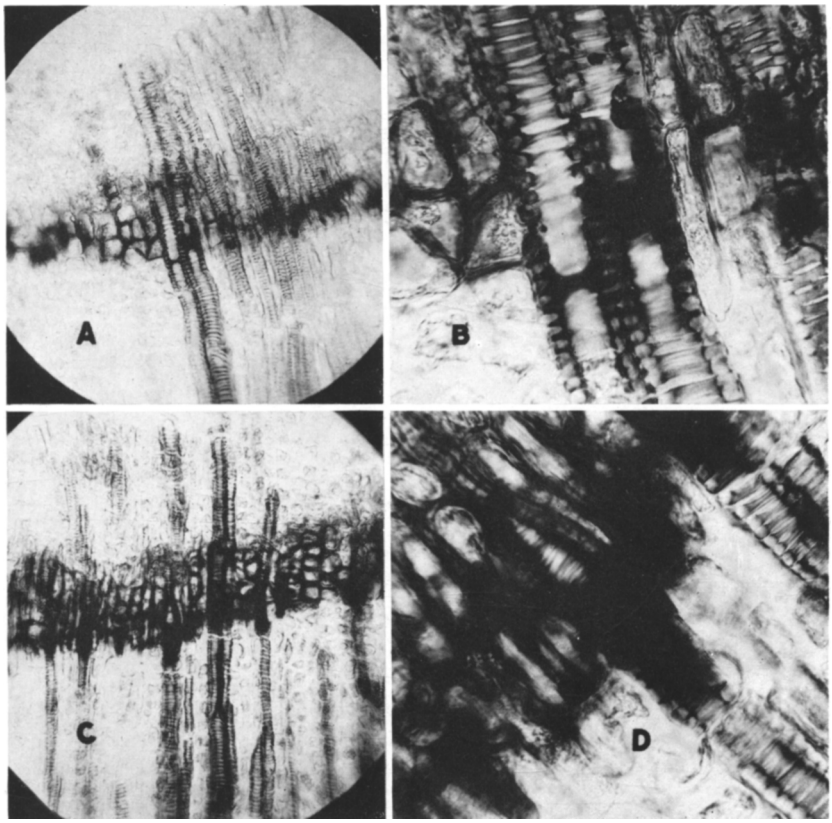

Plate 2.—Vessels plugged by wound gum, and wound gum in the walls of the parenchyma cells: *A*, From the same longitudinal section of a leaf scar as photograph *B* in plate 1. *B*, A portion of the same section as *A*. This shows more clearly the wound gum in the vessels and walls of parenchyma cells. *C*, From the same longitudinal section of leaf scar as photograph *C* in plate 1. *D*, A portion of the same section as *C*. (*A* and *C*, × 192; *B* and *D*, × 694.)

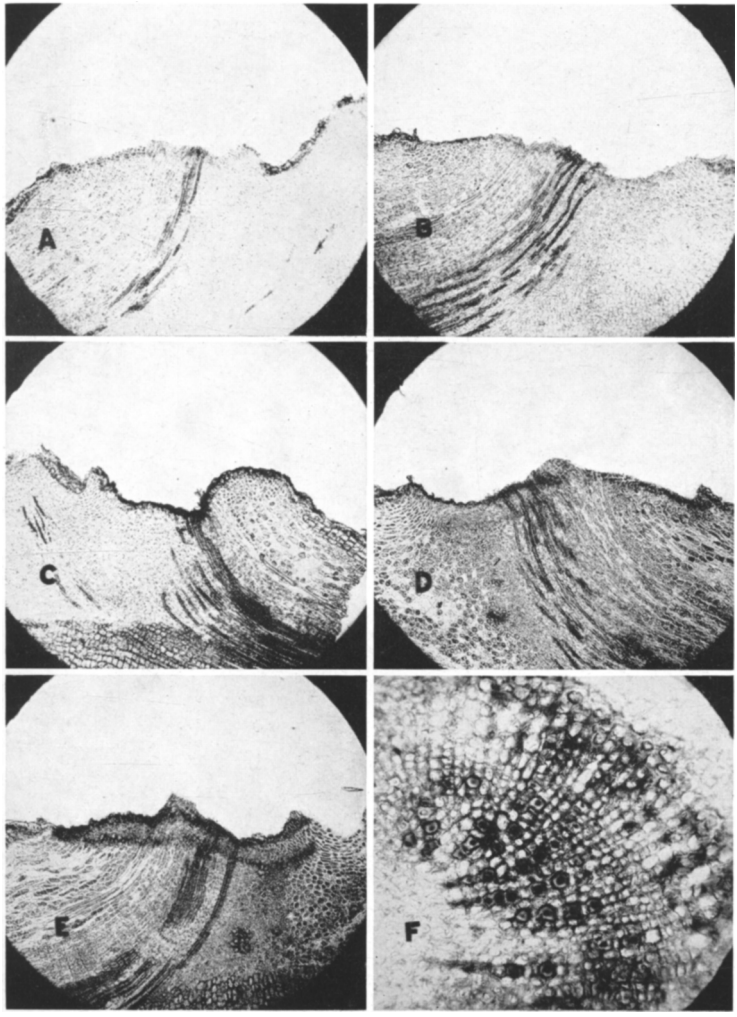

Plate 3.—Sections of leaf scars kept in moist chambers during healing, showing stages in the process of wound-gum formation. Refer to plate 1 for explanation. *A*, Longitudinal section, 2 days old; note the deep staining of the cell contents of the cells bordering the edge of the scar due to a yellow amorphous substance. A trace of wound gum has developed in the upper right edge of the scar. *B*, Longitudinal section, 2 days old, showing a trace of wound gum along the left side in the cells bordering the edge of the scar. *C*, Longitudinal section, 4 days old. The wound-gum layer borders the enire edge of the leaf scar and has completely plugged all vessels and intercellular spaces. *D*, Longitudinal section, 7 days old. The second layer of wound gum has begun to form 3–5 cells interior to the first wound-gum layer. *E*, Longitudinal section, 8 days old. The second layer of wound gum is formed completely. *F*, A portion of a transverse section through the wound-gum zone of a leaf scar 7 days old, showing wound gum plugging some of the vessels. (All × 36.)

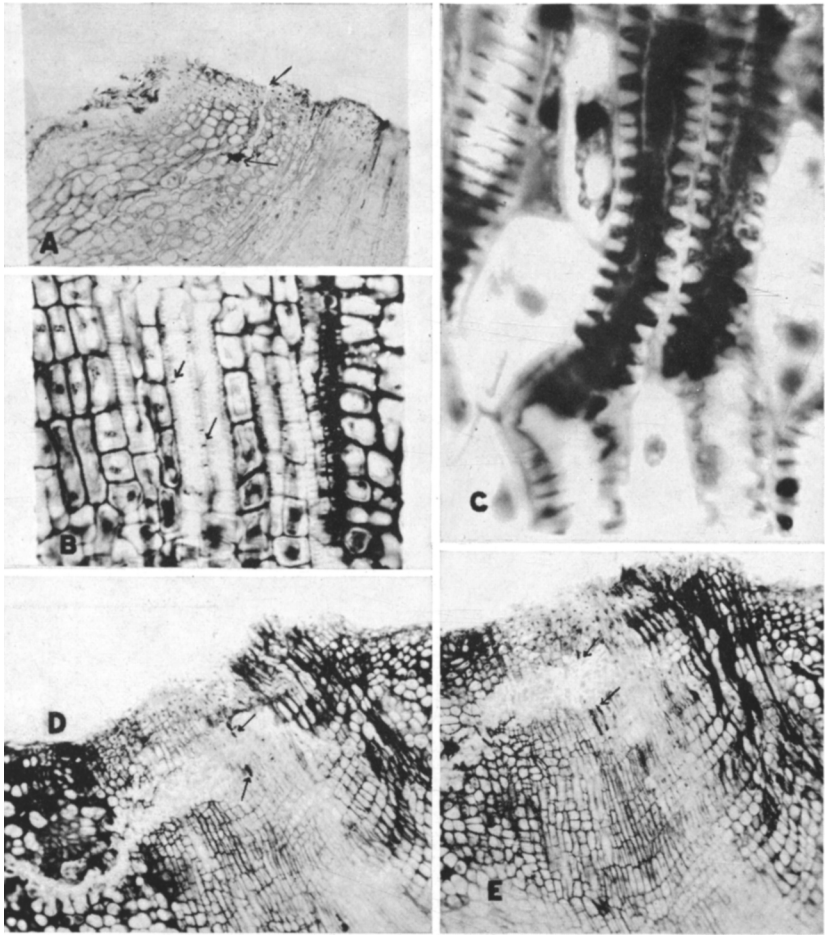

Plate 4.—*A*, Longitudinal section of a leaf scar 12 days old. The leaf scar was inoculated immediately after the leaf was removed. The upper arrow indicates a fissure between the cortex cells through which infection took place, and the lower arrow points to a colony of bacteria. *B*, Vessels from a longitudinal section of leaf scar 4 days after inoculation. The vessels contain colonies of bacteria between the secondary thickenings of the wall, as indicated by arrows. *C*, Vessels from a longitudinal section of a leaf scar collected 7 days after inoculation. They are partly filled with bacterial colonies. The portion of the vessels shown are located just below the wound-gum zone. *D*, *E*, Longitudinal sections of leaf scars collected 12 days after inoculation. Note portions of vessels filled with bacteria and pulled apart and separated by the active division of the periderm cells. The walls from the cells derived from the periderm are not clearly stained. (*A*, × 74; *B*, × 304; *C*, × 1004; *D* and *E*, × 109.)

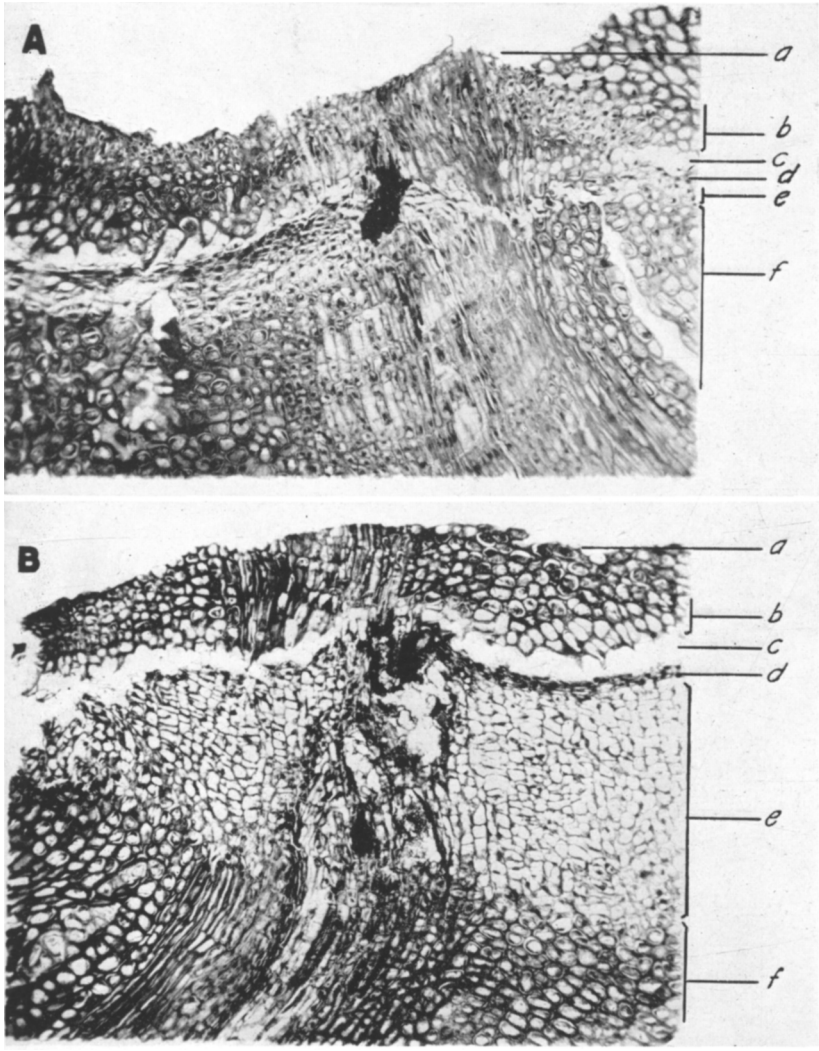

Plate 5.—*A*, Longitudinal section of a leaf scar collected 16 days after inoculation. Note the pocket of bacteria formed among the cells of the periderm. The bacteria in the upper portion of the pocket are extended into vessels. *B*, Longitudinal section of a leaf-scar collected 26 days after inoculation, showing large pockets of bacteria which have developed in the tissue apparently derived from the phellogen. Details are: *a*, leaf-scar surface; *b*, region of the wound-gum zone; *c*, phellem; *d*, phellogen; *e*, tissue derived from the phellogen; *f*, leaf petiole tissue. (All × 133.)