HISTOPATHOLOGY OF BACTERIAL LEAF SPOT OF ALFALFA

by

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A MASTER'S THESIS

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MASTER OF SCIENCE

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INTRODUCTION

Bacterial leaf spot of alfalfa (Medicago sativa L.), incited by Xanthomonas alfalfae (Riker, Jones and Davis) Dows. can cause extensive damage to alfalfa in warm and humid weather. The pathogen causes postemergent damping-off and stunting of seedlings, necrosis and defoliation of leaves and necrosis of stems. X. alfalfae is a rod-shaped, gram-negative, non-sporeforming bacterium, with a polar flagellum (11,12).

History of the disease. This disease was first reported by Riker et al. (12) in August 1930 on the alfalfa varieties Turkestan and Grimm at Madison, Wisconsin. The disease was noticed in experimental rows which had been splashed with water. In October, 1931, the disease was again observed. After studying morphological and physiological characters of single cell isolates they suggested the name Phytomonas alfalfae for the incitant. This name was later changed to X. alfalfae by Dowson (6).

Bacterial leaf spot was noted in 1938 in an Agronomy greenhouse at Kansas State University where it caused extensive damage. The following year the disease appeared on alfalfa at the Agronomy Farm at Manhattan (9). The disease was reported as "bacterial leaf and stem spot" in a Kansas report to the Plant Disease Survey, which apparently is the source of the disease name listed in the Index of Plant Diseases (16,17).

In 1949, Patel et al. (11) noted the disease in India. They reported that peas (Pisum sativum), Melilotus indica and Trigonella foenum-graecum were readily infected by X. alfalfae. Dye (7) added 'Canadian Wonder' bean (Phaseolus vulgaris) to the host list. Stuteville and Sorensen (14) found 'Clark' soybean (Glycine max), 'Dixie' crimson clover (Trifolium incarnatum), 'Common' white sweetclover (Melilotus alba), 'Common' yellow sweetclover (Melilotus officinalis), black medic (Medicago lupulina), and common vetch (Vicia sativa) readily infected by X. alfalfae.

Brigham (1,2) reported the disease in a nursery in Ames, Iowa, in June 1956 and in several alfalfa fields in central Iowa in September 1956.

Stuteville and Sorensen (14) reported that bacterial leaf spot was widespread and caused serious damage in localized areas in Kansas in 1964 and 1965. They also reported that the disease killed over 50% of first year 'Ladak' alfalfa transplants on the Ashland Agronomy Farm at Manhattan, Kansas in July and August 1964. The living plants showed stunting, leaf and stem lesions and defoliation, although the roots appeared healthy. In September 1965, the disease appeared mainly responsible for loss of seedling stands of alfalfa at the Ashland Agronomy Farm. They also reported that, in greenhouse trials, infected seedlings often had necrotic hypocotyls and a stunted appearance. After 25 days, 73% of emerged plants in infested soil were dead in comparison

to none dead in the noninfested soil. Also they noted variation in susceptibility to the disease and suggested that mass screening might produce resistant varieties.

Disease symptoms. Leaf spots at first are small and water-soaked. These spots may enlarge and coalesce, resulting in large spreading lesions with dry centers and water-soaked edges. But leaf spots remain small and become necrotic on some plants. Diseased stems are marked by elongate water-soaked areas. There is also considerable variation in the size of lesions in stems. The spots enlarge to a length of 1-6 cm in some alfalfa plants whereas the spots become necrotic and remain small in others.

Infected susceptible seedlings show stunting and damping-off in addition to the leaf and stem symptoms listed above. Stuteville and Sorensen (14) reported a significant difference in height between infected and noninfected seedlings one month after inoculation. The hypocotyls of damped-off seedlings are marked by a necrotic area near the soil level.

Histological studies involving bacterial diseases. Several histopathological studies have been used to elucidate the mechanisms involved in pathogenicity of bacteria in plants. In their histological study with bacterial blight of cotton, incited by X. malvacearum, Thiers and Blank (15) reported penetration through stomata in the lower epidermis, intercellular invasion, destruction of spongy mesophyll, and finally, destruction of palisade cells. No morphological

barrier was observed which would account for resistance, although, in old stem lesions, a layer of cork tissue occasionally was observed.

Bugbee and Anderson (3) made histological studies of leaf spots of geranium (Pelargonium hortorum) incited by X. pelargonii. Development of bacterial blisters on infected leaves began with an enlargement of mesophyll cells directly under the point of penetration. Cambial activity was initiated in the enlarged mesophyll cells just inside the lower epidermis. The blister was composed of crushed mesophyll cells and cork cells derived from cambial activity. The cork cells apparently prevented the spread of the bacteria.

Williams and Keen (18) used histological methods to study mechanisms of pathogenesis of Pseudomonas lachrymans in cucumber. They found a low number of bacteria in the host tissue during the initial water-soaking stage of development (24-48 hr after inoculation). However, 48 hr after inoculation, the bacteria multiplied rapidly in the spongy mesophyll. Bacteria were seldom seen in the palisade layer, apparently because a closely packed layer of cells between the spongy mesophyll and palisade layer restricted the bacteria.

Zaumeyer (19) reported a comparative histology of three bacterial diseases of bean. He found that X. phaseoli and Pseudomonas medicaginis var. phaseolicola entered stomata but Corynebacterium flaccumfaciens entered stems and leaves

only through wounds. All three of these pathogens moved intercellularly and eventually invaded parenchyma cells apparently by dissolving cell walls. The three pathogens were found in xylem vessels although C. flaccumfaciens did more damage there than did the other two.

Several researchers (3,15,18) used safranin and orange G for their histopathological studies. Hematoxylin and safranin (18), hematoxylin and orange G (10), thionin-orange G (3) and Giemsa stain-fast green or orange G (19) staining combinations also have been used.

Objectives. The objectives of this study were to find the mechanisms of penetration and invasion of alfalfa by X. alfalfae, the morphological nature of resistance to the bacterium, and the morphological damage caused by X. alfalfae within stunted and damped-off seedlings.

MATERIALS AND METHODS

Isolate KX-1 of X. alfalfae obtained from D. L. Stuteville was used throughout these studies. It was maintained on potato-dextrose agar (PDA) slants. Inoculum was prepared by transferring a loop of bacteria from the slant to 100 ml of potato-dextrose broth (PDB) in a 250 ml flask and incubating 3-5 days on a shaker at room temp. Then the broth was diluted to 400 ml with distilled water, resulting in $1\text{-}2\text{x}10^9$ viable cells per ml as determined by the dilution plate method. Inoculum was applied to plants with a No. 152 DeVilbiss atomizer

at 40 psi.

Seedlings of 'Cody' alfalfa were used to study penetration, invasion, damping-off, and stunting. Resistant and susceptible alfalfa seedlings, a resistant clone (KS12X35), and a very susceptible clone (KS12X25) were used to study the morphological nature of resistance. Resistant and susceptible seedlings and clones were selected by screening KS12, an experimental synthetic. Plants were labeled resistant if leaf and stem lesions were restricted to small necrotic spots and susceptible if the lesions became large and remained active.

Seeds were planted in sterilized sand and seedlings were periodically fertilized with Hyponex. Seedlings were inoculated at the cotyledon stage to study stunting. Other seedlings were inoculated at the unifoliolate-leaf stage. Plants were kept in a plastic bag for 4 days to maintain a humid environment. All studies were conducted in a growth chamber of 70 F with continuous fluorescent lighting at 400 ft-c.

Seedlings were removed and preserved at 24 hr intervals for 5 days after inoculation. Leaves from resistant and susceptible plants were preserved 7 days after inoculation. Lodged diseased seedlings were preserved also.

To determine the morphological nature of stunting, three-week-old inoculated and noninoculated seedlings were studied. Lengths of internodes were measured macroscopically. Lengths and numbers of epidermal cells of the internodes were determined

by microscopic examination.

Plant materials were preserved in formalin propionic acid (FPA) (13) for at least 24 hr. Then the materials were dehydrated by a tertiary butyl alcohol (TBA) series. A series of six paraffin changes was used to infiltrate the tissue. After blocked in paraffin, the tissue was cut 12 μ thick with a microtome and the ribbon affixed to a slide by Haupts' adhesive (13). Safranin-fast green (13), thionin-orange G (8), safranin-fast green-orange G (4), and Harris' hematoxylin-safranin-fast green (8) staining series were used. The stained slides were transferred to xylene and then mounted in Permount.

RESULTS

Staining. Xylem cells, chloroplasts, and some diseased tissue stained red with safranin and parenchyma cell walls, cytoplasm and bacteria stained green with fast green. Bacteria stained brown with Harris' hematoxylin and orange with orange G. Bacteria stained purple and cell walls stained golden-brown with thionin. Safranin-fast green proved best for observation of host tissue and bacteria and therefore was used for most studies.

Invasion. Water-soaked lesions on leaves and cotyledons were noted 48 hr after inoculation and the inoculated seedlings were obviously smaller than noninoculated ones. Bacterial masses were evident in sections of upper and lower substomatal

chambers of cotyledons and leaves 36- and 48 hr after inoculation (Fig. 1A and 2A). Some sections showed bacteria intercellularly in a limited area of the mesophyll 48 hr after inoculation (Fig. 1B). Cells appeared intact at this stage of disease development. The bacteria were intracellular and many cells were completely destroyed 72 hr after inoculation (Fig. 1C and 3A). Thickened, red-stained walls were usually present within this diseased tissue but did not delimit infection.

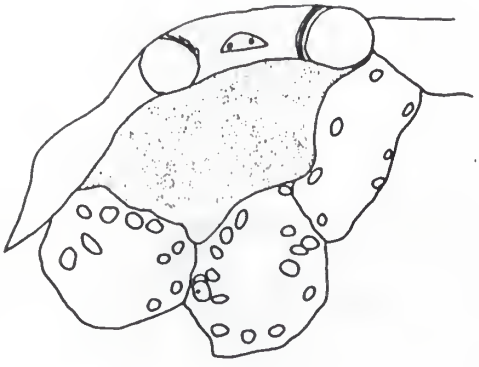
Stem (Fig. 4) sections of inoculated plants revealed bacteria in substomatal chambers, between cells, and occasionally within cells of the cortex.

Sections of infected shoot apical meristems from seedlings showed bacteria surrounding but not invading the meristematic tissue 48 hr after inoculation. But recently produced leaves had bacteria between and within cells. Bacteria were abundant between meristematic cells and had destroyed many of the cells of the young leaves by 96 hr after inoculation (Fig. 5A). Meristematic cells of seedlings that had not developed any leaves for 14 days after inoculation were macerated (Fig. 5B).

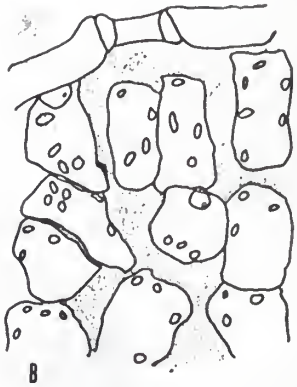
Hypocotyls of lodged diseased seedlings had bacteria in substomatal chambers, in intercellular spaces, and in destroyed cortex cells (Fig. 6). Bacteria were not seen in vascular tissue.

Stunting. Three-week-old inoculated and noninoculated plants were in the third-trifoliolate-leaf stage of growth

Fig. 1. Sections of alfalfa leaves infected with Xanthomonas alfalfae (drawn with aid of camera lucida). A) Accumulation of bacteria in substomatal chamber 36 hr after inoculation. B) Intercellular invasion 48 hr after inoculation. C) Cell destruction 72 hr after inoculation. D) Deposition of debris and delimitation of infection during necrotic stage typical of resistant plants.



A



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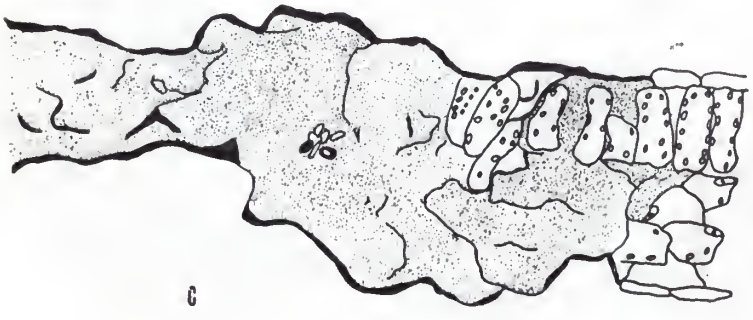
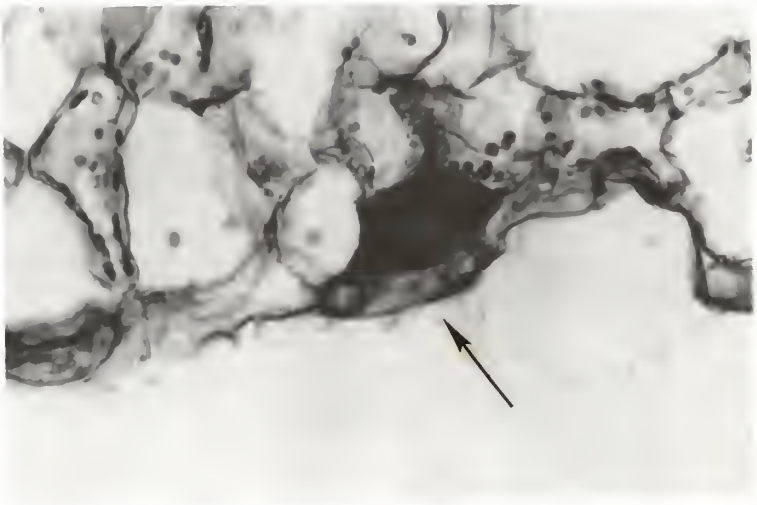


C

D

Fig. 2. Photomicrographs of sections of alfalfa cotyledons infected with Xanthomonas alfalfae. A) 36 hr after cotyledon was inoculated, showing bacterial mass in substomatal chamber and absence of bacteria between adjacent mesophyll cells. B) 48 hr after cotyledon was inoculated, showing bacteria between and within mesophyll cells.



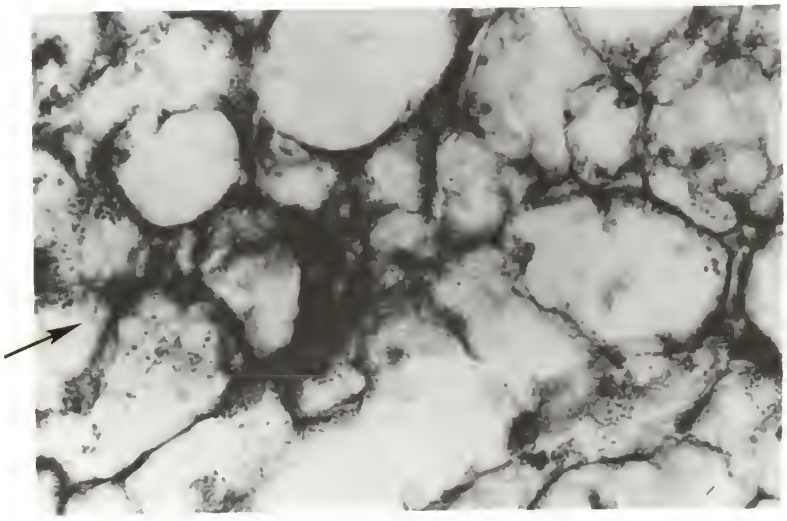


Fig. 3. Photomicrographs of sections of alfalfa leaves infected with Xanthomonas alfalfae in late stages of disease development. A) Water-soaked leaf spot, showing high populations of bacteria in destroyed cells. B) Necrotic leaf spot, showing debris deposition (arrow), low bacterial populations, and delimitation of infection typical of more resistant plants.

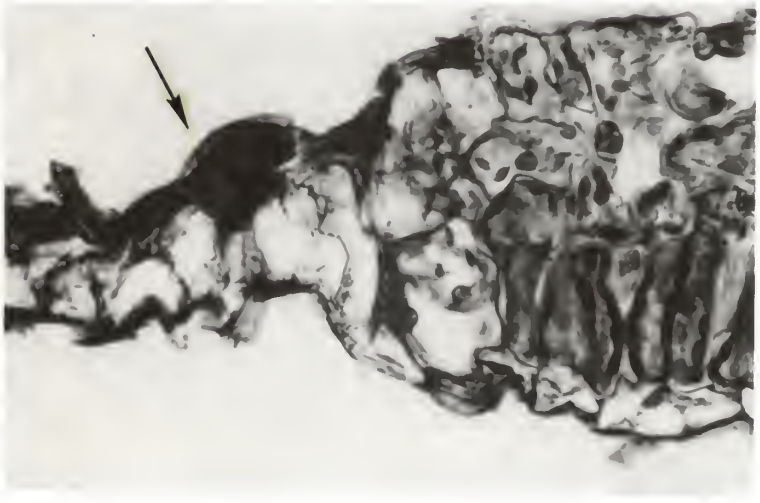


Fig. 4. Section of alfalfa stem 72 hr after inoculation with Xanthomonas alfalfae, showing bacteria inter- and intracellularly in the cortex (drawn with aid of camera lucida).

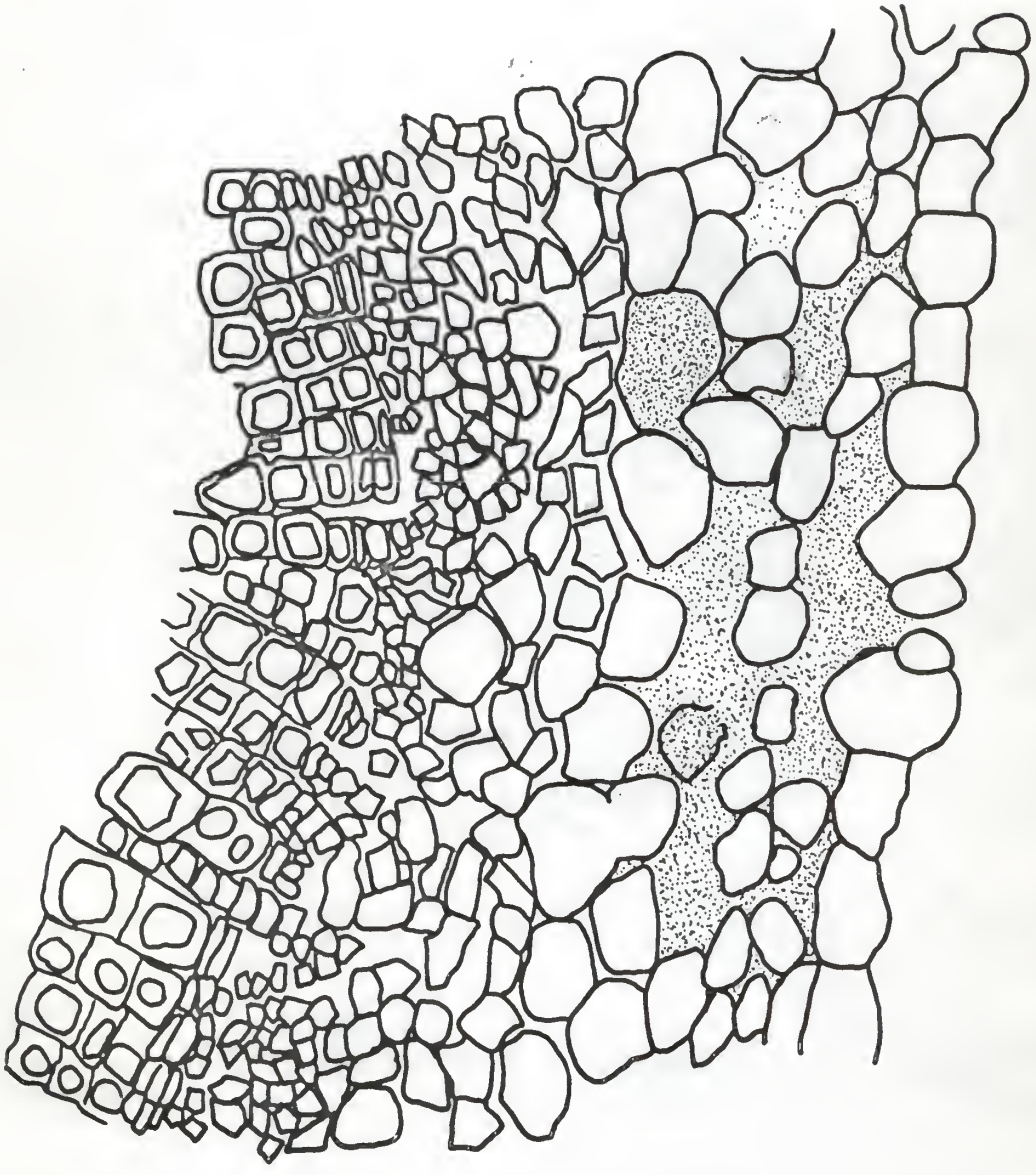
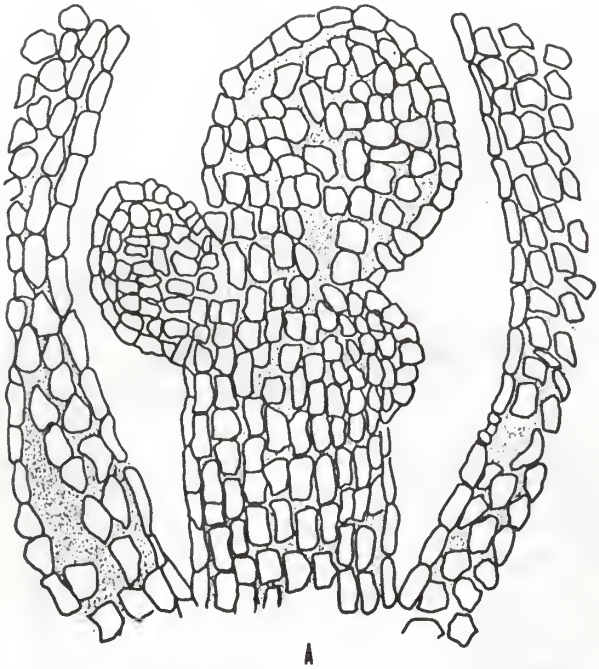
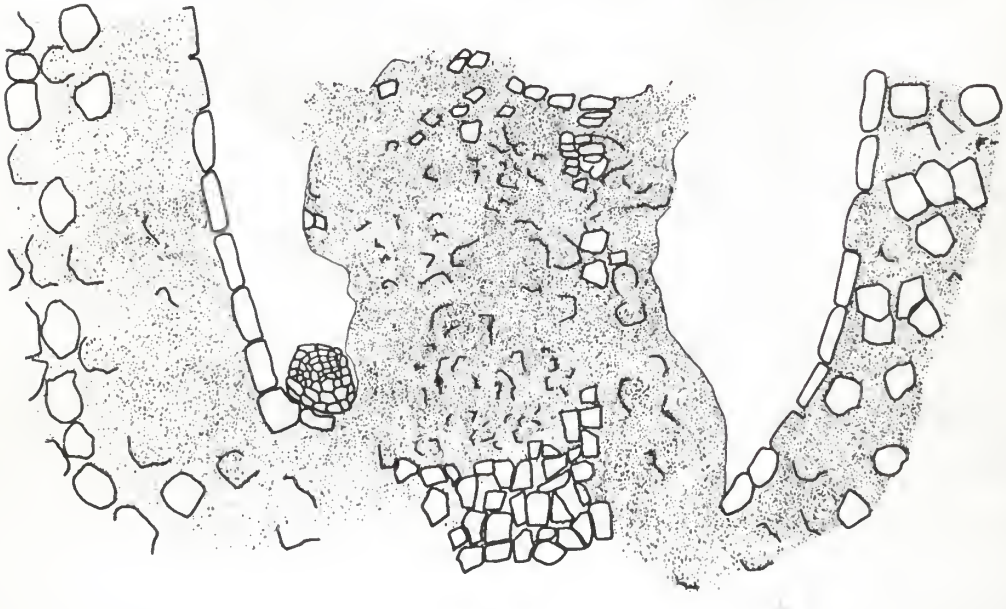


Fig. 5. Sections of apical meristem of shoot of alfalfa seedling infected by Xanthomonas alfalfae (drawn with aid of camera lucida). A) 4 days after inoculation, showing bacterial masses between cells. B) 14 days after inoculation, showing meristematic tissue destroyed by X. alfalfae.

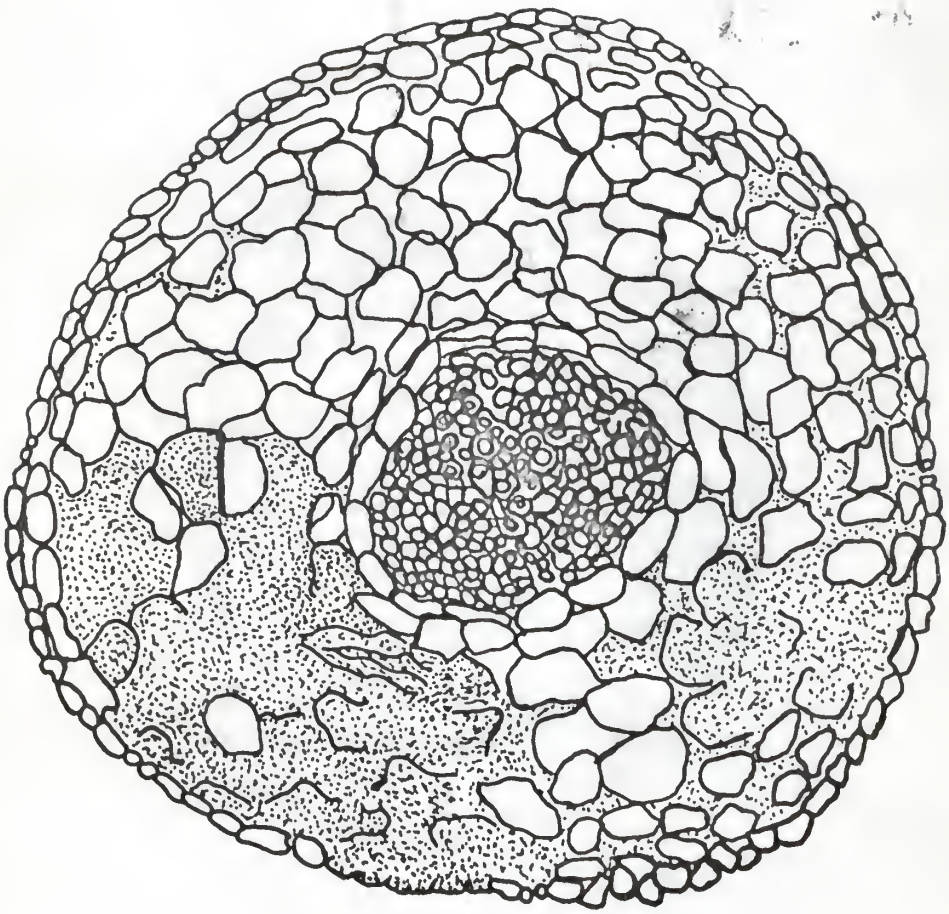


A



B

Fig. 6. Section of hypocotyl of damped-off alfalfa seedling, showing invasion of cortex by Xanthomonas alfalfae (drawn with aid of camera lucida).



but inoculated plants were only 2-3 cm tall whereas the control plants were 7-12 cm tall. The leaves of stunted plants were smaller than those of the control plants. The average length of stem epidermal cells per internode in the stunted plants was approximately one-half the length of the epidermal cells of control plants. In the 2nd and 3rd internode the number of epidermal cells was approximately one-third of those in the control plants. But in the first internode there was no consistent difference between the stunted and control plants in number of epidermal cells.

Resistance. Some thick, red-stained material was found in lesions of susceptible (KS12X25) and resistant (KS12X35) plants. There was more of this material in diseased tissue of resistant plants. Greater populations of bacteria were observed in resistant plants. Cells along outer edges of necrotic lesions, especially in resistant plants, appeared to be destroyed but no bacteria were observed within them (Fig. 1D and 3B).

DISCUSSION

The presence of bacterial masses in the substomatal chambers often without bacteria in surrounding tissue, strongly suggests that penetration occurs through stomata. This common means of penetration by bacteria (3,15,19) occurred in the leaves, stems, cotyledons and hypocotyls. Therefore the splashing of rain drops in the field could

spread the bacteria to the plant parts for penetration through the stomata.

The bacterial population appears to increase in the substomatal chamber. When the population reaches a certain level the bacteria migrate in all directions between the cells. Although the cells are not tightly packed in the leaf, the bacteria are probably breaking through the middle lamella.

The next stage of disease development appears to be the destruction of the cell wall and maceration of the cells. This destruction of cells probably results in the water-soaked spots on the host.

Finally the bacterial population appears to decrease greatly and the mesophyll collapses. This is the necrotic stage of disease development. The thickened, red-stained materials found in the collapsed mesophyll could be interpretted as cell wall remnants that have been pushed together with other debris. Since lignin is found in secondary cell walls, and is more difficult to digest than cellulose, perhaps the lignin from the collapsing cells is combined. Lignin stains red in safranin (13) so this explanation is plausible. Yet it is possible that the host cells are lignified as a response to the infection. The argument is confused by the presence of bacteria observed in some of the red areas. Outer edges of a lesion with high bacterial populations were characterized by collapsing cells. Perhaps this rapid collapsing and the collections of lignin restricted the bacteria. The

bacterial population would then reach a peak and decline due to competition in this restricted region or as a result of self-produced toxins.

It would follow that the difference between susceptible plants, in which water-soaked spots enlarge and coalesce, and resistant plants, in which the spots remain small and become necrotic, would be the amount of the cell collapse and lignification. But, this host reaction did not always succeed in confining the bacteria, even in resistant plants, suggesting that it is not, at least, the only factor involved in resistance.

Damping-off is evidently caused by the destruction of cortical cells in the infected hypocotyls. This damage plus the destruction of meristematic tissues could account for the large number of dead seedlings in soil infested with X. alfalfae as reported by Stuteville and Sorensen (14).

Stunting is associated with a lack of cell elongation and cell division in infected plants. Since cell elongation and cell division are dependent upon the presence of auxins, the bacteria probably interfer with the auxin balance in the host plant.

SUMMARY

This study revealed penetration through stomata, inter- and intracellular invasion and cell destruction in leaves, stems, cotyledons, hypocotyls and shoot apical meristems of

alfalfa by X. alfalfae. The amount of deposition of cell debris in lesions was more prominant in alfalfa plants resistant to X. alfalfae. Destruction of hypocotyl cortex tissue by the bacteria caused damping-off of seedlings. A reduction of cell elongation and cell division accounted for stunting in infected seedlings.

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LITERATURE CITED

1. Brigham, Raymond D. 1956. Bacterial leaf spot of alfalfa in Iowa. Plant Dis. Reptr. 40:830.

2. Brigham Raymond D. 1957. Stem lesions associated with Xanthomonas alfalfae. Phytopathology 47:309-310.

3. Bugbee, W. M. and N. A. Anderson. 1963. Whitefly transmission of Xanthomonas pelargonii and histological examination of leafspots of Pelargonium hortorum. Phytopathology 53:177-178.

4. Conn, H. J. 1953. Biological stains 6th ed. Commission on Standardization of Biological Stains. Geneva, N. Y.

5. Creager, D. B. and E. P. Matherly. 1962. Bacterial blight of poinsettia: histopathological studies. Phytopathology 52:103-110.

6. Dowson, W. J. 1943. On the generic names Pseudomonas, Xanthomonas, and Bacterium for certain bacterial plant pathogens. Trans. Brit. Mycol. Soc. 26:4-14.

7. Dye, D. W. 1958. Host specificity of Xanthomonas. Nature 182:1813-1814.

8. Johansen, D. A. 1940. Plant microtechnique. McGraw-Hill Book Co. New York 523 p.

9. Melchers, L. E., H. Fellows, D. B. Creager, and C. H. Ficke. 1940. Cereal and forage crop disease investigations. In Kansas Agr. Exp. Sta. Tenth Bien. Rept 1938-1940. pp. 105-106.

10. Nelson, P. E. and R. S. Dickey. 1966. Pseudomonas caryophylli in carnation. II. Histological studies of infected plants. Phytopathology 56:154-163.

11. Patel, M. K., Y. S. Kulkarni, and G. W. Dhande. 1949. Bacterial leaf spot of lucerne. Indian Phytopath. 2:166-167.

12. Riker, A. J., F. R. Jones, and Marguerite C. Davis. 1935. Bacterial leaf spot of alfalfa. J. Agr. Res. 51:177-182.

13. Sass, J. E. 1958. Botanical Microtechnique. Iowa State University Press, Ames, Iowa.

14. Stuteville, D. L. and E. L. Sorensen. 1966. Distribution of leaf spot and damping-off (Xanthomonas alfalfae) of alfalfa in Kansas, and new hosts. Plant Dis. Reptr. 50:731-734.

15. Thiers, H. D. and L. M. Blank. 1951. A histological study of bacterial blight of cotton. Phytopathology 41:499-510.

16. United States Department of Agriculture. 1952. Index of Plant Diseases in the United States. Part IV. The Plant Dis. Survey.

17. United States Department of Agriculture. 1960. Index of Plant Diseases in the United States. Agriculture Handbook No. 165.

18. Williams, P. H. and N. T. Keen. 1967. Histology of infection by Pseudomonas lachrymans. Phytopathology 57:254-256.

19. Zaumeyer, W. J. 1932. Comparative pathological histology of three bacterial diseases of bean. J. Agr. Res. 44:605-632.

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ABSTRACT

Bacterial leaf spot of alfalfa (Medicago sativa L.), incited by Xanthomonas alfalfae (Riker, Jones, and Davis) Dows., results in leaf and stem spotting, defoliolation, and seedling stunting and damping-off.

The objectives of this study were to find the mechanisms of penetration and invasion of alfalfa by X. alfalfae, the morphological nature of resistance to the bacterium, and the morphological damage caused by X. alfalfae within stunted and damped-off seedlings.

Plants were inoculated with X. alfalfae, kept in a humid environment at 70 F, preserved, sectioned, and stained. X. alfalfae penetrated through stomata, accumulated in substomatal chambers, then invaded inter- and intracellularly within hypocotyls, cotyledons, leaves, stems, and shoot apical meristems of 'Cody' alfalfa seedlings. No definite morphological barrier was observed but leaf spots in resistant plants contained more red-stained debris. This might indicate the presence of more lignin in resistant than susceptible plants. Destruction of hypocotyl cortex by the bacteria caused damping-off of seedlings. Reduction of cell elongation of cell division accounted for the stunting of diseased alfalfa seedlings.