

Histopathology of *Colletotrichum trifolii* on Alfalfa

Miguel D. M. Porto, Craig R. Grau, Gustaaf A. de Zoeten, and Gary Gaard

First author, associate professor of Fac. Agronomia-UFRGS, CP776, 90000 Porto Alegre, RS, Brazil; other authors, professors and research specialist, respectively, Department of Plant Pathology, University of Wisconsin, Madison 53706.

Research was supported by the Brazilian National Council for Scientific and Technological Development (CNPq) and the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

The authors express their gratitude to Martin Garment and Steve Vicen for their technical assistance with the scanning microscope and photo lab, respectively.

Accepted for publication 10 August 1987 (submitted for electronic processing).

ABSTRACT

Porto, M. D. M., Grau, C. R., de Zoeten, G. A., and Gaard, G. 1988. Histopathology of *Colletotrichum trifolii* on alfalfa. *Phytopathology* 78:345-349.

The invasion of alfalfa stem tissue by *Colletotrichum trifolii* was studied using light, electron transmission, and scanning electron microscopy. Conidia were found in depressions on the stem. Appressoria were formed in these depressions but were not associated with stomata. Penetration was accomplished by appressoria and also by hyphal strands without the formation of appressoria. Some epidermal cells were killed before penetration. Chloroplasts were highly sensitive and were usually destroyed in advance of the hyphae. Prior to penetration, epidermal cells showed

pronounced swelling, and some were killed. After penetration, the fungus progressed inter- and intracellularly towards the xylem vessels and, in 4 to 6 days, destroyed the pith and endodermis. Acervuli were produced 5 to 8 days after inoculation. Genotypic differences in alfalfa did not affect spore germination and the production of appressoria, but marked differences were observed in the number and distribution of acervuli associated with diseased stem tissues.

Anthracnose of alfalfa (*Medicago sativa* L.), caused by *Colletotrichum trifolii* Bain & Essary, is a highly destructive disease of stem and crown tissues in many regions of the world (12,19). The stem disease phase can result in yield and quality reductions (2,5,7), and the crown phase can cause a reduction in the number of stems per plant and plant mortality, which leads to depletion of alfalfa stands (2,14). Past research on anthracnose of alfalfa dealt with its agronomic impact (2,5,7,14), etiology, epidemiology (28), pathogen variability (20,27), physiology of *C. trifolii* (11,18), and host resistance to *C. trifolii* (5,8,9,27). Although the anatomy and morphology of alfalfa are well known (13), few studies have been directed at histological aspects of the interaction between alfalfa and *C. trifolii* (4).

Sampson (23) reported the importance of appressoria for the penetration of red clover (*Trifolium pratense* L.) by *C. trifolii*, but the literature is incomplete in regard to the infection of alfalfa by this fungus. Thus, the objective of this research is to provide information on the penetration and colonization of alfalfa stem tissues by *C. trifolii*.

MATERIALS AND METHODS

Selection of plant material. In order to determine which plant material to use for our studies, we evaluated the degree of infection in stems of the cultivars Saranac, Saranac AR, and Arc and the clones T19 (derived from the cultivar Trumpetor) and W38 (derived from the cultivar WL-316), using the following scale: 0 = no symptoms; 1 = acervuli only on dead stipules; 2 = acervuli on green stipules or leaves; 3 = acervuli on one or both ends of the stem segment; 4 = acervuli on the internode, away from the cut ends. Plants were maintained in a growth chamber, with a day length of 14 hr, at 21 C during the day and 16 C during the night. The cultivar Saranac was observed to be most susceptible to *C. trifolii* and was used in this study.

Isolate of *C. trifolii*. The isolate of *C. trifolii* used in this study was recovered from a stem lesion of an alfalfa plant collected at the Arlington Experimental Station of the University of Wisconsin. The isolate was grown on acidified potato-dextrose agar, pH 5.5, and maintained in an incubator at 24 C. The isolate was deter-

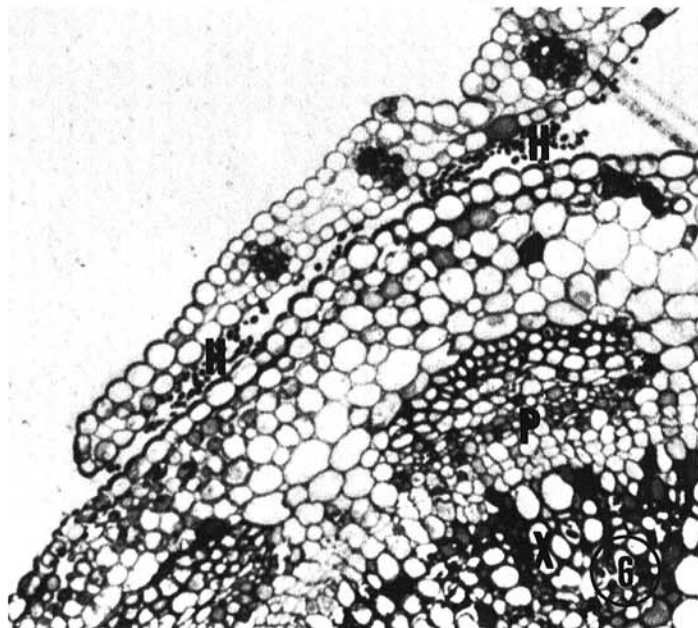
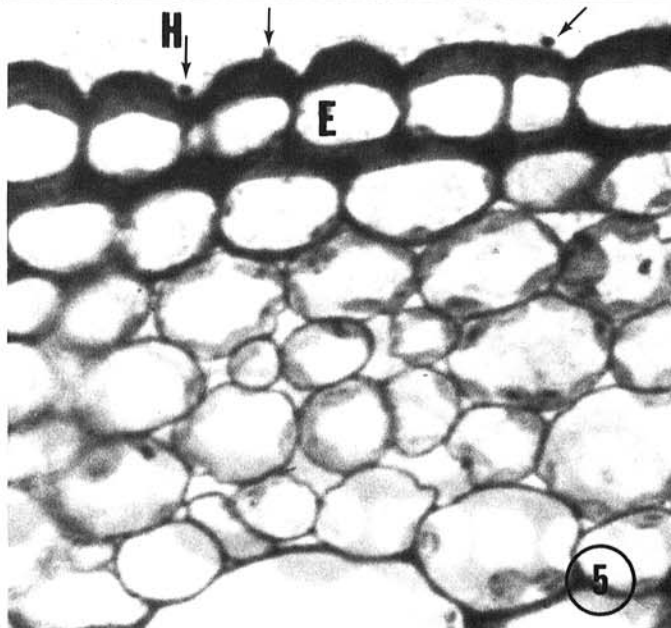
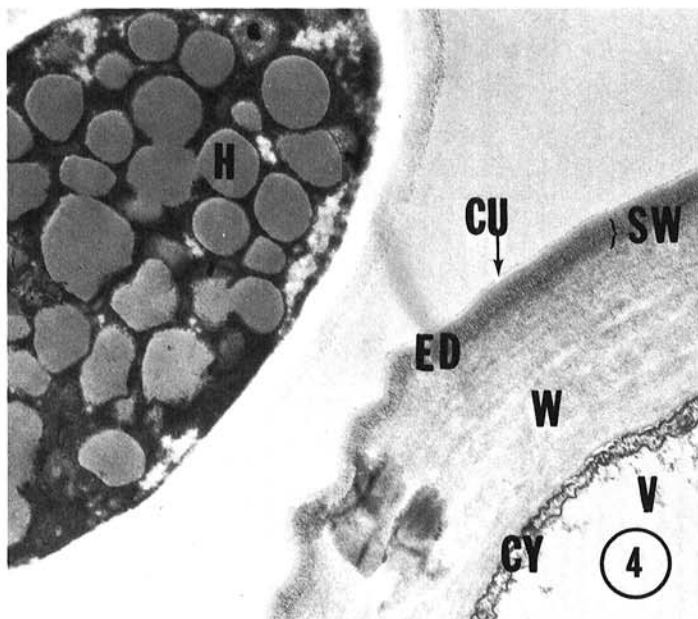
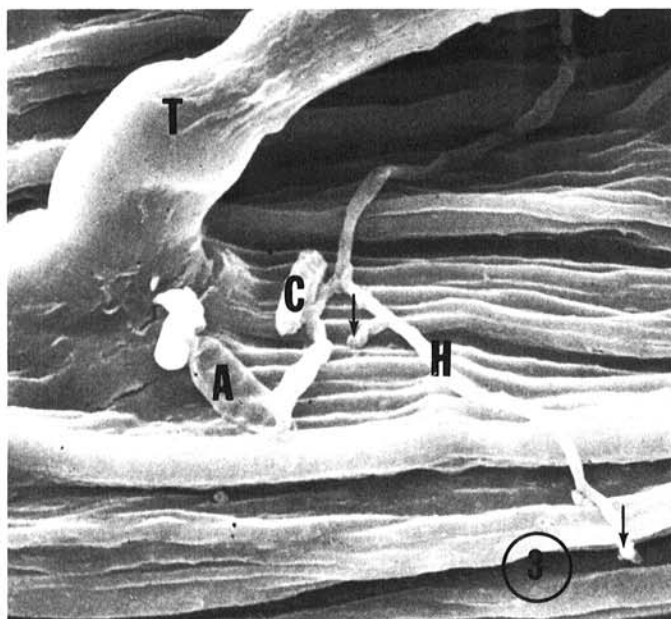
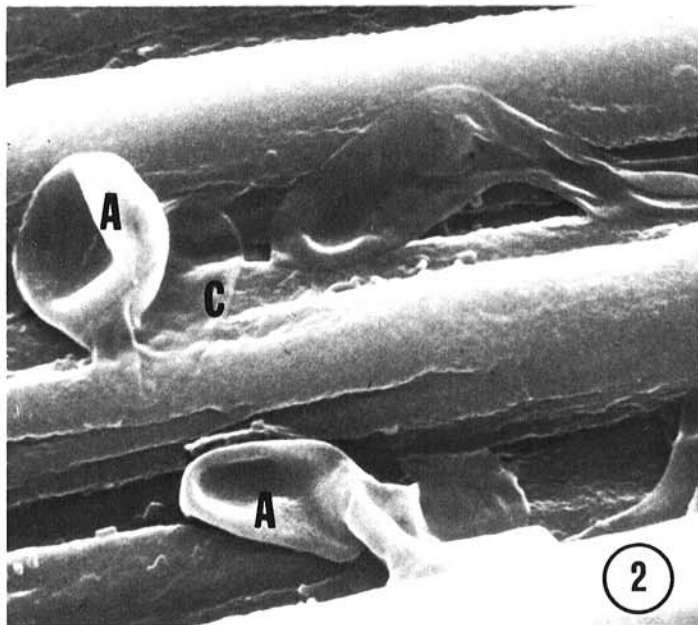
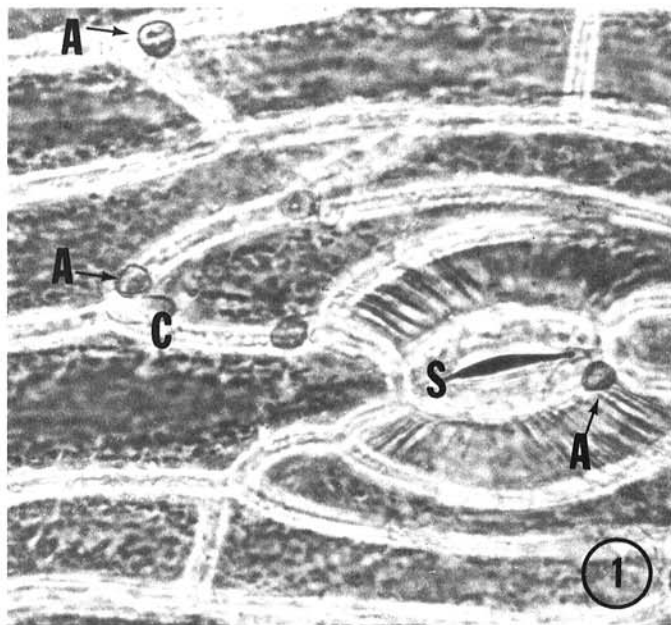
mined to be race I by the standard set of differential cultivars (19,27).

Inoculation procedures. Stems with six to eight internodes were collected and prepared for inoculation as follows. The upper internode and all trifoliate leaves were removed aseptically and discarded. Each stem was cut into pieces in such a way as to have a node in each segment. Then five to seven such stem segments were placed in a petri plate lined with moist filter paper.

Stem inoculation was performed with an atomizer held 15 cm above the stems. A spore suspension of 1.5×10^6 conidia per milliliter, obtained from 10-day-old cultures, was atomized over the stem segments. Plates were sealed with Parafilm (American Can Co., Greenwich, CT) and incubated at room temperature (about 22 C). Microcultures were established on microscope slides coated with potato-dextrose agar, sprayed with a suspension of 1.5×10^6 spores per milliliter, placed in petri dishes sealed with Parafilm, and incubated in the lab.

Scanning electron microscopy. Alfalfa stems of the cultivar Saranac were prepared for scanning electron microscopy (SEM) at different times after inoculation (6, 12, 24, 48, 72, 96, and 120 hr). Small segments (5 mm long) were vacuum-infiltrated with 5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4, and the solution was changed 12 hr later. The samples were rinsed, 24 hr after vacuum infiltration, with 1.5% sucrose in 0.08 M cacodylate buffer, pH 7.4. The samples were kept in fresh sucrose solution for 24 hr. Following this treatment, the material was slowly dehydrated in a graded ethanol series. After the last change of ethanol, the samples were immediately submitted to critical-point drying, coated with gold, and examined with a JEOL-JSM/U3 scanning microscope.

Transmission electron microscopy. For transmission electron microscopy (TEM), small segments (5 mm long) of Saranac alfalfa stems, taken at 12, 24, 48, 72, 96, 120, and 144 hr after inoculation, were treated with glutaraldehyde and sucrose in the same way described for the SEM. Following the treatment with sucrose, the samples were treated with 2% osmium tetroxide in phosphate buffer, where they remained overnight at 4 C, and then dehydrated in an acetone series (30, 50, and 70%, for 30 min in each) at room temperature. After the last change of acetone, the samples were treated for 24 hr at 4 C with a saturated solution of uranyl acetate in 70% acetone. All samples were thoroughly rinsed in 70% acetone, and the dehydration was continued in 90, 100, 100, 100, and 100% acetone. Once the dehydration was completed, the stem segments



were infiltrated, under vacuum, with increasing concentrations of epoxy: epoxy and 100% acetone (1:2) for 1 hr, epoxy and 100% acetone (2:1) for 1.5 hr, and 100% epoxy for 1 hr. The epoxy-infiltrated samples were poured into plastic containers (also with epoxy) and placed in a vacuum oven at 60 C for 36 hr.

Embedded tissue was sectioned with a diamond knife on a Reichert OMU3 ultramicrotome. The ultrathin sections were stained with Reynolds lead citrate (22) and examined with a JEM7 at 80 kV.

Light microscopy. Three types of specimens were examined with a Universal light microscope. First, thin sections were prepared from embedded tissue (as described for TEM), which was sectioned with glass knives on a Reichert OMU3 ultramicrotome. The sections (0.7–1.0 μm thick) were stained with 1% toluidine blue in 1% sodium borate solution (16) and examined under bright field. Second, microcultures were examined under phase contrast, 12, 24, and 48 hr after inoculation. Third, epidermal strips from inoculated alfalfa stems were taken 12, 24, and 48 hr after inoculation and examined under bright field. Some strips were kept in a moist chamber and reexamined on the eighth day after inoculation.

RESULTS AND DISCUSSION

Germination of spores and formation of appressoria. The rate and final number of spores that germinated on stems did not differ among the cultivars and clones, regardless of their phenotypic differences for resistance to *C. trifolii*. After 24 hr of incubation, six to eight germinated spores per square millimeter were observed on the stem surface of all cultivars and clones. More spores germinated in the microcultures than on inoculated stems.

In general, spore germination on epidermal strips was lateral, with very short germ tubes. Appressoria that formed on the junctions of the epidermal cells (Fig. 1) were dark, round structures with a diameter similar to the width of the spore. The pattern of spore germination, shape, color, and position of the appressoria agree with what has been described for *C. trifolii* (18,23), *C. orbiculare* (Berk. & Mont.) v. Arx (1), and the genus (26). No appressoria were formed on the stomatal openings, even when spores were on the guard cells. Germ tubes did not form appressoria in microcultures.

Stems examined with SEM showed some variation in relation to what was observed on epidermal strips. Spores were found in the depressions of the stem, and the appressoria were formed in the same depressions. Not all germinated spores produced appressoria, and the germ tubes were not always short. Inward folding of the appressoria and spore walls was common (Fig. 2). Such deformation may represent the collapse of these structures as a normal phase of the process, or they may have been damaged by the preparative procedures used for SEM. Observations with TEM also showed the presence of appressoria on the junction of two epidermal cells. Hyphopodia, as defined by Emmett and Parbery (10), were not observed.

Penetration. Isolations from the inoculated alfalfa stems revealed that *C. trifolii* penetrated the host tissue between 12 and 24 hr after inoculation. The time of penetration varies according to the host-parasite interaction involved. For instance, *Peronospora tabacina* Adam penetrates tobacco leaves 1.5 to 2.5 hr after inoculation (17), whereas *C. gloeosporioides* (Penz.) Sacc. forms infection pegs on papaya fruit 3 to 4 days after inoculation (3).

Some reports indicate that spore germination and appressorial formation are prerequisites for successful invasion of alfalfa tissues by *C. trifolii* (18). We observed by SEM that penetration was apparently also accomplished by hyphal strands, without the formation of appressoria (Fig. 3). Therefore, our results lead to the conclusion that appressoria are not always essential for invasion. Chau and Alvarez (3) reported that *C. gloeosporioides* was capable of penetrating papaya fruit by way of germ tubes or appressoria. In our study, penetration of alfalfa stems by *C. trifolii* was preceded by swellings of the epidermal cell wall (Fig. 4), disruption of the cuticle, and rupture of the cell wall. The swelling of the epidermal cell wall prior to penetration has been reported for other host-parasite interactions (1), and there are also reports that this deformation occurs after penetration (15). Swollen cell walls appear to be due to the action of pectic (15) or cellulolytic enzymes (24). Swollen cell walls are observed commonly in many different types of invaded tissues (15). In the present research, however, swollen cell walls were restricted to the epidermal layer.

Rupture of the cuticle has been attributed to the pressures exerted by swollen cell walls (16) and also by enzymatic degradation or physical pressure exerted by expanding hypha (15,21). For the interaction between *C. trifolii* and alfalfa, the disruption of the cuticle was characterized by its separation from the cell wall at sites adjacent to a hypha or spore. Occasionally some epidermal cell walls also showed discoloration, which could be due to enzymatic dissolution. Penetration through the glandular trichomes was also observed.

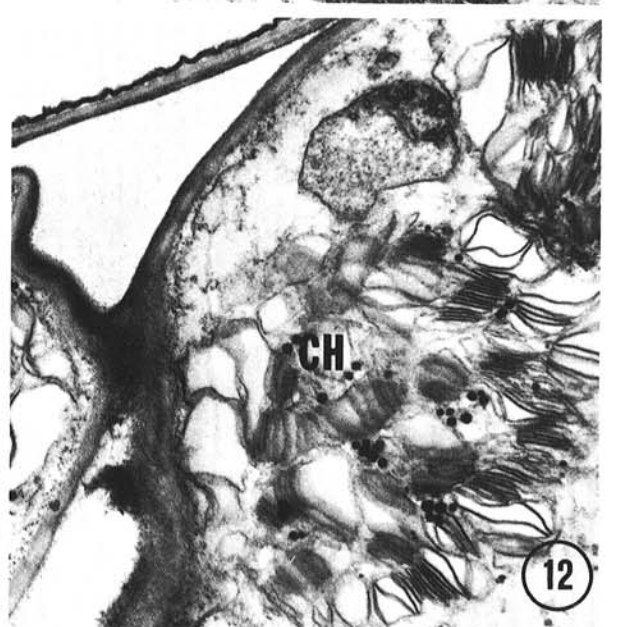
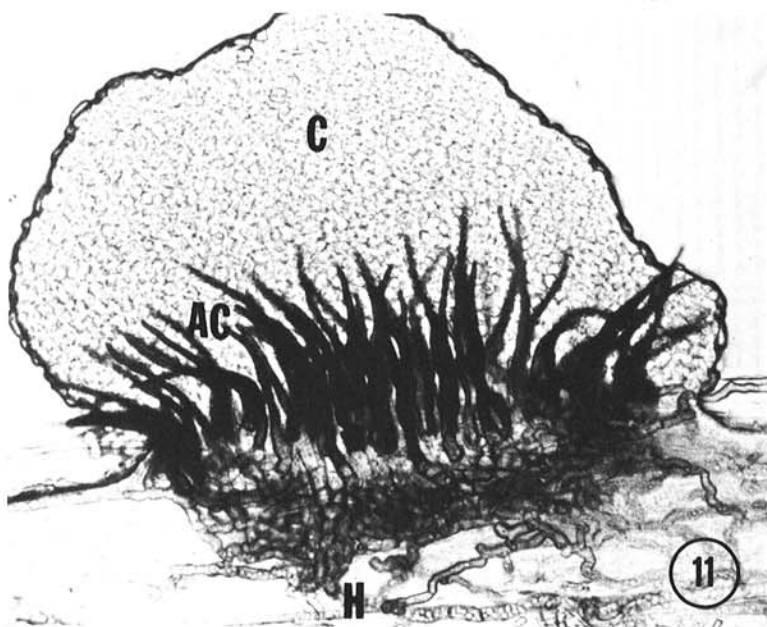
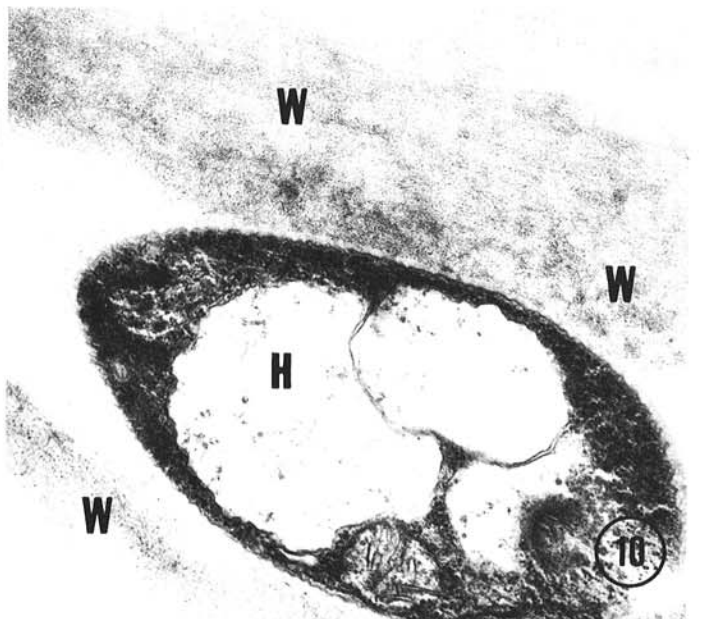
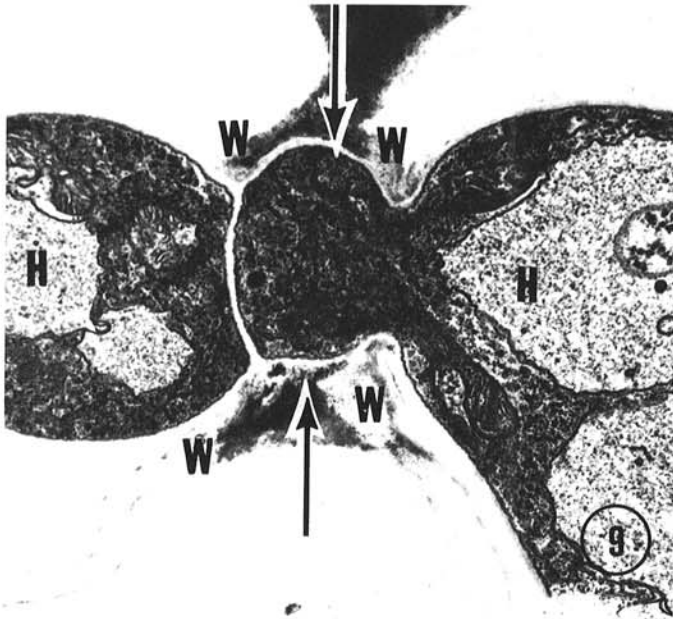
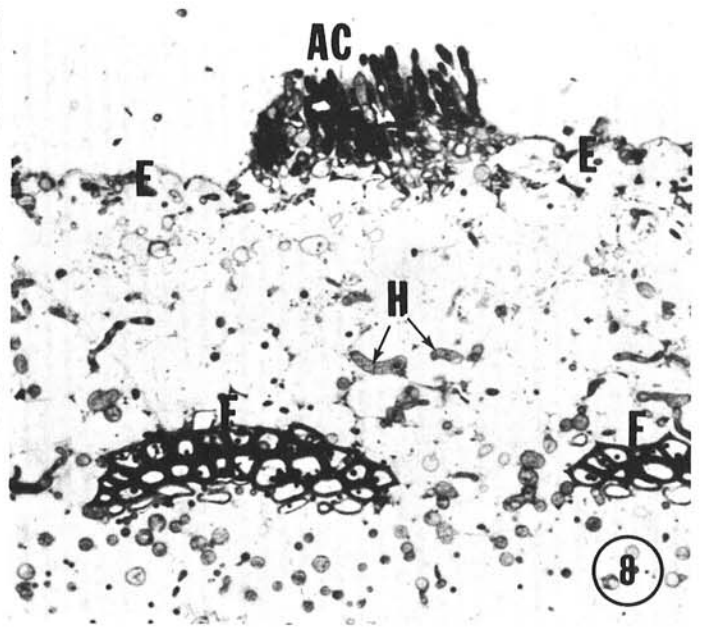
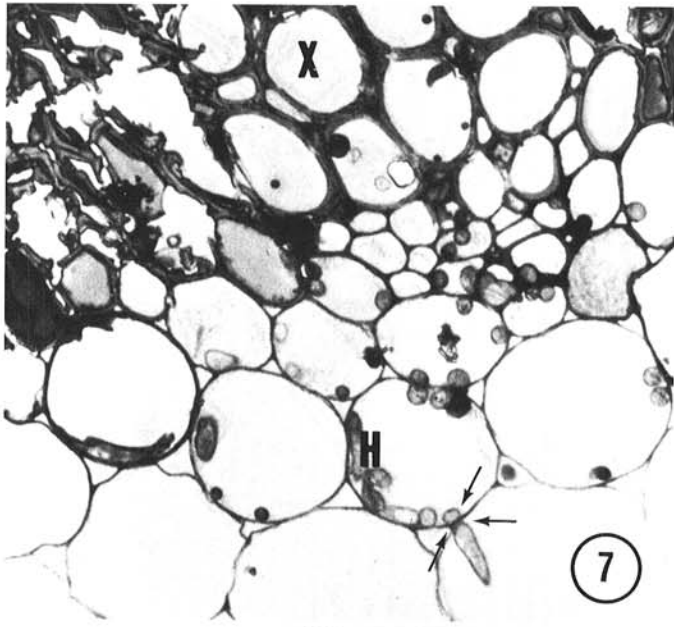
Development of the infection process. A series of observations made by light microscopy provided some information on the sequence of events involved in the development of *C. trifolii* in the stem tissues of alfalfa. Fungal structures were detected on the junctions of epidermal cells 24 hr after inoculation (Fig. 5). Extensive mycelial growth was observed on the outer surface of the host 48 hr after inoculation (Fig. 6), and at the same time some xylem vessels already were invaded. The fungus progressed to the pith (Fig. 7), and some hyphal growth was detected in the endodermal tissues 96 hr after inoculation (13). Pith and endodermal tissues (13) were completely destroyed after 120 hr, and the presence of acervuli was pronounced 144 hr after inoculation (Fig. 8).

This sequence was also studied with TEM. After penetration, the fungus progressed toward the vascular system, in a manner similar to that described for watermelon anthracnose (1). Intra- and intercellular hyphal growth was observed. The passage of *C. trifolii* from cell to cell occurred at any cell layer, and generally there was a constriction of the hypha at the point of passage (Figs. 7 and 9). The formation of septa also was common at these points.

Once the fungus reached the endoderm, destruction of the epidermal layer became evident, and it was common to observe hyphal growth within the epidermal cell wall (Fig. 10). The presence of hyphae within the epidermal cell wall was also reported in other host-parasite interactions, but its occurrence was detected soon after penetration (6). Pronounced concentrations of hyphal strands occurred just below the epidermal layer. New acervuli arose from these hyphal concentrations. Epidermal cell walls around the acervuli were sometimes destroyed, but in other cases they remained intact. A large number of spores immersed in a gelatinous matrix were produced in each acervulus (Fig. 11). The number of acervuli was greatest on stems of Saranac and clone



Figs. 1–6. Early stages of infection of alfalfa by *Colletotrichum trifolii*. **1**, Germinating conidia (C) of *C. trifolii* produce appressoria (A) mainly on cell walls, as seen by light microscopy. Note that the stomates (S) are not preferential pathways for fungal entrance to the host. ($\times 800$) **2**, Appressoria (A) of *C. trifolii*, as seen by scanning electron microscopy. The collapse of the structures may be caused by preparatory manipulation or be related to the age of the appressoria. C = conidia. ($\times 4,800$) **3**, A germinated conidium (C), appressorium (A), and hyphae (H) of *C. trifolii*, as seen by scanning electron microscopy. Note the apparent penetration by hyphae (arrows). T = trichome. ($\times 2,600$) **4**, Transmission electron micrograph of the host-pathogen interphase during early penetration. Note the cuticle (CU) and the layer of enzymatic degradation (ED) directly under the cuticle in the cell wall (W) resulting in swelling (SW). At this early state of ingress the integrity of the cytoplasm (CY) of the cell has not been affected yet. H = hypha; V = vacuole. ($\times 21,000$) **5**, Light micrograph showing hyphae (H, arrows) associated with junctions of epidermal cells (E), 24 hr after inoculation. ($\times 300$) **6**, Extensive growth of hyphae (H) on the surface of the stem, 48 hr after inoculation. P = phloem; X = xylem. ($\times 750$)



Figs. 7-12. Late stages of infection of alfalfa by *Colletotrichum trifolii*. **7**, Light micrograph (transverse section) of the pith area of the stem, showing invasion of the fungus progressing deep into the tissue, 96 hr after inoculation. Note the constrictions (arrows) of hyphae (H) where the fungus apparently passes from cell to cell. X = xylem. ($\times 1,050$) **8**, Light micrograph of infected alfalfa stem tissue, 144 hr after inoculation. Note the complete obliteration of cellular detail except in the area of the fibers (F). AC = acervulus; E = epidermis; H = hypha. ($\times 1,050$) **9**, Transmission electron micrograph of a *C. trifolii* hypha (H) passing through a cell wall (W). Note the hyphal constriction (arrows). ($\times 15,000$) **10**, Transmission electron micrograph of a hypha (H) growing within an epidermal cell wall (W). ($\times 28,000$) **11**, Light micrograph of a mature acervulus (AC). Note the containment of the conidia (C) in the gelatinous matrix produced by the fungus. H = hyphae. ($\times 1,500$) **12**, Transmission electron micrograph in the proximity of invading hyphae, showing the cytopathological breakdown of the cellular content, specifically the chloroplast (CH). ($\times 18,000$)

T19, compared to the cultivars Arc and Saranac AR and the clone W38.

Cytopathology. Two cytopathological effects were observed. Some epidermal cells, which showed deformation of the walls, were killed before any penetration had taken place. Second, breakdown of the stem tissue, mainly the endodermal layer, appeared to have started by the separation of the cells along the middle lamellae. In concurrence with descriptions of watermelon anthracnose (1), chloroplasts were highly sensitive and usually broke down in advance of the hyphae (Fig. 12). Such alterations in chloroplast structure are commonly observed in infected tissues (25).

LITERATURE CITED

- Anderson, J. L., and Walker, J. C. 1962. Histology of watermelon anthracnose. *Phytopathology* 52:650-653.
- Barnes, D. K., Ostazeski, S. A., Schillinger, J. A., and Hanson, C. H. 1969. Effect of anthracnose (*Colletotrichum trifolii*) infection on yield, stand, and vigor of alfalfa. *Crop Sci.* 9:344-346.
- Chau, K. F., and Alvarez, A. M. 1983. A histological study of anthracnose on *Carica papaya*. *Phytopathology* 73:1113-1116.
- Churchill, A. C. L., Baker, C. J., O'Neill, N. R., and Elgin, J. H., Jr. 1986. Histopathology of *Colletotrichum trifolii* on alfalfa. (Abstr.) *Phytopathology* 76:1124.
- Devine, T. E., and McMurtrey, J. E., III. 1975. Performance of anthracnose-resistant alfalfa strains. *Crop Sci.* 15:505-508.
- Ehrlich, M. A., and Ehrlich, H. G. 1971. Fine structure of the host-parasite interfaces in mycoparasitism. *Annu. Rev. Phytopathol.* 9:155-184.
- Elgin, J. H., and Barnes, D. K. 1981. Anthracnose resistance increases alfalfa yields. *Crop Sci.* 21:457-460.
- Elgin, J. H., and Ostazeski, S. A. 1982. Evaluation of selected alfalfa cultivars and related *Medicago* species for resistance to race 1 and race 2 anthracnose. *Crop Sci.* 22:39-42.
- Elgin, J. H., Jr., and Ostazeski, S. A. 1985. Inheritance of resistance to race 1 and race 2 anthracnose in Arc and Saranac AR alfalfa. *Crop Sci.* 25:861-865.
- Emmett, R. W., and Parbery, D. G. 1975. Appressoria. *Annu. Rev. Phytopathol.* 13:147-167.
- Frantzen, K. A., Johnson, L. B., and Stuteville, D. L. 1982. Partial characterization of phytotoxic polysaccharides produced in vitro by *Colletotrichum trifolii*. *Phytopathology* 72:568-573.
- Graham, J. H., Stuteville, D. L., Frosheiser, F. I., and Erwin, D. C. 1979. A Compendium of Alfalfa Diseases. American Phytopathological Society, St. Paul, MN. 65 pp.
- Grove, A. A., Jr., and Carlson, G. E. 1972. Morphology and anatomy. Pages 103-122 in: *Alfalfa: Science and Technology*. C. H. Hanson, ed. American Society of Agronomy, Madison, WI.
- Jones, E. R., Carroll, R. B., Swain, R. H., and Bell, K. W. 1978. Role of anthracnose in stand thinning of alfalfa in Delaware. *Agron. J.* 70:351-353.
- Mansfield, J. W., and Richardson, A. 1981. The ultrastructure of interactions between *Botrytis* species and broad bean leaves. *Physiol. Plant Pathol.* 19:41-48.
- McKeen, W. E. 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. *Phytopathology* 64:461-467.
- McKeen, W. E., and Svircev, A. M. 1981. Early development of *Peronospora tabacina* in the *Nicotiana tabacum* leaf. *Can. J. Plant Pathol.* 3:145-158.
- Miehle, B. R., and Lukezic, F. L. 1972. Studies on conidial germination and appressorium formation by *Colletotrichum trifolii* Bain & Essary. *Can. J. Microbiol.* 18:1263-1269.
- Ostazeski, S. A., Avendano, R. E., Rodriguez, J., and Fernandez, J. P. 1978. Reaction of Argentine and U.S. alfalfas to Argentine isolates of *Colletotrichum trifolii*. *Plant Dis. Rep.* 62:474-476.
- Ostazeski, S. A., Elgin, J. H., Jr., and McMurtrey, J. E., III. 1979. Occurrence of anthracnose on formerly anthracnose-resistant 'Arc' alfalfa. *Plant Dis. Rep.* 63:734-736.
- Pring, R. J., and Byrde, R. J. W. 1981. An ultrastructural study of the infection of pear fruit by *Monilinia fructigena*. *Physiol. Plant Pathol.* 19:1-6.
- Reynolds, E. G. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17:208-212.
- Sampson, K. 1929. Comparative studies of *Kabatiella caulivora* (Kirch.) Kara and *Colletotrichum trifolii* Bain and Essary, two fungi which cause red clover anthracnose. *Trans. Br. Mycol. Soc.* 13:103-142.
- Suzuki, K., Furusawa, I., Ishida, N., and Yamamoto, M. 1982. Chemical dissolution of cellulose membranes as a prerequisite for penetration from appressoria of *Colletotrichum lagenarium*. *J. Gen. Microbiol.* 128:1035-1039.
- Sziráki, I., Mustárdy, L. A., Faludi-Dániel, Á., and Király, Z. 1984. Alterations in chloroplast ultrastructure and chlorophyll content in rust-infected pinto beans at different stages of disease development. *Phytopathology* 74:77-84.
- von Arx, J. A. 1981. The Genera of Fungi Sporulating in Pure Culture. Strauss & Cramer, Vaduz, Liechtenstein.
- Welty, R. E., Gurgis, R. Y., and Rowe, D. E. 1982. Occurrence of race 2 of *Colletotrichum trifolii* in North Carolina and resistance of alfalfa cultivars and breeding lines to races 1 and 2. *Plant Dis.* 66:48-51.
- Welty, R. E., and Rawlings, J. O. 1985. Effects of inoculum concentration and temperature on anthracnose severity in alfalfa. *Phytopathology* 75:593-598.