A Histological Study of Infection of Host Tissue by *Sclerotium rolfsii*

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ABSTRACT

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Hyphae from germinating sclerotia of *Sclerotium rolfsii* ramified over various host tissues (sugarbeet leaves, bean hypocotyls, and carrot roots or petioles) within 24–48 hr following inoculation. Hyphae frequently coalesced to form mycelial aggregates that functioned as infection cushions. Crystals of calcium oxalate were associated with these aggregates and with individual hyphae growing over the host surface. Penetration occurred after death and collapse of cells beneath infection cushions and from appressoria that formed at the tips of individual hyphae. Penetration pegs (1–3 μm thick) formed below infection cushions and from appressoria. The cuticle was apparently not dissolved, but was pulled away from the epidermis, and subsequent subcuticular hyphal growth occurred both inter- and intracellularly. Hyphal growth was parallel to the longitudinal axis of host cells and to the tissue surface. Cell wall components that stained positive with ruthenium red and alizarin red S (pectic materials and calcium, respectively) were depleted from cell walls in advance of the mycelium. Cells distal to the hyphae were necrotic and stained positive with thionin. Crystals of calcium oxalate stained black with $AgNO_3$-dithiooxamide and were produced in abundance in necrotic tissues. Infection cushions produced by *S. rolfsii* appeared to facilitate infection of host tissue by secreting enzymes and oxalic acid that macerated and killed tissue in advance of fungal penetration.

Additional key words: calcium oxalate, cell wall degrading enzymes, infection cushion, pathogenesis.

Sclerotium rolfsii Sacc. is an economically important pathogen on numerous crops worldwide (3). Previous histopathological studies have reported that *S. rolfsii* penetrates host tissue by formation of appressoria (12,20), followed by apparent tissue necrosis in advance of the mycelium (20). *Rhizoctonia solani* Kühn (15), *R. tuliparum* Whetzel and Arthur (11), *Sclerotinia sclerotiorum* (Lib.) de Bary (16), and *Sclerotium cepivorum* Berk. (1) also cause cell death before penetration. Although "hyphal aggregates" have been reported to form during infection by *S. rolfsii* (12), the role of these aggregates in pathogenesis has not been determined, and tissue death in advance of mycelial growth has not been conclusively demonstrated.

During pathogenesis, *S. rolfsii* may produce cell wall degrading enzymes such as endo-polygalacturonase (endo-PG) (6,7), cellulase (5), and polymethylgalacturonase (24) in conjunction with oxalic acid (7,24). Cutinolytic enzymes also have been induced in vitro (4). Endo-PG and oxalic acid were reported to act synergistically in tissue destruction (7). Other pathogens such as *Sclerotinia sclerotiorum* and *R. solani* that are similar in pathogenic behavior to *S. rolfsii* also produce cell wall degrading enzymes during pathogenesis (16,30). One of the first observed responses of cotton to infection by *R. solani* was depletion of ruthenium red-positive substances from host cell walls, which indicated enzymatic dissolution of pectic compounds (30). In apple fruit infected by *Sclerotinia sclerotiorum*, dissolution of cell walls occurred one or two cells in advance of the hyphae (16). Mechanical pressure exerted by infection cushions of *Sclerotinia trifoliorum* Erikss. caused ruptures in the epidermal cell wall of clover, which allowed entry of the fungus; this was followed by enzymatic dissolution of the cuticle and epidermis (21). Infection cushions of *R. solani* also exert mechanical pressure on the host and subsequently degrade host tissue enzymatically (8,9).

The objective of this study was to examine infection of host tissue by *S. rolfsii*. The role of hyphal aggregates during pathogenesis and the action of oxalic acid and enzymes at the cellular level were investigated using light and scanning electron microscopy and various histochemical techniques. A preliminary report of this work has been published (29).

MATERIALS AND METHODS

Inoculation procedure. Isolate 159 of *S. rolfsii* from sorghum in North Carolina (courtesy of R. S. Gurkin, North Carolina State University) was used throughout this study. This isolate was pathogenic on all host plants included in this study. Sclerotial inoculum was obtained from 2- to 6-mo-old oat cultures (22). Sclerotia were dried for 24 hr at 15–20% relative humidity before use to induce eruptive germination (22).

Greenhouse-grown host plants included in this study were bean (*Phaseolus vulgaris* L. 'Red Cloud'), sugarbeet (*Beta vulgaris* L. 'USH-10'), and carrot (*Daucus carota* L. 'Camden'). Five-day-old hypocotyls of bean, excised leaves from 2-mo-old sugarbeet plants, petioles from 2-mo-old carrot plants, and mature carrot roots were rinsed in tap water and blotted dry. Tissues (except carrot roots) were placed on moistened filter paper in 100 × 15-mm petri dishes and inoculated with one or several sclerotia. Carrot roots were inoculated with 6-mm-diameter agar plugs taken from a 4-day-old culture of *S. rolfsii* growing on potato dextrose agar. The tissues were atomized with sterile distilled water. Dishes were sealed in polyethylene bags and incubated at 24–28 C with a 12-hr photoperiod under cool-white fluorescent lights.

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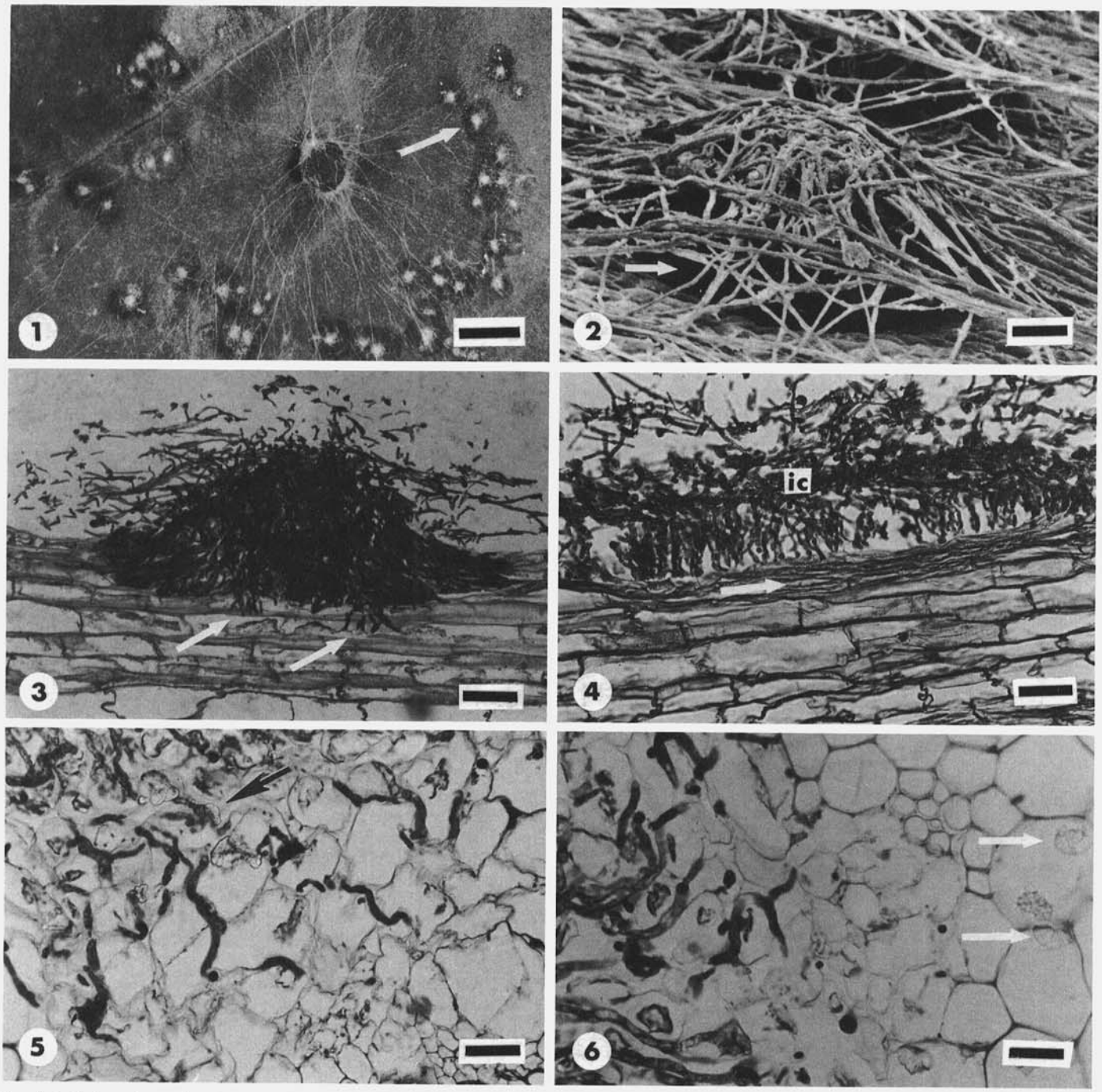
Scanning electron microscopy. Tissues were fixed for 24 hr in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2), dehydrated through a graded ethanol series to 100% ethanol and critical-point dried. Tissues were coated with gold on a Hummer-II sputter coater (Technics EMS, Inc., Springfield, VA) and examined with a JEOL JSM T-200 electron microscope (Japan Electronic Optics Ltd., Peabody, MA) operating at 12 kV. Photomicrographs were taken on Polaroid Type 55 film.

Light microscopy. At approximately 12-hr time intervals from 24 to 72 hr after inoculation, pieces of infected tissues were fixed for 24 hr in FPP (37% formaldehyde-propionic acid-50% isopropanol [5:5:90, v/v]) (14), dehydrated through a graded isopropanol series to 100% isopropanol, and embedded in paraffin. Paraffin blocks were stored in softening solution (2) for 24–72 hr at 4 C. Longitudinal sections of bean hypocotyls and transverse sections of sugarbeet leaves and carrot petioles or roots were cut (12-μm thick) using a rotary microtome, mounted on glass slides with Haupt's adhesive (14) and stained with safranin-fast green-trypan blue (28). Alternatively, tissues were stained with ruthenium red (14) and hydroxylamine-ferric chloride (13) for



Figs. 1–6. Light and scanning electron micrographs (SEM) of pathogenesis by *Sclerotium rolfsii* on bean, sugarbeet, and carrot. Unless otherwise stated, tissues for light photomicrography were stained with safranin-fast green-trypan blue. **1,** Hyphae from a germinating sclerotium ramified over the surface of a sugarbeet leaf. Arrow indicates infection cushion. Water-soaking of the tissue was evident around the infection cushions. Bar = 2 mm. **2,** SEM of infection cushion on a sugarbeet leaf surface. A depression (arrow) was observed on the host surface. Bar = 50 μm. **3,** Cross section of infection cushion on bean hypocotyl. Penetration hyphae (arrow) were formed beneath the infection cushion and invaded the tissue 36–48 hr after inoculation. Bar = 100 μm. **4,** Development of parallel hyphae perpendicular to the host surface under an infection cushion on a carrot root. Necrotic tissue below the infection cushion (ic) stained postive with thionin (arrow). Bar = 100 μm. **5,** Inter- and intracellular growth in bean hypocotyl tissue approximately 48 hr after inoculation. Crystals of calcium oxalate (arrow) were observed among the hyphae. Bar = 50 μm. **6,** Crystals of calcium oxalate (arrow) in carrot petiole (48–60 hr after inoculation) formed in advance of mycelial growth. Bar = 50 μm.

pectic substances, and alizarin red S for calcium (19), and thionin for necrotic areas (27). Pieces of infected sugarbeet leaf tissue (5 × 5 mm) also were stained for calcium oxalate crystals with a $AgNO_3$-dithiooxamide mixture in 70% ethanol containing NH_4OH (31). Whole mounts of $AgNO_3$-stained tissues and sections were examined with a Leitz Ortholux microscope and photographed on Kodak Plus-X-Pan film using a Leitz camera.

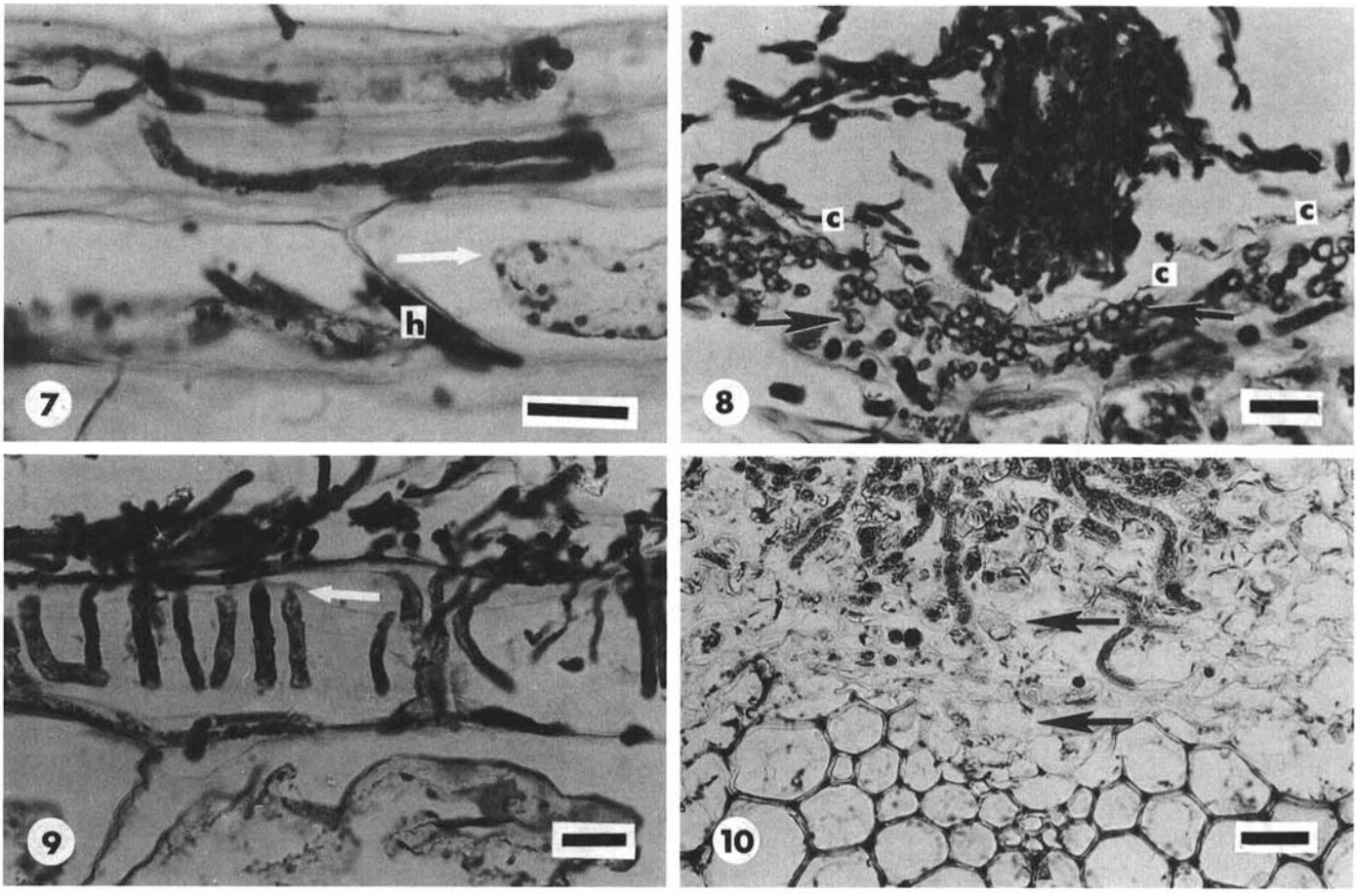
RESULTS

Macroscopic observations. Hyphae from germinating sclerotia of *S. rolfsii* ramified over the surface of all inoculated tissue 24–48 hr after inoculation. Hyphae frequently coalesced to form dome-shaped aggregates (0.5–2 mm in diameter), which were visible to the naked eye. Water soaking of host tissues was associated with these aggregates (Fig. 1).

Scanning electron microscopy. The host surface was sunken (concave) below the aggregates, which were comprised of tightly interwoven masses of hyphae (Fig. 2).

Light microscopy. Numerous penetration hyphae were produced from aggregates 36–48 hr after inoculation (Fig. 3) and from appressoria on the surface of the host. Hyphae also penetrated the host through stomata. Fine infection pegs, about 1 × 3 μm, were observed at the tips of some penetration hyphae. The host cuticle beneath the aggregates apparently remained intact. The host surface below the aggregates was depressed and penetration hyphae, measuring approximately 5.5 μm in diameter, grew perpendicular to the host surface (Fig. 4). Necrotic tissue could be delineated by the thionin stain reaction, because necrotic tissue adjacent to hyphae stained dark purple and healthy tissue 3–5 cell layers away from the hyphae stained light-orange to green (Fig. 4). Subsequent hyphal growth from aggregates 48 hr after inoculation occurred both intra- and intercellularly (Fig. 5). Abundant crystals 10–20 μm in diameter formed in the tissue 48–60 hr after inoculation, and were usually observed 3–5 cell layers in advance of hyphal growth (Fig. 6). Crystals were shown histochemically to be those of calcium oxalate (see below). Hyphae grew parallel to the longitudinal axis of the cells (Fig. 7) and to the surface of the tissue (Fig. 8). Occasionally, hyphae at the point of penetration into cells were constricted to approximately half of the prepenetration diameter (Fig. 9). In the cell, the hyphae regained prepenetration size of approximately 5.5 μm in diameter. Hyphal contents 48–60 hr after inoculation were more granular than during the earlier stages of infection.

Histochemical tests demonstrated that components of the middle lamellae of the cell wall were depleted in advance of mycelial growth. Cells adjacent to the leading edge of the mycelium were not stained with ruthenium red, whereas those distal to the hyphae retained the stain (Fig. 10). Depletion of ruthenium red-positive substances, presumably pectic materials, was observed in the 3–5 cell layers adjacent to the hyphae. As a negative reaction does not always indicate the absence of pectic materials, hydroxylamine-ferric chloride was employed to substantiate the ruthenium red reaction. Cells near the hyphae did not stain with this method, which indicated the absence of pectic materials. Cells near hyphae also did not take up alizarin red S, although tissues 3–5 layers away did. Inter- and intracellular crystals stained light orange with alizarin red S and black with $AgNO_3$, indicating a positive reaction for calcium and for calcium oxalate, respectively (31).



Figs. 7–10. Light micrographs of infection of bean hypocotyl by *Sclerotium rolfsii*. **7,** Growth of hyphae parallel to the longitudinal axis of the cells. Cells in advance of the hyphae (h) were plasmolyzed (arrow). Bar = 25 μm. **8,** Cross sections of hyphae (arrow) growing parallel to surface of the tissue. The cuticle (c) appeared to be intact. Bar = 25 μm. **9,** Constrictions in hyphae (arrow) at the point of penetration into the tissue. Hyphae were granular at this stage of infection, 48–60 hr after inoculation. Bar = 25 μm. **10,** Ruthenium red-positive substances, presumably pectic materials, were depleted in advance of the hyphae. The zone of depletion (between arrows) was usually three to five cell layers. Bar = 50 μm.

DISCUSSION

Infection cushions are produced by many plant pathogenic fungi and these structures were reported to facilitate infection of the host (1,8,11,15,16,21,30). Infection cushions formed by *R. solani* aided in pathogenesis by exerting mechanical pressure on the host surface or by producing enzymes (8). In some cases, however, mechanical pressure was a less important function of the infection cushions than production of cell wall degrading enzymes (30). In *Sclerotinia* spp., mechanical pressure on the host is followed by enzymatic action (17,18,21). Only two previous studies (12,20) have reported the occurrence or presumed function of hyphal aggregates that formed during infection of host tissue by *S. rolfsii*. In the present study, infection cushions of *S. rolfsii* aided in penetration of host tissue by secretion of enzymes and oxalic acid resulting in cell death and collapse. Infection also occurred by formation of penetration pegs from appressoria. Infection also was reported to occur from appressoria produced by germinating basidiospores of the teleomorph of *S. rolfsii* (23). Appressoria are necessary for infection of host tissue by ascospores of *S. sclerotiorum* (26), but do not appear to be solely involved during infection of host tissue by *S. rolfsii*.

The formation of infection cushions may depend on an interaction between the fungus and the host tissue. Host exudation and point of exudate origin on the host surface may stimulate infection cushion formation in *R. solani* (10). Materials that stimulate infection cushion formation may diffuse through the cuticle from cells below. Infection cushions of *S. cepivorum* formed by curling of single hyphae, from tufts of short branches, or from aggregates of branches of hyphae (1). Similar patterns of infection cushion formation from hyphae were reported for *R. solani* (15). *Sclerotinia* spp. may develop infection cushions from single hyphae, with perpendicular hyphae beneath the infection cushions functioning as penetration hyphae (18). Vesicles may serve as focal points for infection hyphae of *Sclerotinia sclerotiorum* (17). In the present study, infection cushions of *S. rolfsii* formed on all tissues and no specific site on the host or hyphal configuration appeared to favor infection cushion formation. We were unable, however, to induce formation of infection cushions on cellophane membranes in the presence of host exudates (authors, *unpublished*), as was reported for *Thanatephorus cucumeris* (Frank) Donk (10). The texture of the host surface or exudation pattern may influence infection cushion formation by *S. rolfsii*.

Constrictions in penetrating hyphae of *S. rolfsii* were observed in the present study. Constrictions at the point of penetration through cell walls have been observed in hyphae of *R. tuliparum* (11). Infection pegs of *R. solani* also were constricted at penetration sites but subsequently enlarged to normal size (8). After entry into the cell, hyphae of *S. rolfsii* enlarged to normal diameter and were granular in appearance. This observation suggests that a small point of ingress is necessary for the fungus to initiate substantial inter- and intracellular growth. The granular appearance of the hyphae may indicate some physiological difference in the penetrating hyphae at this stage of infection. *S. rolfsii* grew through the tissue both inter- and intracellularly, but it did not penetrate vascular elements. A similar observation was reported for *R. solani* (15). Hyphal growth of *S. cepivorum* occurred along longitudinal junctures on the surface of epidermal cells (1). Although *S. rolfsii* did not have this type of surface growth, after penetration it grew parallel to the longitudinal axis of cells in bean hypocotyls. Hyphae in advanced stages of infection also ramified through the tissue in other orientations.

Previous investigators have shown that oxalic acid produced by *S. rolfsii* acts in conjunction with cell wall degrading enzymes in causing tissue destruction (7,24). In bean, characteristic symptom expression was observed only when oxalic acid and endo-PG were applied to the plant (7). Application of either substance alone did not induce characteristic tissue maceration, but when these substances were applied simultaneously, plants displayed lesions typical of those resulting from fungal infection. Virulence in *S. rolfsii* has been highly correlated with endo-PG production, but only weakly correlated with the production of oxalic acid (24). Oxalic acid was produced early during growth of the fungus in culture (24), suggesting that sequestering of calcium in cell walls by oxalic acid may be necessary before the middle lamellae can be enzymatically degraded. Cutinolytic enzymes are produced by *S. rolfsii* (4), but we found no visual evidence of cuticle degradation in this study.

The histological observations presented here substantiate the results of a previous in vitro study (24). In the present study, alizarin red S-positive substances and ruthenium red-positive substances, presumably calcium and pectic materials, respectively, were depleted from cell walls, usually three to five cell layers in advance of the mycelium. This suggests that the oxalic acid secreted by the fungus precipitated calcium from the middle lamellae to form calcium oxalate crystals (24,25); thus the pectic materials that remained after removal of calcium were more susceptible to enzymatic degradation. The loss of integrity of the middle lamellae could have resulted, in part, in the observed death of tissue in advance of hyphal growth. The extensive hyphal growth within the tissue and the production of oxalic acid and cell wall degrading enzymes are major factors that contribute to tissue maceration characteristic of plants infected by *S. rolfsii*.

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