HELMINTHOSPORIUM SOLANI DUR. & MONT. DEVELOPMENT ON POTATO PERIDERM[1]

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Abstract

Development of *Helminthosporium solani* Dur. & Mont. on artificially inoculated, excised potato tuber periderm, incubated in darkness and high humidity at 20 to 24°C, was studied using light and scanning electron microscopy. Spore germination occurred within 16 hours, and appressoria were observed 2 days after inoculation. Penetration of periderm was evident 4 days after inoculation. Conidiophores with young conidia developed on the periderm surface within 7 days of inoculation. Well-developed conidia were visible often by 9 days after inoculation. Rudimentary stromata were observed beneath conidiophore groupings at later stages of development.

In histological studies, *H. solani* hyphae were found in the phellem, phelloderm, and cortex of infected tuber sections. Basal cells of conidiophores or stromata were observed on the surface and in the outer tangential cork cell layers. Several layers of suberized and sometimes collapsed cortical cells were observed beneath disrupted and collapsed, infected periderm. Cavities sometimes formed between periderm and cortex, and callosities were observed on cell walls of some cortical cells beneath infected periderm.

Resumen

Con microscopía de luz y de exploración electrónica se estudió el desarrollo de *Helminthosporium solani* Dur. & Mont. sobre peridermis extirpada de tubérculos de papa, artificialmente inoculadas e incubada bajo condiciones de obscuridad, alta humedad y 20 a 24°C. La germinación de esporas tuvo lugar alrededor de 16 horas después de la inoculación. A los dos días de la inoculación se observaron apresorios. La penetración en la peridermis fue evidente cuatro días después de la inoculación, y a los siete días se desarrollaron conidióforos con conidias jóvenes sobre la superficie de la peridermis. Se vieron conidias bien desarrolladas comúnmente nueve días de-

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spués de inocular el patógeno. Se observaron estromas rudimentarios debajo de los grupos de conidióforos en estadíos posteriores de desarrollo.

En estudios histológicos, fueron detectadas hijas de *H. solani* en la peridermis, la felodermis y la cortex de secciones infectadas de tubérculos. Se observaron células basales de conidióforos o estromas sobre la superficie y en las capas corchosas externas y tangenciales (epidermis). Se observó suberización y a veces colapso de varias capas de células corticales por debajo de la peridermis infectada, desorganizada y flácida. Se formaron a veces cavidades entre la peridermis y la cortex, y se observaron callosidades sobre las paredes celulares de algunas células corticales debajo de la peridermis infectada.

Introduction

Helminthosporium solani Durieu & Montagne is the causal agent of silver scurf of potato tubers. Silver scurf is considered a storage disease of potatoes, although infection often takes place before harvest (21, 27). Unsightly blemishes are readily observed on washed, infected tubers (17). During prolonged storage, excessive fresh weight losses may result from increased permeability of infected periderm (17, 19, 21).

Information concerning the early development pf *H. solani* is essential to understanding the disease process. However, previous reports of *H. solani* development on periderm were vague or lacking in photographic evidence (3, 27, 31). In the present study, the growth of *H. solani* on potato tuber periderm was observed closely. Histologic and morphologic aspects of infection occurring in artificially inoculated and naturally infected tissues were studied with light (LM) and scanning electron (SEM) microscopes. A preliminary report of this study has been published (10).

Materials and Methods

Inoculation and Incubation Procedures

Tuber periderm was inoculated with *H. solani* using a modification of Nnodu's technique (24). Blocks of tuber tissue, including periderm, cortex, and some storage parenchyma were cut from apparently healthy areas on potato tubers of the Norchip cultivar. Tissue blocks were surface-sterilized for 2 minutes in 5% Clorox (The Clorox Company, Oakland, Calif. 94612), and rinsed in sterile distilled water. To maintain humidity, blocks were placed aseptically on sterile water agar in a closed plastic Petri dish.

The isolate of *H. solani* used for inoculations was obtained from a Norchip tuber harvested near Ault, Colorado. Cultures of *H. solani* were maintained on lima bean agar (LBA) plates (30). A few milliliters of sterile distilled water were poured aseptically on the agar surface adjacent to a 3-to 4-week-old culture of *H. solani.* Conidia and water were mixed aseptically using an alcohol-flamed glass slide. The end of a flamed glass slide was dipped in the

inoculum suspension, transferred aseptically to a separate, sterile water agar plate, and stroked over the agar surface. Sections containing inoculum were cut immediately from the water agar and inverted on the periderm of the tissue blocks. Water agar was then removed from the blocks with flamed forceps. Microscopic examination of periderm surface revealed many conidia and some hyphal and conidiophore fragments. Periderm sections were incubated in darkness at 20 to 24°C. Tissue blocks and supporting agar were removed from Petri dishes at hourly or daily intervals for LM examination of surfaces. Young hyphae of *H. solani* were stained with lactophenol cotton blue (Banco Standardized, Anderson Laboratories, Fort Worth, Texas) to improve contrast with the brown periderm.

Slide Preparation and Staining

Naturally infected and healthy periderm and associated cortex and storage parenchyma from the potato cultivars Norchip and Red McClure were prepared for histological examination with the LM. Tissue blocks approximately 3 mm square and 1 to 2 mm thick were fixed in Craf III (26) for at least 24 hours. After fixation, tissues were dehydrated in an alcohol-xylene series and infiltrated with embedding wax according to standard techniques (18, 26). Sections cut 20 μ in thickness with a rotary microtome were expanded in 4% formalin and mounted on glass slides with Haupt's adhesive (18). Embedding wax was removed with xylene prior to staining. Staining procedures included ammoniacal gentian violet (2) for detection of suberization, substituting methyl violet 2B for gentian violet (24); Pianese IIIb (13, 28); Stoughton's Thionin and Orange G (5); and safranin-fast green (18). After staining, slides were dehydrated and coverslips mounted with Harleco Synthetic Resin (Hartman-Leddon Company, Philadelphia, Penn.).

SEM Techniques

Tissues inoculated as described previously and sections prepared as for LM were further treated for observations with the SEM. A fixation chamber was created by inverting a plastic Petri dish bottom on Parafilm so that the edges were sealed (Parafilm "M" Laboratory Film, American Can Company, Dixie/Marathon, Greenwich, Connecticut). A double layer of filter paper less than 8.5 cm in diameter was placed on the Parafilm inside the chamber and saturated with a 2% glutaraldehyde solution. For this study, tissue blocks approximately 3 mm square and 1 to 2 mm thick were secured with short lengths of #00 insect pins to small pieces of rubber or polyethylene previously cleaned in acetone. The tissue was placed within the fixation chamber in an elevated, pierced plastic cup for approximately 2 hours at about 20°C.

Fixed tissue was dehydrated by passing through a graded concentration series of acetone in water. Tissue blocks and dewaxed tissue sections mounted on glass coverslips were critical point dried in a Polaron E3000

Manual Unit using CO_2 as the transitional fluid. Pressure was raised slowly. The liquid level was never allowed to drop below the tissue. Four or five 15-minute soaks were used prior to reaching the critical point. Blocks were mounted on pieces of copper adhesive tape previously fastened to aluminum stubs with conductive silver paint. Coverslips were attached directly to stubs with silver paint. After coating the specimens with about 75 Å of gold-palladium in a Technics Hummer V sputtering unit with argon as the source gas, they were stored in a bell jar under vacuum until observation. Specimens were examined with an Hitachi HHS-2R SEM at an accelerating potential of 20 KV with a 20 degree tilt of the specimen stage and approximately 15 mm working distance.

Results

Germination Patterns

Since *H. solani* conidia did not germinate simultaneously, many stages of development were found during the incubation period. Some conidia are reported to germinate within 8 hours on water agar (9). A few conidia required long periods of incubation on periderm before germination, while some germinated within 16 hours after inoculation (Fig. 1). *H. solani* conidia germinated from either one or both ends, although a germ tube most commonly protruded from the narrow, or distal, end of a conidium (Fig. 1a). Conidia sometimes germinated on artificially inoculated surfaces while still attached to conidiophores. Germination was observed from an interior cell of a conidium only once during the study. Cotton blue stain was absorbed by the cytoplasm of young hyphae. The blue coloration sometimes extended for a short distance into germinated conidia. Brown pigment which developed later in older hyphae was not obscured by lactophenol cotton blue staining.

Appressorium Formation

On artificially inoculated periderm, knob-like structures which resembled appressoria were observed at some hyphal tips as early as 2 days after inoculation (Fig. 1c). Not all hyphae formed these structures, and hyphae were of various lengths before the structures formed. Some of these structures contained a light brown pigment and did not absorb stain well. Knobs were observed on the surface of cork cell faces as well as at cell wall intersections. Within 5 days of inoculation, one *H. solani* conidium had produced a hyphal network with at least seven appressoria-like structures.

Periderm Penetration

Four to 5 days after inoculation, some appressoria-like structures appeared to germinate, producing hyphae which either continued growth on the periderm surface or penetrated the periderm and grew within the outer tangential cork cell layer (Figs. 2a, b, c). The failure of hyphae to absorb the cotton blue stain and the necessity of focusing down into the periderm to ob-

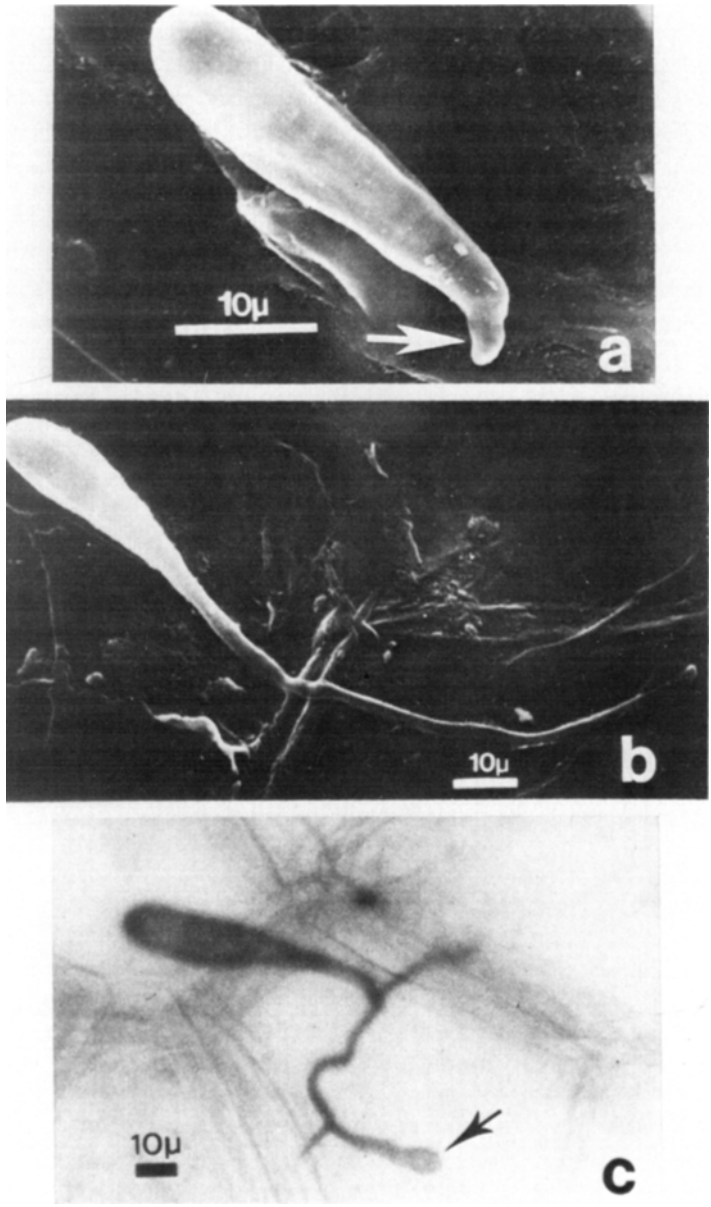

FIG. 1. Early stages in development of *Helminthosporium solani* on artificially inoculated potato (cv. Norchip) periderm. a) Scanning electron micrograph: Germ tube (arrow) protruding from narrow end of conidium, 3 days after inoculation. b) Scanning electron micrograph: Developing germ tube, 16 hours after inoculation. c) Light microscopy: Appressoirum-like structure (arrow), 2 days after inoculation.

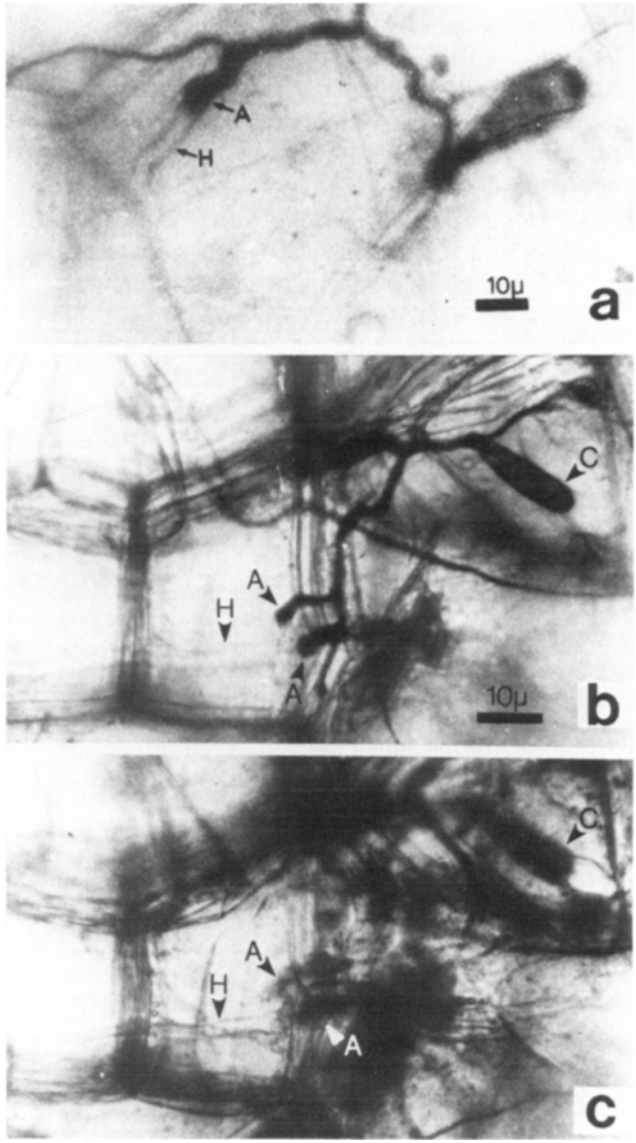

FIG. 2. Appressoria-like structures connected to hyphae beneath surface of potato tuber periderm, 5 days after inoculation with *Helminthosporium solani*. Surface hyphae stained with cotton blue. a) Appressorium-like structure (A) connected to hypha (H) beneath surface. b) Focus on conidium (C) and appressoria-like structures (A). Note slightly out-of-focus hypha (H) beneath the cell surface. c) Same as 2b, except focus is on hypha (H) within cork cell.

serve hyphae indicated that some hyphae were inside the cork cells. Hyphae were not observed beneath the surface of periderm on uninoculated, healthy controls.

Conidiophore Development

Thickened hyphae were observed on periderm within 6 days after inoculation. Many became rudimentary stromata which gave rise to conidiophores (Fig. 3a). Young conidiophores developing from surface hyphae were observed as early as 7 days after inoculation, some with immature conidia attached. Conidiophores were clustered around a stromal grouping of cells (Fig. 3a) or connected to one another by a thick hyphal strand on the surface of the periderm. Sporulating structures sometimes developed on hyphae near attached, germinating conidia (Fig. 3a). In one case 8 days after inoculation, an appressorium-like structure attached by a thin hyphal strand to the base of a conidiophore was observed. Hyaline hyphae growing from the bases of some conidiophores seemed to disappear into the lumena of adjacent cells. Whether arising from a stroma or not, conidiophores typically had a bulb-like base (Figs. 3c, d). Each conidiophore was composed of 5 to 14 elongate, vacuolate cells, and conidia were not produced from the lowest one or two cells of a conidiophore. Under the incubation conditions maintained, conidiophores with well-developed conidia were visible by 9 days after inoculation on many of the periderm blocks. Although the sequence of conidia development was not followed in this study, it was probably similar to that of *H. solani* on LBA as described by Hunger and McIntyre (13). Percurrent conidiophore proliferations (20) were not observed, although germination, which produced normal hyaline hyphae, occurred near the tips of some conidiophores deposited with inoculum.

Rudimentary stromata sometimes occupied entire cork cells in both naturally infected and artificially inoculated tissues (Fig. 3b). Confinement along cell walls was an indication that some stromata were within the cells, rather than on the surface of the periderm. Stromata connected between cells by mycelial strands eventually penetrated adjacent cork cells. Conidiophores then arose from within the periderm.

A summary of *H. solani* development is presented (Fig. 4).

Histological Findings

Infected periderm was often collapsed compared to non-infected periderm (Figs. 5a, b). The degree of collapsing was apparently dependent on how far the disease had progressed. Hyphae of *H. solani* were observed in cork cells and the phelloderm of naturally infected tuber periderm. Hunger (12) also reported finding hyphae tentatively identified as *H. solani* in phelloderm. In tangential sections of *H. solani* infected tissue in cv. Red McClure tubers, abundant hyphal networks were observed within cork cells and connected to other hyphae in adjacent cells (Fig. 6a). At the point of hyphal contact with cork cell walls, bulb-like swellings formed in hyphae (Fig. 6a), perhaps in response to a pressure accumulation at the growing tips. Hawkins and Harvey (8) described a similar phenomenon with *Pythium debaryanum* in potato tuber cortex. Evidence of conidiophore production

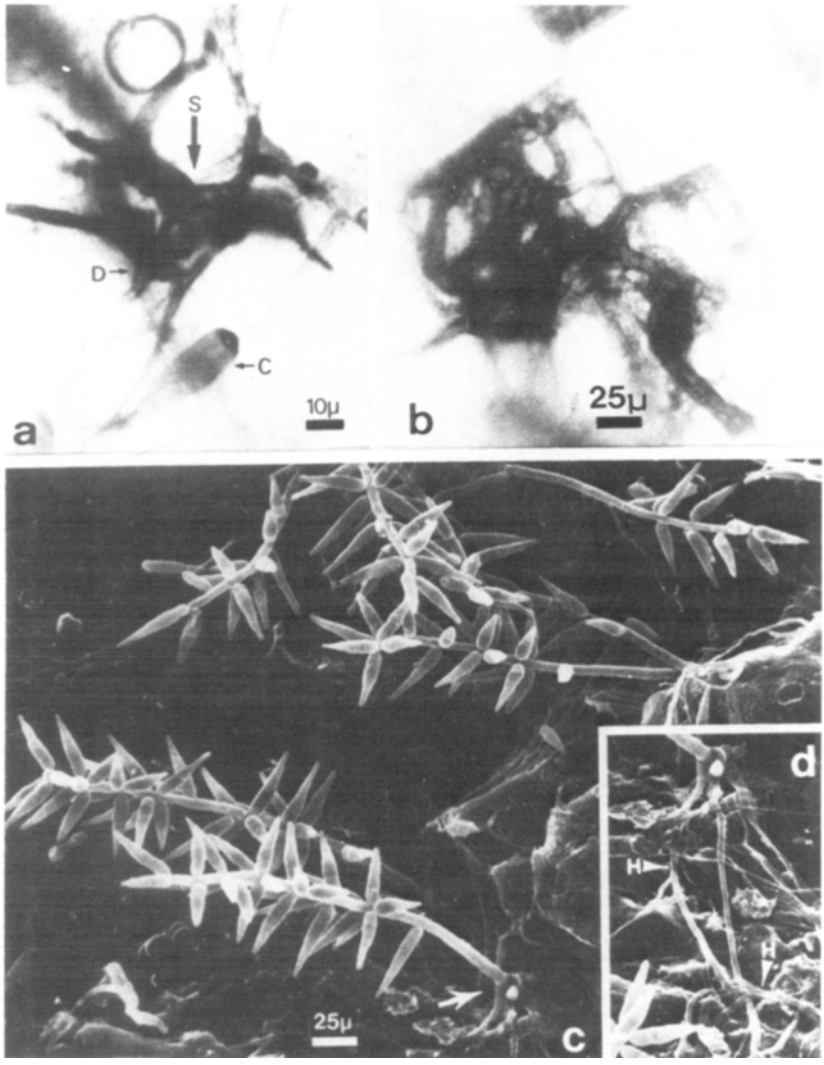

FIG. 3. Late stages of *Helminthosporium solani* development on potato (cv. Norchip) tuber periderm. a) Light microscopy: Conidium (C) connected (D) to rudimentary stroma (S) at base of conidiophore cluster, 9 days after inoculation. b) Light microscopy: Rudimentary stromata within cork cells, 4 weeks after inoculation. c) Scanning electron micrograph: Well-developed conidiophores and conidia, 20 days after inoculation. Arrow indicates conidiophore base featured in insert 3d. d) Close-up of conidiophore base in 3c, showing attached hyphae (H) which are abundant on the periderm surface.

was present in cross sections of naturally infected periderm, as the bases of conidiophores or rudimentary stromata were located on the periderm surface and embedded within the outer tangential cork cell layer (Fig. 6b).

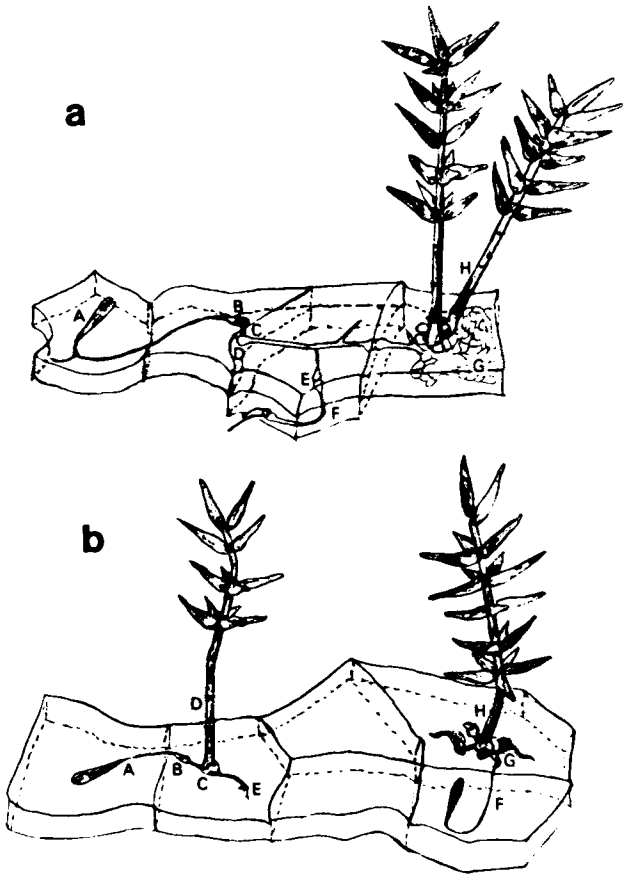

FIG. 4. Illustrations summarizing development of *Helminthosporium solani* on potato tuber periderm. Hyphae, conidia, and conidiophores on the periderm surface are indicated by shading. Mycelium within cork cells is not shaded. a) Growth within cork cells. From left to right: A) Conidium germinates on periderm surface and the germ tube branches. B) One branch forms an appressorium-like structure (in this case, near a cell junction). The appressorium-like structure produces a short infection peg (C) or hypha which penetrates the cork cell wall of the periderm surface. At the next developing septum, a swelling forms in the hypha (D). The hypha proliferates within the cell and contacts cell walls, swelling again (E). Hyphae penetrate cell walls and enter adjacent cell lumina (F). Rudimentary stromata (G) within cork cells give rise to conidiophores (H) which push through the periderm surface to the exterior as they develop. Conidia emerge through pores beneath septa in the conidiophores. b) Growth on periderm surface. From left to right: A) Conidium germinates on periderm surface. Appressorium-like structure develops on short germ tube (B). Hypha growing from appressorium-like structure produces bulb-like base on periderm surface (C) which grows into a conidiophore (D). Hyphae from base of conidiophore may eventually penetrate the periderm surface (E). Alternatively, a conidium germinates, producing a germ tube (F) which develops directly into a rudimentary stroma (G) on surface of the periderm. Conidiophores (H) develop from the stroma.

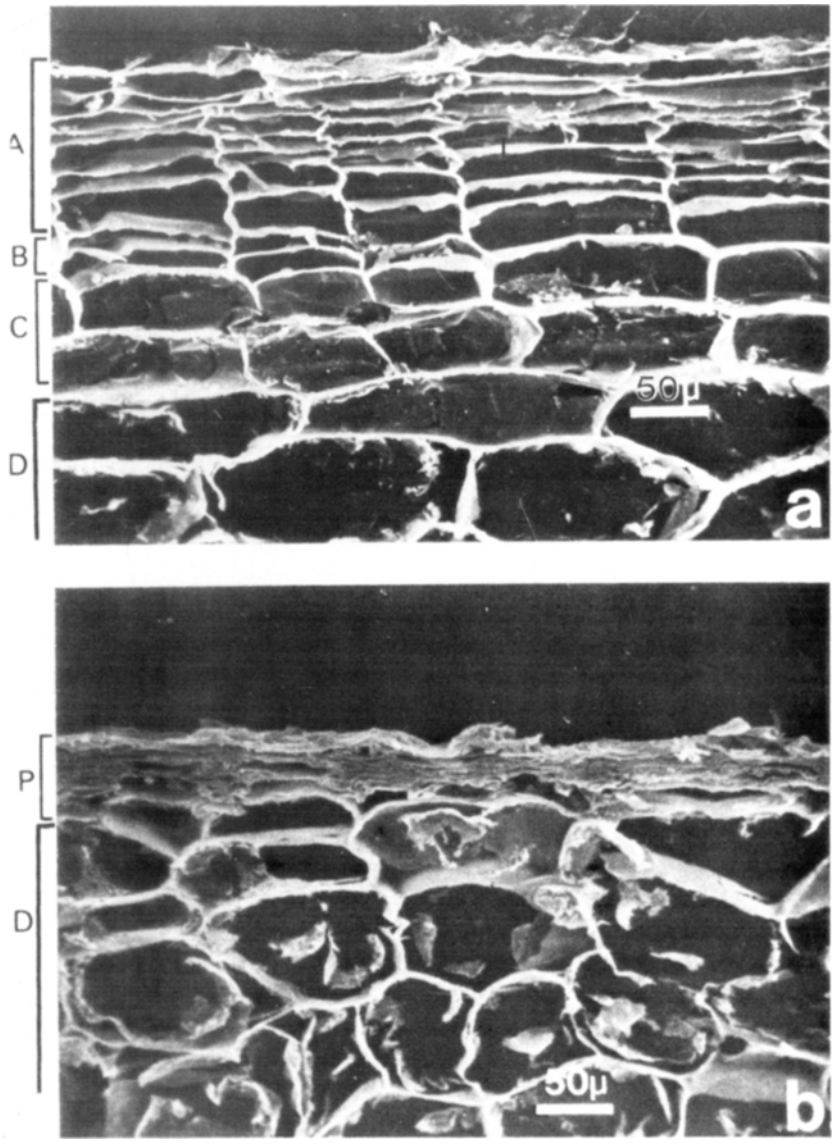

FIG. 5. Scanning electron micrographs of cross sections of healthy and *Helminthosporium solani* infected potato (cv. Norchip) tuber periderm. a) Noninfected Norchip periderm, showing regions of cork (A), phellogen (B), and phelloderm (C), and underlying cortex (D). b) Collapsed periderm (P) due to *H. solani* infection. Underlying cortex (D) is shown.

Septate hyphae, believed to belong to *H. solani,* were observed in the cortex of naturally infected tubers (Fig. 6c). This is the first report of *H. solani* hyphae in cortical tissue. It is possible that *H. solani* penetrated the

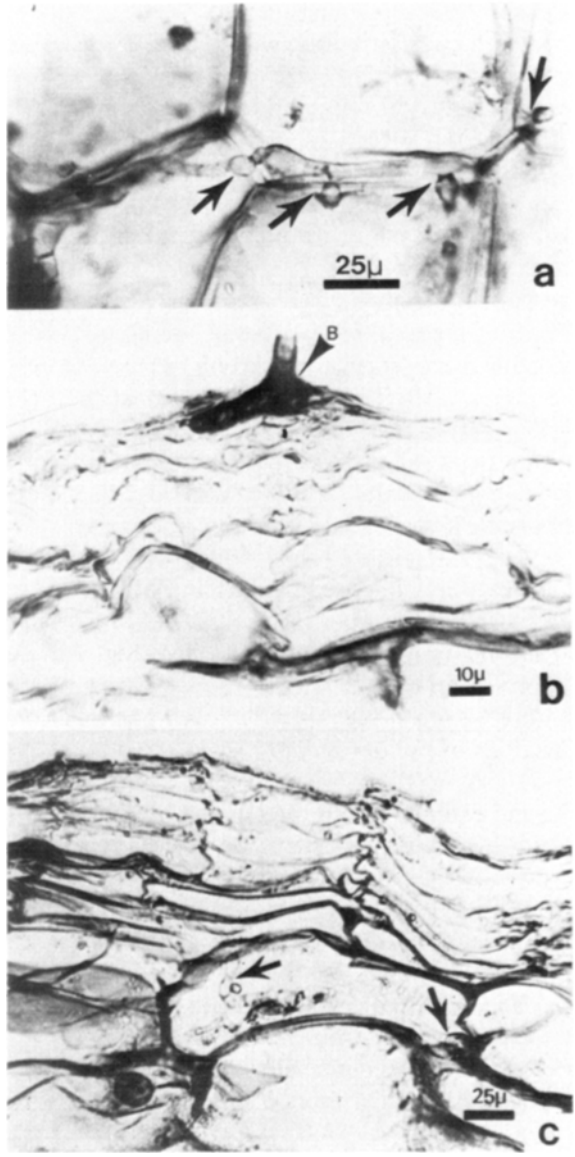

FIG. 6. Light microscopy of *Helminthosporium solani* in section of infected potato tuber tissue. a) Tangential section through naturally infected periderm of a Red McClure potato tuber. Close-up of hyphal knobs penetrating cell walls (arrows). b) Radial section through naturally infected periderm of a Norchip potato tuber, showing base of conidiophore (B) embedded in outer tangential cell layer. c) Radial section through naturally infected Norchip tuber tissue showing hyphae (arrows) in cortical cell.

cortical cells following their death by desiccation or other causes. Previous reports (3, 27, 31) indicated that *H. solani* was confined to the periderm.

In some sections of severely disrupted periderm, cavities or "air pockets" (3, 16) formed from destruction of radial cell walls (Figs. 7a, b). *H. solani* hyphae were evident within these areas. Cavities occurred at the phelloderm level, effectively separating periderm from cortex. Air pocketing is credited with causing the silvery appearance of silver scurf symptoms (3, 16). Shedding of cork cells above the cavities is likely to occur. Fragments of conidia observed in the section shown (Fig. 7b) may be *in situ* or they may have fallen into the cavities through a break in the periderm surface during fixation or dehydration.

Certain areas of tuber tissue were more heavily damaged by infection than others. Beneath areas where periderm was greatly disrupted, two to four, or occasionally more, cortical cell layers were stained by methyl violet (24), malachite green in the PIIIb stain (28), or safranin (18), suggesting suberin deposition (9) (Fig. 7b). In healthy periderm, staining of suberin did not extend inward from the cork beyond the outer tangential wall of the phellogen or of the phelloderm. In infected periderm, the outer tangential layer of cortex or phelloderm sometimes remained nearly normal in radial width while underlying cortical cells collapsed. In other areas, both periderm and cortex were collapsed. Collapsing of cortical cells seemed to contribute to shriveling of the tuber surface (9). Beneath injured or collapsed parenchyma, a secondary periderm often formed (Fig. 7b). Suberization of the exposed tuber parenchyma surface as observed beneath disrupted periderm may have been a prerequisite for cork phellogen formation (25).

In many sections of periderm naturally infected by *H. solani,* growths 4.0 to 5.4 μ wide and up to 10.8 μ in length were observed on the outer tangential walls and extending into the lumena of cortical cells located one or two cell layers beneath the phelloderm. Some cells contained several of the ingrowths on one wall (Fig. 8). Descriptions, drawings, and photographs of similar structures observed by others indicated the appropriate term to describe each growth was "callosity" (33) or "lignituber" (7). Close examination of prepared slides revealed that hyphae often were located close to cell walls on which callosities had formed, but in adjacent cells (Fig. 8), suggesting a host cell reaction to contact with the fungus.

Callosities formed on the outer cell walls of cortical cells located two to three cell layers inside the cut surfaces of incubated, inoculated tuber tissue blocks. Some hyphae proximal to *H. solani* conidia, with dimensions and appearance of *H. solani* hyphae, were observed near callosities in sections of artificially inoculated tissue blocks. Developing callosities were visible 6 days after inoculation, and were numerous within 7 days.

Discussion

Development of H. solani

Although appressoria are well-known in other species formerly classified under *Helminthosporium,* e.g. *H. carbonum* (22) and several other

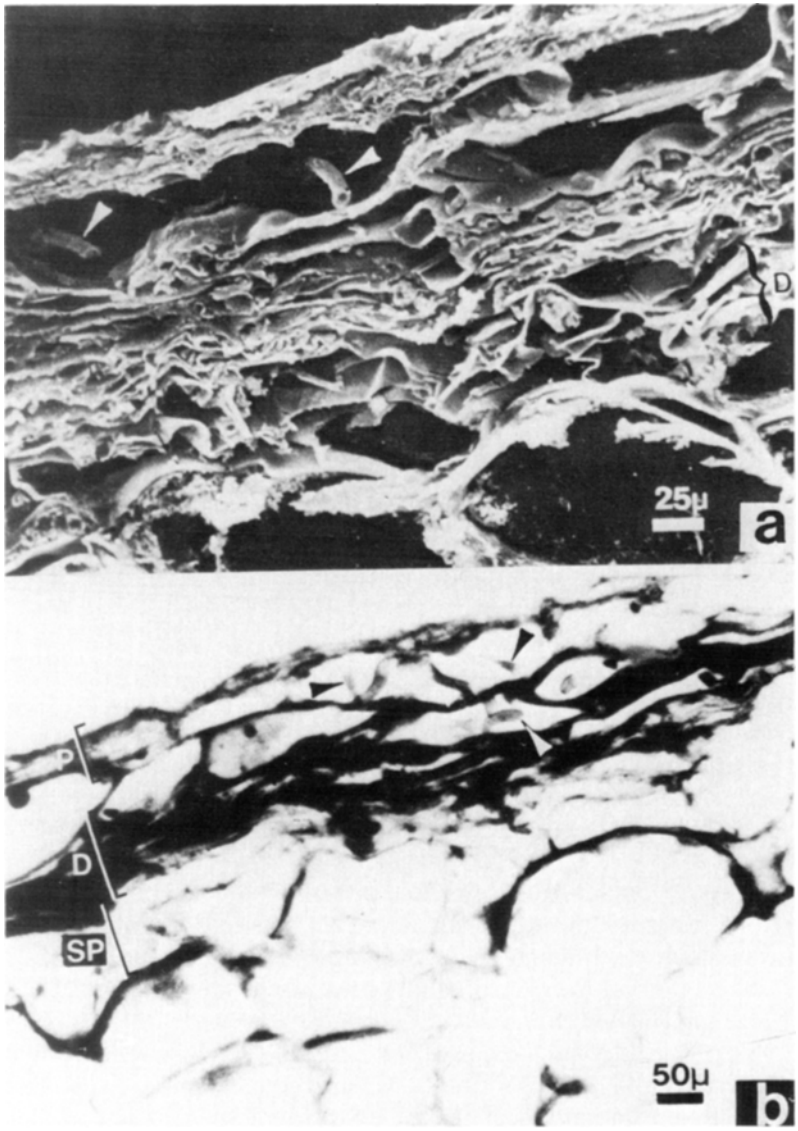

FIG. 7. Radial sections of cavities in *Helminthosporium solani* infected potato (cv. Norchip) tuber tissue. a) Scanning electron micrograph of radial section showing *H. solani* hyphae (arrowheads) located in cavities in or beneath the periderm. Some damage to cortex is indicated (D). b) Light microscopy: Disrupted periderm with hyphae, conidiophore and conidia fragments (arrowheads) of *H. solani* in cavities. Brackets delimit original periderm (P), collapsed cortical cells (D), and secondary periderm (SP).

pathogens of cereals and grasses (23), this is the first report of their occurrence in an isolate of *H. solani*. Burke (3) reported that no holdfast organ

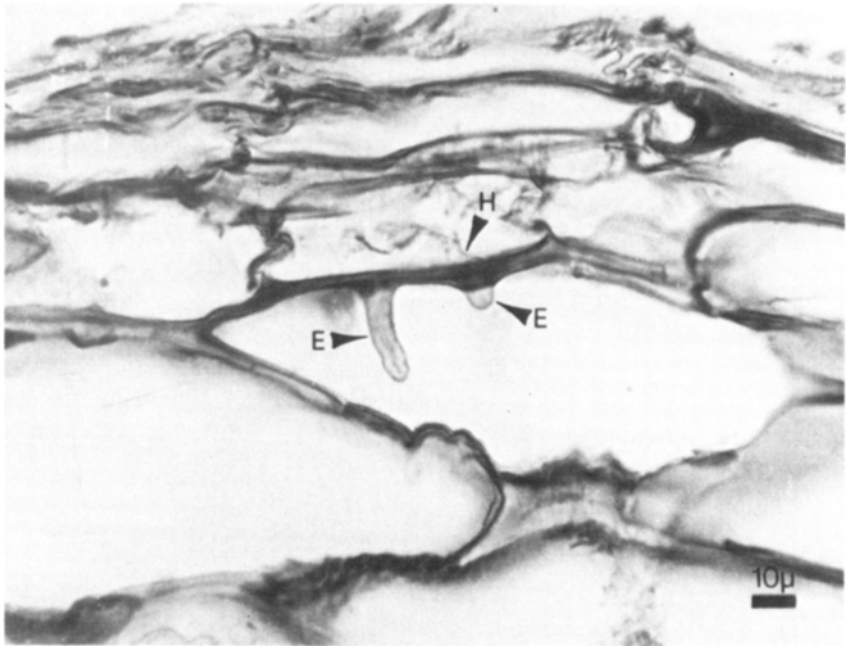

FIG. 8. Radial section of *Helminthosporium solani* infected potato (cv. Norchip) tuber tissue, showing callosities (E) on wall of suberized cortical cell. A hypha (H) is evident in cell adjacent to a callosity.

was observed at the point of entrance of the fungus into the periderm, although "slight enlargement" occurred in most instances. The enlargement Burke described may have been an appressorium similar to those pictured (Fig. 2).

Burke (3) reported that entrance into the periderm by *H. solani* required 48 hours following inoculation at an unspecified temperature and relative humidity. Although specimens were observed daily beginning 1 day after inoculation in the present study, penetration was not evident until 4 days after inoculation. Several investigators (3; 15, cited in 17; 27) reported that production of conidia required 3 to 5 weeks after *H. solani* inoculation of whole tubers. In this study, fully developed conidia appeared on conidiophores within 9 days of inoculation on periderm of tissue blocks, with incubation in water agar plates at 20 to 24°C. Germination of conidia and subsequent growth require high humidity (approximately 93% (3)), which was easy to maintain in small Petri dishes. Maintaining high humidity would be more difficult in the larger containers required for whole tubers, and this may account for the slower growth reported previously.

Histological Studies

Examination of cross sections of *H. solani* infected periderm revealed that the cortex may be more directly involved in the silver scurf disease than

previously believed. Collapsed cortical cells next to disrupted periderm can be difficult to distinguish and can lead to incorrect interpretation of tuber anatomy. What has been called a loss of cork cell layers may have actually been the collapse of radial width of infected periderm. When a loss of cork cells was evident in sections, several layers were usually lost at once, exposing the cavities or air pockets described previously (Fig. 7b). In other reports (3, 16), pocketing was associated with the silvery symptoms of the disease. However, in this study not all areas showing symptoms exhibited pocketing. Hunger made similar observations (12). Other phenomena may be occurring besides pocketing to cause the symptoms of silver scurf on tubers from thin- or red-skinned cultivars. Hunger and McIntyre (13) speculated that deposition of phytoalexins and other phenolic compounds in the periderm may be partially responsible for silver scurf symptoms. Perhaps enzymic degradation of pigments, periderm disruption, cortical collapse, and suberization in response to *H. solani* infection also influence lesion color.

In the present study, walls of cortical cells beneath periderm disrupted by *H. solani* infection appeared to be suberized. Suberization of outer cortical cell walls would be expected after any type of damage to adjacent periderm (6). Wenzl (32) reported that most of the starch was dissolved in several layers of the storage parenchyma in parts of tubers infected by *H. solani.* This was probably a result of the conversion of starch to sugars which are used in the production of fatty acids needed for suberin formation (29). The collapsed and suberized cortical cells did not seem to present any greater barrier to *H. solani* development than the periderm itself. As secondary periderms form beneath the suberized parenchyma, it is conceivable that *H. solani* could move into those areas, continually creating conditions which would lead to water vapor loss in the tuber.

Appositions encasing haustoria of *Phytophthora infestans* in potato tuber parenchyma and callosities in sweet potato have been described previously (11; 14, cited in 1). The discovery of callosities in *H. solani* infected potato tuber tissue adds a new dimension to information concerning silver scurf. If callosities were formed as a result of host cell contact with *H. solani* hyphae, then *H. solani* has a direct effect on living cells. The formation of callosities is evidence that cells were alive at the time of initial hyphal contact. Wall appositions are considered by some (4, 11) to result from an unspecific and general response to infection or mechanical injury.

Future Studies

This study suggests several areas which merit further investigation, particularly with regard to appressoria of *H. solani* and callosities in tuber parenchyma. The stimuli required for appressorium formation are unknown. Because appressoria were not observed on agar media but formed on glass slides and tuber periderm, contact with a relatively firm surface appeared necessary for development. However, appressoria did not form at a specified

distance from germinated conidia, so other influences, such as fluctuating humidity, temperature or nutrient availability, may also be involved. Transmission electron microscopy may reveal whether *H. solani* hyphae penetrate cork cells solely by mechanical force, or whether enzymes are important. Mucilaginous sheaths surrounding *H. solani* conidia were evident in transmission electron micrographs (9), and may be necessary for attachment of conidia to periderm. Electron microscopy could also supplement information on callosities, including their relationship to cell wall components, whether they form around sites of plasmodesmata, and whether *H. solani* is directly responsible for their formation. Although callosities did not appear to greatly hinder invasion of cortical cells by *H. solani* in the cultivar Norchip, it is possible other cultivars develop callosities which impede the infection process.

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