Histopathology of Sclerotinia sclerotiorum Infection of Bean

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ABSTRACT

Infection cushions were formed on bean hypocotyls within a few hours after inoculation with *Sclerotinia sclerotiorum*-infested oat kernels. The dome-shaped cushions were composed of three safranin-stained hyphal types: (i) dark red hyphae on the upper surface; (ii) light red, granular, inflated hyphae in the center; and (iii) light red, granular, dichotomously-branched, penetration hyphae next to the host surface. Multiple penetration pegs from the latter hyphae forcibly entered the host through the cuticle and formed inflated, lightly stained vesicles between the cuticle and epidermal cells. The vesicles were focal points for production of lightly stained subcuticular and cortical "infection" hyphae (modal diam 17.0 μm). The subcuticular hyphae were oriented parallel to one another and formed organized infection fronts. These hyphae eventually became oriented parallel to the long axis of the hypocotyl and grew up the stem more rapidly than the cortical hyphae and generally on the same side as the point of penetration. Cortical infection hyphae also were oriented parallel to the hypocotyl, and girdled the stem. In contrast to the infection hyphae, "ramifying" hyphae (modal diam. 8.5 μm) were stained dark red with safranin and grew throughout the invaded tissue both inter- and intracellularly. These hyphae arose as branches, ca. 55 μm from the apex of the infection hyphae. A primary role in pathogenesis is suggested for the intercellular infection hyphae, and a secondary and perhaps nutritional role is suggested for the ramifying hyphae. Upon maturation of lesions (48 to 72 hr), ramifying hyphae moved to the surface layer of hypocotyl cells and, being unable to penetrate the still intact cuticle, the hyphae emerged through stomata. On the surface, tufts of mycelia developed into dense cottony mats of mycelia or formed sclerotium initials. The vascular and pith regions of the stem were not colonized until the cortex was thoroughly invaded.

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The pathogen-suscept relationship of diseases caused by *Sclerotinia sclerotiorum* (Lib.) d By. has been of interest since de Bary first investigated the infection process of this fungus (4, 5). He described the formation of appressoria from germinating ascospores and the dependence of the invading mycelium on its prepenetration nutritional status. Hyphae provided with adequate nutrients were capable of penetrating the superficial living cells of the host by piercing their cell walls, growing indiscriminately within, between, and through the living cells, soon killing them. The ability to infect the host was controlled by the ability to penetrate the "membranes", which were dissolved at the point of penetration.

According to Boyle (2), penetration of the cuticle of leaf tissue was attributed solely to mechanical pressure, not enzymatic dissolution. Boyle described the penetration process, indicating that hyphae entered the epidermal cell and swelled into vesicles within the cell.

Purdy (19) also used leaf tissue to study formation of appressoria by germinating ascospores. He confirmed the dependence of appressoria formation on nutrients and a firm surface. In addition, mycelia from germinating sclerotia were capable of initiating infection of healthy tissue, but only when a nutrient source of nonliving organic matter was available. Other reports (16, 20, 21) have firmly established the importance of a food base in the infection process.

Sclerotinia trifoliorum Eriks. [considered synonomous with *S. sclerotiorum* (18)], unlike *S. sclerotiorum*, produces distinct, organized, infection cushions (17) similar to those described for *Thanatephorus cucumeris* (7). Otherwise the development of *S. trifoliorum* within the host tissue is reportedly the same as that of *S. sclerotiorum*, including the indiscriminate inter- and intracellular penetration by hyphae (3, 17).

In this report we provide a more definitive account of the early stages of infection by *S. sclerotiorum* than is presently available, and define more clearly the various histologic stages of development of the fungus during the course of pathogenesis. A preliminary report has been published (13).

MATERIALS AND METHODS.—Two isolates of *S. sclerotiorum* were used in this investigation. Isolate Ss-3, from a diseased bean plant in Maryland (14), is the large-sclerotium form, or *S. sclerotiorum* "major" (18). This isolate was used for most of this investigation. Isolate Ss-13, from an infected lettuce plant in New Jersey, is the small sclerotium form, or *S. sclerotiorum* "minor" (18). It was used mainly for comparative purposes.

Inoculum was prepared from these isolates, with the use of autoclaved oat kernels (50 g oats: 50 g water) to which mycelial fragments of *S. sclerotiorum* in 10 ml water were added and incubated 4 to 5 days at 24 C.

Infested oat kernels were placed at the base of

young bean (*Phaseolus vulgaris* L. 'Topcrop') hypocotyls and incubated in a humid environment as previously described (11). Hypocotyls were harvested 6 hr to 7 days after inoculation. They were rated with a disease index (D.I.) from 0 to 5 (14), where 0 indicated healthy tissue and 5 indicated complete invasion of the tissue, with the hypocotyl collapsed and dried.

At various times after inoculation, hypocotyls were harvested and placed in chloroform until they

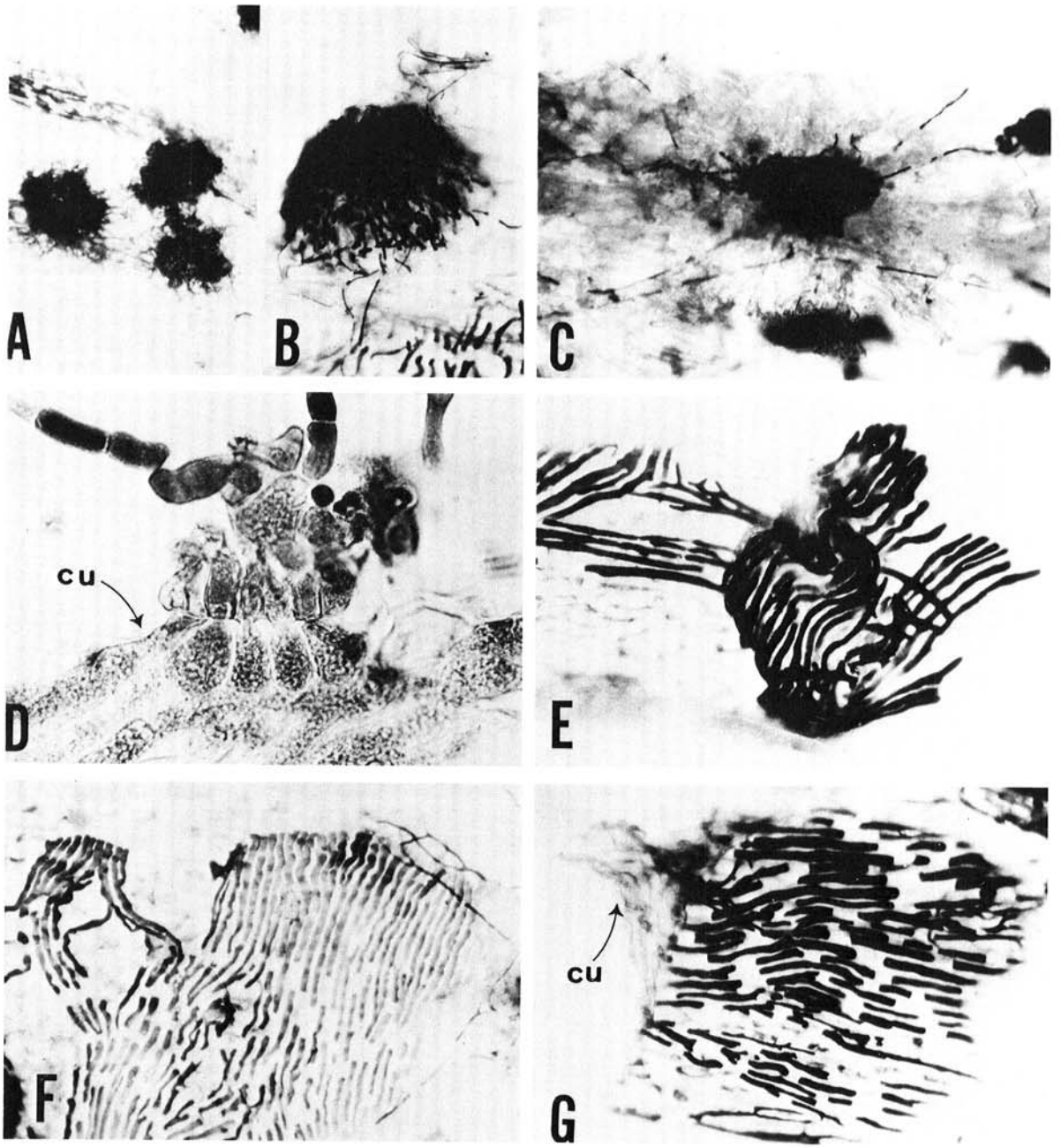

Fig. 1. (A-G). Penetration and early stages of infection of bean hypocotyls by *Sclerotinia sclerotiorum* (Ss-3). **A, B)** Infection cushions stained with aniline blue-lactophenol, × 125 and × 380, respectively; **C)** infection cushion and surface hyphae stained with aniline blue-lactophenol with nonstained radiating hyphae beneath the intact cuticle, × 220; **D)** longitudinal section (× 1,890) of cushion stained with safranin-fast green, cu = cuticle; **E, F)** organized subcuticular hyphae stained with aniline blue-lactophenol, × 380; **G)** subcuticular hyphae stained with aniline blue-lactophenol only where the cuticle (cu) has been pulled up, × 380.

were examined microscopically with incident-light. Small areas of cuticle were removed by the chloroform soak, but in order to examine large areas of subcuticular hypocotyl tissue, a modification of a method for removal of cuticle (10) was employed. Repeated treatment with boiling ammonium oxalate-oxalic acid solution (1.6%:0.4%), or refluxing with chloroform in a Soxhlet extractor, usually loosened large enough areas of hypocotyl cuticle for examination. Aniline blue-lactophenol solution (0.02 g aniline blue in 11.2 ml 88% phenol, 10 ml glycerin, 10 ml lactic acid, and 8.8 ml distilled water) stained the mycelia in the subcuticular and upper cortical areas an intense blue, whereas the plant cuticle and underlying tissues remained relatively clear.

Infected hypocotyl tissue was also examined microscopically after sectioning. Hypocotyl pieces 0.5 cm long were killed and fixed in formalin-acetic acid-alcohol, dehydrated with tertiary-butyl alcohol and embedded in Histowax (Matheson, Coleman and Bell, mp 53 to 55 C) (9). Longitudinal and cross sections (10-μm thick) were stained with safranin-fast green (9).

Anatomical features of the bean plant were identified from the work of Doutt (6).

RESULTS.—*Penetration and initial infection.*—Mycelia from oat-kernel inoculum formed finger-shaped infection cushion initials at random on the surface of bean hypocotyls within 6 hr after inoculation. These initials developed into dome-shaped infection cushions (Fig. 1-A, B, C) composed of three distinct types of hyphae (Fig. 1-D): (i) dark red-stained hyphae near the top of the cushions; (ii) inflated, granular, light red-stained hyphae in the center of the cushion; and (iii) dichotomously branched penetration hyphae that stained approximately the same color as the hyphae in the interior of the cushion. The walls of the penetration hyphae, however, were often stained brown, especially next to the cuticle of the invaded plant. The penetration hyphae formed infection pegs that penetrated the cuticle and developed into swollen, light red-stained, granular vesicles above the epidermal cells. Considerable force is apparently exerted during penetration, since the infection cushions often appeared to pinch the surrounding suscept tissue into a slightly convex mound and the cuticle itself was pushed inward at the point of penetration.

Large, granular "infection" hyphae extended from the vesicles and grew radially from the infection cushion (Fig. 1-C) between the cuticle and the epidermal cells. These subcuticular hyphae were oriented parallel to one another and formed an organized infection front beneath the cuticle (Fig. 1-C, E, F, G). This organization was striking and a certain amount of coherence among hyphae was apparent (Fig. 1-E). Hyphal tips were sometimes lobed. Branching of infection hypha occurred with the formation of fan-shaped infection fronts.

Cortical tissue was also invaded by the infection hyphae developing from the infection cushions. All movement of infection hyphae in the cortex was intercellular.

Development of the lesion at advancing margins.—After the initial 12 to 24 hr of infection, the radial hyphal fronts appeared to break up into clusters of 8 to 20 hyphae (Fig. 1-F). These clusters became oriented parallel to the hypocotyl axis and developed rapidly up the hypocotyl. Upward growth was more rapid than downward or in a transverse direction. This often resulted in peak-shaped advancing margins of the lesions.

Longitudinal and cross sections of hypocotyls revealed that the swollen subcuticular, infection hyphae (Fig. 2-E) continued to maintain an infection front beneath the cuticle (Fig. 2-A, B) as the infection progressed up the hypocotyl. These subcuticular hyphae grew well in advance of the infecting hyphae in the cortex. However, subcuticular growth was usually on one side of the hypocotyl and generally on the side of initial infection. Complete girdling of the hypocotyl by the pathogen occurred through cortical invasion of the remaining tissue. The cortical infection hyphae grew more rapidly in the deep cortical tissue than near the epidermis (Fig. 2-C, D) and strictly in an intercellular manner (Fig. 2-F).

Incident-light microscopic examination of fresh lesions (X 40) enabled close examination of the extensive water-soaking of tissues. Areas containing subcuticular infection hyphae were blistered and filled with copious fluid beneath the cuticle. The cuticle surface was ridged and roughly adhered to the contour of the underlying infection hyphae. The margin of the blister, which corresponded to the macroscopically visible margin of the lesion, was slightly in advance of the tips of the subcuticular hyphae. An average distance of 10.0 μm was recorded, with a range of 2.5 to 24.9 μm in 100 observations. Attempts to delineate the margin in fresh tissue by marking it with a small cut, then fixing and sectioning the tissue for examination, were not successful. Although the cut was usually close to the tips of the advancing mycelia, measurements were not definitive.

Water-soaked areas containing underlying cortical infection hyphae were not blistered. Moreover, the tissue was slightly more firm than near subcuticular hyphae, and fluids were not as copious.

Mature portions of lesions.—Striking variations in cortical hyphal size and in intensity of staining with safranin were evident in hyphae in older parts of lesions when compared with the subcuticular hyphae and with hyphae on the surface of the suscept tissue (Fig. 3). Hyphal measurements tended to group around one or more distinct modes. Hyphae on the surface of lesions at the 24- and 72-hr harvests ranged in size from 3.4 to 17.0 μm (Fig. 3-A). A distinct mode at 8.5 μm was evident, at which point 30% of the hyphal measurements were present. These hyphae usually stained an intense red with safranin. Hyphae at the margin of fresh, actively growing 3-day-old cultures of *S. sclerotiorum* on potato dextrose agar had about the same range in size as those on the host surface (Fig. 3-B). There also was a predominance of

hyphae (23%) at a mode of 8.5 μm. Hyphae in the center of fresh cultures (Fig. 3-B), and in 8-day-old cultures, also varied considerably in size but had a large percentage (29%) of small-diameter hyphae at a mode of 3.4 μm.

Infection hyphae beneath the cuticle (subcuticular hyphae) at the margin of lesions ranged in diameter from 8.5 to 27.2 μm for 24-hr lesions and from 11.9 to 34.0 μm for 48-hr lesions (Fig. 3-C). Distinct modes at 17.0 μm were prominant for hyphal diameters with both ages of lesions. Hyphae at this mode apparently increased in diameter with the age of the lesion (Fig. 3-D). This was reflected by a major shift in this predominant mode in the margins of 24-

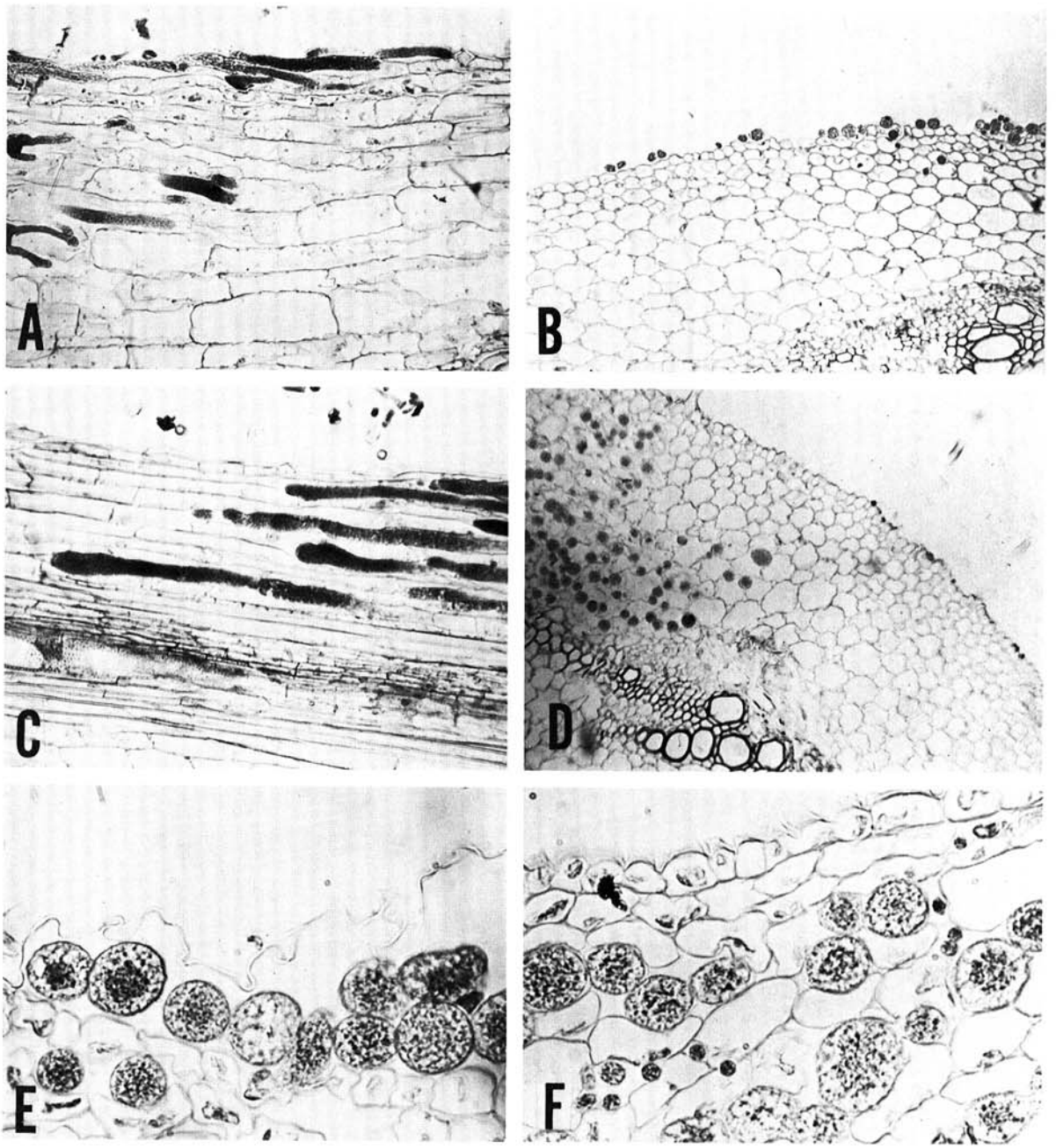

Fig. 2. (A-F). Development of infection hyphae of *Sclerotinia sclerotiorum* (Ss-3) in bean hypocotyl tissue stained with safranin-fast green. **A, B)** Subcuticular hyphae at margin of lesion in advance of cortical hyphae, ×325; **C, D)** cortical hyphae at margin of lesion in advance of subcuticular hyphae, ×325; **E)** large, granular infection hyphae beneath raised cuticle, ×1,250; **F)** large, granular, intercellular infection hyphae and small ramifying hyphae in cortex, ×1,250.

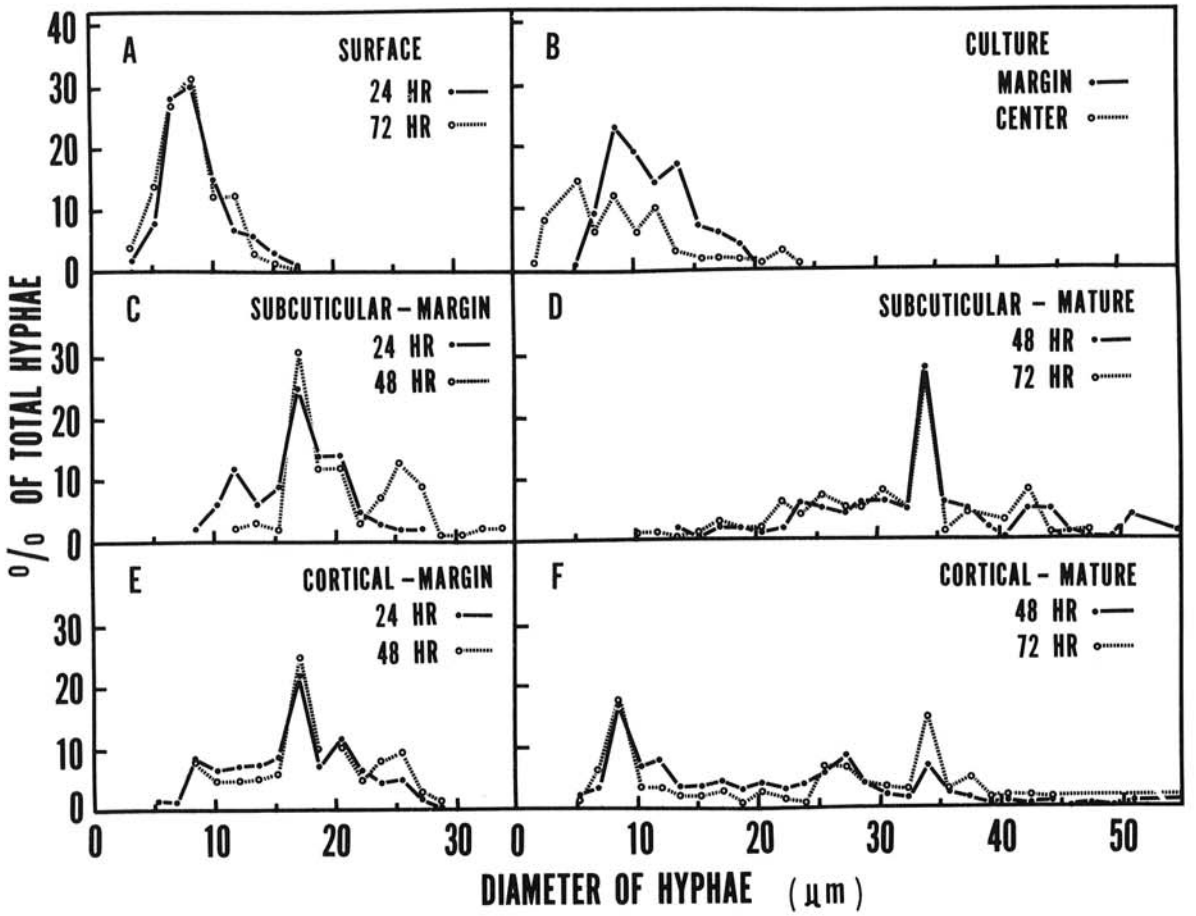

Fig. 3. (A-F). Diameter of hyphae of *Sclerotinia sclerotiorum* in culture and in infected bean hypocotyls. The ordinate expresses the percent of the total hyphae in 100 observations made at each location and each time period. **A)** Diameter of hyphae stained with safranin-fast green and located on the surface of hypocotyls harvested 24- (D.I.= 1) and 72-hr (D.I.= 3) after inoculation; **B)** diameter of hyphae in fresh 3-day-old potato dextrose agar cultures of *S. sclerotiorum* at the margin and near the center of the colony; **C)** diameter of stained hyphae located beneath the cuticle of hypocotyls (subcuticular) at the margin of 24- and 48-hr-old (D.I.= 2) lesions; **D)** same as above but located in the mature portion of 48- and 72-hr-old lesions; **E)** diameter of stained hyphae located near the margin of the lesion in the cortex (cortical) of hypocotyls harvested 24 and 72 hr after inoculation; **F)** same as above except located in the mature portion of 48- and 72-hr-old lesions. D.I.= disease index where 1= 1 cm lesion; 2= 2-4 cm lesion; 3= lesion on 3/4 of hypocotyl up to the cotyledonary scar.

and 48-hr-old lesions (25 and 31% occurrence of hyphae, respectively) to a predominant mode at 34 µm for subcuticular hyphae in mature portions of 48- and 72-hr-old lesions (29% occurrence of hyphae). In addition, hyphal diameters increased in range over those in lesion margins. Mature portions of 48-hr-old lesions contained hyphae with diameters ranging from 11.9 to 55.0 µm and hyphae in 72-hr-old lesions ranged from 10.2 to 47.6 µm. Mature portions of these lesions were located at the base of the hypocotyls near the points of inoculation.

As with those of subcuticular hyphae, measurements of diameters of cortical hyphae in 24- and 48-hr-old lesions also fell into distinct modes (Fig. 3-E). A mode at 8.5 µm reflected the presence of small, deep red-stained hyphae similar in size to the surface hyphae. These small hyphae were even more abundant in maturing parts of lesions (48 and 72 hr, cortical-mature) (Fig. 3-F). A second mode of 17.0 µm was present in measurements near the margin of cortical lesions (Fig. 3-E), and corresponded to the data from the light red-stained, inflated, subcuticular infection hyphae (Fig. 3-C). These too appeared to increase in size in maturing lesions (48 and 72 hr, cortical-mature) (Fig. 3-F). A third mode of 34 µm was observed with cortical hyphae, but only in maturing parts of 48- and 72-hr lesions. This mode corresponded to subcuticular measurements made at the same harvest times (Fig. 3-D). The range in hyphal size in the mature portions of the cortical lesions was from 5.1 to 64.6 µm.

The small-diameter hyphae (mode= 8.5 µm) in the cortex of hypocotyls originated from branching of the large infection hyphae (mode= 17.0 µm) (Fig.

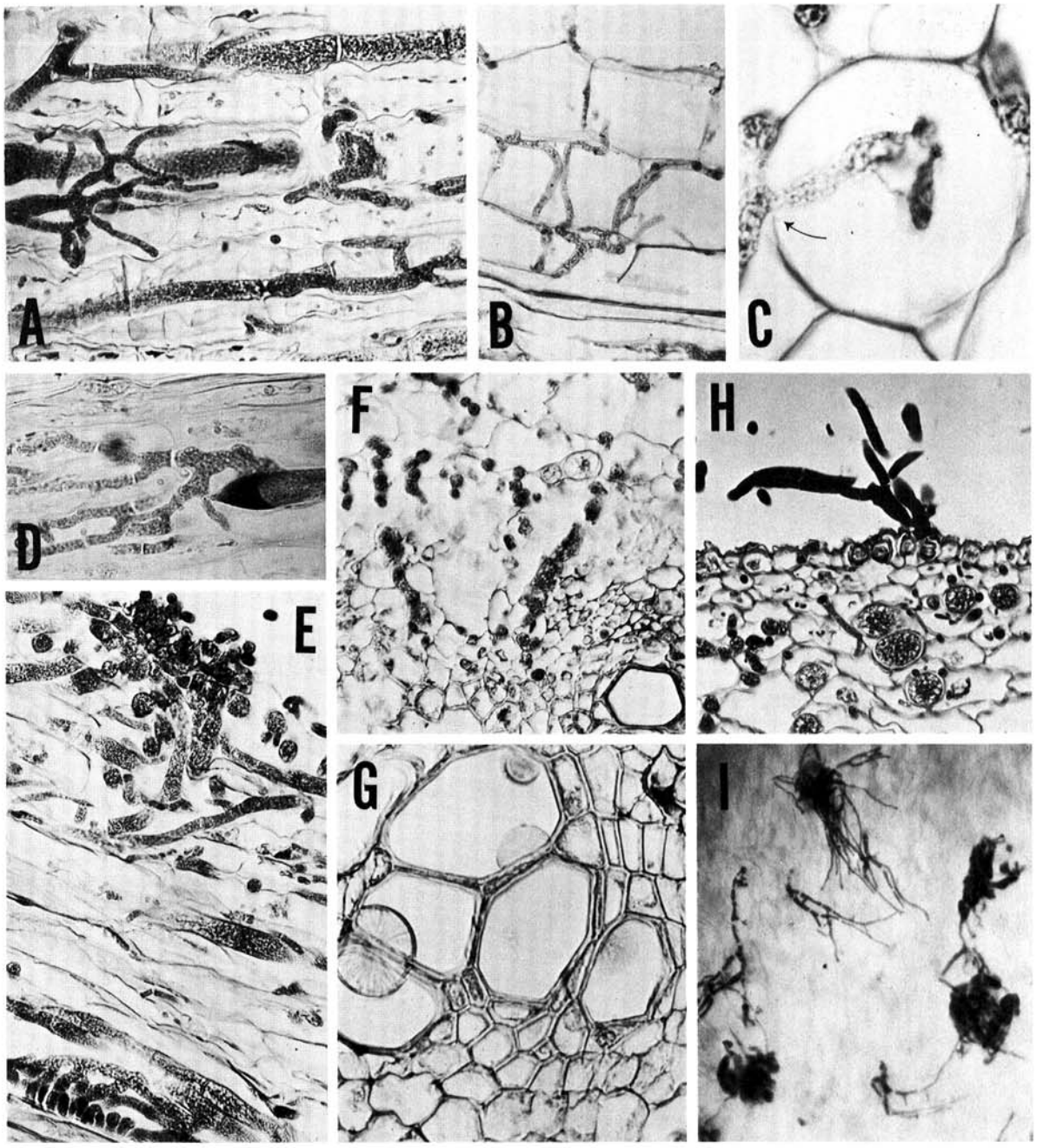

Fig. 4. (A-I). Sections of maturing lesions in bean hypocotyls infected with *Sclerotinia sclerotiorum*, and stained with safranin-fast green. **A)** Longitudinal section of cortex with ramification and branching of small hyphae of isolate Ss-3, × 800; **B)** intracellular hyphae (Ss-3) in pith cells, ×800; **C)** intracellular hyphae (Ss-3) in cortical cell, ×3,120 (oil); **D)** ramifying hypha branching from infection hypha (Ss-3), ×900; **E)** structure invading phloem tissue (lower left) resembling infection cushion (upper right), ×900, isolate Ss-3; **F)** hyphal clusters (Ss-3) formed at resistant xylem and phloem tissues, ×900; **G)** crystals in xylem cells, ×1,250; **H)** hyphal tuft (Ss-3) emerging through stomate, ×900; **I)** sclerotial initial on the surface of the hypocotyl (Ss-13), ×380.

4-D). The branching occurred 12 to 24 hr after penetration and 54.5 μm (range 29.0 to 85.0 μm) behind the advancing infection hyphae. These "ramifying" hyphae often branched profusely (Fig. 4-A) and therefore were capable of extensive colonization of the tissue already invaded by the infection hyphae. Movement in the tissue was largely intercellular, but also intracellular (Fig. 4-B, C).

When approaching phloem and xylem tissues, the inflated infection hyphae concentrated in the intercellular spaces next to these tissues (Fig. 4-F). At this barrier point, structures were often formed that in longitudinal section resembled infection cushions, with numerous dichotomously branched hyphae (Fig. 4-E, upper right–infection cushion; lower left–barrier structure). This clumping of hyphae at barriers occurred early in infection–often as early as 24 hr after inoculation, near the site of initial infection.

In spite of early attempts to penetrate vascular tissues, invasion of the xylem did not usually occur until 3 days after inoculation (D.I.= 3.0), and pith tissue was usually not invaded until even later in pathogenesis (D.I.= 4.0 to 5.0).

Before the xylem was invaded by hyphae, numerous crescent-shaped crystals formed on the walls of the lumen of the xylem cells (Fig. 3-G). These crystals often occluded the xylem vessels. They were formed abundantly also in the cortical and epidermal tissues, but these were smaller than in the xylem. Crystals were often found in noninvaded tissues in advance of lesions but not in comparable noninoculated healthy tissue.

After extensive colonization of the tissue (D.I.=3.0 to 4.0), ramifying hyphae emerged from the suscept and formed mycelial wefts and eventually cottony growth on the surface of these mature lesions. Often clumps of short, barrel-shaped cells were formed, which were sclerotial initials (Fig. 3-I), that gave rise to mature sclerotia in 3 to 7 days after inoculation if the remaining food base was adequate.

Close examination of the surfacing hyphae revealed that they exited only through stomata (Fig. 3-H) or tears in the cuticle. The cuticle, even in advanced stages of pathogenesis, was an effective barrier to hyphae exiting the tissue. In several instances, hyphae advanced through the cortex and epidermis only to be diverted by the cuticle. Hyphae were often able to penetrate into the lumen of trichome basal cells, but could not break the cuticle barrier. The imperviousness of the cuticle was also indicated by the failure of the aniline blue-lactophenol stain to penetrate it. Figure 1-C shows unstained subcuticular hyphae beneath a stained infection cushion, and Fig. 1-G shows stained subcuticular hyphae only in the area where the cuticle was pulled away or dissolved by the chemical treatment.

Comparison between isolate Ss-3 and Ss-13.–Isolate Ss-3 and isolate Ss-13 are dissimilar morphologically. Nevertheless, the two isolates behaved similarly during pathogenesis in bean hypocotyls.

Major differences between the two isolates were few: (i) Ss-13 was somewhat slower to infect and invade tissue than Ss-3; and (ii) the small sclerotia of Ss-13 formed more abundantly on the surface of infected hypocotyls than the large sclerotia of Ss-3. In general the same sequence of events occurred with both isolates during the course of disease development.

DISCUSSION.–*Sclerotinia sclerotiorum* requires a source of exogenous nutrients in order to infect plant tissues (16, 19, 20, 21). In spite of this primitive characteristic, the pathogen has developed an effective means of breaching the cuticle barrier through production of infection cushions. The cushions are similar in appearance to those of *T. cucumeris* (7), and probably identical to those of *S. trifoliorum* (17). The cushions have considerable organization, with three types of hyphae composing the structure (Fig. 1-D). Presumably the dome=shape of the cushion and the differentiated hyphae enable the infection pegs to forceably penetrate the cuticle. The effectiveness of the cuticle in resisting penetration is emphasized by the fact that even hyphae in mature parts of invaded tissue are unable to penetrate the still–intact cuticle, but exit the suscept tissue through stomata. In addition, aniline blue=lactophenol could not penetrate the intact cuticle to stain the underlying mycelia. The resistance of the cuticle to penetration even this late in pathogenesis supports the contention that *S. sclerotiorum* penetrates the cuticle mechanically, and not by enzymatic dissolution (1, 2, 17). The cushions apparently form, at least partially, in response to the physical contact offered by the cuticle (19). This is evident from the fact that other tissues, namely the phloem and xylem, also elicit the formation of similar penetration structures (Fig. 3-E, F).

Once ingress into the suscept is accomplished, another seemingly specialized type of hypha becomes apparent. Inflated, granular, lightly stained vesicles form immediately beneath the cuticle, but above the epidermal cell layer. These vesicles are undoubtedly identical to those described by Purdy (19). The vesicles are focal points for the rapid invasion of tissue by the similar, greatly inflated infection hyphae. The infection hyphae, too, are specialized, in that their growth is strictly intercellular and more rapid in the subcuticular area than in the underlying cortex. Perhaps the subcuticular region offers less resistance to penetration by the hyphae, thus accounting for rapid, aggressive advance up the hypocotyl. Moreover, the organized infection front and the orientation of hyphae parallel to the long axis of the hypocotyl also probably contribute to the rapidity of lesion development by *S. sclerotiorum*.

In contrast to the distinctive infection hyphae that initiate the infection process, the much smaller, intensely staining ramifying hyphae appear to have a secondary role in pathogenesis. These hyphae originate as branches from the infection hyphae ca. 55 μm from the apices of the latter, and ramify in the tissue both inter- and intracellularly.

The distinct morphological difference between these two types of hyphae suggests the possibility of distinct physiological differences. Perhaps the infection hyphae are capable of synthesizing enzymatic metabolites essential for softening the middle lamella to allow rapid movement through tissue, and toxic metabolites to weaken or kill cells in advance. *Sclerotinia sclerotiorum* produces in diseased tissue abundant endopolygalacturonase (8,

12), which would fit into the former category, and oxalic acid (14) into the latter. The ramifying hyphae may have a nutritional role in pathogenesis, possibly producing cellulolytic or other cell wall-degrading enzymes that provide nutrients essential for the intensive metabolic activity of the fungus as infection hyphae move rapidly through the tissue. A secondary or nutritional role for these hyphae in pathogenesis is suggested by their appearance after infection has been established (12 to 24 hr after initial infection), and their ability to develop in tissues intracellularly. Infection hyphae do not have this capability.

The work of Maxwell et al. (15), also with isolate Ss-3, revealed cytological differences between regions of hyphae and suggested physiological differences between these regions. Light and electron microscopy showed abundant lipid bodies in the first 80 μm of the hyphal tip. In contrast, microbodies with crystal inclusions were most abundant 80 to 160 μm from the apex. The hyphal tips used by Maxwell et al. (15), produced in culture, may not be the same type as the infection hyphae present in infected tissue. Nevertheless, a difference in diameter (Fig. 4-B) between hyphae at the margin of the colony and those in older parts, also suggests differences in morphology and perhaps physiology of hyphae in culture. The host tissue may influence the overall diameter of the hyphae and account for the difference between a modal diameter of 8.5 μm at the margin of colonies vs. 17.0 μm for infection hyphae at lesion margins. Regardless of the relationship between hyphae produced in vitro and in vivo, it is interesting that two apparent types of specialized hyphae are produced by *S. sclerotiorum* in diseased tissue that perhaps also possess physiological specialization. This possibility needs further investigation.

Although only two isolates of *S. sclerotiorum* were used in this study, the similarity in histological pathology between the *S. sclerotiorum* 'major' isolate and the *S. sclerotiorum* 'minor' isolate, two morphologically distinct isolates, was striking when compared on the same host species.

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