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A Histopathological Study of *Rhizoctonia solani* Kuhn Infection of Resistant and Susceptible Lines of Lima Bean (*Phaseolus limensis* Macf.)

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A HISTOPATHOLOGICAL STUDY OF *RHIZOCTONIA SOLANI* KUHN INFECTION OF RESISTANT AND SUSCEPTIBLE LINES OF LIMA BEAN (*PHASEOLUS LIMENSIS* Macf.)

by

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ABSTRACT

A Histopathological Study of Rhizoctonia solani Kühn Infection

of Resistant and Susceptible Lines of Lima Bean

(Phaseolus limensis Macf.)

by

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The effects of Rhizoctonia solani on the hypocotyls of resistant and susceptible lines of lima bean were studied. The fungus attacks lima bean at one or more stages during host development and causes pre-and-postemergence damping-off, root rot, and foliage blight. The isolate of the fungus used in this study was obtained from infected radishes grown in Salt Lake County, Utah.

The formation of infection cushions and modes of penetration by this fungus was no different on the resistant and susceptible lima bean hypocotyls. The infection process was studied under laboratory conditions. Differential staining showed that the fungal hyphae were closely appressed to the host surface, and at first along the longitudinal axis of the epidermal cells. Later, hyphal branches grew in oblique and transverse directions. Aggregated hyphal tips formed infection cushions, which gave rise to one or more infection pegs

that penetrated the host directly. Following penetration, both intercellular and intracellular hyphae were formed. The build-up of masses of hyphae occurred as club-shaped structures in the cortex of the susceptible lima bean hypocotyls, but were not observed in the resistant hypocotyls. The extents of infection in the resistant and susceptible lima bean hypocotyls were different at 7 days after inoculation. The hyphae in the susceptible hypocotyl had penetrated into vascular bundles and pith, whereas the hyphae had penetrated the cortex and pith of the resistant hypocotyl but were not observed in the vascular tissue.

Stomatal penetration from individual hyphal side branches was rare and apparently of minor importance as a means of penetration by this isolate.

(65 pages)

INTRODUCTION

Rhizoctonia solani Kühn is one of the most widely distributed soil-inhabiting pathogenic fungi. The increasing interest in diseases caused by this fungus and its extremely wide host range testify to its economic importance. This fungus attack the host at one or more stages of development with the result that more than one distinct phase of the disease have been described on a single host species. Four phases of the common Rhizoctonia disease are (1) pre- and post-emergence damping-off, (2) stem and root rot, (3) storage-organ decay, and (4) foliage blight or spot. The fungus, Rhizoctonia solani, causes stem and root rot, foliage blight or spot, and also incites severe pre- and post-emergence damping-off of lima bean.

Symptoms on lima bean, Phaseolus limensis Macf., caused by R. solani are expressed at two stages of plant growth. The highest incidence of Rhizoctonia disease occurs when the seedlings are at an early, and probably the most susceptible stage of development, immediately after germination. On the emerged seedings, the fungus attacks the subterranean part of the hypocotyl, causing brown, deeply sunken lesions. There is no abnormal appearance on the above ground portions at the early stage of infection. The infection develops until the brown discoloration of collapsed

tissues becomes apparent. After a period of time, the bean plants suddenly wilt and die. Infected beans grown in the greenhouse die faster than similar plants in the field, due to the conditions in the greenhouse favoring the pathogen more than the host.

Generally, when microorganisms are introduced into plants, the microorganisms die, and the wound through which they were introduced heals, Resistance of plants to microorganisms is the rule and susceptibility is the exception. Many terms have been used to describe and interpret various aspects of disease resistance. The interaction between a host and pathogen has been divided into three consecutive phases which although interdependent are physically separated by the host surface, namely, growth of the pathogen prior to penetration, penetration, and growth within the host. It is possible that in many interactions, resistance or susceptibility is determined at an early stage, prior to or at penetration, rather than at a later stage in pathogenesis.

In order to gain entry, the pathogen must first reach the host and develop active growth on its surface. The pathogen may eventually enter the host in one of three ways, directly through the intact surface, through natural openings, or through wounds. The penetration of host tissues by microorganisms is a complex process that is not well understood. The mode of penetration and establishment of physiological contact with the host can be highly specific with the formation of structures such as appressoria,

infection cushions, infection pegs, haustoria, and primary and secondary hyphae.

Many workers have studied the infection by Rhizoctonia solani on many different parts of plants. The hypocotyl is usually considered to be the primary and principal infection site of this fungus. It may be injured even when the fungus hyphae do not parasitize the tissues.

A lima bean breeding program at the Utah Agricultural Experiment Station has produced breeding lines of lima beans that differ in their susceptibility to R. solani. Resistance to the stem and root rot diseases induced by R. solani is controlled by a single dominant gene, but the nature of resistance has not heretofore been determined.

Therefore, the objectives of this study were i) to determine the formation of infection cushions by Rhizoctonia solani on the resistant and susceptible lima bean hypocotyls, ii) to study the mode of penetration by this fungus on both lines of lima bean, iii) to determine the development of Rhizoctonia solani within the hypocotyl tissues, and iv) to study the histopathology of the infected bean hypocotyl tissues.

REVIEW OF LITERATURE

Rhizoctonia disease on bean plants may be observed at almost any stage of plant growth. The first record of Rhizoctonia injury to bean seedlings was given by Atkinson (1892). He reported that the pathogen, Rhizoctonia solani, attacked the bean seedlings causing pre- and post-emergence damping-off. On the emerged seedlings, the fungus attacked the subterranean part of the hypocotyl causing brown deeply sunken lesions.

Bateman and Lumsden (1965) reported that the hypocotyl tissue of "Red Kidney" bean was highly susceptible to Rhizoctonia solani during the first two weeks of plant growth and then became resistant with age. They further stated that the changes in resistance were associated with elongation and maturation of the hypocotyl and concomitant changes in the pectic substances and calcium content.

Bateman (1964a) has presented evidence that the conversion of pectins to calcium pectate, a material resistant to attack by the fungal enzyme, polygalacturonase, around the lesion areas may contribute to the limitation of lesion growth and the lack of progressive invasion of the suscept by the pathogen. The young and growing tissues of seedlings contain large amount of pectic substances, and the main enzymatic

process operating during initial invasion of tissues is apparently the degradation of pectic substances by the invading fungi.

Matsumoto (1921), Barker and Walker (1962), Garret (1962), and Bateman (1963, 1964b) agree that Rhizoctonia solani produces both pectinolytic and cellulolytic enzymes which play an important role in pathogenesis by this pathogen. Mahadevan et al. (1965), stated that neither pectinolylic nor cellulolytic activity was detected in hypocotyls of inoculated resistant plants.

Many papers have been published dealing with the nature of resistance of plants to Rhizoctonia disease, and several extensive reviews have been complied. Walker and Stahmann (1955), Akai (1959), Müller (1959), and Tomiyama (1963) indicated that in various diseases there was no difference in the manner of penetration of epidermis between susceptible and resistant varieties. But the difference is the interactions between host cells and the invading pathogen after penetration.

Walker (1959) stated that it is entirely possible for the host to carry the gene for high resistance but its expression may be completely or partially inhibited by another gene, or may be prevented entirely by the absence of an essential epistatic gene.

Steinswat (1966), and Steinswat et al. (1967), have found that resistance in lima bean to Rhizoctonia solani was controlled by a single dominant gene.

Hadwiger and Schwochau (1969) proposed that the dominant host genes for resistance represent a genetic potential for altering or disorganizing host metabolism in a manner that is detrimental to the symbiosis essential to the susceptible reaction. They further proposed that the corresponding genes of the pathogen that determines avirulence are expressed through the production of specific inducers which activate the genes for resistance of the host.

Recent works by Müller (1961), Cruickshank (1963), and Tomiyama (1963, 1968) have dealt extensively with toxic materials produced at the site of infection in response to infection. The possible presence of toxic materials within the host as fungus inhibitors has been explored in numerous cases. The term "phytoalexins" was given to such compounds by Müller (1958). He postulated that phytoalexins play an important role in disease resistance.

Pierre (1966), and Pierre and Bateman (1967) indicated that bean lines responded to inoculation with Rhizoctonia solani by producing substances, two of which were found ot inhibit germination and growth of fungus. One of these substances is phaseollin which previously had been identified by Cruickshank (1963). They revealed that greater quantities of both inhibitory substances were induced in resistant than in susceptible lines.

Biehn (1968) found that an accumulation of several phenolic compounds is associated with the hypersensitive reaction produced on hypocotyls of etiolated seedlings of soybean, Glycine max; snap bean, Phaseolus vulgaris; and lima bean. Phaseolus limensis; after penetration by Helminthosporium carbonum.

Deverall and Wood (1961) indicated that the phenoic metabolism of diseased host tissues may produce toxic materials which may inhibit pectic enzymes secreted in host tissues by pathogenic fungi. Müller (1958) concluded that the basic response that occurs in resistant and susceptible hosts is similar but the basic difference between them is the speed of the formation of phytoalexins.

Various workers have reported on one or another of the sequential stages of the infection process of R. solani on several of its numerous hosts. Allen (1959) stated that the infection caused by this fungus required a characteristic organization of hyphae on the surface of the host epidermis which is called "infection cushion."

Nakayama (1941) reported in his study of infection of cotton seedlings by R. solani that the formation of infection cushions is its principal means for hypocotyl penetration. The entry is then accomplished by penetration at the site of such an infection cushion. This is in agreement with the findings of Townsend and Willetts (1954), Kerr (1956), Kerr and Flentje (1957), Flentje (1957), Khadga et al. (1963), and Dodman et al.

(1968a, 1968b). Descriptions of the mode of penetration from infection cushions developed by R. solani have been reported by Flentje (1957), Gonzales and Owen (1963), Khadga et al. (1963), Chi and Childers (1964) Van Etten et al. (1967), and Dodman et al. (1968a, 1968b).

Dodman et al. (1968a, 1968b) observed numerous fine infection pegs, developing at the base of the infection cushions on bean hypocotyls, penetrating the cuticle and then enlarging into the space between the cuticle and epidermal cell walls. Further penetration is then inter- or intracellular.

Flentje (1957), in his study of host penetration by R. solani, reported that the infection cushion occurred before any penetration. The penetration occurred 24-36 hr after the initiation of the infection cushion on the host surface by adhering to the host and forming an appressorium from which a very thin infection peg developed simultaneously from several hyphal tips and penetrated the epidermal cells. The infection pegs regained normal hyphal diameter immediately after they passed through the cell wall. These hyphae grew both within and between the host cells and were confined almost entirely to the cortex, having little extension into the vascular system. Collapse and breakdown of cell contents occurred at or just behind the advancing hyphal tips. The hyphae advanced both upward and downward leading to complete collapse of the seedlings.

Kerr (1956) and Kerr and Flentje (1957) investigated the theory of a contact stimulus initiating appressorial formation by using seedlings of radish, lettuce, and tomato grown in cellophane bags buried in soil inoculated separately with the various pathogenic strains of R. solani. Under those conditions hyphae of the strain pathogenic to each host aggregated on the cellophane immediately opposite the roots of the seedlings in a growth pattern very similar to that shown by appressoria, indicating a response to substances diffusing from the roots through the cellophane. Strains which were not pathogenic to the host showed no such response, growing evenly over the cellophane surface.

Martinson (1965) conducted an experiment that supported Kerr's. He observed that R. solani produced numerous infection cushions on all synthetic film surfaces continguous to germinating seeds or seedlings. The infection cushions adhered firmly to the films. The factor(s) from seeds or seedlings that stimulated infection cushion formation passed through only cellophane and nylon films.

Khadga et al. (1963) studied the infection of seedling cotton hypocotyls by R. solani and reported that infection cushions were formed by either a single hyphae; by an aggregation of short, stubby hyphal branches; by proliferation of hyphal branches; or by the aggregation of several hyphae. Christou (1962) believed that the combination of more than one hypha was necessary to initiate an infection cushion. Khadga et al. also observed that

R. solani penetrated the host cells directly either through epidermal cell walls or through intercellular spaces between epidermal cells by means of infection pegs that arose from swollen hyphal tips on the underside of infection cushions. They further reported that all tissues of cotton hypocotyls, except the xylem elements, were invaded. Cells in advance of invading hyphae showed granulation and were often discolored.

Similar observations were made by Boosalis (1950) on soybean, Christou (1962) on bean varieties Pinto and Small White, and Gonzales and Owen (1963) on tomato. They observed that within 48 hrs after contact with the fungus, the infected regions completely disintegrated. Penetration was favored by enlargement of glandular cells in cortical tissues, by presence of glandular hairs on the epidermis, and by epidermal injuries.

Penetration may also take place through stomata beneath infection cushions, and this sometimes may occur before cuticular penetration. Ullstrup (1936) described stomatal penetration by R. solani on leaves of china aster, sugar beets, and Begonia sp.

Christou (1962) observed stomatal penetration on bean hypocotyls. R. solani hyphae had apparently entered the stomatal openings, but formed no swollen appressorial structures and made no further growth after reaching the substomatal chamber. Dodman et al. (1968b) found penetration through stomata on both radish and bean by R. solani

hyphae. They reported that further penetration may occur both intra- and intercellularly.

Histological investigation of Rhizoctonia-infected tissues of bean hypocotyl by Christou (1962) showed that the pathogen, after infection, may spread through the tissues by growing intercellularly in the early phases of pathogenesis. Bateman (1963) stated that this type of hyphae development in the host tissues is undoubtedly facilitated by the secretion of pectic enzymes.

Barker and Walker (1962), and Christou (1962) suggested that intercellular growth of the hyphae in the host may be followed by intracellular penetration and growth. Collapse of the invaded cells results in small localized necrotic lesions.

Van Etten et al. (1967), revealed that the fungus penetrated bean hypocotyl in both inter- and intracellular manners during all phases of pathogenesis. Christou's isolate however, generally invaded epidermal and outer cortical cells in an intracellular manner, followed by intercellular penetration within the underlying cortical tissues during the early phase of pathogenesis. Extensive intracellular development and sclerotial formation occurred in the later phases of pathogenesis.

MATERIALS AND METHODS

The infection process of Rhizoctonia solani Kühn was studied on seedling hypocotyls of resistant and susceptible lines of lima bean (Phaseolus limensis Macf.). The isolate of R. solani used in this study was obtained from Dr. O. S. Cannon, Department of Plant Pathology, Utah State University. The study was made on greenhouse-grown inoculated lima bean hypocotyls.

Seeds were free of mechanical damage and infection. As a precaution against surface contamination, all seeds were treated for 10 minutes in 2:1 diluted solution of clorox (5.25 percent of sodium hypochlorite). After treatment, seeds were washed in distilled water several times and dried at room temperature (22-32 C).

Seeds were sown in vermiculite and maintained at room temperature. Five-to-seven-day-old healthy seedlings were removed from the vermiculite, surface-sterilized by dipping in 1:1000 mercuric chloride solution for a few seconds, and washed in distilled water. Care was taken at all times to minimize handling injury. Seedlings were, then, transferred to a 4-day-old culture of R. solani growing on potato-dextrose-agar in petri dishes. One or two seedlings were placed in

proximity to the fungus hyphae in the petri dishes and incubated at room temperature.

Two types of hypocotyl tissue samples were used for these studies:

1) The first sample was taken for immediate observations. Epidermal tissues in contact with the fungus for at least 36 hr were used to study fungal growth on the host surface, the formation of infection cushions, and the beginning of the infection process. Plant materials for this study were prepared in the following ways:

a. Epidermal strips were removed with a scalpel and forceps and placed on a microscope slide.

b. Sections of hypocotyl were split longitudinally, the internal tissues separated from the epidermis, so that the epidermal cells had their upper surfaces in contact with the slides.

All strips of fresh epidermal tissues were stained with aniline blue (lg/100ml of 50 percent ethyl alcohol) mixed in lactophenol. Sections stained in aniline blue for 2-3 min and then washed in 50 percent ethyl alcohol had better contrast between host and parasite than did materials stained with any other dyes. All fresh materials were mounted in Aman's lactophenol as described by Sass (1958).

c. Epidermal strips, with the fungal hyphae growing over the surface, were floated on water and agitated to determine the tenacity of the fungus and infection cushion formation.

2) The second sample was used for fixation and subsequent microtome sectioning. Portions of seedling hypocotyls, in contact with the fungus for at least 36 hr, were taken at appropriate intervals up to 120 hr or until the infected hypocotyls became completely disintegrated. Hypocotyl samples were cut into approximately 5 mm pieces and fixed in formalin acetic acid alcohol (FAA) for at least 36 hr. After fixation the sections were dehydrated in a tertiary butyl alcohol series (TBA) and embedded in paraffin as described by Sass (1958). Microtome sections were cut between 10-15 μ and affixed to microscope slides.

The sections on slides were passed through a regular xylo-alcohol series down to water, and stained with an squeous solution of safranin (0.5 g/100 ml of 50 percent ethyl alcohol) and fast-green (0.5 g/100 ml of 100 percent ehtyl alcohol). All sections were mounted in Canada balsam. Tissues bearing lesions at east stage of the three characteristic stages of maturation were examined microscopically.

RESULTS

The first symptom of lima bean hypocotyl infection was the appearance of a slight water-soaking of the affected area, quickly followed by a partial collapse of the hypocotyl. At high temperature, approximately 30 C, R. solani lesion development was rapid and passed through three characteristic phases of lesion maturation. These stages in lesion maturation were designated young, intermediate, and mature by Van Etten et al. (1967). Under the conditions used in this study, the young phase of lesion was usually evident within 24-36 hours after inoculation. It was characterized by a water-soaked appearance of the susceptible tissues, which remained colorless or turned slightly fawn in color and exhibited no microscopic evidence of tissue collapse.

Lesions at the intermediate stage differed from those in the young stage only in that the lesion surface had become brown to dark brown in color and in some instances there was slight microscopic evidence of tissue collapse within the lesion areas. This stage was reached within 48-60 hr after inoculation. Mature lesions were characterized by dry appearances of the lesion surfaces and the collapse of the invaded tissues. Lesions assumed the characteristic concave appearance and the surface coloration ranged from dark brown to brick red. Mature lesions were apparent with in 96-120 hr after inoculation.

The hyphae grew inside and outside the host tissues of both resistant and susceptible lima bean hypocotyls. No specific differences were observed as to the type of fungal growth that appeared in the lesions of various degree of lesion maturation. There was, however, considerably more cell disintegration in the older lesions.

Development of the fungal hyphae on the host surface

Examination of epidermal strips of fresh and fixed hypocotyls of both susceptible and resistant lima bean seedlings 5 and 7 days after inoculation, revealed that young hyphae were closely appressed to the epidermis and were generally growing along the hypocotyl following the longitudinal walls of continguous epidermal cells (Figure 1). Sometimes, these appressed hyphae changed their course and grew in an oblique or transverse direction over the epidermal cells.

In either case, after growing for a distance in this fashion, they produced side branches. The side branches produced by this fungus grew either oblique or at right angles to the present hyphae, across a few epidermal cells and then followed the longitudinal axis of the host cells. Branches arising from hyphae growing over the hypocotyl either continued to elongate or initiated infection cushions. Elongation was suppressed and branches appeared short, stubby, curved or coiled and closely septate (Figure 1).

Formation of infection cushions

The continued growth, branching, curling, and aggregation of side branches in the same area led to the formation of a discrete, tightly compacted infection cushion. Usually, cushions were formed by the aggregation of side branches from different elongating hyphae (Figure 2). These cushions developed parallel to and apparently closely appressed to the cuticle (Figure 3A-B), consisting of a roughly hemospherical mass of hyphae with the hyphal tips in contact with the cuticle. The hyphal tips, in contact with the host surface under the infection cushions, were swollen and slightly flattened on the ventral side. Infection cushions remained firmly attached to the host surface by these swollen hyphal tips. When epidermal strips, with the hyphae growing on the surface, were floated on water and agitated, the infection cushions, even though in an early stage of development and with no evidence of penetration, remained in place whereas other hyphae could be shaken loose from the epidermal cells. Infection cushions also remained attached during the rigor of the dehydration, infiltration, and staining processes. The infection cushions showed wide variation in shape and size, and covered 2-10 epidermal cells of the lima bean hypocotyl.

Figure 1. Initiation of infection cushion of Rhizoctonia solani from hyphae closely appressed to the surface of the epidermis of lima bean hypocotyl. The side branches are not always at a right angle, but tend to follow the longitudinal junctures between the epidermal cells. (Epidermal strip 66x)

Figure 2. Advanced stage of organization of infection cushion of Rhizoctonia solani on the epidermis of lima bean hypocotyl. (42x)

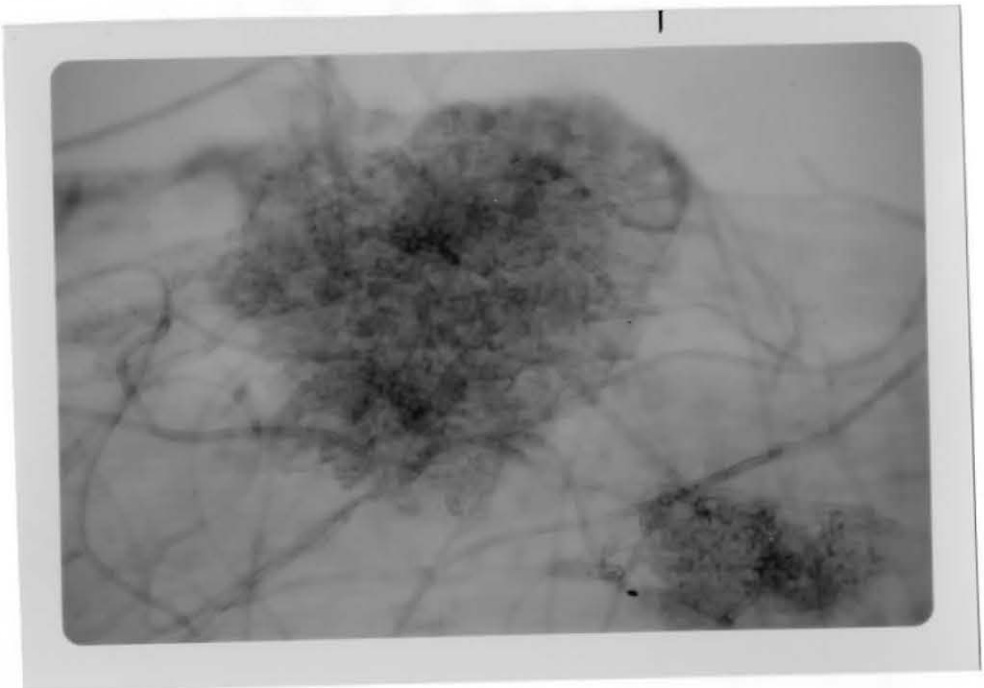
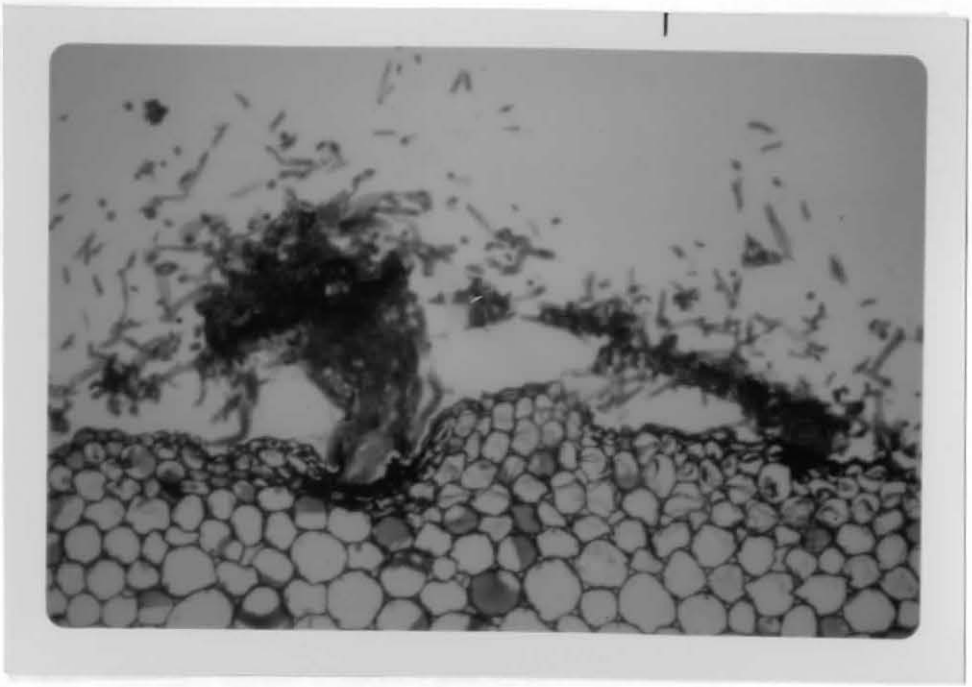
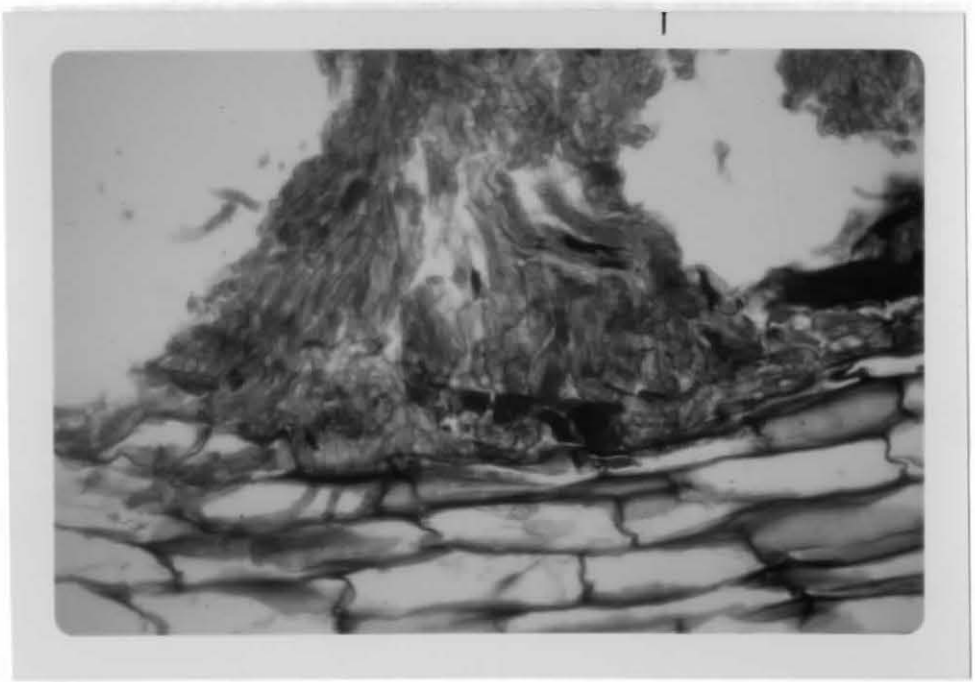


Figure 3. Transverse section (A), and longitudinal section (B) of lima bean hypocotyls through the center of a dome-shaped infection cushion of R. solani appressed to the host epidermis. Notice the numerous hyphae originating from it and invading the host tissues inter- and intracellularly into the cell lumen and across the cells. (42x, 84x)





Penetration of cuticle and epidermal cell walls by infection pegs from the swollen tips in the infection cushions

Following the formation of an infection cushion on both resistant and susceptible lima bean hypocotyls, the swollen hyphal tips produced slender peg-like structures which penetrated the cuticular layer covering the epidermis. In the next stage of the penetration process, a few thick hyphae grew from the outside of the infection cushion through the cuticle directly into an epidermal cell and finally emerged into the cell lumen (Figure 3B). The slender infection peg enlarged in the cell lumen and grew across the cell. After penetrating the opposite cell wall, it emerged in the intercellular space between the epidermal and cortidal cells (Figure 2), and resumed its normal diameter. In another instance, the hyphae pushed the cell walls out, but no sign of breaking was observed. From there, the fungus invaded neighboring cells; at the same time, an increased number of hyphae penetrated from the infection cushion through the epidermis. Usually, each epidermal cell was invaded by more than one infection peg under the same infection cushion.

When the infection cushion was established, a pronounced darkening of the region directly below was always observed. This discoloration involved the epidermal cells and a few layers of the cortex (Figure 3A). Curving of the epidermal cells toward the cortex (Figure 3A-B, 10), coincident with the penetration, also was evident.

Stomatal penetration

The hyphae commonly penetrated through stomata on bean. This, sometimes, occurred before cuticular penetration. The hyphal tips grew through the stomatal openings and developed intercullularly (Figure 5). No consistent attraction of the hyphae tips toward stomata was observed, and the influence of stomatal opertune on penetration was not determined.

Formation of primary hyphae within epidermal cells

As soon as the slender infection peg penetrated the epidermal cell walls and reached the cell lumen, it swelled and resumed normal hyphal diamter, giving rise to a so-called primary hypha. It continued growing in the lumen of the penetrated epidermal cells, forming a primary septum a short distance from its point of origin (Figure 4). Primary septa were consistently produced as essentailly the same distance from the point of origin by each primary hypha.

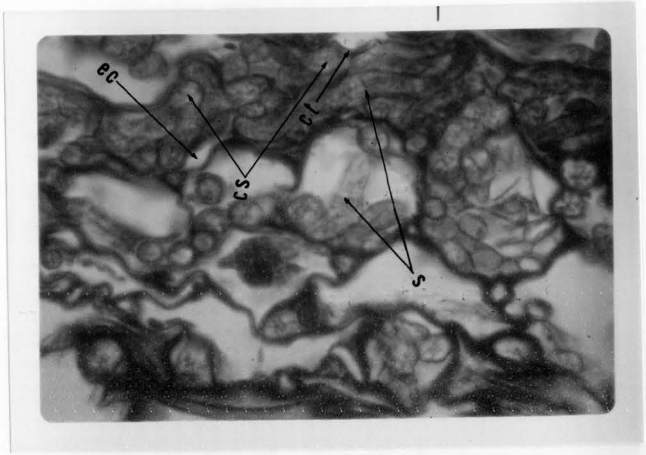
Penetration between epidermal cells

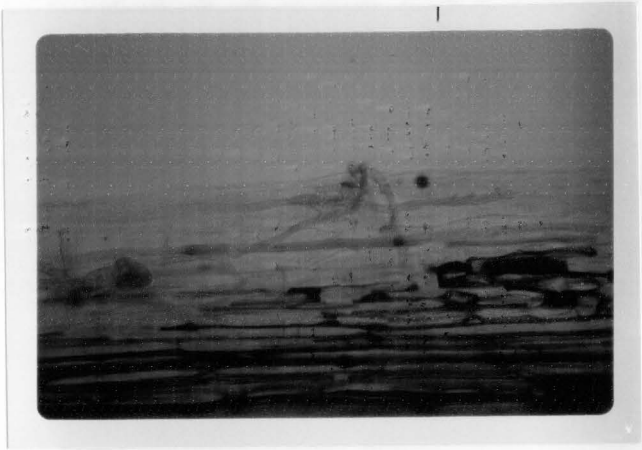
Occasionally, when cuticular penetration took place at the boundary between the epidermal cells, the primary hyphae formed under the cuticle by the infection pegs grew along the middle lamella. Finally, the contiguous epidermal cells were separated (Figure 4).

These infection processes were in early stages of pathogenesis. After the intial penetration, the fungus continued to send the infection pegs into the

Figure 4. Transverse section of lima bean hypocotyl in an advanced stage of infection by R. solani. The prominent primary hypha at the center apparently extends between the epidermal and cortical cells and shows the characteristic first septum(s) near its point of origin. In the epidermis and outer cortex, the hyphae usually are intracellular. Many are shown in cross section (cs), including those located between the lifted cuticle (ct) and the epidermal cells (ec). (264x)

Figure 5. Stomatal penetration by R. solani hyphal tip penetrate through stomatal opening on lima bean hypocotyl. (66x)


EC
CS
CT
S



host tissues. Some of these passed directly through the epidermal cells, whereas others entered through intercellular spaces produced by breaks in the epidermal layer. This intercellular infection caused the epidermal cells to spread apart (Figure 4), thus, allowing more invading hyphae to enter the host.

Cortical and vascular tissues invasion

Under the epidermal cells, the infecting hyphae tended to grow and branch in the space between the outer layer of the cortical cells and epidermis (Figure 4). This growth separated the epidermal cells from the cortical cells (Figure 4). Eventually, epidermal cells became completely disintegrated, allowing more hyphae to enter the host. Entrance of more hyphae and their subsequent branching disorganized and disintegrated the outer layers of cortical cells. Thus, the intercellular spaces in this region of the cortex provided an easy entrance for additional invading hyphae (Figure 6).

In all types of infection, vigorous hyphae rapidly invaded the epidermal cells and the first few layers of cortical cells. Growth was mostly intercellular by fascicles of hyphae growing in the longitudinal intercellular spaces of both resistant (Figure 7) and susceptible (Figure 8) lima bean hypocotyls. Some hyphae were found growing intracellularly in these regions (Figures 6, 7, 8). These hyphae were sometimes so numerous that they caused

Figure 6. Transverse section of lima bean hypocotyl showing cortical invasion by R. solani with the hyphae mostly in the intercellular spaces. (165x)

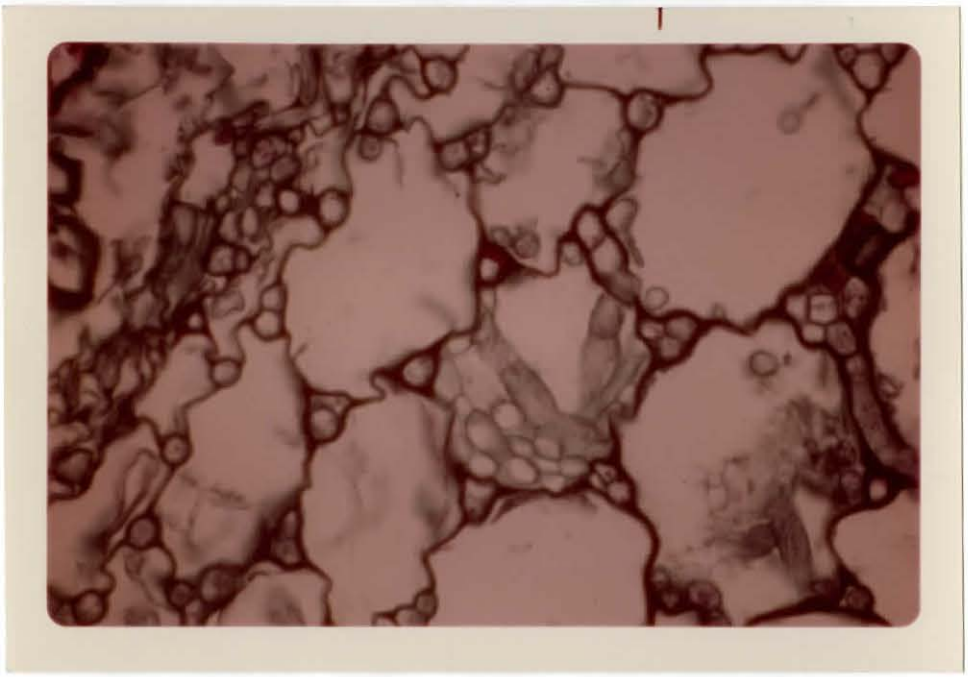
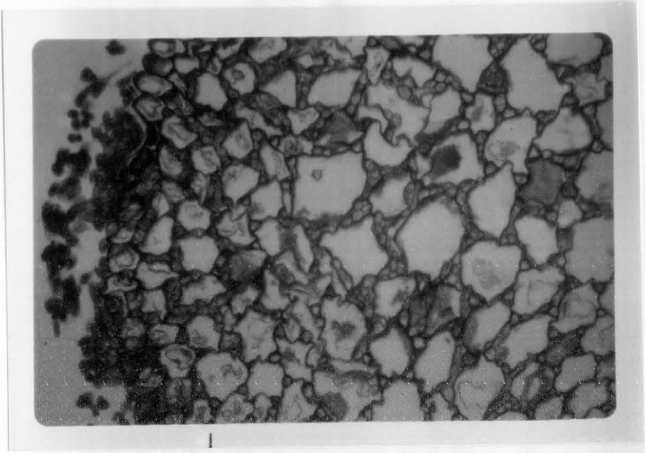
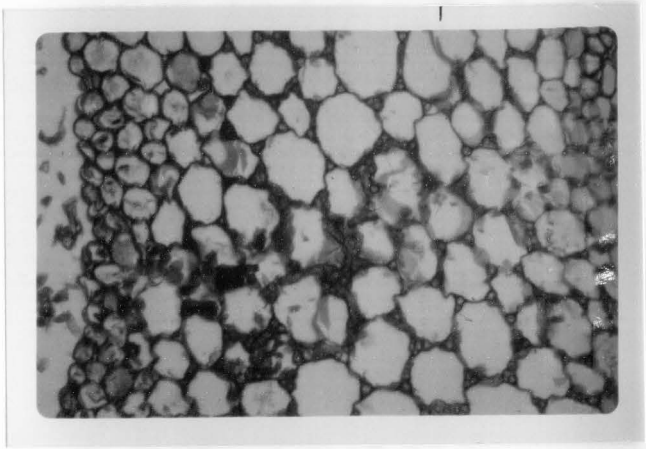


Figure 7. Transverse section of Rhizoctonia-infected resistant lima bean hypocotyl showing mostly intercellular and a few intracellular hyphae in the epidermal and cortical cells. These hyphae were so numerous that they caused completely separation of the adjacent cells. They were thick, did not stain deeply with safranin and showed more granulation in appearance. (84x)

Figure 8. Transverse section of susceptible lima bean hypocotyl showing hyphae growing in the longitudinal intercellular spaces and a few intracellular hyphae in the epidermis and cortex. (66x)





complete separation of the adjacent cells (Figures 7, 8). Branches of the longitudinal hyphae turned inward and enveloped the cortical cells growing along the middle lamella and the intercellular spaces (Figures 7, 8). The hyphae found in the infected areas of the resistant hypocotyl tissues were often thicker, did not stain as deeply with safranin, and showed more granulation (Figure 7) than those in the infected areas of the susceptible lines (Figure 8). Most of the hyphal growth was intercellular; in certain places, the walls of the cells were pushed apart by thick strands of several hyphae growing together. There was also more hyphal growth inside the cells at this stage.

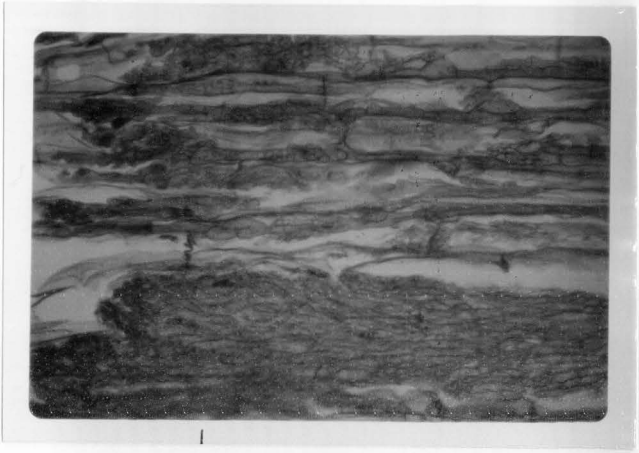
In this study, a build-up of masses of hyphae occurred as club-shaped structures in the cortical region (Figure 9) of the susceptible lima bean hypocotyls. The hyphae practically involved the entire cortical region (Figures 9, 10,), and continued growing inward both inter- and intra-cellularly into the vascular tissue (Figures 11, 12) and the pith (Figures 13, 14). The fungal hyphae were also found to invade the pith of the resistant hypocotyl tissue (Figure 15) but were not observed in the vascular tissue.

Sclerotia formation within cortical tissues of lima bean hypocotyls

The long hyphae in the longitudinal intercellular spaces and those packed within the cells produced side branches of a different morphological appearance. At this point, the formation of sclerotia began. These side

Figure 9. Longitudinal section of susceptible lima bean hypocotyl showing club-shaped structure of R. solani hyphae forming in the cortical region. (66x)

Figure 10. Transverse section of susceptible lima bean hypocotyl showing the hyphae of R. solani located throughout the entire thickness of the cortex. Curving of the epidermal cells toward the cortex is evident along with the penetration. (42x)



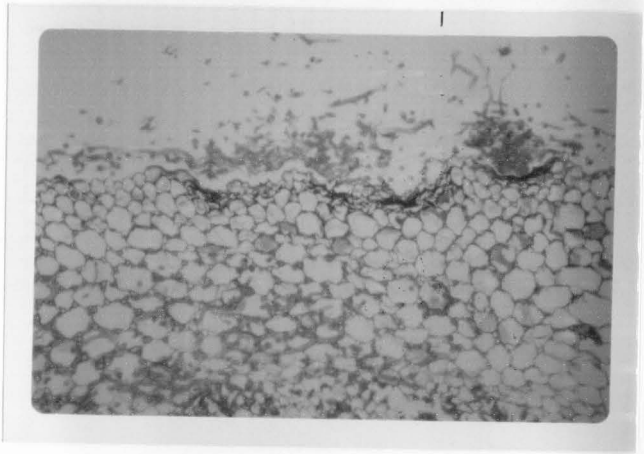
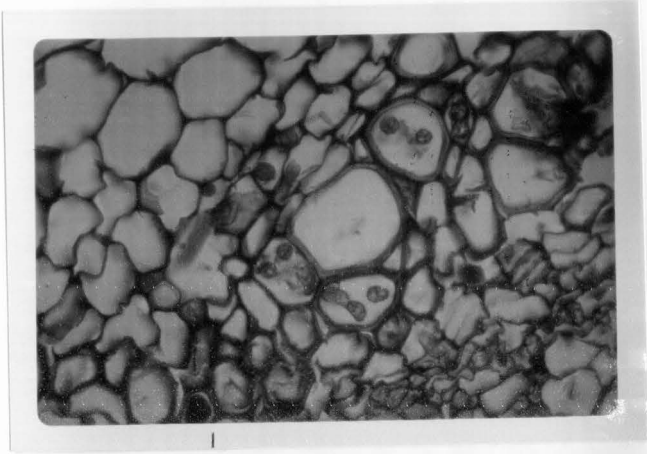


Figure 11. Transverse section of susceptible lima bean hypocotyl showing the hyphae of R. solani invading the vascular tissues (xylem elements). (165 x)

Figure 12. Longitudinal section of susceptible lima bean hypocotyl showing the hyphae of R. solani invading the vascular tissues (xylem elements). (165x)



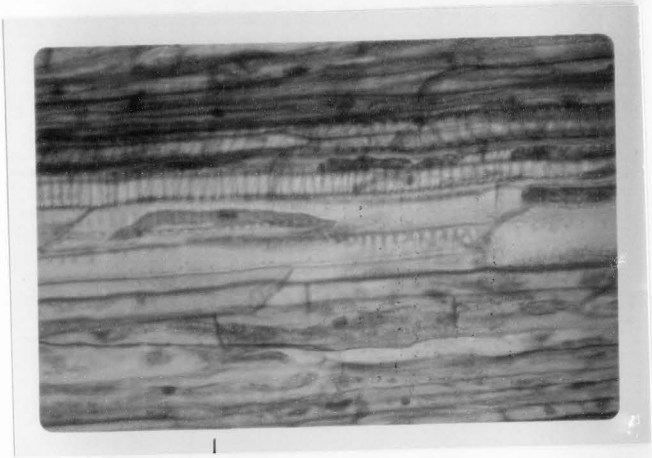
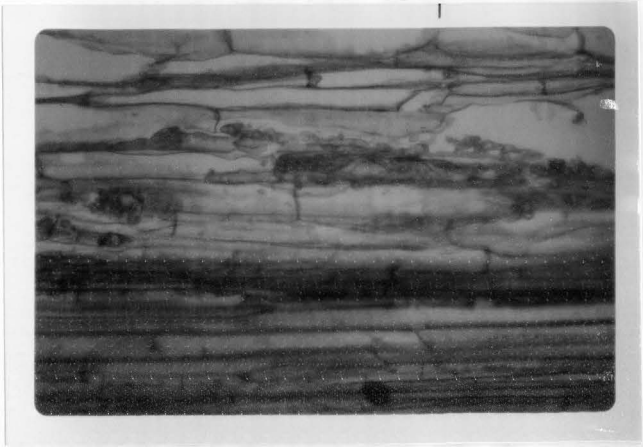


Figure 13. Longitudinal section of susceptible lima bean hypocotyl showing R. solani growing inward both inter- and intracellularly into the pith. (66 x)

Figure 14. Transverse section of susceptible lima bean hypocotyl showing the R. solani hyphae located throughout the entire thickness of cortical region and into the pith. (66 x)



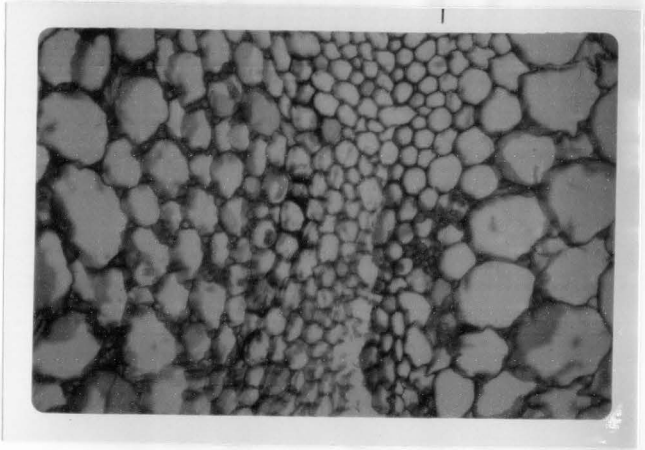
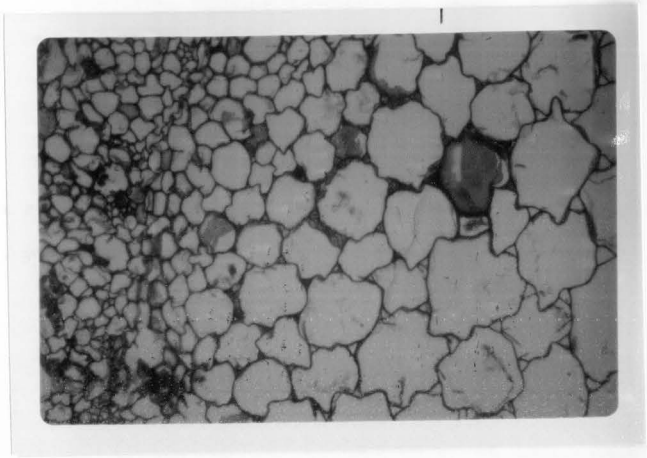


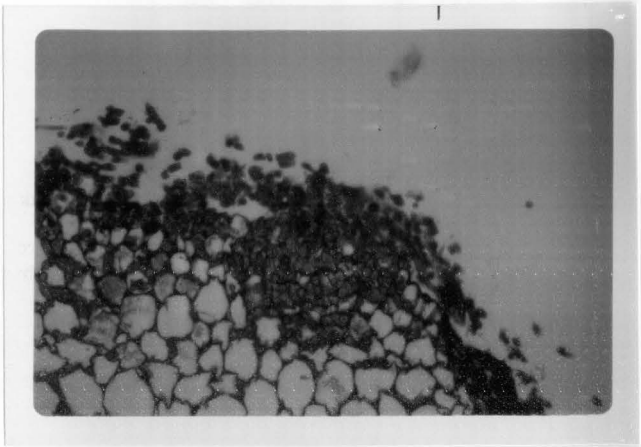
Figure 15. Transverse section of resistant lima bean hypocotyl showing the R. solani hyphae having penetrated through the pith intercellularly, but not invading the vascular tissues. (66x)

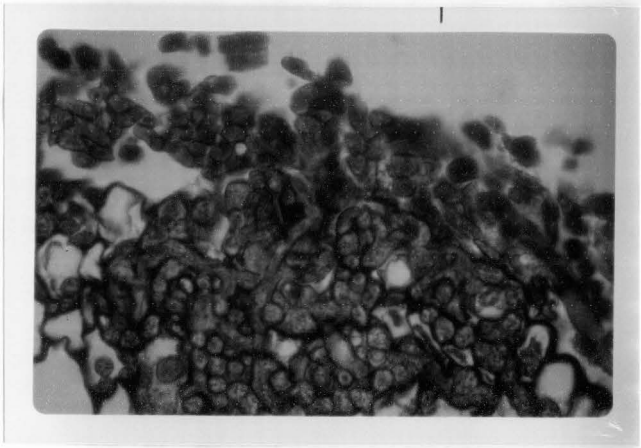


branches were much greater in diameter, and their individual cells were short, and barrel-shaped (Figure 17), in contrast to the very long, more or less uniformly cylindrical cells of the invasion or vegetative hyphae. These hyphae, composed of barrel-shaped cells, continued branching in this manner and became aggregated in the lumen of dead cortical cells and in large spaces resulting from the collapse of infected cortical cells. Finally, an aggregated mass of intertwined hyphae, mostly of the type just described, enclosing remnants of the collapsed cortical cells, became the sclerotium which protrudes from the surface of the lesions (Figure 16).

Figure 16. Transverse section of lima bean hypocotyl through a sunken lesion caused by R. solani. Under the darken layers of collapsed cortical cells in a nonelevated portion of sclerotium in cross section, which is composed of packed barrel-shaped celled hyphae within and between cortical cells. (165 x)

Figure 17. Transverse section of a sclerotium of R. solani, showing the barrell-shaped, densely granular cells of intertwined hyphae. (66 x)





DISCUSSION

The observation of the orientation of R. solani hyphae along the longitudinal (anticlinal) walls of the epidermal cells of resistant and susceptible lima bean hypocotyls was similar to the observations reported by earlier workers. Flentje (1957, 1959), Kerr (1956), Kerr and Flentji (1957), and Martinson (1965) suggested that this phenomenon might be due to some chemical stimulus. Morphological organization for direct penentration through cuticle and epidermis usually involves infection structures on the host surface. R. solani-formed infection cushions developed into dome-shaped structures, consisting of a roughly hemispherical mass of hyphae with the hyphal tips on the underside. Khadga et al. (1963) indicated that that the infection cushion could be formed either by a single hypha; by an aggregation of a short, stubby branches; by proliferation of branches; or by the aggregation of several hyphae. In this study, the formation of infection cushions has supported the work by Christou (1962) in which he stated that the combination of more than one hypha was necessary to initiate an infection cushion.

There were no differences between the formation of infection cushions on resistant and susceptible lines of lima beans. Although there was evidence that exudates stimulate the formation of dome-shaped cushions, Dodman et al.

(1968b) found that the hyphae failed to develop on glass surfaces and collodion membranes either with or without exudate from a susceptible host. It appears that a contact stimulus alone is insufficient to induce cushion formation.

The function of the infection cushion is primarily as the main seat of host invasion. Ullstrup (1936) concluded that the infection cushion appeared to act as a fulcrum against which a force was exerted during penetration. Nakayama (1941) thought that the formation of infection cushions enabled the fungus to enter the host in mass, which he termed "mass action." In this study, masses of R. solani hyphae were found in club-shaped structures in the cortex of susceptible hypocotyls. Christou (1962) suggested that the infection cushion had a survival value, because it facilitated host invasion and subsequent parasitic life. Since almost all infections observed in this study were from the hyphal tips under the infection cushions, the above explanations were probably valid. Infection pegs arose from any part of the infection cushion. This observation did not agree with Nakayama (1941), who reported infection pegs arising only from the center of the infection cushion.

Besides being the main seat of the host tissue invasion, the infection cushion also served as a holdfast for the fungus, as shown in its tenacity during agitation on water and the rigors of staining.

In previous reports, there has been considerable emphasis on dome-shaped infection cushions as a means of penetration by R. solani (Dodman, et al. 1968b; Flentje, 1957). From this study, it appears that this type of infection structure is important, especially in the penetration of young seedlings prior to or just after emergence. However, it should be recognized that other isolates of this fungus may penetrate by other means.

Dodman et al. (1968a) observed that the modes of penetration by R. solani are different among different isolates. When isolates were grouped according to their modes of penetration, it was found that dome-shaped infection cushions were usually formed by isolates which had originally been collected from the stem or roots of plants. The isolate of R. solani used in study was obtained from the roots of radishes grown in Salt Lake County, Utah.

Person (1944) reported that R. solani can cause damping-off and stem rot of bean under a rather wide range of temperature and soil moisture. Temperature is one of the most important environmental variables affecting the development of biological systems. Temperature not only has a marked effect upon seed germination, but it determines to a significant degree the susceptibility of plants to disease and the rates at which diseases develop. Moser (1968) reported that both temperature and inoculum density are important factors affecting the pathogenicity of R. solani on radish. Booslalis (1950) also found from his field and laboratory studies that certain isolates can parasitize germinating seeds or seedlings of soybeans. The reason that

lima beans were invaded at the early stage of plant growth was because the tissues were mostly meristematic in nature and had relatively little significantion or suberization of cell walls allowing the pathogen to invade easily.

Penetration occurred on both resistant and susceptible lines of lima bean hypocotyls with no significant difference between them. Walker (1959) reported that the metabolic activity of cells surrounding the invaded cells is much greater in resistant than in susceptible tissues and in the reactive resistant tissues phenols became more prominent followed by rapid necrosis. Most of the disorganized cells of resistant hypocotyl tissues had a grandular appearance (Figure 7) and a yellowish color, which changed to dark brown or brick red in older lesions. Cell collapse in resistant bean hypocotyl tissues could be due to a hypersensitivity of the cells as expressed by various workers, or to Müller's active principle which he called "phytoalexins" (Müller, 1958). The hypersensitive reaction by R. solani occurred only where infection structures were produced, even though no penetration occurred. Klement and Goodman (1967) concluded that hypersensitive necroses appear earlier in resistant varieties than typical symptoms in susceptible ones.

R. solani has been shown to produce toxic materials as reported by Bateman (1963), Sherwood and Lindberg (1962), and Wyllie (1962). In the susceptible host, while spreading from the site of infection, the pathogen destroys the tissues of the host with the dissolution of middle lamellae, often

in advance of the hyphae. The loosening of the middle lamella in the cortical region by the presence of numerous invading hyphae may be attributed to the ability of the fungus to produce exoenzymes after infection, as suggested by the work of Bateman (1963). Barker and Walker (1962), Garret (1962), and Bateman (1963) showed that the fungus produced both pectinolytic and cellulolytic enzymes. After invading the first few layers of cortical cells, the rapidly growing fungus became intracellular, perhaps because the middle lamella in this region of the cordical cells were less affected by the enzymes or enxyme production began after penetration. Discoloration and disintegration were characteristic of penetrated epidermal cells, probably resulting from the production of toxic materials, however, there was no evidence to determine whether these phenomena occurred during or immediately after penetration (Chi and Childers, 1963). Kernkamp et al. (1952) indicated that on the roots of sweet clover Pellieularia filamentosa killed and discolored host cells before penetration. Similar observations were reported by Boosalis (1950) and Wyllie (1962) on soybean roots. Flentje (1957) and Christou (1962), on the other hand, suggested that before penetration there was no apparent discoloration or death of the host cells beneath the infection cushions.

No attempt has been made in this study to determine what chemical changes take place during discoloration and disintegration. This rapid breakdown of the host tissue is probably accompanied by the action of enzymes or toxins.

The curving of the epidermal cells toward the cortex below the infection cushions before penetration suggests that this process was brought about by considerable mechanical pressure of the infection hyphae, as described by Gonzales and Owen (1963) and Nakayama (1941).

Histological observations of hypocotyl sections 5 days after inoculation of lima bean seedlings of both resistant and susceptible lines revealed that there were no significant differences in the extent of fungus hyphal development. The fungus was limited to the outer regions of the nonvascular region (cortex) of the hypocotyl, with intercellular hyphae penetrating to the same depth (Figures 7, 8). Microscopic observations made 7 days after inoculation revealed slight differences between resistant and susceptible lines. The hyphae in the susceptible hypocotyls had penetrated the vascular regions (Figures 11, 12, 13, 14), and was readily distinguishable in that region, whereas the hyphae of R. solani had penetrated only the phloem and the pith of resistant hypocotyls but did not invade the xylem (Figure 15). A build-up of masses of hyphae in club-shaped structures was occurred in cortex of the susceptible lima bean hypocotyls (Figure 9), but was not observed in the resistant lima beans.

Steinswat et al. (1967) made hybrids between a resistant and susceptible line and found that F_1 progeny was resistant to stem rot in the field. Segregation in the F_2 showed a very good fit to the 3 resistant:1 susceptible ratio when grown in the field. The progeny of a resistant plant

found in a susceptible line also segregated 3:1 ratio. Resistance to stem rot in lima beans appears to be inherited as a single dominant factor. When infected susceptible plants were grown in the greenhouse, they died earlier than similar plants grown in the field. This may have been due to conditions in the greenhouse being generally more favorable to disease development than host development. In this study the only difference between the resistant and susceptible was the restriction of fungal growth in vascular tissue of the resistant. This may be associated with either the creation of a chemical environment within the suscept that is hostile to the pathogen, or with induced changes in the chemical constituents of the invaded tissues that render them resistant to enxymatic degradation by the pathogen.

Hyphae found in less advanced positions of the infected area of resistant hypocotyls were often thicker and did not stain deeply with safranin, and showed more granulation (Figure 7) than in most advance areas of the suceptible hypocotlys (Figure 8). Most of the hyphal growth was intercellular, in certain places the walls of the cells were pushed apart by thick strands of several hyphae growing together (Figure 7). There was also some hyphal growth inside the cells at this stage. Numerous hyphae grew through the cell wall and branched inside the cell, without producing constrictions or swellings on either side of the cell wall.

The hyphae extended in all directions from the place of penetration, branching profusely. Those found near the more advanced margin of infection were growing almost entirely in the intercellular spaces (Figures 7, 8, 10).

Invaded cortical cells subsequently collapsed and the tissues became compressed. This may explain why the lesions in the hypotocyl tissues became sunken.

R. solani produces sclerotia within the cortex of the hypocotyl. Internal sclerotial formations similar to those which have been observed by Christou (1962) and Masumoto (1921).

It is of interest to note that this isolate of R. solani rarely penetrated through stomata during the early stages of dome-shaped infection cushion formation, even though numerous hyphal branches are formed, often close to stomata.

SUMMARY AND CONCLUSIONS

Rhizoctonia solani rapidly invaded the hypocotyls of resistant and susceptible lima bean seedlings causing discrete water-soaked lesions within 24-36 hr after inoculation under the conditions employed in this study. The observations on the growth of R. solani revealed that the penetrating hyphae gradually developed into a bundle and finally into a dome-shaped infection cushion. Thus, the hyphae of the cushion gain entrance into the epidermis and cortex and cause rot of the hypocotyls.

Histological studies of infected lima bean hypocotyls were made to determine the extent of fungal growth in host tissues and reaction of the host to infection. Following penetration of the epidermal cells and the formation of primary hyphae, a few layers of the cortex changed in appearance. The primary hyphae developed intercellularly, branched profusely, and formed normal septate hyphae, which penetrated deeply into the host tissues. Some hyphae have been found to penetrate the host tissues intracellularly.

Conclusions that can be drawn from the results of the study are: (1) There were no differences on the formation of infection cushion between the resistant and susceptible lima bean hypocotyls. Although there were evidences that exudates from resistant and susceptible varieties stimulated the formation of dome-shaped cushions, it appears that a contact stimulus

alone is not sufficient to induce their formation. (2) Similar modes of the penetration occurred on both resistant and susceptible lima bean hypocotyls with no significant differences between them. (3) The extents of hyphal development of R. solani on hypocotyls 5 days after inoculation were similar between the resistant and susceptible lines. The hyphae were limited to the outer region of the cortex with intercellular hyphal penetrating at the same depth. There were slight differences between the ectents of infection in the resistant and susceptible hypocotyl tissues 7 days after inoculation. The hyphae in the susceptible hypocotyl had penetrated the cortical tissues through the vascular tissues and the piths, whereas the hyphae had penetrated the cortex and grew inward to the phloem and the piths of the resistant hypocotyls, but did not invade the xylem. (4) Hyphae found in less advanced positions of the resistant hypocotyls were often thicker and did not stain deeply with safranin, and showed more granulation than in more advanced areas of the susceptible hypocotyls. (5) The build-up of masses of hyphae occurred as club-shaped structures in the cortical region of the susceptible lima bean hypocotyls, but were not observed in the resistant hypocotyls.

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