Initial Host Responses in Cotton to Infection by Rhizoctonia solani

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ABSTRACT

A histochemical and morphological study was made of the initial processes of infection of cotton hypocotyls by *Rhizoctonia solani*. Emphasis was placed on the relationship between pathogen location and host damage, and the stage of infection at which the effects of cell wall degrading enzymes could be detected. *Rhizoctonia solani* used in this study formed dome-shaped infection cushions on the surface of the hypocotyls. The first host response that could be detected was removal of ruthenium red positive substances, presumably pectic materials, from the cell walls. This occurred as early as 18 hr after the pathogen came in contact with the host plant. At this stage there was extensive hyphal branching, and infection cushions were beginning to form. Observation of cells below newly formed cushions, using polarized light, revealed a loss of birefringence in the cell walls, indicating damage to the crystalline structure of the cellulose. Twenty-four to 30 hr after host contact, an extensive cavity had formed beneath the cushions, but penetration had not occurred and the cuticle could be observed tightly appressed to the cushions. Later, penetration occurred by growth of numerous hyphae directly from the base of the infection cushions into the tissue macerated by the pathogen.

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Additional key word: histochemistry.

Critical events that may determine the subsequent course of a host-pathogen interaction occur during the initial stages of infection. An understanding of the nature and sequence of these events could provide valuable insight into pathogenic mechanisms. Primary host reactions to infection occur at the cellular level, and must be observed in relation to the pathogen. Therefore, when examining this relationship it is necessary to use histochemical techniques.

There have been numerous studies of host penetration by *Rhizoctonia solani* Kuehn (3, 4, 5, 6, 7, 8, 9, 10, 11, 13). These studies were primarily concerned with the morphology of penetration, and the host reaction to infection was not emphasized. The involvement of pectolytic enzymes in pathogenesis by *R. solani* has been well established (2). However, details of the initial events following contact of the host by *R. solani* have not been defined. Areas that require clarification include the relation between pathogen location and host damage, and the stage of infection

at which pectolytic enzymes are produced and their effects become detectable.

The purpose of this study was to determine alterations in host structure associated with infec-

tion by *R. solani* and the relationship of these changes to the location of the pathogen.

MATERIALS AND METHODS.—Five-day-old seedlings of cotton, *Gossypium hirsutum* (L.) 'Acala
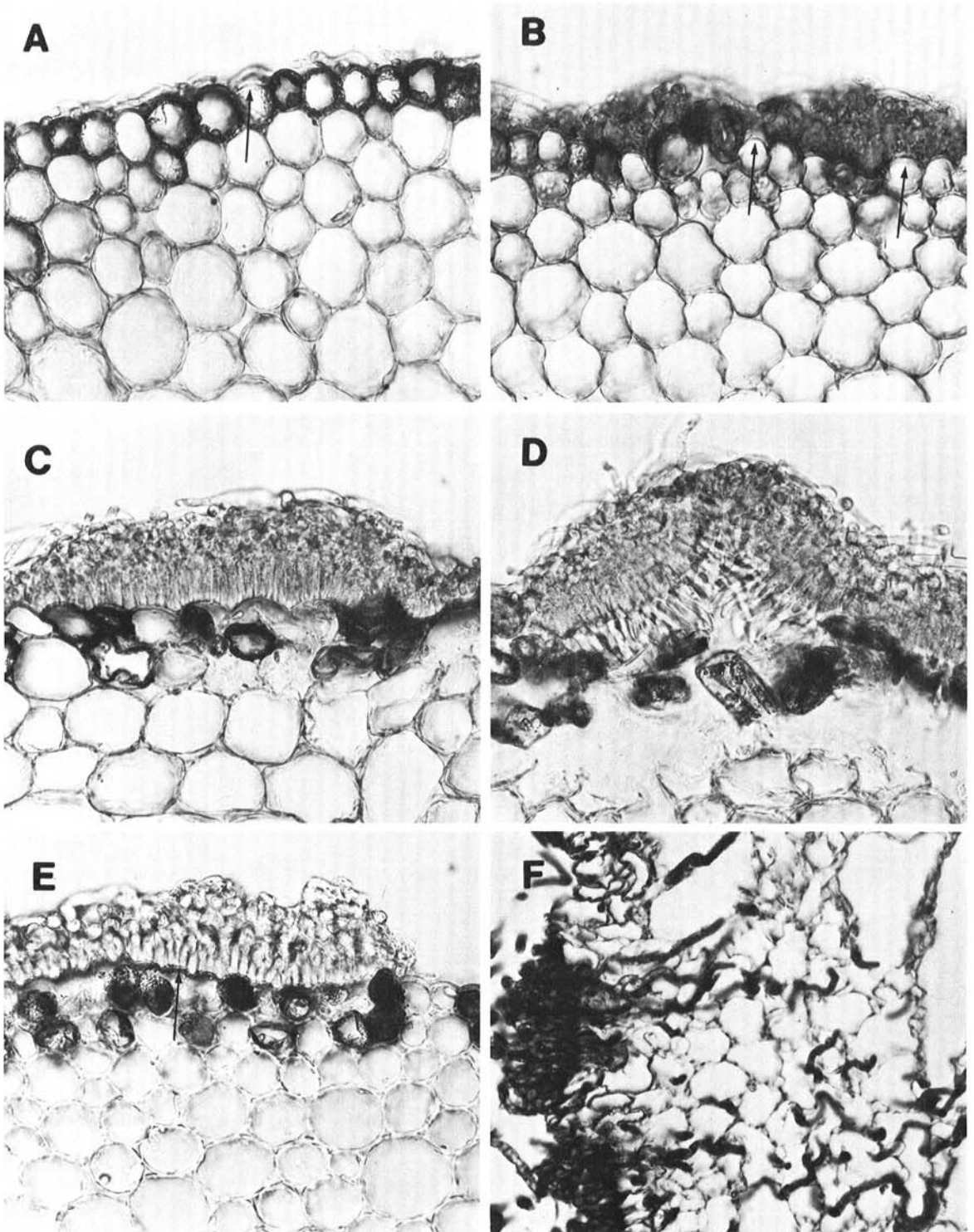
4-42', family 77, that had been grown in the greenhouse, were inoculated in the laboratory as previously described (13). Inoculum of *R. solani* was grown for 5 days at 28 C on a liquid medium containing essential salts, 2.0 g asparagine, and 20 g glucose/liter of distilled water (13).

Mycelial discs 2 mm in diam were placed next to the hypocotyls, and the plants were incubated at 28 C with continuous light. At intervals, hypocotyl tissue was removed and fixed for 18-24 hr in 3% glutaraldehyde in 0.13 M phosphate buffer at pH 7.0. After fixation the tissue pieces were transferred to buffer (0.13 M phosphate, pH 7.0) and stored until they were sectioned.

Transverse serial sections 50-75 μ thick were cut with an Oxford vibratome. The sections were treated to stain various structural components and observed using a light microscope. To stain pectic substances, the sections were placed in a 1:5,000 aqueous solution of ruthenium red for 30-40 min, rinsed, and mounted in glycerine. Lipid substances were stained by placing sections in 70% ethanol saturated with Sudan IV for 20 min. Sections were observed using a polarizing microscope to detect changes in the crystalline nature of cell wall cellulose. Tissue at later stages of infection were embedded in paraffin using standard Formalin-acetic acid-alcohol fixation and tertiary butyl alcohol dehydration. Sections were cut at a thickness of 20 μ and stained with Delafield's haematoxylon.

The results reported in this study are based on two isolates of *R. solani*. More than 60 isolates from cotton and potato were tested for pathogenicity. The two isolates used in the study were typical of those that were strongly pathogenic to cotton, bean, potato, and sugar beet. They were representative of the predominant pathogenic *Rhizoctonia* isolated in California. This fungus grows rapidly in culture, forming a white, mealy colony with relatively few sclerotia.

RESULTS.—The time between inoculation and lesion formation varied, depending upon incubation temperature. However, the developmental stages progressed in a definite sequence. The development of the pathogen and early symptoms on the hypocotyl could be separated into five stages. The stages were defined as: I. hyphae from inoculum contacted hypocotyl and grew along surface; II. extensive branching of hyphae; III. formation of infection cushions; IV. first sign of discoloration beneath cushions; V. distinct lesions with tissue maceration. At 28 C, the time interval between inoculation and stage I was ca. 10-12 hr. After the pathogen contacted the host plant, development was rapid. The

average time intervals between inoculation and stages II, III, IV, and V were 15, 18, 24, and 30 hr, respectively.

The first host response that could be detected with the techniques used in this study was the removal of ruthenium red positive substances from the cell walls. To determine the nature of the ruthenium red positive substances, sections were incubated in a 0.1 M acetate buffer (pH 4.5) solution containing 1 mg/ml commercial pectinase (Calbiochem). After 2 hr, all ruthenium red positive substances were removed. Although the pectinase undoubtedly contained other enzymes that may have removed materials from the cell walls, the activity of commercial pectinase indicates that most of the compounds stained with ruthenium red were pectic substances.

It was possible to detect the loss of pectic substances in epidermal cell walls during stage II of pathogen development (extensive hyphal branching) (Fig. 1-A). As cushions began to develop, many cells beneath the pathogen exhibited loss of pectic materials (Fig. 1-B). The initial loss of these materials beneath developing cushions was not uniformly distributed but occurred in discrete, localized areas. These areas did not appear to be related to any structures such as stomates or trichomes. A few hours after cushion formation (stage III), the protoplast of the cells beneath the structures became slightly yellow. At this stage, removal of pectic substances and loss of cell wall structure was readily apparent (Fig. 1-C). This was followed by increased discoloration and tissue breakdown and growth of the pathogen into the resulting cavity (Fig. 1-D, 2-C). During lesion development, the pathogen grew throughout the macerated tissue and for a distance of a few cells into the adjacent tissue. Sections through the edge of a macerated cavity revealed hyphae in the relatively intact cells (Fig. 1-F). There was no evidence that infection pegs were involved in penetration by *R. solani*.

Throughout the initial processes of lesion formation, there was no penetration of host tissue by the pathogen. Sections stained with Sudan IV (Fig. 1-E) show this clearly, with the cuticle well defined beneath the infection cushion. The pathogen was very tightly appressed to the cuticle, and there was no evidence of cuticle damage.

Observation of host tissue sections with polarized light revealed considerable change in the crystalline structure of the cell wall cellulose prior to penetration by *R. solani*. A series of sections showing an early (Fig. 2-A) and an advanced stage (Fig. 2-B) of cushion formation and hyphal penetration (Fig. 2-C)

Fig. 1. Progressive steps in attack of cotton hypocotyl by *Rhizoctonia solani*. Sections in A-D stained with ruthenium red. **A)** Stage II of pathogen development; extensive hyphal branching but infection cushions have not formed. Some loss of ruthenium red positive materials (arrow) in epidermal cell walls can be detected. **B)** Stage III. Initial infection cushion formation with loss of ruthenium red positive materials (arrow) more pronounced. **C)** Stage IV. Infection cushions were well developed and loss of cell wall structure was apparent. **D)** Extensive damage to host cells with pathogen growing into cavity formed below infection cushion. **E)** Pathogen development comparable to C but section stained with Sudan IV. Cuticle (arrow) is intact and tightly appressed against base of infection cushion. Extensive cell wall damage but no penetration. **F)** Section through edge of macerated cavity after penetration showing hyphae in relatively intact cells (X 300).
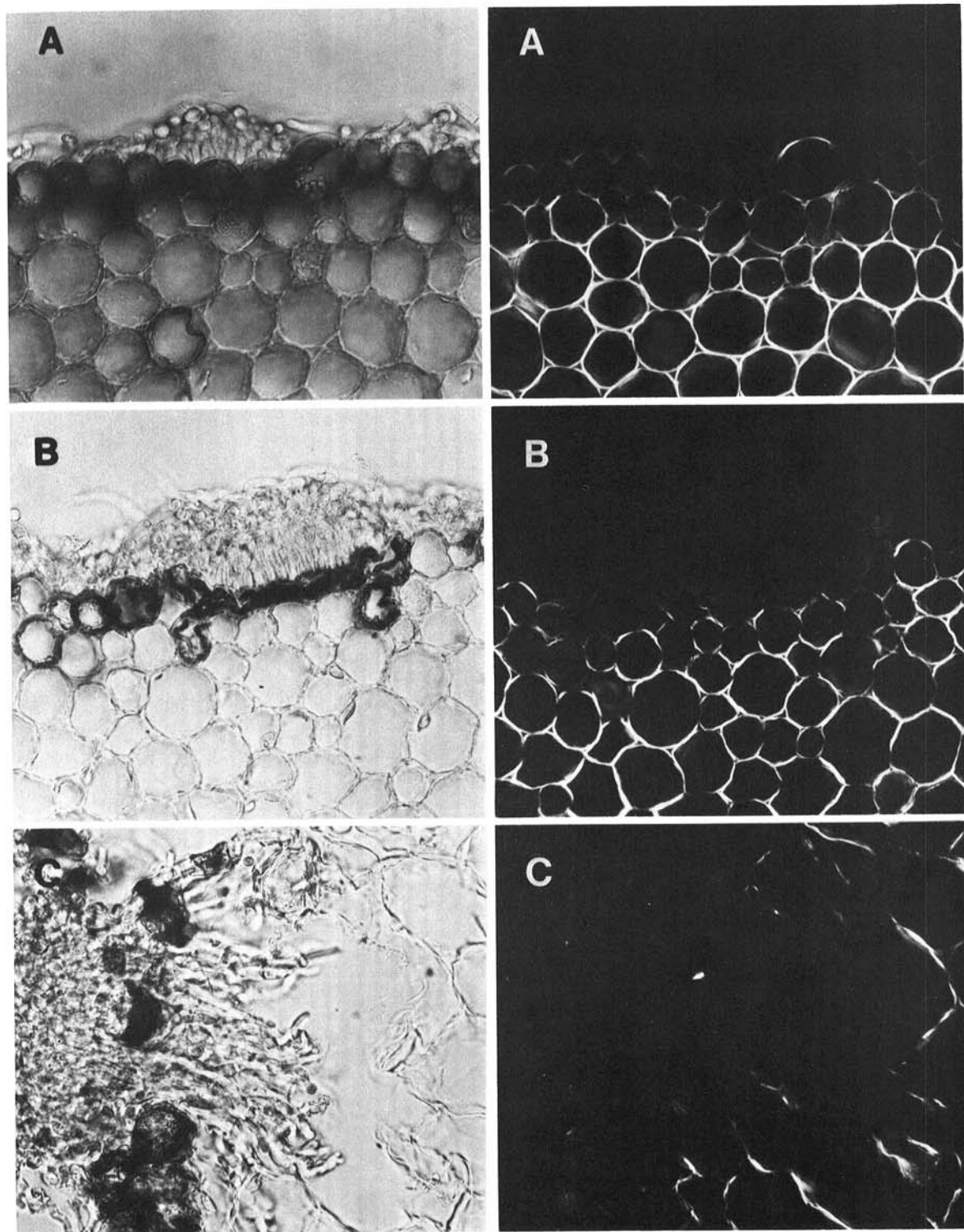
Fig. 2. Progressive steps in attack of cotton hypocotyl by *Rhizoctonia solani*. Paired plates are identical. Those on the right were taken with polarized light. Sections were not stained. A) Stage III. Early infection cushion formation. Note loss of birefringence in cell walls beneath the infection cushion. B) Stage IV. Cell wall damage extending into second layer of epidermal cells. C) Pathogen growing into macerated cavity. Extensive cell wall damage is occurring in advance of the pathogen (X 300).

were photographed in both plane and polarized light. A comparison of each pair of photographs reveals a loss of birefringence in the epidermal cell walls beneath the developing cushions that progresses until, at penetration, an extensive area of cortical tissue is damaged. Some of the loss of birefringence may be due to interference by the yellow-to-brown substances that accumulate in cells shortly after the initiation of cushion formation. In Fig. 2-B, however, there are areas in the walls of cells one and two layers below the damaged epidermis which show loss of birefringence. These do not appear to be associated with pigmentation.

The infection process in bean and radish was observed and found to be similar to that occurring in cotton.

DISCUSSION.—The first host reaction that could be detected, in response to infection by R. solani, was the removal of pectic substances from cell walls. This occurred during infection cushion formation, and was followed a few hours later by cell wall degradation. Penetration was by growth of hyphae into damaged tissue beneath the cushion, without the formation of infection pegs.

The species R. solani encompasses a group of fungi with diverse pathogenic capabilities. Therefore, it is not unexpected that investigators have observed different modes of penetration and differences in the relation between penetration and damage to the host. A detailed study of infection of cotton by R. solani has been published by Khadga et al. (10). They reported that the fungus formed dome-shaped infection cushions and penetrated directly by means of infection pegs. Similarly, Christou (5), Van Etten et al. (12), and Dodman et al. (6) reported that, on bean, penetration occurred directly by numerous infection pegs arising from cells at the base of infection cushions. The penetration of host tissue by infection pegs arising from cushions has been reported by other investigators (7, 8). On radish, Dodman et al. (6) found that one or two hyphal tips in the center of the cushion penetrated the cuticle without the formation of constricted infection pegs. A similar method of penetration was observed by Nakayama (11) on cotton. Chi & Childers (4) reported that roots of red clover and alfalfa were penetrated by infection hyphae that developed from cushions.

The question of whether host damage occurs before or after penetration has been considered by several investigators. Chi & Childers (4) reported that discoloration and disintegration were characteristic of penetrated epidermal cells. Kernkamp et al. (9) observed that cells of sweet clover roots were killed and discolored prior to penetration. Similar observations on soybean roots were reported by Boosalis (3). In contrast, Flentje (7) and Christou (5) reported that before penetration there was no discoloration or death of host cells beneath infection cushions on bean and other hosts. They also suggested, as did Gonzales & Owen (8), that the depression of epidermal cells reflected mechanical pressure. In the present study, cell wall disintegration occurred

beneath infection cushions prior to penetration. The cuticle appeared to remain intact. Although mechanical pressure may have been involved in penetration of the cuticle, it would appear to be less important than enzymatic breakdown of the host cell walls.

The involvement of pectolytic enzymes in the damage of host plants by R. solani is well established (2). Van Etten et al. (12) emphasized the importance of investigating the early stages of infection. They detected endopolygalactoronase and cellulase in extracts of R. solani-infected bean hypocotyls 35 hr after inoculation. Khadga et al. (10) observed that the cuticle is often separated from the epidermis by invading hyphae. This was probably due to the production of pectolytic and cellulolytic enzymes. Dodman et al. (6) also observed that the cuticle commonly became detached from the epidermis after penetration. Bateman (1), studying R. solani on bean, has presented evidence that cellulolytic enzymes are not important until the later stages of disease development.

In the present study, it was clearly shown that cell wall-degrading enzymes are produced, and that they damage host cell walls before penetration and before symptoms are apparent. By as early as 12 hr after host-pathogen contact, infection cushions had formed and removal of pectic materials from host cell walls and loss of birefringence could be detected. Based upon loss of birefringence in host cell walls, it is suggested that, on cotton, cellulase may be produced by R. solani prior to penetration. The mode of infection by our isolates involved infection cushion formation and enzymatic disintegration of host cell walls followed by growth of the pathogen into the cavity beneath the cushion.

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