Ultrastructure of *Rhizobium*-Induced Infection Threads in Clover Root Hairs

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Ultrastructural studies of *Rhizobium*-induced infection threads in clover root hairs show that the infection thread is initiated by an invagination process. Root hair wall growth is redirected at a localized point, resulting in the formation of an open pore. There is no direct penetration through the wall, and the bacteria remain extracellular within the root hair.

The first stage in the establishment of the *Rhizobium*-legume symbiosis is the infection of the root of the host legume by the appropriate *Rhizobium* species. A recent and complete review of the infection process has been given by Dart (1). In the clover symbiosis, infective strains of *Rhizobium trifolii* enter the host through root hairs. A characteristic deformation is curling at the root hair tip to produce a "shepherd's crook" (4). The bacteria enter the root hair and are enclosed in a tubular structure, the infection thread, which is the first visible, microscopic sign of a successful infection (10). The majority of infected root hairs have the shepherd's crook at the infection thread origin; however, exceptions exist (4, 16) and will be reported in this paper.

Three theories have been proposed regarding the entry of the bacteria into the root hair. Nutman (15) has advanced the hypothesis of root hair cell wall invagination. An invagination is the redirection of plant cell wall growth at a localized point, resulting in the wall growing back into the root hair to form the tubular infection thread. There is no penetration through the wall at the point of entry, and the bacteria remain extracellular; i.e., there is no contact with the host cytoplasm. Ljunggren and Fahraeus (12) have proposed a "polygalacturonase" hypothesis, in which the rhizobial exopolysaccharide increases plant pectic enzyme activity and a single bacterial cell softens and subsequently penetrates the plant cell wall without pronounced structural disruption. The infection thread is presumably initiated once the bacterium penetrates to the plant plasmalemma. Dart and Mercer (2) have proposed the entry of small cocoid forms of the rhizobia through gaps in the cellulose microfibrils.

The light microscope has been invaluable in studying the growth of the infection thread through root hairs (1). However, the point of entry of the bacteria into the root hair, and thus the mechanism, cannot be resolved by the light microscope. In this study, electron microscopy of serial sections of infected clover root hairs has provided evidence to support the invagination theory of infection.

MATERIALS AND METHODS

Bacteria-host interaction. *Trifolium fragiferum* (Palestine strawberry clover) seeds were surface sterilized, rinsed, and cold treated for 48 h at 4 °C (14). Seeds were germinated overnight (inverted water agar plates) into humid air at 22 °C and transferred to Fahraeus glass-slide assemblies (4) inoculated with 3-day-old cultures of *R. trifolii* (NA30) grown on yeast extract-mannitol agar (14). The assemblies were incubated at 22 °C, with a 12-h photoperiod, and examined for infection threads using phase-contrast microscopy.

Transmission electron microscopy. Three- and 7-day-old inoculated whole clover seedlings from the Fahraeus assemblies were fixed at 22 °C for 2 h with 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8) and postfixed at 22 °C for 1.5 h with 1% buffered osmium tetroxide. Seedlings were dehydrated through a graded ethanol series (25, 50, 75, 95, and 100%) followed by acetone, infiltrated with Spurr resin (20), and polymerized overnight at 50 °C. The seedlings were flat embedded in rectangular, Peel-a-way disposable embedding molds (22 by 40 mm; Peel-a-way Scientific, South El Monte, Calif.). The embedded seedlings were viewed under phase-contrast microscopy, and areas containing infection threads were selected for sectioning. Serial sections were cut on a Sorvall MT2 ultramicrotome with a diamond knife. The sections were picked up on Formvar-coated, one-hole grids and stained with Reynolds lead citrate (18). Grids were examined in a Hitachi HU11E electron microscope at 75 kV.

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RESULTS

A typical infection thread is shown in Fig. 1. The arrow indicates a floc of bacteria on the curled root hair tip. Single cells of *R. trifolii* (NA30) outside a root hair are shown in Fig. 2. The bacteria were surrounded by capsules of various sizes. *R. trifolii* has been reported to produce a capsular acidic polysaccharide (F. Dazzo and D. Hubbell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, N28, p. 189) and cellulose microfibrils (11). Figure 3 shows

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**Fig. 1.** Phase-contrast microscopy of a root hair containing an infection thread (IT). The top arrow indicates a floc of bacteria on the root hair tip. ×500.

**Fig. 2.** Encapsulated bacteria outside the plant cell wall (PCW). Bar, 2 μm.

**Fig. 3.** Groups of bacterial cells enclosed within a common slime layer (S) outside the root hair cell wall (PCW). Several groups appear segmented. Bar, 1 μm.
groups of bacterial cells enclosed within a common slime layer; some groups were segmented. The fibrillar slime layer completely enclosed the bacteria, as evidenced by serial sections. *R. trifolii* (NA30) cells in the rhizosphere were pleomorphic only when enclosed in slime; otherwise they had a fairly uniform morphology.

**Fig. 4.** Diagrammatic illustration of the sectioned root hair. The infection thread (IT) originated midway on the root hair and branched past the nucleus (N). A floc of bacteria (F) was attached to the root hair at the origin of the infection thread. Sectioning began at the floc (indicated by the top arrow) and continued through the root hair.

**Fig. 5-11.** Serial sections through the infection thread origin. Bar, 2 μm.

**Fig. 5.** The slime wall (arrow) of the attached floc was grazed by the knife.

**Fig. 6.** The floc was sectioned and bacteria were revealed inside. The arrow indicates the slime surrounding the floc.

**Fig. 7.** The root hair wall was grazed by the knife. The arrows indicate the interface between the root hair wall and the attached floc.
Serial sections through an infected root hair are shown in Fig. 5 through 11. Figure 4 is a diagrammatic illustration of the root hair based on serial sections. The root hair was slightly curled. The infection thread originated midway on the root hair and branched past the nucleus, and each branch grew into the base of the root hair. A floc of bacteria, enclosed within a common slime layer, was attached to the root hair at the origin of the infection thread. The sectioning began at the floc (arrow) and continued through the root hair. The sections in Fig. 5 through 11 were selected from the serial sections. The slime wall of the attached floc

**Fig. 8.** The root hair wall appeared to be invaginated (arrows). The attached floc had a segmented appearance.

**Fig. 9.** The root hair wall invaginated to form the infection thread. The plant cell wall (arrows) was continuous with the wall of the infection thread. Bacteria were within the floc and infection thread.

**Fig. 10.** This is a section past the middle of the invagination. The plant cell wall is indicated by arrows.

**Fig. 11.** The back wall (arrows) of the invagination was grazed by the knife and the attached floc ended.
was grazed (Fig. 5), and then bacteria were revealed inside (Fig. 6). Several sections were cut through the floc before the root hair wall was sectioned (Fig. 7). The arrows in Fig. 7 indicate the interface between the root hair wall and the floc. The floc had a segmented appearance (Fig. 7 and 8). As sectioning continued through the root hair, the wall appeared to be invaginated (Fig. 8). Sectioning through the area where the infection thread originated revealed a pore filled with and surrounded by the floc (Fig. 9). The wall of the root hair was continuous with the infection thread wall (Fig. 9–11). The floc decreased in size past the middle of the pore (Fig. 10) and ended as the back wall of the pore was grazed (Fig. 11). Bacteria were seen within the infection thread (Fig. 9–11).

The infection thread was seen beside the nucleus (Fig. 12) and then branched. The infection thread was composed of two distinct layers, an outer fibrillar layer similar to the plant cell wall and an inner amorphous layer surrounding the bacteria (Fig. 12). This amorphous material was occasionally absent, possibly due to incomplete fixation and/or penetration of the plastic. The outer layer of the infection thread and the plant cell wall stained with the periodic acid-silver hexamine stain for polysaccharide (17). However, the inner layer did not stain, and its composition is unknown at this time. The process of the infection thread crossing from one cell into another in the cortex (Fig. 13) appears to be a repetition of the invagination, which occurred at the site of infection. There was no disruption in the continuity of the infection thread; the wall of the infection thread and the plasmalemma are continuous.

Five infected root hairs having the shepherd's crook at the infection thread origin were serially sectioned, and in every case an invagination formed a pore. Figure 14 is a diagrammatic illustration of a sectioned root hair based on a serial section sequence, from which Fig. 15 through 17 were selected. The invagination was seen before, through, and past the pore (Fig. 15, 16, and 17, respectively). Bacteria were seen within the pore and in the infection thread. This infection thread was probably newly initiated, as it had not progressed far into the root hair. The nucleus was seen in close association with the infection thread.

DISCUSSION

Nutman's theory of invagination (15) has been challenged on several points. First, how the cell wall invaginates against the high hydrostatic pressure of the root hair is unknown (3). Secondly, invagination would form an open pore, which has not been shown in earlier

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**Fig. 12.** The nucleus (N) of the root hair was next to the infection thread. The infection thread was composed of two distinct layers, an outer fibrillar layer (OL) and an inner amorphous layer (IL). Bacteria (B) were within the infection thread. Bar, 2 μm.

**Fig. 13.** The infection thread crossed from the root hair cell into another cell in the cortex of the root. There were no breaks in the plant cell walls (PCW) or infection thread. ML is the middle lamella. Bar, 1 μm.
Fig. 14. Diagrammatic illustration of the root hair. The infection thread (IT) origin was in the tightly curled root hair tip. The root hair nucleus (N) was in close association with the infection thread. The sectioning began at the arrow and continued through the invagination.

Fig. 15-17. Serial sections through the invagination. The invagination was seen before, through, and past the pore (Fig. 15, 16, and 17, respectively). The arrows in the figures indicate the invaginated plant cell wall. Bar, 2 μm.

electron micrographs (5, 19). However, serial sections of root hairs were not used in these studies. Additionally, an open pore would allow simultaneous entry of different cell types, which would result in different *Rhizobium* strains isolated from one nodule. Early studies indicated that only one strain of *Rhizobium* was isolated from a nodule when the host had been inoculated with a mixture of infective *Rhizobium* differentially marked by antibiotic resistance (13) or serological type (6, 8). Recent studies (7, 11) have shown that several cell types can be isolated from one nodule. Our study indicated that invagination resulted in the formation of a pore, but serial sections revealed that either the tightly curled root hair tip or a floc of bacteria surrounded the pore. In this way, entry of the bacteria into the root
hair is restricted to cells enclosed in the shepherd’s crook or to cells comprising the floc.

The majority of infected root hairs have the tightly curled root hair tip, but infection will occasionally occur in a relatively undeformed root hair. We believe that the attached bacterial floc on the slightly curled root hair served the same function as the tightly curled root hair tip, localizing and concentrating the biochemical interactions of the plant and bacteria which initiate infection. The enclosure of a number of R. trifolii (NA30) cells within a common slime layer has been observed only in the presence of the clover host and not in pure culture. The appearance of the small flocs suggests that cell division has occurred but a common capsule has remained. The segmented appearance of the flocs suggests that several smaller flocs may have fused to form a larger one.

Bacteria enclosed within slime were pleomorphic, whereas bacteria outside slime enclosures had a fairly uniform rod morphology. The pleomorphism could be due to incomplete penetration of the fixative through the slime. However, it could also be a host-induced change, since the bacteroids in nodules are highly pleomorphic (9). We are currently examining the effect of clover root exudates on R. trifolii in pure culture.

In accordance with the invagination theory, the wall of the infection thread is synthesized by the host plant and is an extension of the plant cell wall. The observation that rhizobia produce cellulose (14) raises the interesting question of whether or not bacterial cellulose contributes to the assembly of the infection thread wall. This question is being investigated in our laboratory.

The infection thread wall at the point of invagination is difficult to see in Fig. 9 through 11 and 15 through 17. However, the infection thread walls away from the invagination are clearly recognizable. This may reflect a physical and/or chemical alteration of the cell wall structure at the invagination origin, where the specific bacteria-plant interactions resulted in infection thread initiation.

Ultrastructure evidence presented in this paper strongly supports the invagination theory. Serial sectioning of the root hair at the point of origin of the infection thread indicates that R. trifolii enters through a pore rather than by direct penetration through the plant cell wall. The tightly curled root hair tip or a floc of bacteria provides a complete enclosure around the bacteria where the unknown, specific biochemical interactions involved in the infection process take place.

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LITERATURE CITED


