Root hair deformation in the infection process of *Alnus rubra*

ALISON M. BERRY
Department of Biology, Carleton University, Ottawa, Ont., Canada K1S 5B6
AND
JOHN G. TORREY
Cabot Foundation, Harvard University, Petersham, MA, U.S.A. 01366

Received December 1, 1982

BERRY, A. M., and J. G. TORREY. 1983. Root hair deformation in the infection process of *Alnus rubra*. Can. J. Bot. **61**: 2863–2876.

Structural and cell developmental studies of root hair deformation in *Alnus rubra* Bong. (Betulaceae) were carried out following inoculation with the soil pseudomonad *Pseudomonas cepacia* 85, alone or in concert with *Frankia*, and using axenically grown seedlings. Deformational changes can be observed in elongating root hairs within 2 h of inoculation with *P. cepacia* 85. These growing root hairs become branched or multilobed and highly modified from the single-tip growth of axenic root hairs. The cell walls of deformed hairs are histologically distinctive when stained with the fluorochrome acridine orange. Filtrate studies using *P. cepacia* 85 suggest that the deforming substance is not a low molecular weight compound. Root hair deformation and the associated wall histology are host specific in that *Betula* root hairs show none of these responses when grown and inoculated in the experimental conditions described. The bacterially induced changes in root hair cell walls during deformation may create a chemically and physically modified substrate for *Frankia* penetration, and the deformation itself may serve to entrap and enclose the filamentous organism, allowing wall dissolution and entry. Thus these events represent a complex host response as a precondition to successful nodulation.

BERRY, A. M., et J. G. TORREY. 1983. Root hair deformation in the infection process of *Alnus rubra*. Can. J. Bot. **61**: 2863–2876.

Des études de la structure et du développement cellulaire ont été poursuivies sur la déformation des poils racinaires de l'*Alnus rubra* Bong. (Betulaceae) après l'inoculation à des plantules axéniques d'une pseudomonade du sol, *Pseudomonas cepacia* 85, seule ou de concert avec *Frankia*. Des déformations dans les poils racinaires en croissance peuvent être observées moins de 2 h après l'inoculation du *P. cepacia* 85. Ces poils racinaires deviennent ramifiés ou multilobés et ils sont fortement modifiés par rapport à la croissance terminale simple des poils racinaires en condition axénique. La paroi cellulaire des poils déformés est histologiquement distinctive lorsqu'elle est colorée au fluorochrome acridine orange. Des études de filtration utilisant le *P. cepacia* 85 montrent que la substance responsable des déformations n'est pas un composé de faible poids moléculaire. La déformation des poils racinaires et les conditions histologiques de la paroi cellulaire qui lui sont associées sont spécifiques à l'hôte, car les poils racinaires de *Betula* ne présentent aucune de ces réactions lorsqu'ils croissent en présence du même inoculum et dans les mêmes conditions expérimentales. Les changements provoqués par la bactérie dans la paroi cellulaire des poils racinaires au cours de la déformation pourraient modifier chimiquement et physiquement le substrat de manière à permettre la pénétration du *Frankia*; la déformation elle-même pourrait aider à piéger et à entourer l'organisme filamenteux, permettant la dissolution de la paroi et la pénétration. Cette suite d'événements constitue donc une réaction complexe de l'hôte et serait une condition préalable au succès de la nodulation.

[Traduit par le journal]

Introduction

The initial infection of the host root tissue leading to nodule formation in actinorhizal plants occurs via the invasion of a deformed root hair by the filamentous actinomycete *Frankia*. For *Alnus*, both Pommer (1956) and Angulo Carmona *et al.* (1976) published micrographs of entire deformed root hairs in which infection threads can be traced very roughly to probable points of entry at the base of the lobed apical region of the hair. Lalonde (1977) postulated on the other hand that infection in *Alnus* occurs at the site of attachment of "exoencapsulation threads," shown attached at the outside wall of the hair cells. In only one study (Callaham and Torrey 1977) is the actual zone of penetration of the root hair wall clearly delimited, in the deeply folded region at the base of lobes of the deformed hair (in *Comptonia peregrina*). At the presumed site of penetration, an amorphous mass was evident within the hair, apparently resulting from the deposition of wall polysaccharide continuous with the root hair wall in the deeply folded region (Callaham *et al.* 1979).

The deformation of host root hair cells during the course of infection is a distinctive and characteristic change in host cellular structure and has been frequently reported. Deformation has been observed to be a consequence of inoculation with preparations of the nodule actinomycete (von Plotho 1941; Pommer 1956; Lalonde 1977; Callaham *et al.* 1979) and to coincide spatially with sites of nodule formation on the roots of actinorhizal plants (Pommer 1956; Käppel and Warten-

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

berg 1958; Angulo Carmona 1974; Lalonde 1977; Callaham *et al.* 1979). Angulo Carmona (1974), Lalonde (1977), and Callaham *et al.* (1979) described a progressive increase acropetally in the degree of curl in root hairs proximal to the root apex for several actinorhizal species, within a day after inoculation in nonaxenic conditions. Only one or two hairs subsequently become infected (Pommer 1956; Callaham and Torrey 1977).

The reported abundance of root hair deformation in apparently uninfected regions of the *Alnus* root system (Krebber 1932; von Plotho 1941; Taubert 1956; Kappel and Wartenberg 1958) was used as the basis for suggesting that root hair deformation is not a consequence of infection (Taubert 1956) but, further, that "ein Zusammenhäng Zwischen deformierten Wurzelhaaren und Infektion scheint nicht zu bestehen" (Taubert 1956, p. 155).

Recent evidence from work using *Frankia* isolate strains as inoculant for axenically grown *Alnus* seedlings (Knowlton *et al.* 1980) has strengthened the argument (Pommer 1956; Callaham *et al.* 1979) that the changes in root hair cells associated with root hair deformation are a necessary precondition for subsequent infection. On axenic seedlings, *Frankia* alone produced only low levels of nodulation. Root hairs remained undeformed in the nonnodulated plants. When microorganisms which were found to cause root hair deformation were introduced with *Frankia*, very high levels of nodulation resulted. Lalonde *et al.* (1981) rightly pointed out that *Frankia* infection can occur in axenic conditions without helper organisms as had been previously stated (Knowlton *et al.* 1979, 1980). Higher rates of infection are found when, for example, the soil pseudomonad strain *Pseudomonas cepacia* 85 is introduced with *Frankia* in axenic conditions (Knowlton and Dawson 1983).

Moreover, since a range of soil bacteria successfully induce root hair deformation in the host plant, such complex interactions leading to nodulation may well occur in nonaxenic circumstances in the soil, even when the relationship is not an obligate one.

A bacterial strain such as *P. cepacia* 85 (PC 85), which causes extensive root hair deformation in *A. rubra* seedlings on agar slants (Knowlton *et al.* 1980), provides a useful tool for examining root hair surface and cellular preconditions as phenomena separable from, but necessary for, actual root hair infection.

Root hair penetration as a means of initial infection has been described for other host–microorganism interactions, both pathogenic (*Plasmodiophora brassicae*, Aist and Williams 1971) and symbiotic (legumes, cf. Callaham and Torrey 1981; certain mycorrhizae, cf. Burgeff 1909; Pommer 1956). Only in the case of the legume–*Rhizobium* symbiosis is root hair deformation reported to occur as a regular feature of infection, suggesting that extensive host cellular modification may be important for successful infection in these systems.

The changes in root hair structure which comprise deformation have been little investigated. Studies usually begin with the events of root hair invasion (Nutman et al. 1973; Callaham and Torrey 1981). Turgeon and Bauer (1982) placed root hair deformation and infection within a framework of root cellular differentiation for soybean, although they did not examine individual hair deformation.

The present structural and developmental study was carried out under controlled conditions to delineate the process of root hair deformation in the actinorhizal species *Alnus rubra* in response to inoculation with PC 85, as a cellular interaction between host root tissue and rhizosphere microorganisms and as a prelude to root hair infection by *Frankia*.

Materials and methods

Plant culture

Agar slants

Fruits of *Alnus rubra* Bong. collected by B. Rottink of Crown Zellerbach Corp. from Molalla, OR, were selected, scarified, and surface sterilized in 30% H_2O_2 as described previously (Knowlton *et al.* 1980). A 24-h presoak in aerated distilled water prior to sterilization was found to enhance germination percentages. Fruits were sown either on mineral–agar plates or slants. In either case the mineral solution was quarter-strength Hoagland's N-free with pH adjusted to 6 or 6.5, consisting of the following per liter (from Hoagland and Arnon 1950): K_2SO_4 (0.5 *M*), 1.25 mL; $MgSO_4 \cdot 7H_2O$ (1.0 *M*), 0.5 mL; $Ca(H_2PO_4)_2$ (0.05 *M*), 2.5 mL; $CaSO_4 \cdot 2H_2O$ (0.01 *M*), 50 mL; micronutrient stock (H_3BO_3, 2.86 g/L; $MnCl_2 \cdot 4H_2O$, 1.81 g/L; $ZnSO_4 \cdot 7H_2O$, 0.22 g/L; $CuSO_4 \cdot 5H_2O$, 0.08 g/L; $Na_2MoO_4 \cdot 2H_2O$, 0.025 g/L; $CoCl_2 \cdot 6H_2O$, 0.025 g/L), 1 mL; and chelated iron stock ($FeSO \cdot 7H_2O$, 5.56 g/L; Na_2EDTA, 7.45 g/L), 5 mL.

Seedlings were transferred to fresh 0.8% agar slants or other axenic containers 14–21 days after sowing and allowed to recover for 3 days or more before further treatment. Axenic conditions were checked by incubating discarded fruit coats in yeast–glucose broth (0.5% Difco yeast extract, 0.5% glucose, pH 6–7) and rejecting seedlings corresponding to contaminated fruit coats.

Slide cultures

For time-course studies of root hair development, modified Fahraeus-type slide cultures (Fahraeus 1957) were used (Fig. 1). Using sterile procedures, 0.5% agar made up with quarter-strength Hoagland's solution, as described above, was gelled on sterile washed 25 × 100 mm glass slides. Five-millimetre-wide wicks of Whatman No. 1 filter paper were added. Axenic seedlings were transferred onto the slides, which were then placed on end between glass supports (washed used glass knives) in quarter-strength Hoagland's solution in glass jars. Jars were covered with glass petri dishes and sealed with Parafilm.

Glass was found to be a reliably inert substance, while metals, plastics, inks, and resin glues were tested and found to

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

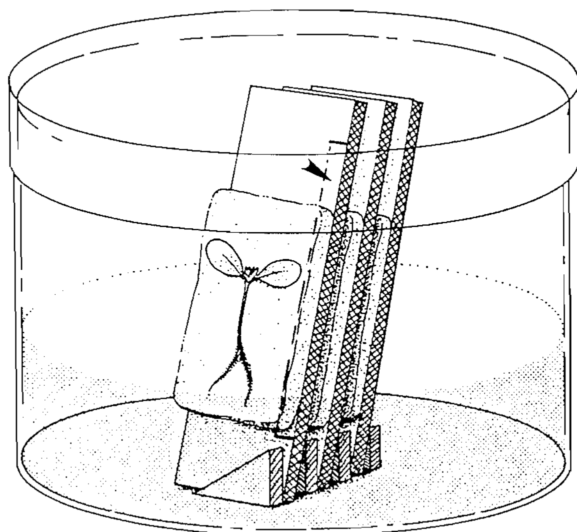


FIG. 1. Method of preparing axenic seedlings for time-course studies. Germinated seedlings are placed atop gelled, 0.5% mineral agar, using sterile procedure. A filter-paper wick (arrowhead) is added atop the agar to extend into the nutrient solution.

be toxic to seedlings. Pouch cultures (Lalonde 1979; Bauer 1981) and other culture methods were tested but could not be kept axenic prior to inoculation.

Root growth was monitored by removing each slide to a sterile petri dish in the laminar-flow hood and measuring the root on a millimetre rule under a dissecting microscope. For observations of root hair deformation, seedling root systems were covered with a sterile cover slip. Roots were examined and photographed with a Zeiss WL microscope using phase-contrast or Nomarski interference-contrast optics.

Sterile clay particles

"Turface" (International Minerals and Chemical Corp.), a particulate clay growth medium, was placed in 24-mm-diameter test tubes and moistened to saturation with quarter-strength Hoagland's N-free solution, pH 6.5. Sterilized seeds or pregerminated seedlings were transferred aseptically into the autoclaved tubes. Tubes were slanted at 45°.

Axenically grown seedlings were germinated and maintained in a controlled-environment chamber with 16-h day length (combined fluorescent and incandescent illumination, approximately 3000 ft-c (1 ft-c = 10.763 91 lx)) and actual temperatures of 27:24°C (day:night).

Inoculation of Betula *root hairs*

Seedlings of *Betula* spp. (*populifolia* and *pendula*) were inoculated with PC 85 or with PC 85 plus *Frankia* in both axenic slide setups as for *Alnus* and in aeroponics (Zobel *et al.* 1976). In aeroponics, *Betula* spp. were grown in the same tank with seedlings of *Alnus rubra* and *Alnus crispa*. Both *Alnus* species were heavily nodulated within 7–14 days after inoculation, when both PC 85 and *Frankia* were present. Extensive root hair deformation was noted for *Alnus* within 24–48 h.

Bacterial culture and preparations

Frankia *sp. ArI3, CpI1*

The *Frankia* isolate strains ArI3 (Berry and Torrey 1979) and CpI1 (Callaham *et al.* 1978) were maintained and subcultured as described previously (Berry and Torrey 1979). Inoculum was prepared as a washed suspension of 3- to 4-week-old cultures, tissue homogenized and applied with a sterile Pasteur pipette at a rate of 2×10^{-4} mL packed cell volume per plant.

Pseudomonas cepacia

Strain 85 (Stanier *et al.* 1966) was obtained from T. Lessie at the University of Massachusetts, Amherst, and maintained in culture on yeast–glucose agar (0.5% Difco yeast extract, 1% glucose, 1% Difco Bacto-agar, pH 6–7, unadjusted). For inoculations, new growth at the colony margins was removed 16 h after streak plating and was suspended in sterile distilled water. The suspension was visibly turbid.

Bacterial filtrates

(*i*) PC 85 was prepared to obtain cell-free filtrates, following Yao and Vincent (1960). Cells in log phase from agar plates or broth cultures were washed and suspended in mineral salts for 1 h, then filtered through sterile 0.2-μm Nuclepore filters. (*ii*) A filtrate was also prepared from the rhizosphere solution of agar-grown seedlings inoculated with both PC 85 and *Frankia*. The seedlings were nodulated, and motile PC as well as vital *Frankia* were observed in quantity in the solution used. This solution was filtered with 0.2-μm sterile Nuclepore filters and tested directly.

Histological procedures

Transmission electron microscopy (TEM)

Specimens were dissected and fixed in 2% glutaraldehyde in 0.025 *M* sodium phosphate buffer, pH 6.9, overnight at 4°C, and rinsed five times in 0.050 *M* sodium phosphate buffer, same pH. Roots were postfixed for 1 h in 2% OsO_4 and rinsed in distilled water. Dehydration in an acetone series was followed by infiltration in Spurr's resin (Spurr 1969) and polymerization. Thick plastic sections (1–2 μm) were cut with glass knives on a Porter-Blum ultramicrotome and stained with toluidine blue, 0.05 *M* in sodium phosphate buffer, pH 6.9, with 1% sodium borate. Thin sections were cut with glass knives on a Reichert OMU2 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Zeiss EM-9.

Scanning electron microscopy (SEM)

Specimens were fixed and dehydrated as for light microscopy, critical point dried, and coated with gold–palladium.

Ruthenium red

Ruthenium red was made up at 1:5000 aq. (Jensen 1962). Fresh material was stained for 1–5 min and examined with bright-field microscopy.

Acridine orange

Whole seedling roots and other biological materials were stained in acridine orange (AO) at 0.005% (approximately 10^{-5} *M*) in 0.025 *M* sodium phosphate buffer, pH 6.9, for 15 min at 25°C, unless otherwise specified. Specimens were rinsed for 15–30 min in 0.025 *M* sodium phosphate buffer and mounted in distilled water (pH 6–7). Viewing and photography were carried out with a Leitz Ortholux microscope equipped with Ploem epifluorescence (Ploem 1967), and an

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

HBO 200-W mercury vapor lamp. Excitation filters used were BG 12 and BG 38; barrier filters used were K525 and the No. 2 built-in filter. Photography was done on Ektachrome 400 or Ilford HP5 (800 ASA). Further evaluation of AO methodology as applied to this system is presented elsewhere (Berry 1983).

Results

Root hair differentiation in Alnus

In both axenic and inoculated seedling roots, root hair initiation follows the common type reviewed by Cormack (1949). At the locus of root hair initiation, there is no apparent specialization of trichoblast cells (e.g., Cutter and Feldman 1970; Dosier and Riopel 1978) among epidermal cells, in terms of size or cytoplasmic density. Root hair production along a single axis can be extremely variable, but every epidermal cell can initiate a root hair.

Root hairs may be initiated at the apical end or centrally on the outer surface of hair-producing cells. Axial displacement of epidermal cells at the locus of hair initiation is either minimal or has ceased. Mature root hair length is variable, up to 1.0 mm. Papillae, or root hairs which elongate very little, are frequent in a population of mature hair cells.

Root hairs tend to differentiate for a short length (four or five cells) within a single file in *Alnus*. Adjacent cells in different files frequently have hairs differentiated at approximately the same level laterally, giving rise to clusters of root hairs, which when deformed, intertwine extensively (Figs. 4, 5 at arrowheads).

In axenic conditions, strands or sheets of mucilage presumably of root epidermal origin extend among elongating or mature root hairs. The thickness of the layer varies with different growth media and is particularly thick in the clay media (Turface). Ruthenium-red staining gives a pink to red color, indicating the pectic nature of the mucilage (Jensen 1962).

In a fashion reminiscent of that reported by Greaves and Darbyshire (1972) extensive bacterial floc formation occurs after inoculation in strands from hair to hair, probably initially along the mucilaginous sheets (Fig. 5, lower right). These aggregations are most dense in regions where root hairs are closely approximated.

Root hair deformation and root development

Microscopical studies of the time course of root hair response to inoculation with PC 85 were made using axenic seedlings of *Alnus rubra*. The majority of root hairs in the developing epidermis exhibit root hair deformation after inoculation in this system. Root hairs occurring within the zone of root hair elongation show observable deformation within 2 h following inoculation. As observed by other authors (Angulo Carmona 1974; Lalonde 1977; Callaham *et al*. 1979), deforming root hairs form an acropetal gradient based on a progressive modification of tip growth relative to hair elongation. The earlier the root hair is affected by inoculum relative to its elongation, the more elaborate is the deformation sequence that follows (to be described below). Root hairs in the most proximal portion of the root are elongate and undeformed (Fig. 2).

Deformed root hairs are not initiated *de novo*. Progressive deformation within a population of root hairs following inoculation can be traced through cross sections at intervals along the length of roots (Table 1). The percentage of epidermal cells which forms root hairs in the successive cross sections studied remains fairly constant. The number of deformed hairs increased from none at 12 mm proximal to the apex to two-thirds of the hair population at 3 mm.

Root hairs which differentiate entirely after inoculation do not exhibit lobing until some degree of root hair elongation occurs. This is evident from time-lapse sequences of roots which have no root hairs at the time of inoculation (Figs. 6*a*, 6*b*). Newly initiated root hairs at 20 h exhibit deformation only at 48 h.

There is thus a specific stage in root hair differentiation during which deformation can occur. This stage corresponds to a morphological locus on the developing root epidermis, delimited distally approximately where root hairs begin to elongate after a period of initiation and papilla extension. At the proximal end, deformation terminates approximately where root hair elongation ceases. This zone corresponds in normal (axenic) root hair development to the region where root hairs elongate rapidly.

Other events associated with inoculation

Binding

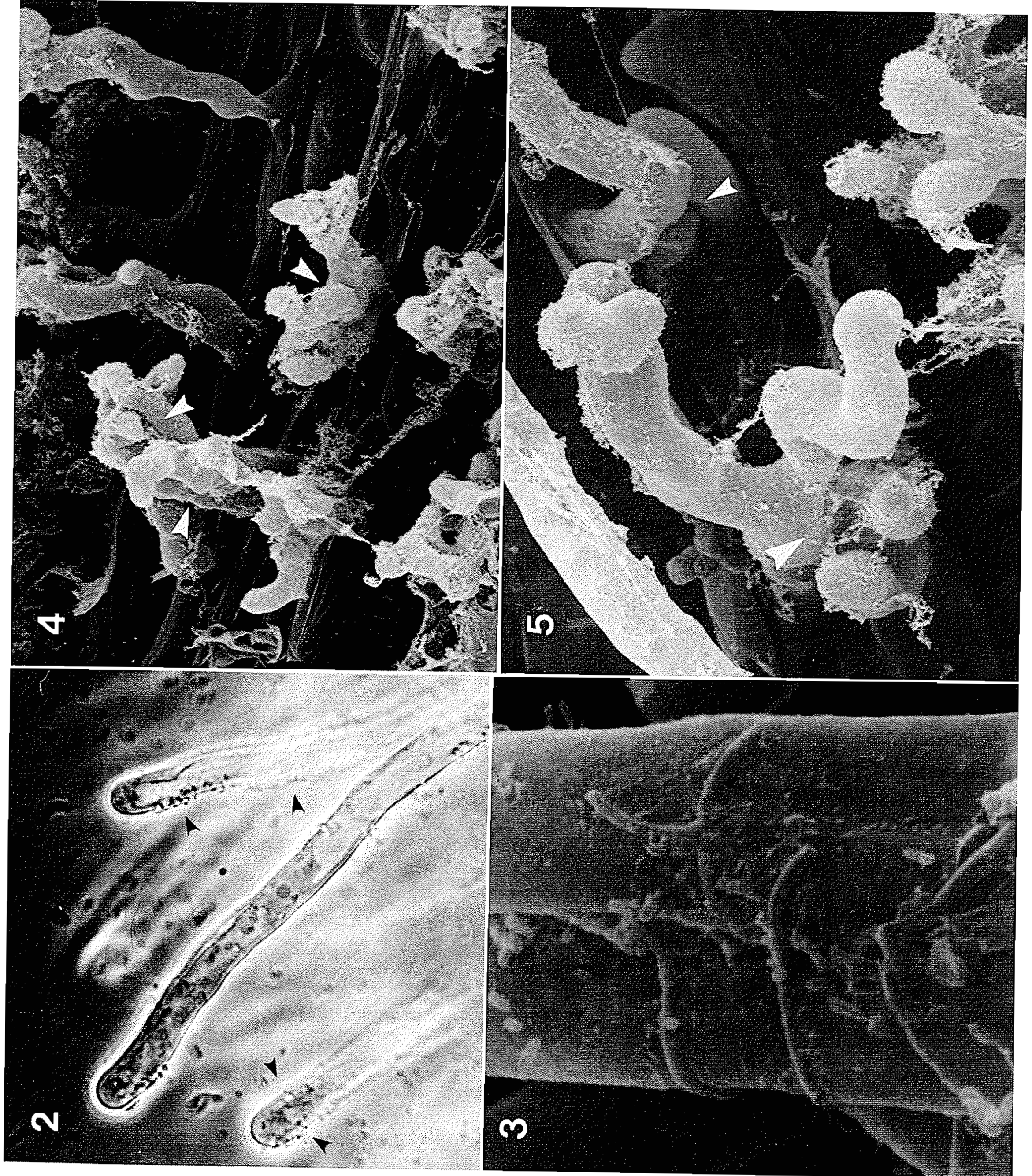
Bacteria can be seen to bind to the root hairs within minutes of inoculation, often in polar fashion. By 20 h

FIG. 2. Phase contrast micrograph of straight root hair from the transitional zone, showing minimal response 20 h after inoculation (nuclear and nucleolar enlargement but no deformation). Neighboring hairs show varying degrees of deformation (not shown). Note extensive nonpolar binding of PC 85 at the hair tips (arrowheads). 600×. FIG. 3. Scanning electron micrograph (SEM) demonstrating binding of *Frankia* on root hair surfaces. PC 85 is also present. (Seedling grown in sterile Turface; postfixed in ruthenium red, 0.05%). 3000×. FIG. 4. SEM of nodulated root axis (inoculated with PC 85 and *Frankia*) showing typical deformed root hairs. Note axial distortion and lateral separation of epidermal cell files owing to the cortical cell enlargement of the prenodule beneath. Every epidermal cell has produced a root hair. Initiation of root hairs at the same levels laterally is observable, and because of lobe intertwining during deformation, there is close lateral association among hairs (at arrows). 420×. FIG. 5. SEM of branched root hairs which occurred frequently with inoculation with "helpers." Bacterial flocs extend typically in strands from branch to branch of the hair. 1000×.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.



RIGHTSLINK

TABLE 1. Straight and deformed root hair formation along an inoculated *Alnus* root axis

Distance from apex, mm	Total no. epidermal cells counted	Hair cells as % total epidermal cells	No. straight hairs	No. deformed hairs	No. deformed hair cells/total no. hair cells (%)
3	62	50	12	19	19/31 (61%)
6	57	46	11	15	15/26 (58%)
9	45	51	12	11	11/23 (48%)
11	59	42	15	10	10/25 (40%)
12	58	50	29	0	0/29 (0%)

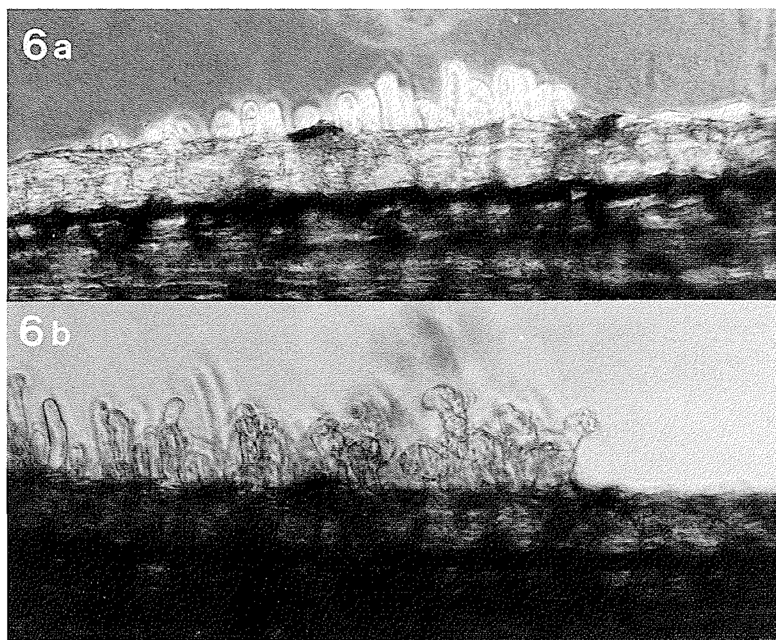


FIG. 6. Time-lapse phase contrast micrographs of a growing root axis in zone of root hair initiation. Root hair deformation following inoculation with PC 85 after (*a*) 20 h and (*b*) 48 h. Apex of root in each case is to the left. There were no root hairs differentiated on this axis prior to inoculation (as seen to the right of the new hairs). By 20 h, new hairs had differentiated, but deformation of these first-formed hairs was only observed at 48 h. 175×.

after inoculation with PC 85, attachment is extensive and especially dense on the straight root hairs in the transitional zone (Fig. 2). By this time, bacterial attachment is primarily nonpolar. Such nonpolar adhesion is consistently more dense on straight than on deformed root hairs, although floc formation is often extremely dense near deformed hairs. *Frankia* also binds tightly to root hair surfaces (Fig. 3).

Root elongation

The rate of growth in length of seedling roots increased significantly ($P = 0.02$) in the first 7 days after inoculation, for one typical set of seedlings measured ($n = 12$). Mean increment per day went from 1.35 mm ± 0.54 (SE) before inoculation to 1.95 ± 0.63 over the week following inoculation. This stimulated growth phase is of limited duration, probably peaking within the 7-day period.

Sequence of root hair deformation

Serial optical sections through a root hair following inoculation with PC 85 were used to follow the course of individual root hair deformation. The hair illustrated in Figs. 7*a*–7*i* was partially elongated at the time of inoculation and was found in the middle of the zone most affected by inoculation, as discussed in the previous section. Within 1 h of inoculation, the tip of the root hair increased in diameter and began to branch. One side of the hair cell dome did not continue to grow (the static left side of the root hair tip can be seen in 2 h in Figs. 7*a* and 7*c* and again in the same position at 8 h, Fig. 7*d*). On the right side, by 2 h after inoculation, the extending lobe

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

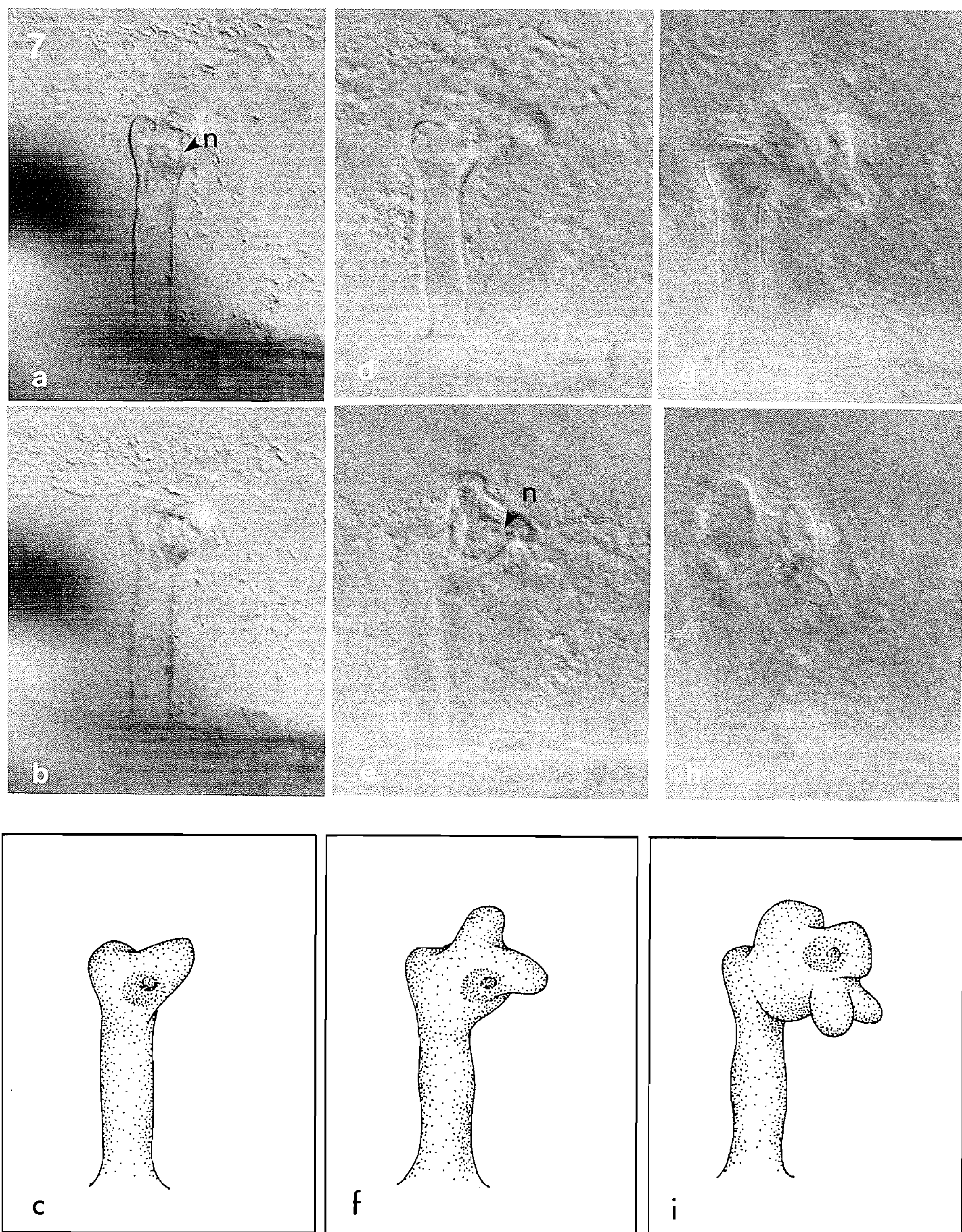


FIG. 7. Time-course study of deformation for a single, living root hair in midelongation at the time PC 85 was introduced. Optical sections, using Nomarski interference contrast microscopy, were photographed at each sampling time: *a–c*, 2 h after inoculation; *d–f*, 8 h after inoculation; *g–i*, 20 h after inoculation. Two sections per sampling period are shown. The schematic representation beneath is a reconstruction of the deformation sequence based on the optical sections. Note the nucleus (*n*, at arrowhead) with prominent nucleolus. 500×.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

had grown outward and slightly vertically. The cytoplasm was dense and the nucleus, with prominent nucleolus, lay at the base of the developing branch. By 8 h after inoculation, the growing tip to the right of the root hair extended outward laterally. The lobe which it produced had enlarged markedly and a second-order branch lobe arose from the original branch. The nucleus migrated to the base of the most lateral growing tip, within the enlarged lobe, and was itself apparently enlarged. By 20 h postinoculation, this region had enlarged further and become vacuolate. Further branching had occurred. It was difficult to discern cytoplasmic contents against the background of bacterial proliferation in the rhizosphere at this time, but the dense, optically refractile appearance which marked the growing tip in earlier sequences was no longer evident. Examination after 20 h revealed no further changes in this root hair. A diagrammatic summary of the deformation sequence is shown in the series Figs. 7*c*, 7*f*, 7*i*, bottom row.

During the course of deformation, no changes were noted in the position of the wall of the root hair laid down previous to inoculation. Only the portion which must have been the apical dome at the time of inoculation showed a growing, or deforming, response. The bulging of the newly formed regions of the hairs, which created the appearance of lobes, was not due to a loss of tip growth but rather to an expansion and vacuolization following an initial tip-type extension. Plasmoptysis (cell bursting) was never observed in deformed root hairs even in distilled water.

This sequence of initial tip outgrowth followed by expansion of the cell behind the growing tip was observed in many root hairs. Often several new tips were initiated progressively from expanding lobes of a single hair cell. The relationship between expansion in an axial direction (that is, in the direction of tip growth) and lateral cellular expansion subsequent to wall deposition appears to determine whether a branching or a lobing pattern will result.

In inoculated root hairs sectioned in regions where cell contents are prominent, the wall fine structure is densely fibrillar (Fig. 8). Small vacuoles, frequent mitochondria, and rough endoplasmic reticulum can be seen commonly in young deforming root hairs. Large plastids of a fine granular density are commonly present. No clearly established axis of microfibrillar orientation can be discerned in the wall in Fig. 8. Nevertheless, these walls, like uninoculated hair walls, are birefringent in the polarizing light microscope, both as whole mounts or sectioned, indicating that cellulose is present in the wall. The fibrillar wall in Fig. 8 is not obviously thinned or degraded. Microtubules, if present, are not easily detected.

Cytological comparisons of inoculated and uninoculated root hairs, using the fluorochrome acridine orange

The fluorochrome acridine orange (AO), used as a stain for biological materials, exhibits a differential in fluorescent maxima, when excited with blue light (480–490 nm), showing a peak at about 530 nm and other peaks from 590 to 650 nm. The differential emission is based on dye complexes formed with selected polyanionic biopolymers; the most frequent use of AO is with nucleic acids. Rigler (1966) analyzed the relationship between fluorescent emission maximum and single or double strandedness in DNA and RNA and determined that the higher order molecular structure produced a greater fluorescent efficiency, i.e., a lower maximum of fluorescent emission. Since the differential fluorescence is qualitatively observed as color shifts, Kinzel (1955) examined the AO fluorescent properties of plant cell wall components and related these properties to wall structural considerations.

Root hair walls of inoculated and uninoculated seedlings were stained with AO and observed as described under Materials and methods. Autofluorescence (Figs. 11*a*, 11*b*) was assayed periodically and found to be low or absent from *Alnus* roots, especially from the root hairs.

Root hair walls

Root hair walls of uninoculated, axenically grown seedlings stained with AO consistently exhibited bright-green fluorescence (Fig. 9). Walls of deformed root hairs (axenically grown, PC-inoculated seedlings) appeared bright yellow–orange upon excitation (Fig. 10). Root hairs of inoculated seedlings which had matured prior to inoculation had green fluorescent walls, although there was occasionally a gradation of green to yellow–orange. Hairs inoculated with *Frankia* only, where no deformation had occurred, also exhibited bright-green fluorescence. But where *Frankia*-inoculated hairs were deformed (on nodulated seedlings), the hair walls showed yellow–orange wall fluorescence (Fig. 12). In no circumstance did deformed root hairs ever have green fluorescent walls, even in the undeformed regions of individual deformed hairs.

Following plasmolysis (25–50% mannitol or sucrose, $NaPO_4$ buffered) to localize staining, there was no loss of fluorescence in the wall region, either for uninoculated or for inoculated, deformed root hairs. There is thus a clear differential in fluorescent emission, localized in the hair wall, between deformed root hair walls and walls of undeformed root hairs, regardless of the circumstances of inoculation.

Infection site

At the site of *Frankia* infection, in a folded region of

RIGHTSLINK

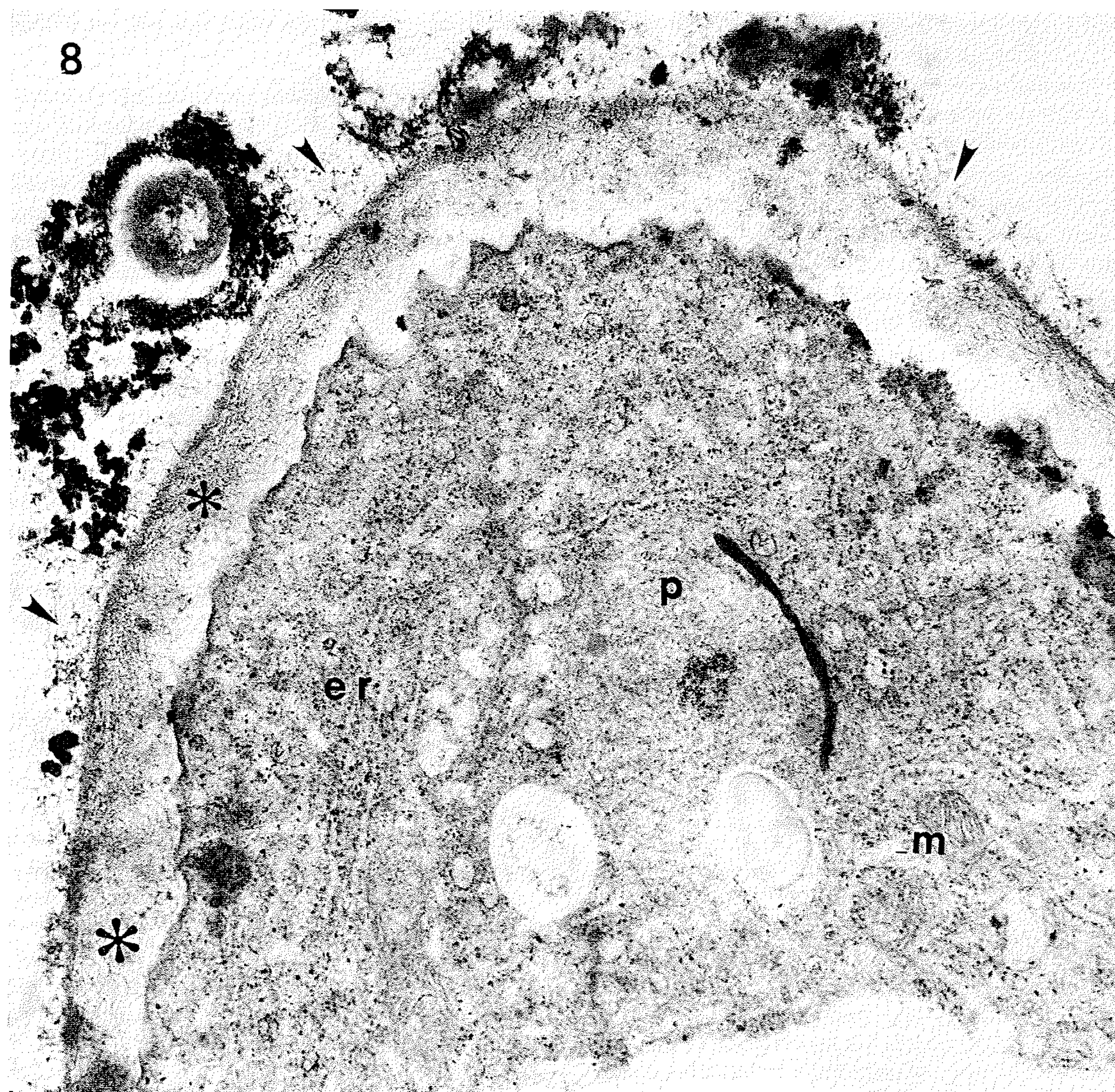


FIG. 8. Transmission electron micrograph of an oblique section through a deformed root hair of *A. rubra* 1 week after inoculation with PC 85. Bacteria adhere to the outer wall surface via a reticulate network (arrowheads; see Greaves and Darbyshire 1972). No mucigel layer is yet in evidence. The root hair wall is densely fibrillar (*) with no axis of orientation evident. Note the presence of endoplasmic reticulum (*er*), mitochondria (*m*), and a typical prominent plastid (*p*) in the dense cytoplasm. 33 000 ×.

the root hair, AO staining produced a bright-yellow–orange fluorescence in the material which seemed to form a matrix around the infecting actinomycete (Fig. 12).

Rhizoplane

Under certain conditions (seedlings grown axenically in Turface or first-formed root hairs of the observed emergent radicle on agar), a bright-orange fluorescent outer layer could be observed on all root hairs, whether deformed or straight, which obscured the green but not the yellow–orange underlying layer. The outer layer corresponded with the ruthenium-red staining, presumed mucigel at the root hair surface, which was particularly thick for Turface-grown seedlings.

Cytoplasm

The cytoplasm of undeformed root hairs stained with AO was clear to light green, with distinctive bright-orange particulate inclusions or aggregates located either within the cytoplasm or paramurally (Fig. 9, arrows). Likewise, non-hair-forming epidermal cells

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

exhibited a light-green or clear cytoplasm, with bright-orange particulate inclusions. The cytoplasm of deformed root hair cells was quite different in fluorescent color, yellow to yellow–orange, and diffuse, without apparent particulate inclusions.

Rhizosphere bacteria

Both PC 85 and *Frankia* appeared bright green in the rhizosphere when stained with AO and were easily distinguishable. *Frankia* vesicles, hyphae, and sporangia were all observed among inoculated root hairs. The extracellular "slime" of PC 85 did not exhibit AO fluorescence.

Root hairs of Betula

Axenically grown root hairs of *Betula*, while finer and longer than those of *Alnus*, had AO fluorescent characteristics identical with those of uninoculated axenic *Alnus* root hairs.

Purified polysaccharides and other biological materials

Efforts were made to identify known plant materials which would show the AO fluorescent characteristics of straight and deformed root hairs (Berry 1983). Cellulosic materials always exhibited bright-green AO fluorescence, as did callose-containing tissue and tissue rich in hemicellulose. The only carbohydrate polymeric substances tested which produced orange AO fluorescence were polyuronides (anhydrous polygalacturonic acid, pollen intine, corn root-cap cell interstices).

Experiment on the host specificity of root hair deformation by PC 85

Seedlings of *Betula* (Betulaceae), a nonactinorhizal genus, were tested both in axenic slide cultures and in aeroponics for response to inoculation with *Frankia* and PC 85 (see Materials and methods). Occasional tip bends or bulges were noted, but no evidence of root hair deformation was seen. Mature hairs in inoculated slide cultures were often observed to adhere to one another at the tips, and the same observation was made for aeroponically grown root hairs but not for axenically grown hair tips.

Application of bacterial filtrates to axenic root systems

Killed cells of PC 85 (lysed or pasteurized) caused root mortality in *Alnus*. Filtrates made from the rhizosphere of actively growing, nodulated seedlings inoculated with both *Frankia* and PC 85 produced no observable changes in seedling root hairs. A filtrate of PC 85 from broth culture, washed and suspended in mineral salts, also failed to trigger root hair deformation.

To permit continuous exposure of axenic seedling roots to exudates of PC 85, dialysis bags (12 000–15 000 molecular weight cutoff) containing the bacteria in a minimal medium were introduced into the liquid phase of the agar slants. No appreciable change in root hair differentiation was observed in the week following introduction of the bags, although the bacterial cultures remained viable within. Thus the deforming substance is likely not to be a low molecular weight species, for example a phytohormone.

Discussion

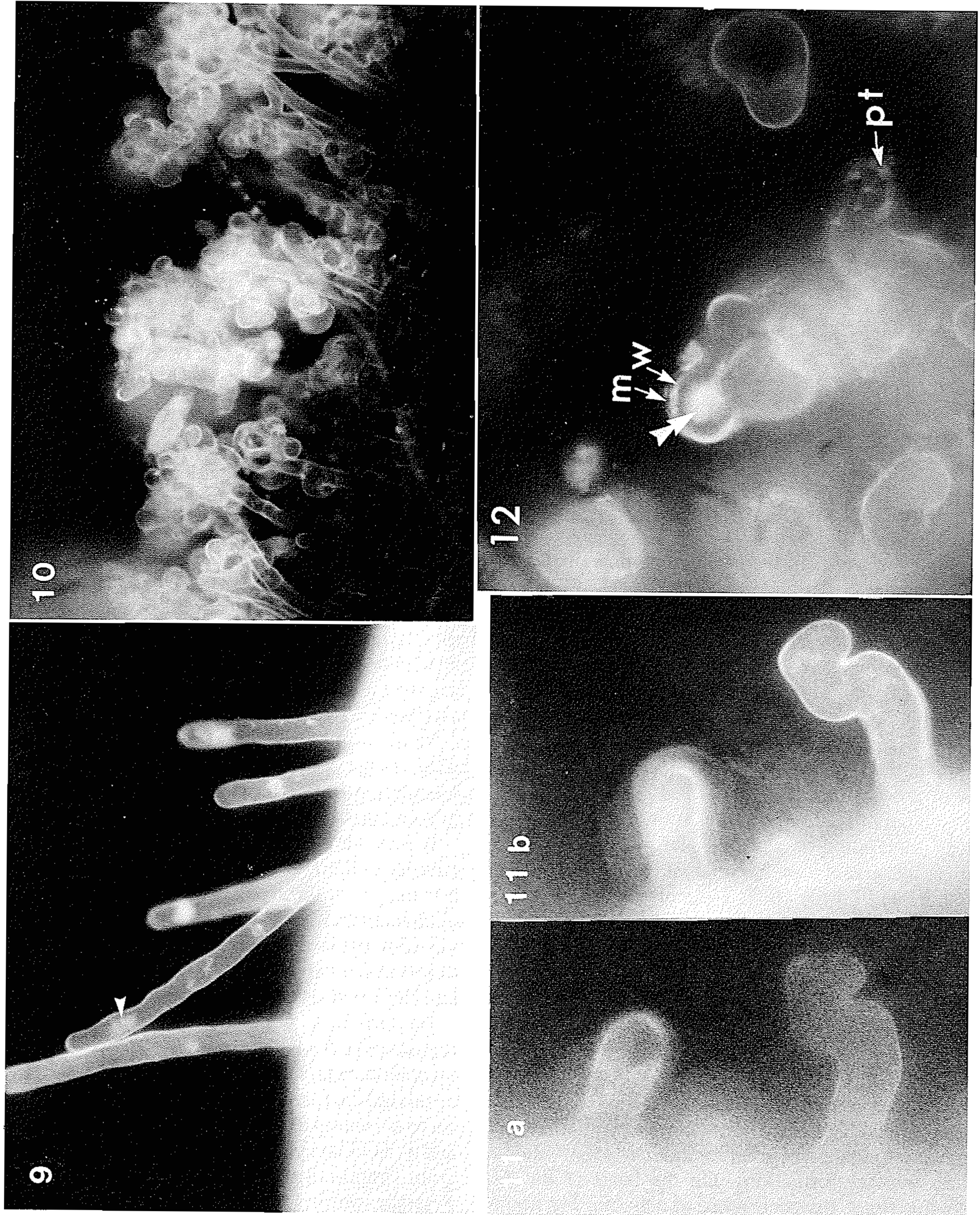
The nodulation studies reported previously (Knowlton *et al.* 1980) demonstrated that root hair deformation, as elicited either by *Frankia* or by "helper" bacteria, is an important precondition for successful root hair infection by *Frankia*. The "helper" bacteria provide a convenient tool for examining these intermediate events separately from the subsequent events of root hair infection. The current study extends the earlier observations to a detailed description of the changes in host root hair cellular development resulting from inoculation with the appropriate bacteria, in this case the "helper" PC 85.

As the present study makes clear, deformation and associated cellular changes in root hairs of *Alnus* are part of a rapid host tissue response to inoculation. The

FIG. 9. Root hairs of living axenic seedlings stained with acridine orange and photographed with Ploem epifluorescence. Bright fluorescent inclusions are not nuclei but apparently cytoplasmic aggregations. Walls fluoresce bright green. 320×. FIG. 10. Deformed root hairs of living seedlings inoculated with PC 85, stained with AO and photographed with Ploem epifluorescence. Note absence of fluorescent inclusions, and bright fluorescence of hair walls. 200×. FIG. 11. Demonstration of minimal wall autofluorescence. (*a*) Deformed root hair, unstained, photographed with Ploem epifluorescence. To obtain any image at all, exposure time was extended beyond 15 s. Note almost total absence of autofluorescence in the walls. (*b*) Same hair photographed with epifluorescence following AO staining. Exposure time: about 1 s. Note bright wall fluorescence. 680×. FIG. 12. AO fluorescence of deformed, infected root hair of *Alnus rubra*. Seedling was grown in Turface and inoculated with a spore-enriched preparation of *Frankia* sp. CpI1. Note the two fluorescent wall layers, the outer of which (*m*) corresponds in fluorescent color (deep orange) and in thickness with the mucigel layer characteristic of seedlings grown in clay media (see text). Clay particles (*pt*) can be seen in the amorphous mucigel. The inner layer (*w*) corresponds to the hair wall. It appears continuous both in fluorescent color (yellow–orange) and structurally with the infection site, which is seen here (at double arrowhead) as a bright fluorescent region in the lower face of the deformed hair wall. Encapsulated hyphae, also fluorescent, extend from the infection site up along the hair lobe and down toward the hair base.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK



Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

maximal deformational response occurs in individual hairs during midelongation phase, and the process continues for between 8 and 20 h. Since the penetration site is in an extensively deformed region of an infected hair, the actual infection must occur after deformation, through the newly deposited wall.

The mechanisms of root hair formation are associated with new wall formation and cellular expansion. Normal root hair differentiation in *Alnus* appears to follow the most common, nonspecialized pattern. There are features of the deformation process, however, which represent departures from the model of normal root-hair tip growth and elongation presented by Newcomb and Bonnett (1965). In *Alnus*, the new wall expands transversely with respect to the vector of tip growth. More than one growing dome is then organized, producing branched hairs or a series of successive lobes. Without a detailed ultrastructural study, it is not clear whether the directed quality of root hair wall deposition and cell elongation is altered, perhaps by some disorganization of microtubular arrays, for example, or whether the redirection of the vector of tip growth which occurs during deformation is a consequence solely of wall softening and expansion.

The cell walls of deformed root hairs show distinctive acridine orange fluorescence, i.e. bright yellow–orange instead of the green characteristic of straight root hair growth. This fluorescence behavior matches with that of the dye complexed with a polyanionic, perhaps polyuronidic, material in the deformed root hair wall. Presumably, the polypectatelike polymer is formed by the root hair cell in response to a bacterial agent, and wall characteristics are modified in such a way as to allow or promote hair deformation.

The general features of the actinorhizal infection process, including root hair deformation, closely parallel the events in legume infection sequences (Callaham and Torrey 1981; Turgeon and Bauer 1982). Sethi and Reporter (1981) found localized increases in Ca^{2+} binding to the fluorescent antibiotic chlorotetracycline at the infection site and root hair wall in clover inoculated with *Rhizobium*, and this may reflect a change in wall deposition for legumes accompanying deformation similar to that suggested here for *Alnus*.

Bauer (1981) speculated that a local inhibition of cellulose microfibril deposition in the β-layer at the site of *Rhizobium* attachment in root hairs of legumes could provide a softened pivot point for hair curling around the bacteria (crozier formation). On the basis of the AO results for *Alnus*, the occurrence of a greater fraction of polyuronidic components relative to other wall components in deforming *Alnus* root hairs cannot be excluded. The limited ultrastructural evidence available for actinorhizal species (this paper; and see Callaham *et al.* 1979) demonstrates a fibrillar wall component rather than a strictly amorphous root hair wall, however.

The nature of the bacterial agent causing deformation is not understood at the present time. For the *Alnus* – PC 85 system, the bacterial promotion of root hair deformation is separable from wall penetration both in time and (since PC 85 binds readily but remains at the rhizoplane) in mechanism. There are several reports that nonhomologous (i.e., noninfective) rhizobial strains cause root hair deformation in legumes (Nutman 1959; Hubbell 1970), but there is apparently a greater or more precise degree of deformation in the presence of the homologous (infecting) strains. Such is not the case in the *Alnus* – PC 85 system reported here. If pectolytic and cellulolytic enzymes figure both in deformation and infection in *Alnus* (as suggested by Hubbell (1981) for legumes), then there must be two different categories of such enzymes, a general type which causes deformation and a *Frankia*-specific enzyme or enzymes which are active in wall penetration. Purified bacterial extracellular polysaccharides have been reported (Hubbell 1970) to cause root hair deformation in clover. *Pseudomonas cepacia*, the "helper" employed in this study, is a pathogenic species known to elaborate pectolytic enzymes (Ulrich 1975), but other extracellular polymers have not yet been examined. It may be that the early events of actinorhizal symbioses are less specialized in regard to promoting root hair deformation than those of legume symbioses. The surprisingly negative results from filtrates of PC 85 may be ascribable to such a lack of precision or specialization.

While root hair deformation in *Alnus* is elicited by a wide range of bacterial species, the results from inoculating *Betula* roots demonstrate that susceptibility of root hairs to deformation is host specific.

It may be possible to bypass root hair deformation entirely in the *Frankia–Alnus* infection process. Infection can occur through a noncurled root hair in legumes (Callaham and Torrey 1981). *Rhizobium* infects directly via root epidermal cells also (Nutman 1959). These observations call into some question the function of root hair deformation in the infection process.

Nevertheless, since the infection through deformed regions of root hairs is greatly enhanced over penetration by other means, sequestering of *Frankia* seems to confer an advantage for successful infection. Deformation may serve to entrap *Frankia*, as proposed by D. Callaham and J. G. Torrey (unpublished data). This could be of great importance in actinorhizal infections, since *Frankia*, unlike *Rhizobium*, is not motile. The crypt formed by deforming lobes, in concert with bacterial floccing, may provide a microenvironment suitable for infection, by counterbalancing the turgor pressure generated by the host cell (Bauer 1981) or by

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK

concentrating the hydrolytic enzymes of the microsymbiont (Callaham *et al.* 1979). The cytological changes in the root hair wall which accompany its deformation could function directly in the infection process as well, by providing a specialized substrate or altered wall structure at the site of penetration which would then be open to further enzymatic action by *Frankia*.

Acknowledgements

This research was supported in part by the Maria Moors Cabot Foundation for Botanical Research of Harvard University and by research grant DEB8106952 from the U.S. National Science Foundation. The advice and assistance of William Ormerod, Ed Seling, Otto Stein, and Patricia Young are gratefully acknowledged.

AIST, J. R., and P. H. WILLIAMS. 1971. The cytology and kinetics of cabbage root hair penetration by *Plasmodiophora brassicae*. Can. J. Bot. **49**: 2023–2034.

ANGULO CARMONA, A. F. 1974. La formation des nodules fixateurs d'azote chez *Alnus glutinosa* (L.) Vill. Acta Bot. Neerl. **23**: 257–303.

ANGULO CARMONA, A. F., C. VAN DIJK, and A. QUISPEL. 1976. Symbiotic interactions in non-leguminous root nodules. *In* Symbiotic nitrogen fixation in plants. *Edited by* P. S. Nutman. Cambridge University Press, Cambridge. pp. 474–484.

BAUER, W. D. 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. **32**: 407–449.

BERRY, A. M. 1983. The development of the actinorhizal association between *Frankia* and *Alnus rubra* Bong. Ph.D. dissertation, University of Massachusetts, Amherst, MA.

BERRY, A. M., and J. G. TORREY. 1979. Isolation and characterization *in vivo* and *in vitro* of an actinomycetous endophyte from *Alnus rubra* Bong. *In* Symbiotic nitrogen fixation and the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. A. Perry. Oregon State University, Corvallis, OR.

BURGEFF, H. 1909. Die Wurzelpilze der Orchideen, ihre Kultur and ihr Leben in der Pflanze. Jena.

CALLAHAM, D., P. DEL TREDICI, and J. G. TORREY. 1978. Isolation and cultivation in vitro of the actinomycete causing root nodulation in *Comptonia*. Science (Washington, D.C.), **199**: 899–902.

CALLAHAM, D., W. NEWCOMB, J. G. TORREY, and R. L. PETERSON. 1979. Root hair infection in actinomycete-induced root nodule initiation in *Casuarina*, *Myrica*, and *Comptonia*. Bot. Gaz. (Chicago), **140**: S1–S9.

CALLAHAM, D., and J. G. TORREY. 1977. Prenodule formation and primary nodule development in roots of *Comptonia* (Myricaceae). Can. J. Bot. **55**: 2306–2318.

——— 1981. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. Can. J. Bot. **59**: 1647–1664.

CORMACK, R. G. H. 1949. The development of root hairs in angiosperms. Bot. Rev. **15**(9): 583–612.

CUTTER, E. G., and L. J. FELDMAN. 1970. Trichoblasts in *Hydrocharis*. I. Origin, differentiation, dimensions, and growth. Am. J. Bot. **57**: 190–201.

DOSIER, L. W., and J. L. RIOPEL. 1978. Origin, development, and growth of differentiating trichoblasts in *Elodea canadensis*. Am. J. Bot. **65**: 813–822.

FAHRAEUS, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **14**: 374–381.

GREAVES, M. P., and J. F. DARBYSHIRE. 1972. The ultrastructure of the mucilaginous layer on plant roots. Soil Biol. Biochem. **4**: 443–449.

HOAGLAND, D. R., and D. I. ARNON. 1950. The water culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. No. 347.

HUBBELL, D. H. 1970. Studies on the root hair "curling factor" of *Rhizobium*. Bot. Gaz. (Chicago), **131**(4): 337–343.

——— 1981. Legume infection of *Rhizobium*. A conceptual approach. BioScience, **31**(11): 832–837.

JENSEN, W. A. 1962. Botanical histochemistry. W. H. Freeman and Co., San Francisco.

KÄPPEL, M., and H. WARTENBERG. 1958. Der Formenswechsel des *Actinomyces alni* Peklo in den Wurzeln von *Alnus glutinosa* Gaertn. Arch. Mikrobiol. **30**: 46–63.

KINZEL, H. 1955. Zur Kauselfrage der Zellwand—fluorochromierung mit acridin orange. Protoplasma, **45**: 73–96.

KNOWLTON, S., A. BERRY, and J. G. TORREY. 1979. The role of rhizosphere microorganisms in nodule formation in *Alnus rubra* Bong. *In* Symbiotic nitrogen fixation in the management of temperate forests. *Edited by* J. C. Gordon, C. T. Wheeler, and D. H. Perry. Oregon State University, Corvallis, OR.

——— 1980. Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. Can. J. Microbiol. **26**(8): 971–977.

KNOWLTON, S., and J. O. DAWSON. 1983. Effects of *Pseudomonas cepacia* and cultural factors on the nodulation of *Alnus rubra* roots by *Frankia*. Can. J. Bot. **61**. This issue.

KREBBER, O. 1932. Untersuchungen uber die Wurzelknollchen der Erle. Arch. Mikrobiol. **3**: 588.

LALONDE, M. 1977. The infection process of *Alnus* root nodule symbiosis. *In* Recent developments in nitrogen fixation. *Edited by* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco. Academic Press, London. pp. 569–589.

——— 1979. Techniques and observations of the nitrogen-fixing *Alnus* root nodule symbiosis. *In* Recent advances in biological nitrogen fixation. *Edited by* N. S. Subba Rao. Oxford University Press, London.

LALONDE, M., H. E. CALVERT, and S. PINE. 1981. Isolation and use of *Frankia* strains in actinorhizae formation. *In* Current perspectives in nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Australian Academy of Sciences, Canberra.

NEWCOMB, E. H., and H. T. BONNETT. 1965. Cytoplasmic microtubule and wall microfibril orientation in root hairs of radish. J. Cell. Biol. **27**: 575–589.

NUTMAN, P. S. 1959. Some observations on root-hair infection by nodule bacteria. J. Exp. Bot. **10**(29): 250–263.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

NUTMAN, P. S., C. C. DONCASTER, and P. J. DART. 1973. Infection of clover by root-nodule bacteria. The British Film Institute, London.

PLOEM, J. S. 1967. The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. Z. Wiss. Mikrosk. Mikrosk. Tech. **68**(3): 129–142.

POMMER, E. H. 1956. Beitrage zur Anatomie und Biologie der Wurzelknollchen von *Alnus glutinosa* Gaertn. Flora (Jena, 1818–1965), **143**: 604–634.

RIGLER, R. 1966. Microfluorometric characterization of intracellular nucleic acids and nucleoproteins by acridine orange. Acta Physiol. Scand. Suppl. **67**: 267.

SETHI, R. S., and M. REPORTER. 1981. Calcium localization pattern in clover root hair cells associated with infection process: studies with aureomycin. Protoplasma, **105**: 321–325.

SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31–43.

STANIER, R. Y., N. J. PALLERONI, and M. DOUDOROFF. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**: 159–271.

TAUBERT, H. 1965. Uber den Infektions-vorgang und die Entwicklung der Knollchen bei *Alnus glutinosa* Gaertn. Planta, **48**: 135–156.

TURGEON, B. G., and W. D. BAUER. 1982. Early events in the infection of soybean by *Rhizobium japonicum*. Time course and cytology of the initial infection process. Can. J. Bot. **60**: 152–161.

ULRICH, J. M. 1975. Pectic enzymes of *Pseudomonas cepacia* and penetration of polygalacturonase into cells. Physiol. Plant Pathol. **5**: 37–44.

VON PLOTHO, O. 1941. Die Synthese der Knollchen an den Wurzeln der Erle. Arch. Mikrobiol. **12**: 1.

YAO, P. Y., and J. M. VINCENT. 1969. Host specificity in the root hair "curling factor" of *Rhizobium* spp. Aust. J. Biol. Sci. **22**: 413–423.

ZOBEL, R. W., P. DEL TREDICI, and J. G. TORREY. 1976. Method for growing plants aeroponically. Plant Physiol. **57**: 344–346.

Can. J. Bot. Downloaded from www.nrcresearchpress.com by HARVARD UNIVERSITY HERBARIA on 08/30/11
For personal use only.

RIGHTSLINK