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Recognition of Leguminous Hosts by a Promiscuous *Rhizobium* Strain

S. SHANTHARAM AND PETER P. WONG*

Division of Biology, Kansas State University, Manhattan, Kansas 66506

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The lima bean (*Phaseolus lunatus* L.) and the pole bean (*Phaseolus vulgaris* L.) are nodulated by rhizobia of two different cross-inoculation groups. *Rhizobium* sp. 127E15, a cowpea-type *Rhizobium*, can induce effective nodules on the lima bean and partially effective nodules on the pole bean. *Rhizobium phaseoli* 127K14 can induce effective nodules on the pole bean but does not reciprocally nodulate the lima bean. Root hairs of the lima bean when inoculated with *Rhizobium* sp. 127E15 showed tip curling and swelling and infection thread formation as observed by light microscopy and scanning electron microscopy. When lima bean root hairs were inoculated with *R. phaseoli* 127K14, no host-specific responses were observed. Pole bean root hairs that had been inoculated with *R. phaseoli* 127K14 or *Rhizobium* sp. 127E15 also showed tip curling and swelling and infection thread formation. Colonization of lima bean root hairs by *Rhizobium* sp. 127E15 and pole bean root hairs by *R. phaseoli* 127K14 or *Rhizobium* sp. 127E15 appeared to involve the elaboration of microfibrils. This study showed that when *Rhizobium* sp. 127E15 nodulates a host of a different cross-inoculation group, it elicits the same specific host responses as it does from a host of the same cross-inoculation group.

Soil bacteria of the genus *Rhizobium* enter into symbiotic partnership with legumes which result in N_2 fixation. As a prerequisite for the formation of the symbiotic association, the two partners come in contact by their cell surfaces, where the phenomenon of specificity and recognition is believed to take place (3). This event is followed by root hair tip curling, formation of infection threads, and the subsequent establishment of N_2-fixing root nodules (14).

Although some rhizobia have a restricted host range (5), others can promiscuously enter symbiotic association with many species of legumes (6). Lange (8), for example, reported promiscuous *Rhizobium* strains which are capable of nodulating legumes of four different cross-inoculation groups. We have shown recently that *Rhizobium* sp. 127E15, which was originally isolated from effective lima bean nodules (J. C. Burton, personal communication), is capable of forming effective nodules on the cowpea (*Vigna unguiculata* L., Walp. cv. California Blackeye), lupine (*Lupinus angustifolius* L., cv. Frost), the lima bean (*Phaseolus lunatus* L., cv. Henderson), and partially effective nodules on the pole bean (*Phaseolus vulgaris* L., cv. Kentucky Wonder). *R. phaseoli* 127K14, which induces effective nodules on the pole bean, does not reciprocally nodulate the lima bean (10, 15). Although the pole bean and lima bean are of the same genus, they belong to different cross-inoculation groups. The pole bean belongs to the bean group, and the lima bean belongs to the cowpea group (1).

Presently it is not known whether a promiscuous *Rhizobium* strain has the same or a different mechanism for recognition and subsequent interactions with hosts of different cross-inoculation groups. In this report we employed light and scanning electron microscopy to study how *Rhizobium* sp. 127E15 and *R. phaseoli* 127K14 interact with the pole bean and the lima bean.

MATERIALS AND METHODS

***Rhizobium* and legume cultures.** *Rhizobium* sp. 127E15 and *R. phaseoli* 127K14 were generous gifts of J. C. Burton, the Nitragin Co., Milwaukee, Wis. *R. japonicum* 311b-110 was obtained from the U.S. Department of Agriculture *Rhizobium* Culture Collection, Beltsville, Md. *R. trifolii* ATCC 10328 was purchased from the American Type Culture Collection, Rockville, Md. All rhizobia were grown in shake culture of a liquid mannitol-yeast extract medium as described earlier (9). Rhizobial cells of late exponential phase were harvested by centrifuging at 12,000 × *g* for 10 min and washed twice with a sterile N-free plant nutrient solution (9). Rhizobia were then suspended in plant nutrient solution to about 1×10^8 cells per ml and used as inoculants.

Rhizobium sp. 127E15 cultures were routinely plated on mannitol-yeast extract medium to make sure that they were not contaminated with *R. phaseoli*. Since *Rhizobium* sp. 127E15 is a slow-growing strain and *R. phaseoli* is a fast-growing *Rhizobium*, the large

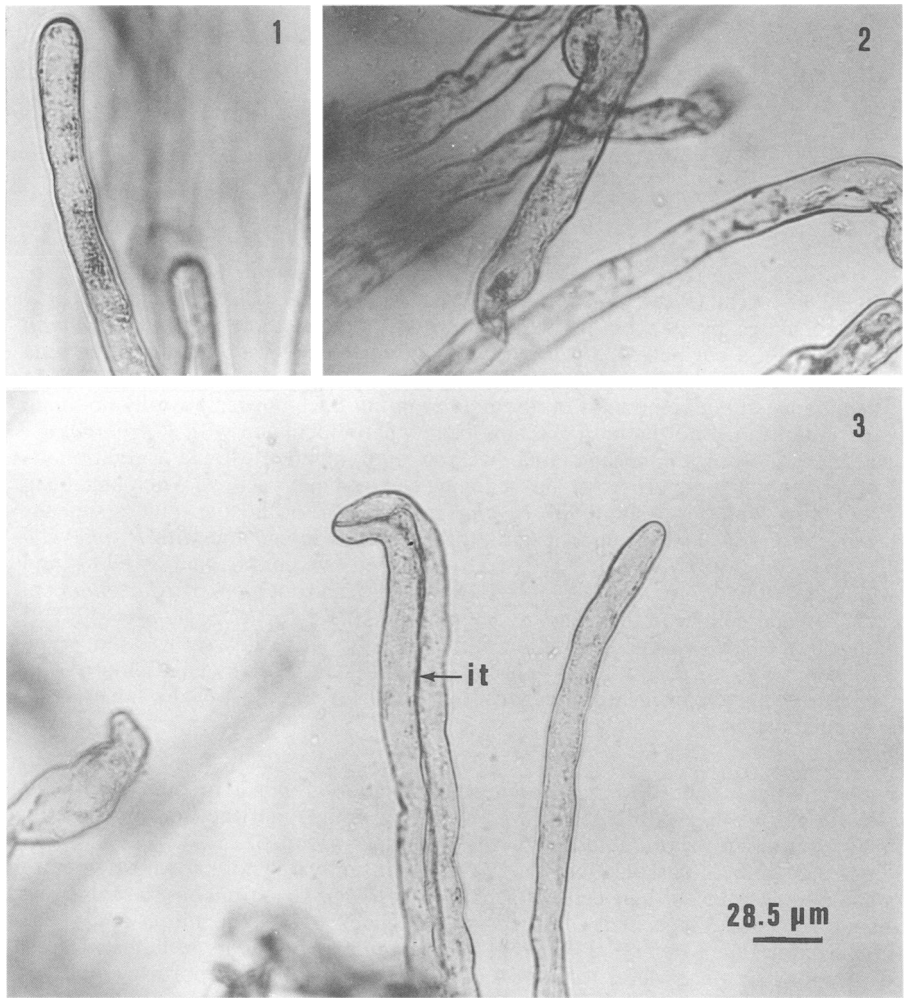

FIG. 1 to 3. Light micrographs of lima bean root hairs as affected by inoculation with *Rhizobium* sp. 127E15. (Fig. 1) Uninoculated root hair. (Fig. 2) Root hairs 2 h after inoculation showing curling and swelling. (Fig. 3) Root hair 2 days after inoculation showing shepherd's crook and an infection thread (it).

variation in the size of the colonies would indicate a mixed culture. Pure *Rhizobium* sp. 127E15 was used for inoculation.

The pole bean and the lima bean were purchased from Burpee Seed Co., Clinton, Iowa. Seeds were surface-sterilized as described earlier (9) and planted in plastic growth pouches (Seed-Pack, Evanston, Ill.). The paper lining of the pouch was moistened with 15 ml of sterile N-free plant nutrient solution. The moisture level was maintained at a constant by adding sterile deionized water throughout the experiment. The pouches were placed in a growth chamber (light intensity, 23,000 lx; photoperiod, 16 h of light and 8 h of darkness; day temperature, 27°C; night temperature 22°C). Three to four days after planting, the seedlings, approximately 5 cm in length, were inoculated with 2 ml of appropriate rhizobial suspension.

Light and scanning electron microscopy. The inoculated seedlings were sampled at 2-h, 2-day, and 5-day intervals. Freehand serial sections (about 1 mm thick) were made acropetally from a marked region of the root where inoculant was applied. The sections were stained with 1% rose bengal and observed under a Carl Zeiss Photomicroscope II fitted with bright-field optics.

Similar root sections were fixed in 2.5% glutaraldehyde–25 mM potassium phosphate (pH 6.8) at 23°C overnight (13). The sections were rinsed twice in 25 mM potassium phosphate buffer and dehydrated in a series of ethanol grades. They were then critical-point-dried (DCP-1; Denton, Cherry Hill, N.J.) and sputter-coated with gold-palladium (DV-502; Denton). The specimens were examined under an ETEC scanning electron microscope at 20 kV.

RESULTS

The root hairs of the uninoculated lima bean were straight and not deformed throughout the experiment (Fig. 1). Root hair tip curling and

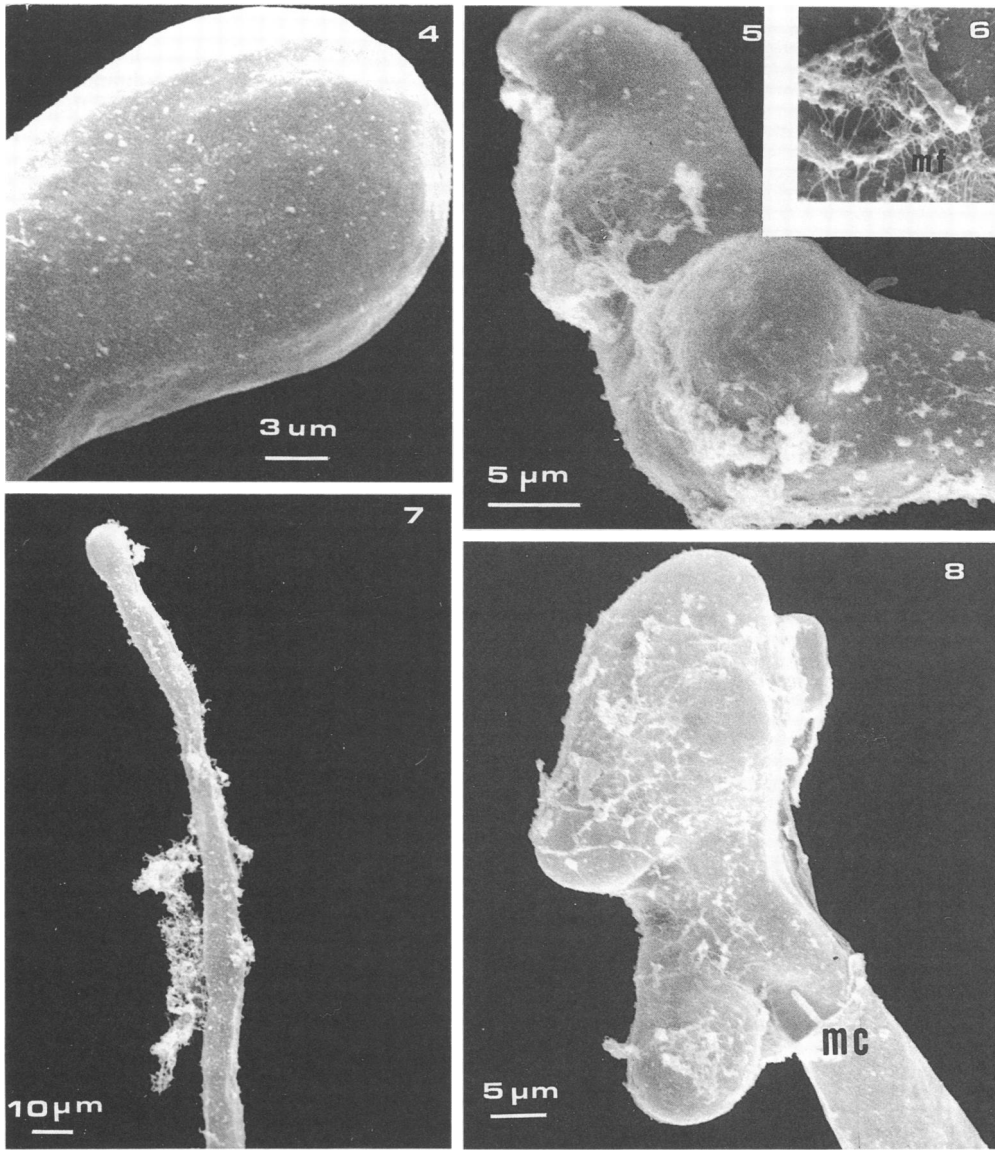

FIG. 4 to 8. Scanning electron micrographs of lima bean root hairs as affected by inoculation with *Rhizobium* sp. 127E15. (Fig. 4) Uninoculated root hair. (Fig. 5) Root hair tip after 2 h of inoculation showing distortion. (Fig. 6) *Rhizobium* sp. 127E15 colonization on root hair surface by means of microfibrils (mf). (Fig. 7) *Rhizobium* sp. 127E15 colonization of an old root hair along its length without eliciting any host-specific responses. (Fig. 8) A highly contorted root hair tip 2 days after inoculation. Note the folding back of a peeled mucigel (mc).

swelling were observed 2 h after inoculating the lima bean with *Rhizobium* sp. 127E15 (Fig. 2). Curling and swelling were seen in 8 out of 10 young and short (150 to 250 µm) root hairs counted randomly. Within 2 days, infection threads were observed (Fig. 3). Samples of 5 days after inoculation showed responses similar to those of samples taken 2 days after inoculation.

The initial colonization of *Rhizobium* sp. 127E15 on lima bean root hair was clearly visualized by scanning electron micrographs (Fig. 5 through 8). The uninoculated control root hair was relatively clean and smooth (Fig. 4). Root hair tip curling and swelling and colonization of *Rhizobium* were clearly seen 2 h after inoculation (Fig. 5 and 6). Colonization of the root hair surface by *Rhizobium* appeared to be aided by microfibrils (Fig. 6). After 2 days, root hair swelling was enhanced, and a layer of peeled mucigel could be seen on the surface (Fig. 8). When bacteria were found attached to the mid-

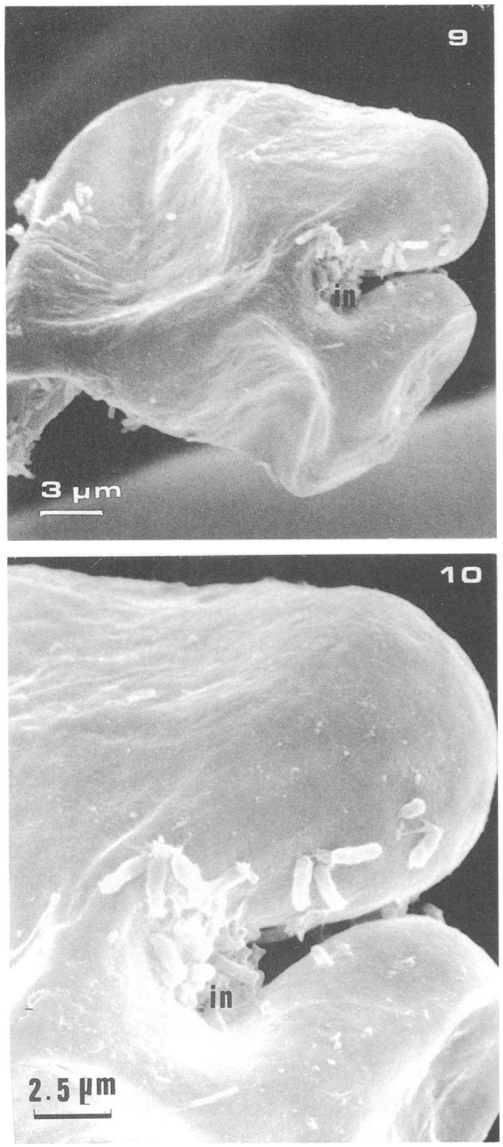

FIG. 9 and 10. Highly magnified scanning electron micrographs of lima bean root hairs as affected by inoculation with *Rhizobium* sp. 127E15. (Fig. 9) Folding of root hair tip 2 days after inoculation. Note the bacteria being entrapped (in). (Fig. 10) A magnified view of the same.

dle of old and long (250 to 350 μm) root hairs, there appeared to be no swelling or any other specific host responses (Fig. 7). Invariably only the young and short root hairs showed host responses to rhizobial contact. At 2 days after inoculation, it was possible to see entrapment of *Rhizobium* cells (Fig. 9 and 10).

The pole bean interacted specifically with *R. phaseoli* 127K14, as shown in Fig. 11 through 14. Uninoculated root hairs were straight and not deformed (Fig. 11). Within 2 h after inoculation, *R. phaseoli* 127K14 cells colonized around the root hairs (Fig. 12). In 2 days, there was visible root hair curling and the formation of shepherd's crook (Fig. 13). Infection thread formation was observed in 5 days (Fig. 14). Approximately 6 out of 10 young and short (150 to 250 μm) root hairs randomly counted showed the specific infection process. The old and long (250 to 350 μm) root hairs showed no response to rhizobial inoculation.

Scanning electron microscopy of *R. phaseoli* 127K14 interaction with the pole bean showed a network of microfibrils anchoring *Rhizobium* to the root hair tips in 2 h after inoculation (Fig. 16). The uninoculated control root hair appeared relatively clean and smooth (Fig. 15). The host plant responded with root hair tip swelling in 2 days (Fig. 17). Folding of the root hair tips was seen in 5 days (Fig. 18). Peeling off of mucigel was also seen clearly (Fig. 16 through 18).

The pole bean was also nodulated by *Rhizobium* sp. 127E15, and it also interacted specifically with this *Rhizobium* strain. The root hairs of uninoculated plant appeared straight (Fig. 19). Root hair tip curling was observed 2 h after inoculation (Fig. 20). Approximately 4 out of 10 young and short (150 to 250 μm) root hairs showed such curling. Old and long (250 to 350 μm) root hairs did not respond. The formation of infection thread and shepherd's crook was clearly seen 2 days after inoculation (Fig. 21 and 22).

Scanning electron microscopy of the uninoculated pole bean root hairs appeared relatively clean and smooth (Fig. 23). Root hair tip curling and swelling were observed 2 h after inoculation with *Rhizobium* sp. 127E15 (Fig. 24). Folding of root hair tip and peeling off of mucigel was seen in 2 days after inoculation (Fig. 25 and 26).

The lima bean responded specifically only to *Rhizobium* sp. 127E15, and the pole bean responded specifically only to *Rhizobium* sp. 127E15 and *R. phaseoli* 127K14. When rhizobia such as *R. japonicum* 311b-110 and *R. trifolii* ATCC 10328, which do not nodulate the lima bean and pole bean, were used as inoculants, they were observed adhering only to the middle of old and long root hairs in the same fashion as shown in Fig. 7. These rhizobia elicited no specific responses from the two legumes. The lima bean also did not show any responses to *R. phaseoli* 127K14.

DISCUSSION

The specificity and host recognition in legume-*Rhizobium* symbiosis are demonstrated by root hair tip curling and swelling, formation of infection thread, and the establishment of N_2-fixing root nodules (14). Some rhizobia, such as

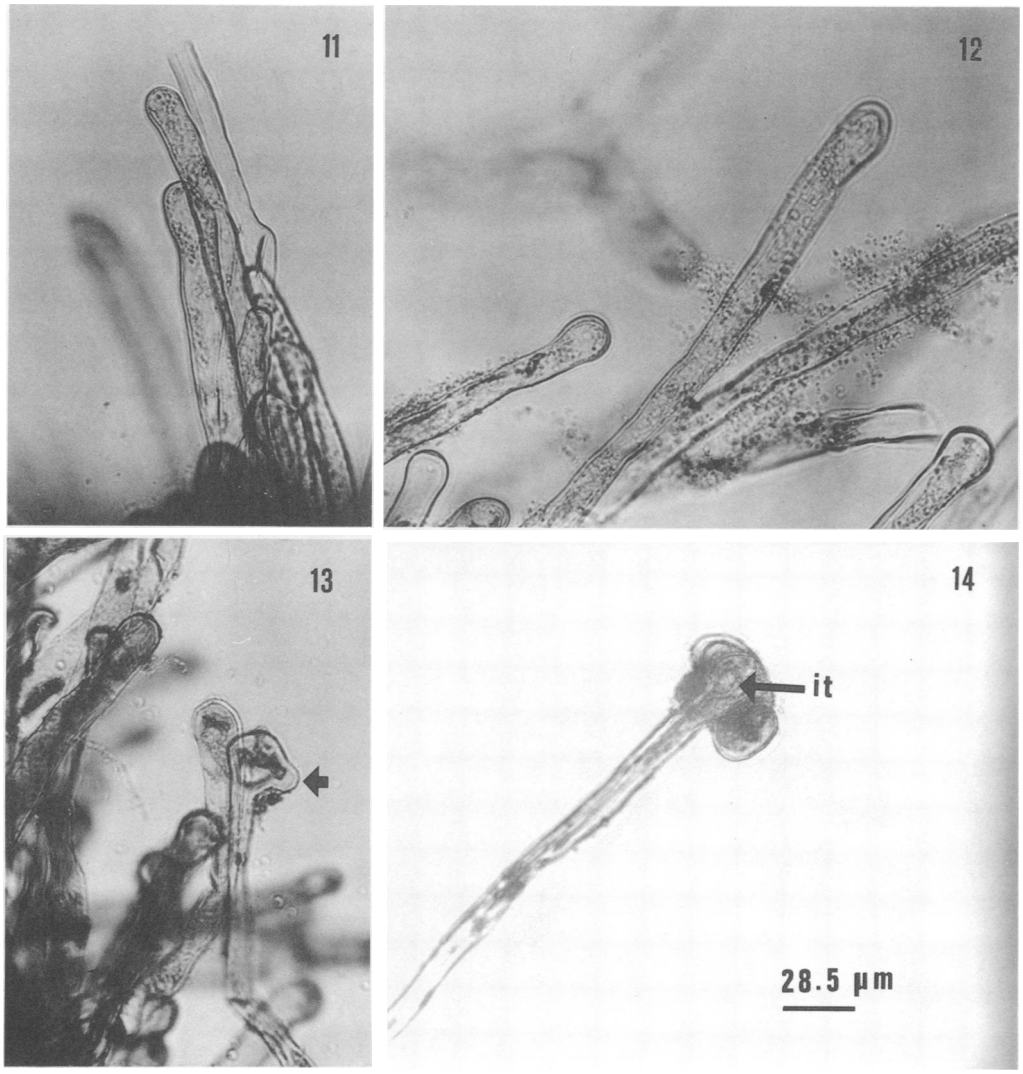

FIG. 11 to 14. Light micrographs of pole bean root hairs as affected by inoculation with *R. phaseoli* 127K14. (Fig. 11) Uninoculated root hairs. (Fig. 12) Root hairs being colonized by *R. phaseoli* 127K14 2 h after inoculation. (Fig. 13) Root hairs showing tip curling and shepherd's crook 2 days after inoculation. (Fig. 14) Root hair 5 days after inoculation showing tip curling and infection thread (it) formation.

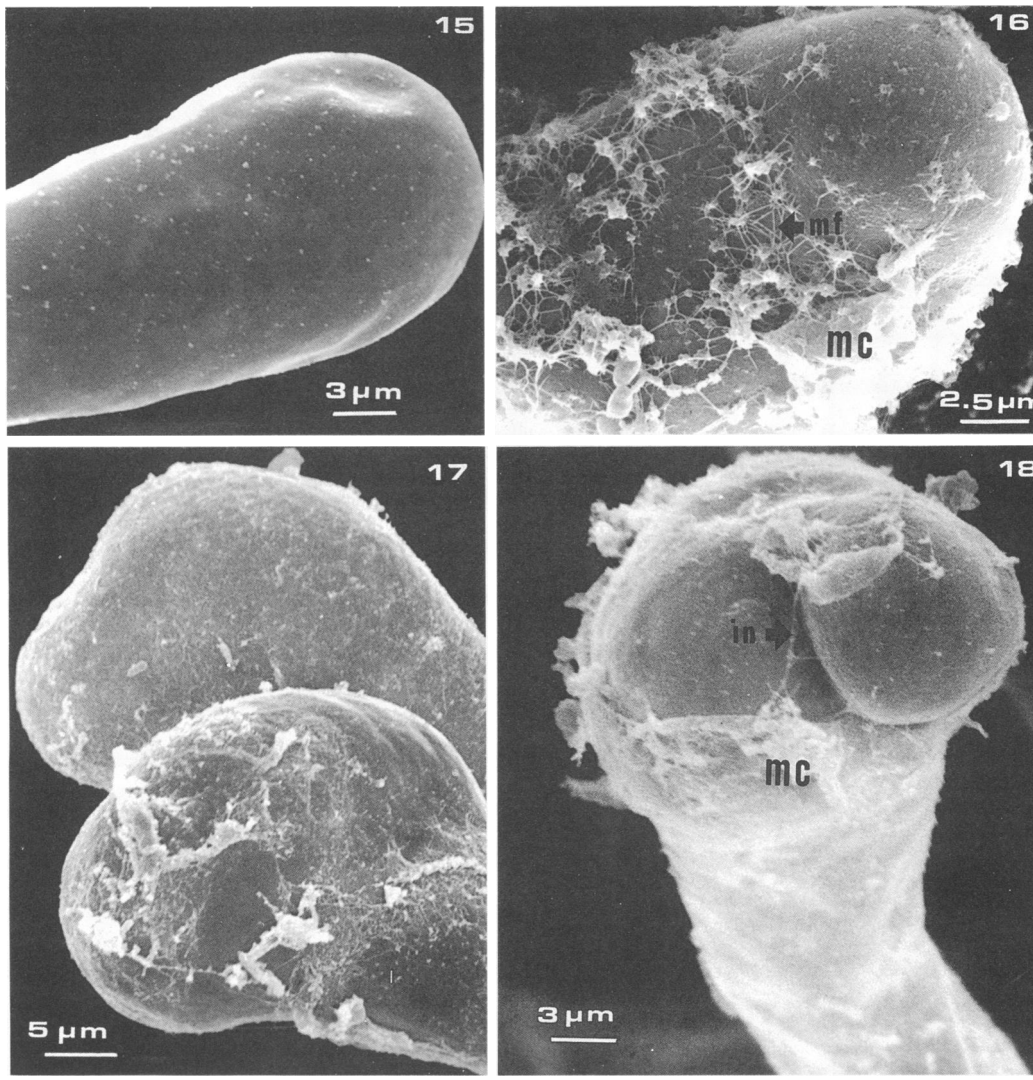

FIG. 15 to 18. Scanning electron micrographs of pole bean root hairs as affected by inoculation with *R. phaseoli* 127K14. (Fig. 15) Uninoculated root hair. (Fig. 16) Root hair tip 2 h after inoculation showing colonization by *R. phaseoli* 127K14 and massive network of microfibrils (mf). Note the peeled mucigel (mc). (Fig. 17) Root hairs 2 days after inoculation showing swelling and rhizobial colonization. (Fig. 18) Root hair 5 days after inoculation showing folding of root hair tip (in) and peeled mucigel (mc).

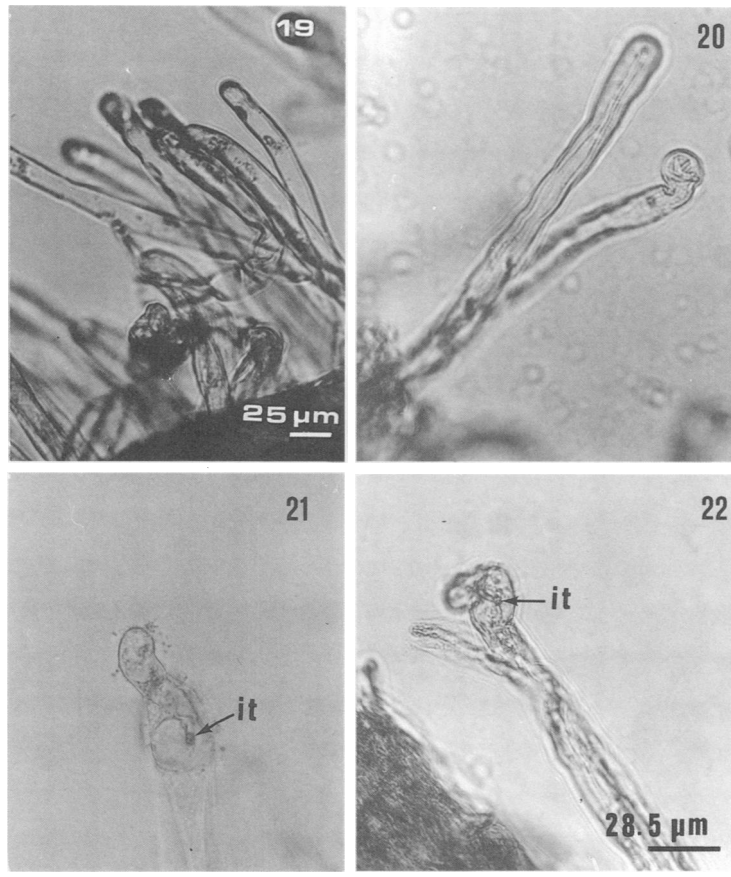

FIG. 19 to 22. Light micrographs of pole bean root hairs as affected by inoculation with *Rhizobium* sp. 127E15. (Fig. 19) Uninoculated root hairs. (Fig. 20) Root hairs 2 h after inoculation showing curling of the tip. (Fig. 21 and 22) Root hairs 2 days after inoculation showing infection thread (it) formation and shepherd's crook.

R. meliloti, have restricted host range in that they can only nodulate legumes within a specific cross-inoculation group (14). Other rhizobia can nodulate legumes of several cross-inoculation groups. *Rhizobium* sp. 127E15, for example, can nodulate not only the lima bean, which belongs to the cowpea group, but also the pole bean, which belongs to the bean group.

Although *Rhizobium* sp. 127E15 induced only partially effective nodules on the pole bean, our microscopical studies revealed that it nevertheless elicits specific host responses, such as root hair tip curling and swelling and infection thread formation from the pole bean. *Rhizobium* sp. 127E15, however, did not elicit responses from the pole bean as vigorously as it did from the lima bean. We observed that 80% of the young lima bean root hairs showed responses after inoculation, whereas the pole bean only showed 40%.

Examination of a marked root region after inoculation by serial sectioning has revealed that only the young and short root hairs showed specific responses. Therefore, it seems that specificity operates only in young root hairs and not in old and long ones. Similar observations have been made in the soybean-*R. japonicum* symbiosis (2).

We have observed also that the colonization of rhizobia on legume root hair tips may involve the formation of a network of microfibrils. Formation of cellulose microfibrils in gram-negative bacteria has been well documented (4), and it has also been reported in *R. trifolii* (12) and *Agrobacterium tumefaciens* (11). The function of the microfibril network may be to anchor rhizobial cells onto the legume root hair surface in the same way that the microfibril network anchors *A. tumefaciens* to its host cell surface (11, 12).

There appears to be a layer of mucigel covering the root hair of both the lima bean and the pole bean. The layer appears to be peeled off during root hair tip swelling. The presence of

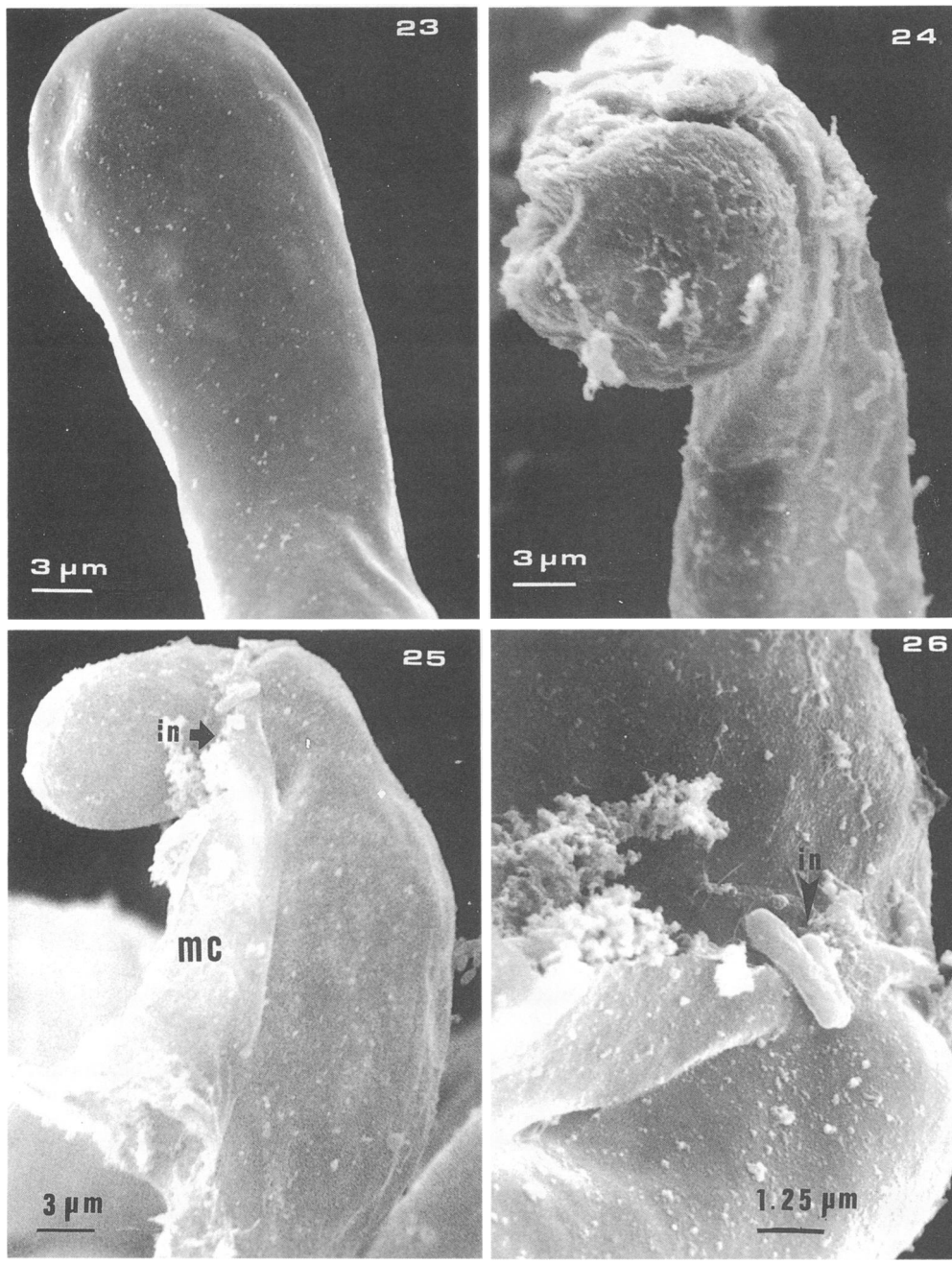

FIG. 23 to 26. Scanning electron micrographs of pole bean root hairs as affected by inoculation with *Rhizobium* sp. 127E15. (Fig. 23) Uninoculated root hair. (Fig. 24) A curled and swollen root hair 2 h after inoculation. (Fig. 25) Root hair 2 days after inoculation showing peeled mucigel (mc) and folding of root hair tip (in). (Fig. 26) A magnified view of the folding area of Fig. 25 showing entrapment of rhizobia at the point of folding (in).

mucigel has been shown in the root hairs of pea by Greaves and Darbyshire (7). Although the significance of mucigel in legume-*Rhizobium* interaction is presently not known, possibly the carbohydrate of the mucigel and rhizobial exopolysaccharide could complementarily react with each other for specificity and recognition.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Allen, O. N., and E. K. Allen.** 1981. The leguminosae, a source book of characteristics, uses, and nodulation, p. 511–515. University of Wisconsin Press, Madison.
2. **Bhuvaneswari, T. V., B. G. Turgeon, and W. D. Bauer.** 1980. Early events in the infection of soybean (*Glycine max* L. Merr.) by *Rhizobium japonicum*. I. Localization of infectible root cells. Plant Physiol. **66**:1027–1031.
3. **Dazzo, F. B., and D. H. Hubbel.** 1975. Cross reactive antigens and lectins as determinants of symbiotic specificity in the *Rhizobium*-clover association. Appl. Microbiol. **30**:1017–1033.
4. **Deinema, M. H., and L. P. T. M. Zevenhuizen.** 1971. Formation of cellulose fibrils by Gram negative bacteria and their role in bacterial flocculation. Arch. Mikrobiol. **78**:42–57.
5. **Fred, E. B., I. L. Baldwin, and E. McCoy.** 1932. Root nodule bacteria and leguminous plants, p. 123–133. University of Wisconsin Press, Madison.
6. **Graham, P. H.** 1976. Identification and classification of root nodule bacteria, p. 99–112. *In* P. S. Nutman (ed.), Symbiotic nitrogen fixation. Cambridge University Press, Cambridge.
7. **Greaves, M. P., and J. F. Darbyshire.** 1972. The ultrastructure of the mucilaginous layer on plant roots. Soil Biol. Biochem. **4**:443–449.
8. **Lange, R. T.** 1961. Nodule bacteria associated with the indigenous leguminosae of South Western Australia. J. Gen. Microbiol. **26**:351–356.
9. **Manhart, J. R., and P. P. Wong.** 1979. Nitrate reductase activities of rhizobia and the correlation between nitrate reduction and nitrogen fixation. Can. J. Microbiol. **25**:1169–1174.
10. **Manhart, J. R., and P. P. Wong.** 1980. Nitrate effect on nitrogen fixation (acetylene reduction) activities of legume root nodules induced by rhizobia with varied nitrate reductase activities. Plant Physiol. **65**:502–505.
11. **Matthysee, A. G., K. V. Holmes, and R. H. Gurlitz.** 1981. Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. J. Bacteriol. **145**:583–595.
12. **Napoli, C., F. Dazzo, and D. Hubbell.** 1975. Production of cellulose microfibrils by *Rhizobium*. Appl. Microbiol. **30**:123–131.
13. **Stacey, G., A. S. Paau, and W. J. Brill.** 1980. Host recognition in the *Rhizobium*-soybean symbiosis. Plant Physiol. **66**:609–614.
14. **Vincent, J. M.** 1974. Root-nodule symbiosis with *Rhizobium*, p. 265–341. *In* D. Quispel (ed.), The biology of nitrogen fixation. Elsevier/North-Holland Publishing Co., Amsterdam.
15. **Wong, P. O.** 1981. Symbiotic nitrogen fixation by *Phaseolus vulgaris* root nodules induced by *Rhizobium* sp. 127E15, p. 470. *In* A. H. Gibson and W. E. Newton (ed.), Current perspectives in nitrogen fixation. Australian Academy of Science Press, Canberra.