Entry of Rhizobia into Roots of *Mimosa scabrella* Bentham Occurs between Epidermal Cells

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The nitrogen-fixing symbiosis between *Mimosa scabrella* and *Rhizobium* Br 3454 was initiated by an infection sequence consisting of (a) surface colonization of roots; (b) penetration of mucigel and primary layers of radial walls of epidermal cells; (c) further penetration through primary wall layers and intercellular air spaces; (d) occasional intracellular penetration of epidermal and cortical cells, always occurring from a boundary between two neighbouring cells. Root hairs, when present, were never seen to be infected by the well-known infection thread mechanism, nor were infections seen to occur through wounds. This newly described method of infection may be common in legumes.

INTRODUCTION

In spite of many years of investigation of infection of legume roots by *Rhizobium*, observations have only been made on a narrow range of species, most of which are herbaceous (Dart, 1977; Newcomb, 1981; Lawrie, 1983). So far, two distinct methods of infection have been described: first, and the most widely studied, through root hairs, with the formation of a definite tube-like structure (the infection thread) which encloses the bacteria as they pass through cells towards the adjacent root cortex (Dart, 1977; Newcomb, 1981); second, through wounds where lateral roots emerge (Chandler, 1978; Chandler *et al.*, 1982), without the formation of infection threads.

We describe here a third type of root infection in *Mimosa scabrella*, a tree with many potential uses in south and south-east Brazil. This species was selected from 86 under study because of its inconsistency in root hair production. Root nodules of *M. scabrella* have indeterminate growth as defined by Sprent (1981), corresponding morphologically to the astragaloid type (Corby, 1981), (Fig. 1). They show an apical meristem and have infected cells interspersed with non-invaded cells. Nodules are not associated with lateral roots.

METHODS

Plants of *Mimosa scabrella* were grown in sterilized pouches or perspex chambers containing, respectively, absorbent paper or vermiculite:perlite (1:1, v/v). Roots were spot-inoculated (Turgeon & Bauer, 1983) with the rhizobial strain Br 3454 originally isolated at EMBRAPA, Km 47, Seropédica, Brazil. Plants were given nutrient solution consisting of CaSO₄·4H₂O 4.01 mM, KH₂PO₄ 2.19 mM, MgSO₄·7H₂O 3.1 mM, ferric citrate (C₆H₅O₇Fe·5H₂O) 5.0 µM, CoSO₄·4H₂O 0.02 µM, CuSO₄·5H₂O 0.1 µM, H₃BO₃ 5.0 µM, MnSO₄·4H₂O 1.0 µM, NaCl 10.0 µM, Na₂MoO₄·2H₂O 0.5 µM, ZnSO₄·7H₂O 0.1 µM. Root segments about 1–2 mm long were harvested from between 2 h and 26 d after inoculation, by which latter time nodules were fully formed. They were fixed in 2.5% (v/v) glutaraldehyde in 50 mM-phosphate buffer pH 7.0. Preparation for light, scanning and transmission electron microscopy was done as described previously (Faria *et al.*, 1986). Antiserum against rhizobial strain Br 3454 was raised in rabbits according to Vincent (1970): partial purification was achieved with 50% saturated ammonium sulphate precipitation, followed by centrifugation at 18000 g; the pellet was resuspended and dialysed against 0.01 M-phosphate buffer pH 7.2 with 0.87% NaCl. Aliquots were kept at –20 °C. Goat anti-rabbit

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globulin, conjugated with fluorescein isothiocyanate (FITC), was purchased from Sigma. For examination of the cross-reactivity between the inoculated rhizobia in the section and the antiserum, root segments were fixed in 4% (w/v) formaldehyde in 50 mM-phosphate buffer pH 7.0 and the resin polymerized under UV irradiation at room temperature. Semi-thin sections (2–5 µm) were cut and stained with aqueous 0.1% toluidine blue in 1.0% borax (pH 4.4). When infection sites had been located, sections were taken for immunofluorescence examination on slides coated with poly(L-lysine), according to Van Noorden (1983).

Root hairs, when present, were examined for infection by the methylene blue method of Vasse & Truchet (1984). Early events in infection were also followed in sterilized seeds inoculated and grown in Petri dishes: in this case root segments were harvested from 1 to 96 h after inoculation and prepared for scanning electron microscopy. Some samples were sonicated (Bransonic, 50 kHz amplitude) for 30 s prior to dehydration.

RESULTS

The results described were taken from observations of 92 root segments from 36 plants from which more than 800 slides were prepared. Only those results in which the presence of rhizobia was confirmed by cross-reactivity with the antibody are given.

Infection and formation of a definite tube (infection thread) such as observed in root hairs of *Glycine max*, *Vicia faba* or *Trifolium* spp. (Newcomb, 1981; Vasse & Truchet, 1984) were never observed, either in sections or using the staining procedure of Vasse & Truchet (1984).

Rhizobia attached to the root soon after inoculation, often in a polar way (Fig. 2). The mucilaginous layer which covers most of root surface was first seen to be ruptured at 24 h, leaving the rhizobia in direct contact with the plant cell wall. When loosely bound bacteria were removed by sonication, it was possible to see mucigel and epidermal walls being penetrated by rhizobia (Fig. 3). At this stage intracellular penetration, such as observed on rare occasions in soybean (Bieberdorf, 1938), was not observed. Although the primary wall layer appears to be relatively easily penetrated, bacteria were never seen to enter the secondary layer (Fig. 4; see also Foster, 1986). Further proliferation of bacteria followed the junctions of the epidermal cells (Fig. 5). Rhizobia then entered between epidermal cells, possibly through degradation and/or digestion of the cell wall (Fig. 6). During invasion, an electron-dense material accumulated between walls surrounding the bacteria (Figs 6 and 7). Similar electron-dense material has been seen during intercellular movement of rhizobia in the non-legume *Parasponia rigida* (Lancelle & Torrey, 1984). Further penetration occurred between cell walls and through intercellular air spaces. Bacteria appeared to progress in the cortical region through the walls rather than by separating the cells at the middle lamella (Fig. 7). Multiplication of bacteria in these regions resulted in cell walls being pushed inwards, sometimes allowing intracellular penetration (Figs 8 and 9). Such rather disorganized intracellular infections were seen in both epidermal cells (including root hairs when present) and cortical cells (Figs 8 and 9), but always from the radial or inner tangential walls, never from external surface. This requirement for two adjacent cell surfaces is the only common feature with infection through curled root hairs: it may be necessary for bacteria effectively to oppose cell turgor (Bauer, 1981; Turgeon & Bauer, 1985).

Once inside the cell, bacteria multiplied repeatedly, but always remained enclosed by cell wall material. As a result of this multiplication, bacteria often occupied a large proportion of the cell volume (Figs 9, 10 and 11). From these groups of bacteria, structures resembling ill-defined infection threads sometimes arose, possibly through further deposition of cell wall material. These structures crossed cell boundaries (Fig. 10), thus infecting adjacent cells. Some cortical cells were also invaded after a massive multiplication of the bacteria between cells and in intercellular air spaces. This bacterial proliferation, unlike that described above, appeared to breach the cell walls and allow intracellular infection (Fig. 12). Bacteria entering these cells were embedded in a matrix of unknown origin: no infection-thread-like structures were seen in this situation, and infected cells appeared very dense and disorganized (Fig. 12). This disorganized mode of intracellular invasion appeared to end in the death of the cell. A similar situation was seen in *Stylosanthes* spp. (Chandler *et al.*, 1982) where, following a wound infection, bacteria entered the root cortex by a progressive collapse of invaded cells.

Normal root hairs (i.e. outgrowths of epidermal cells) were seen only rarely in this study (Fig. 14). However, we have often observed hair-like structures originating in the sub-epidermal layer

Fig. 1. Nodules of *Mimosa scabrella*. Bar, 1 cm.

Fig. 2. Scanning electron micrograph showing attachment of rhizobia (R) to root 4 h after inoculation. Bar, 1 μ m.

Fig. 3. Colonization of root by rhizobia (R). Loosely bound bacteria were removed by sonication: note depressions caused by rhizobia on the root surface, and the penetration into the mucigel layer (M). Scanning electron micrograph; bar, 1 μ m.

Fig. 4. Rhizobia (R) penetrate into the primary wall layer (PW) but not the secondary layer (SW). Transmission electron micrograph; bar, 1 μ m.

Fig. 5. Rhizobia (R) appear to be penetrating between two neighbouring cells, defined by the outside of their radial walls (CW). Scanning electron micrograph; bar, 1 μ m.

Fig. 6. Rhizobia (R) invading the root between epidermal cells (EC). An electron-dense material accumulates around the rhizobia (arrows). Transmission electron micrograph; bar, 5 μ m.

Fig. 7. Rhizobia (R) penetrating between cortical cells, apparently by moving through the primary wall layer (compare with Fig. 4) rather than the middle lamella (ML). They are still associated with electron-dense material (arrows; see Fig. 6). Transmission electron micrograph; bar, 1 μ m.

Fig. 8. Rhizobia (R) occasionally penetrate root hairs (RH), but always from areas adjacent to other cells, never through the external surface. Light micrograph; bar, 10 μ m.

Fig. 9. Epidermal cells may also be infected via radial or internal tangential walls, after intercellular movement of rhizobia (R). Inside the cell they are sometimes confined by host wall material in structures resembling infection threads (IT). Light micrograph; bar, 10 μ m.

(Fig. 13), as found in some monocotyledons (Pinkerton, 1936). These could easily be mistaken for normal root hairs when the epidermal layer has been abraded by soil particles.

Nodules are initiated through cell divisions in the inner cortex. Although it has not been established exactly when this reaction occurs, it often follows intracellular invasion of outer cortical cells (Fig. 13). Progeny of the new meristematic cells are invaded by infection threads, apparently like those formed in root hairs of some species. From these, bacteria are released into

Fig. 10. Inter- and intracellular penetration of rhizobia (R) in the cortex. Ill-defined infection threads (IT) may be formed and cross cell boundaries. Light micrograph; bar, 10 μ m.

Fig. 11. Higher magnification of part of a section adjacent to that shown in Fig. 10, showing profuse multiplication of rhizobia (R), which then occupy a large part of the cell volume. Bacteria appear to remain enclosed within cell wall material (CW). Transmission electron micrograph; bar, 5 μ m.

Fig. 12. Intracellular penetration of rhizobia (R) after massive intercellular multiplication. Cell walls (CW) appear to be breached during this invasion, ending in the death of the cell. Transmission electron micrograph; bar, 5 μ m.

Fig. 13. General view of the cortex region. Sub-epidermal hairs are often invaded by rhizobia, through structures resembling infection threads (IT). Remains of the epidermal layer (EC) are still present. Light micrograph; bar, 20 μ m.

Fig. 14. A normal root hair. Light micrograph; bar, 20 μ m.

membrane-bound envelopes. Development of nodules is then broadly similar to that of other indeterminate forms, (Newcomb, 1981).

Because many legumes have neither root hairs (Sprent *et al.*, 1987; and H. D. L. Corby's discussion of that paper) nor nodules associated with lateral roots (permitting wound infections as in the legume tribes Aeschynomeneae and some Dalbergieae; Sprent *et al.*, 1988), rhizobial infection between epidermal cells may be common. It may occur in the tropical legume *Neptunia* (Schaeede, 1940) and in the piliferous roots of *Aotus* sp., where very extensive searching did not reveal hair infections (Lawrie, 1983).

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