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# The development and structure of root nodules on bambara groundnut [*Voandzeia* (Vigna) *subterranea*]

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**The infection of *Vigna subterranea* (formerly *Voandzeia subterranea*) by *Bradyrhizobium* strain MAO 113 (isolated from *V. subterranea*) was examined by light and transmission electron microscopy. Bacteria accumulated on the epidermis close to root hairs, and subsequently entered the latter via infection threads. Most of the steps involved in nodule formation were generally characteristic of determinate nodules, such as those which form on the closely related *V. radiata*. For example, nodule meristems were induced beneath the root epidermis adjacent to infected root hairs, but prior to infection of the meristem by rhizobia. Moreover, after the infection of some of the meristematic cells by the infection threads, and the release of the rhizobia into membrane-bound vesicles, the infection process ceased and dissemination of the rhizobia was by division of already-infected host cells. However, there were some aspects of this process in *V. subterranea* which have been more commonly described in indeterminate nodules. These include long infection threads entering a number of cells within the meristems simultaneously and a matrix within infection threads which was strongly labelled with immunogold monoclonal antibodies, MAC236 and MAC265, which recognize epitopes on an intercellular glycoprotein. The MAC236 and MAC265 antibodies also recognized material in the unwalled infection droplets surrounding bacteria which were newly-released from the infection threads. The amount of labelling shown was more characteristic of the long infection threads seen in indeterminate nodules such as pea (*Pisum sativum*) and *Neptunia plena*. The structure of mature *V. subterranea* nodules was similar to that described for other determinate nodules such as *Glycine max*, *Vigna unguiculata* and *V. radiata*, i.e. they were spherical and the infected zone consisted of both infected and uninfected cells. Surrounding the infected tissue was an inner cortex of uninfected cell layers containing the putative components of an oxygen diffusion barrier (including glycoprotein-occluded intercellular spaces), and an outer cortex with cells containing calcium oxalate crystals.**

*Key words*: *Bradyrhizobium*, determinate, indeterminate, infection thread, nodule, *Voandzeia subterranea*.

Bambara groundnut [*Vigna subterranea*, formerly *Voandzeia subterranea*; (Verdcourt 1980)] is an important tropical papilionoid grain legume grown in the semi-arid zones of Africa (Hepper 1970; Verdcourt 1980; Allen & Allen 1981). Although it superficially resembles peanut (*Arachis hypogaea*) in growth habit (i.e. production of subterranean fruits) the two species are not closely related; they are in different tribes within the Papilionoideae (Polhill & Raven 1981): *Arachis* in the Aeschynomeneae and *Vigna* in the Phaseoleae. However, in common with peanut, and also with other *Vigna* spp., *V. subterranea* will nodulate and fix $N_2$ in partnership with *Bradyrhizobium* strains (Gueye & Bordeleau 1988), including those isolated from peanut and *Macroptilium atropurpureum* (Kishinevsky *et al.* 1996). In this study we examined the processes involved in the infection of *V. subterranea* by a *Bradyrhizobium* strain (MAO 113) originally isolated from *V. subterranea* (Gueye & Bordeleau 1988) and compared them to those described previously for other *Vigna* spp. e.g. *V. radiata* (Newcomb & McIntyre 1981).

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## Materials and Methods

Seedlings of bambara groundnut were grown singly in sterilized Gibson tubes (Gibson 1963) and inoculated with 1 ml of *Bradyrhizobium* strain MAO 113 (Gueye & Bordeleau 1988) liquid culture containing $10^9$ cells/ml. Plants were then given N-free Jensen nutrient solution and placed at 30 °C in a growth cabinet with a 12 h photoperiod. At 2 day intervals, one seedling was taken and examined for the presence of infection threads. Roots were harvested from 12 to 24 days, by which time nodules were fully formed, and cut into 1 cm sections. They were then fixed in 2.5% (v/v) glutaraldehyde in 50 mM-phosphate buffer pH 6.8 for 72 days. Preparations for light (LM) and transmission electron (TEM) microscopy were carried out according to James *et al.* (1992). In addition, immunogold labelling using the monoclonal antibodies MAC236 and MAC265 (VandenBosch *et al.* 1989) was performed according to James *et al.* (1991). The monoclonal antibodies were supplied by Dr N.J. Brewin, John Innes Centre, Norwich, UK. Sections for LM and TEM were viewed and photographed by using an Olympus BH2 microscope and a JOEL 1200EX transmission electron microscope, respectively.

## Results and Discussion

Entry of *Bradyrhizobium* into the roots of *V. subterranea* occurred via root hairs (Figure 1A–D), as shown previously in other *Vigna* spp. (Newcomb & McIntyre 1981) and in many determinate nodule types e.g. soybean (*Glycine max*) (Newcomb *et al.* 1979; Turgeon & Bauer 1982), *Lotus corniculatus* (Vance *et al.* 1982), and see reviews by Sprent (1989) and Brewin (1991). Prior to the infection of root hairs, there appeared to be production of an incipient nodule meristem below the epidermis of the root (Figure 1A), and rhizobia accumulated on the epidermis before penetrating the root hairs (Figure 1B and C). After the root hairs were penetrated by the rhizobia the latter were conveyed to the nodule meristem via thick-walled infection threads (Figure 1C and D). Within 12–14 days, nodule primordia were macroscopically visible and were similar in structure to those in *V. radiata* (Newcomb & McIntyre 1981). They contained a central core of meristematic cells in the process of being infected, an uninfected cortex surrounding the meristem, and a vascular system connecting the nodule to the parent root (Figure 2A). Within the meristem, infection threads could be seen branching into many host cells simultaneously (Figure 2B–D, Figure 3A and B). These infection threads varied in their size, some being small structures emerging from between cell walls (Figure 2B and C) typical of determinate nodules (Newcomb & McIntyre 1981; Newcomb *et al.* 1979; Vance *et al.* 1982; VandenBosch *et al.* 1989; Rae *et al.* 1992), whilst others were much longer and traversed host cells (Figure 2D, Figure 3A and B). The latter were more typical of infection threads observed within indeterminate nodules, such as those on pea (*Pisum sativum*) (Newcomb 1976; VandenBosch *et al.* 1989; Rae *et al.* 1992). Both infection thread types released rhizobia into membrane-bound vesicles within the host cell cytoplasm (Figure 2C and D, Figure 3A–D), where the bacteria subsequently differentiated into $N_2$-fixing bacteroids, as seen in the development of nodules on many legumes e.g. *P. sativum* (Newcomb 1976), *Trifolium* (Dixon 1964; Roughley *et al.* 1976), soybean (Goodchild & Bergersen 1966), *L. corniculatus* (Vance *et al.* 1982) and *Neptunia* (James *et al.* 1992). Prior to their development into bacteroids, the released rhizobia were contained within 'infection droplets' (Figure 3A and C). In most legume nodules so far studied, the release of bacteria from infection threads involves the localized breakdown of the infection thread wall and the release of the bacteria into the host cells within a 'droplet' of infection thread matrix material which is rapidly absorbed by the host cell as the peribacteroid membrane forms around the rhizobia (Newcomb 1976; Robertson *et al.* 1978; Newcomb & McIntyre 1981; Vance *et al.* 1982; VandenBosch *et al.* 1989; Brewin 1991; Rae *et al.* 1992; James *et al.* 1992). The infection droplets were originally thought to be made up of polysaccharide-like material (Vance *et al.* 1982), although recent data has shown that glycoproteins are a major component (see next paragraph).

An interesting feature of the infection threads and infection droplets within *V. subterranea* nodules was the occurrence of the 95 kDa 'matrix' glycoprotein recognized by the monoclonal antibodies MAC236 and MAC265 (Figure 3B and C). This plant-derived, but nodule-enhanced, glycoprotein was originally observed in pea nodule infection threads, infection droplets and cortical intercellular spaces (VandenBosch *et al.* 1989). Since then it has also been observed in other indeterminate nodules e.g. *Vicia hirsuta* (Rae *et al.* 1992) and *Neptunia* (James *et al.* 1992), and James *et al.* (1996) have recently shown that *S. rostrata* stem and root nodules, which are intermediate between indeterminate and determinate nodules (Ndoye *et al.* 1994), also have infection threads containing the MAC265 antigen. Our paper is the first observation of infection thread glycoprotein in *Vigna* nodules but not the first observation in other determinate nodules, as VandenBosch *et al.* (1989) have reported MAC236 within *G. max* infection threads and James *et al.* (unpublished work) have shown that nodules on *Lotus* spp. express the MAC236 antigen very strongly within infection droplets. However, the matrix material in *G. max* infection threads was very little compared to that of *V. subterranea* and Rae *et al.* (1992) did not observe the MAC265 antigen within *Phaseolus vulgaris* infection threads. Therefore, it may be that infection threads in other tropical legumes with determinate nodules e.g. *P. vulgaris* and *G. max* are different from those in

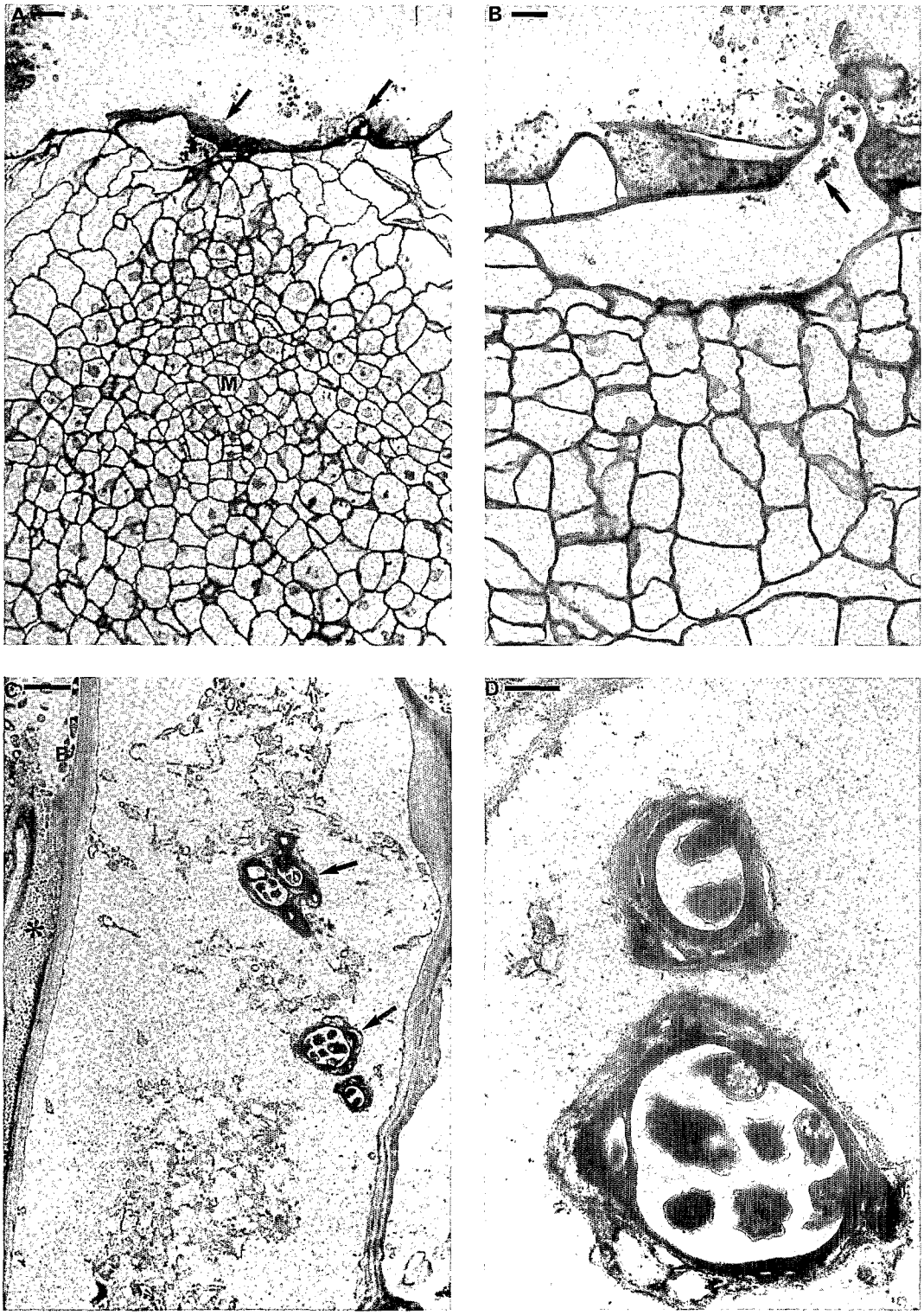


**Figure 1.** (A) Meristematic tissue (M) developing in the root of *Voandzeia*, after infection of root hairs by rhizobia (arrows) (bar = 20 $\mu$m). (B) Root hair containing infection threads (arrows). Note the bacteria accumulating on the root epidermis adjacent to the infected root hair (bar = 10 $\mu$m). (C) Enlarged epidermal cell containing infection threads (arrows). Bacteria (B) can be seen on the epidermis, as well as darkly-stained material (*) (bar = 2 $\mu$m). (D) High magnification view of infection threads in an epidermal cell (see Figure 1B, C) (bar = 500 nm).

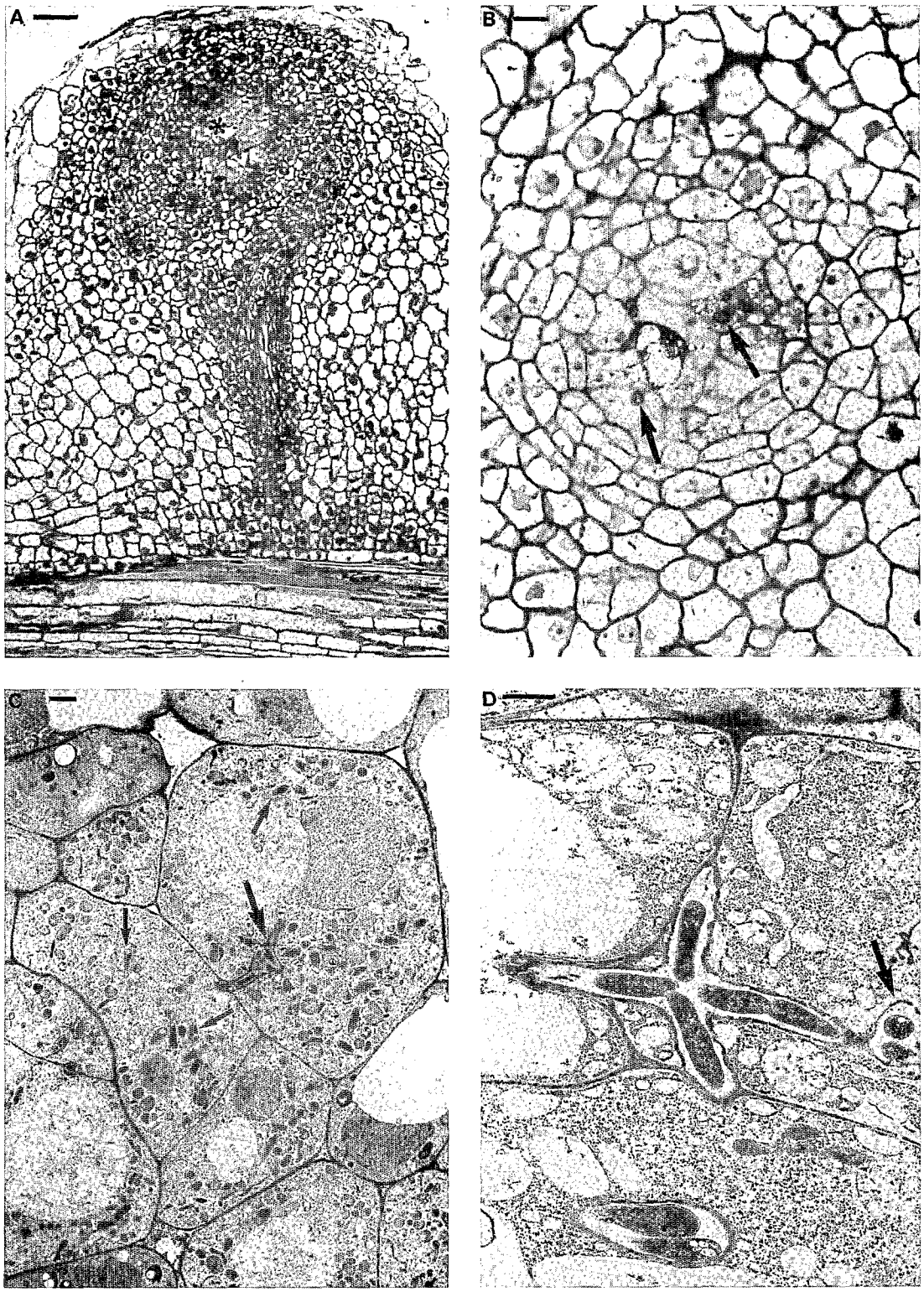


**Figure 2.** (A) Nodule primordium showing newly-infected cells (*) and vascular tissue connecting the nodule to the root (bar = 50 $\mu$m). (B) Meristematic tissue in the centre of a nodule primordium. Some of these cells are being invaded by infection threads containing rhizobia (arrows) (bar = 10 $\mu$m). (C) Transmission electron micrograph (TEM) of meristematic cells containing newly-released rhizobia (arrows) and a small infection thread emerging from between cell walls (large arrow) (bar = 2 $\mu$m). (D) Infection thread penetrating three cells simultaneously. Note that some bacteria have been released (large arrow) (bar = 1 $\mu$m).

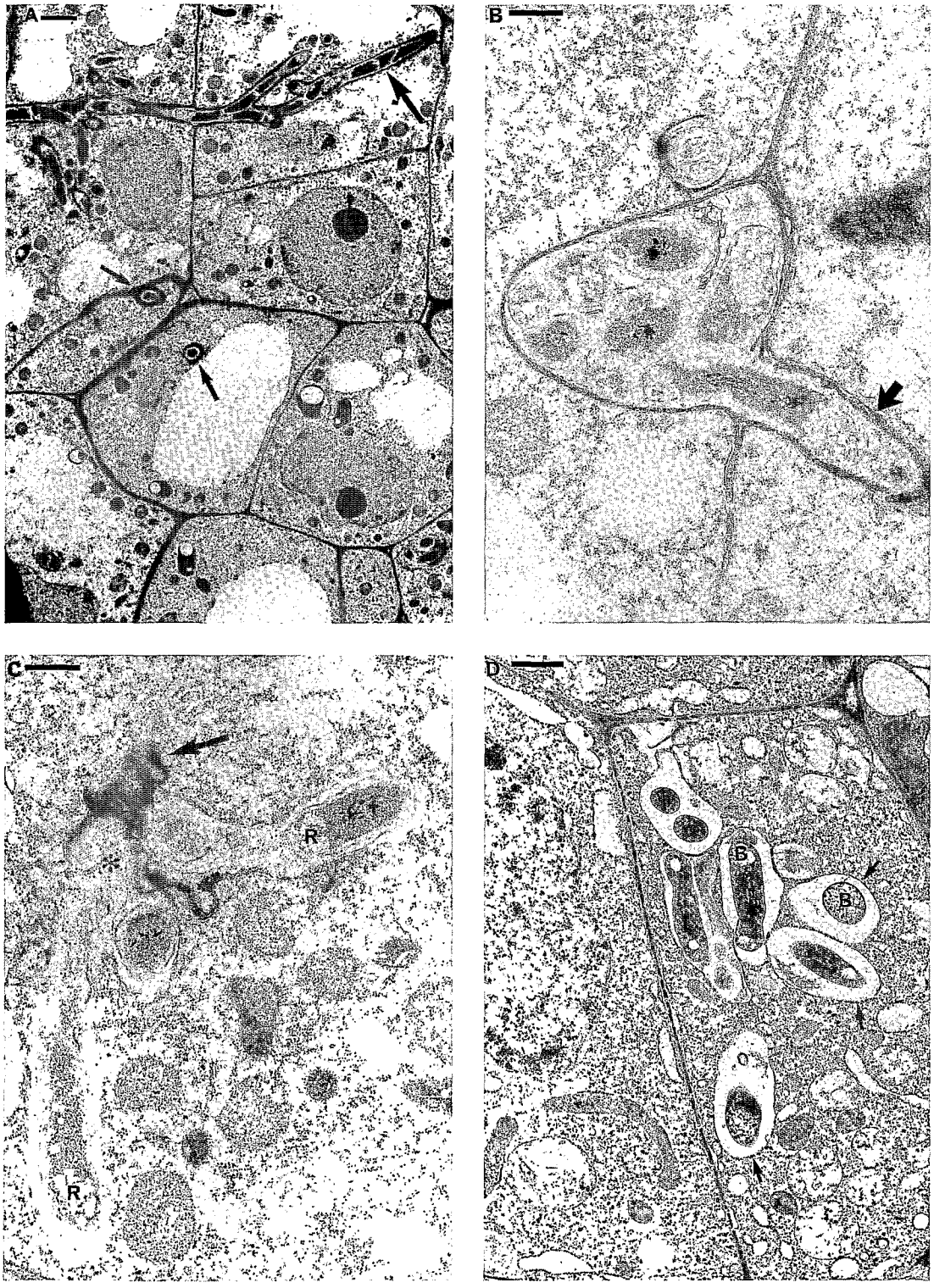


**Figure 3.** (A) Meristematic cells from a nodule primordium. Some of the cells contain small infection threads (arrows), but there is also a large infection thread traversing the host cells and releasing bacteria as it passes through the cells (large arrows) (bar = 2 $\mu$m). (B) Intercellular infection thread entering a host cell (arrow). The matrix of the infection thread is immunogold labelled with the monoclonal antibody, MAC265 (bar = 500 nm). (C) Newly-released rhizobia (R) in a host cell. Note that some of the wall of the infection thread (arrows) and that some immunogold-labelled (with MAC265) infection thread matrix material has also been released into the cell (*) (bar = 500 nm). (D) Bacteroids (B) forming in a host cell in a young nodule. Peribacteroid membranes are fully formed around the bacteroids (arrows) (bar = 1 $\mu$m).

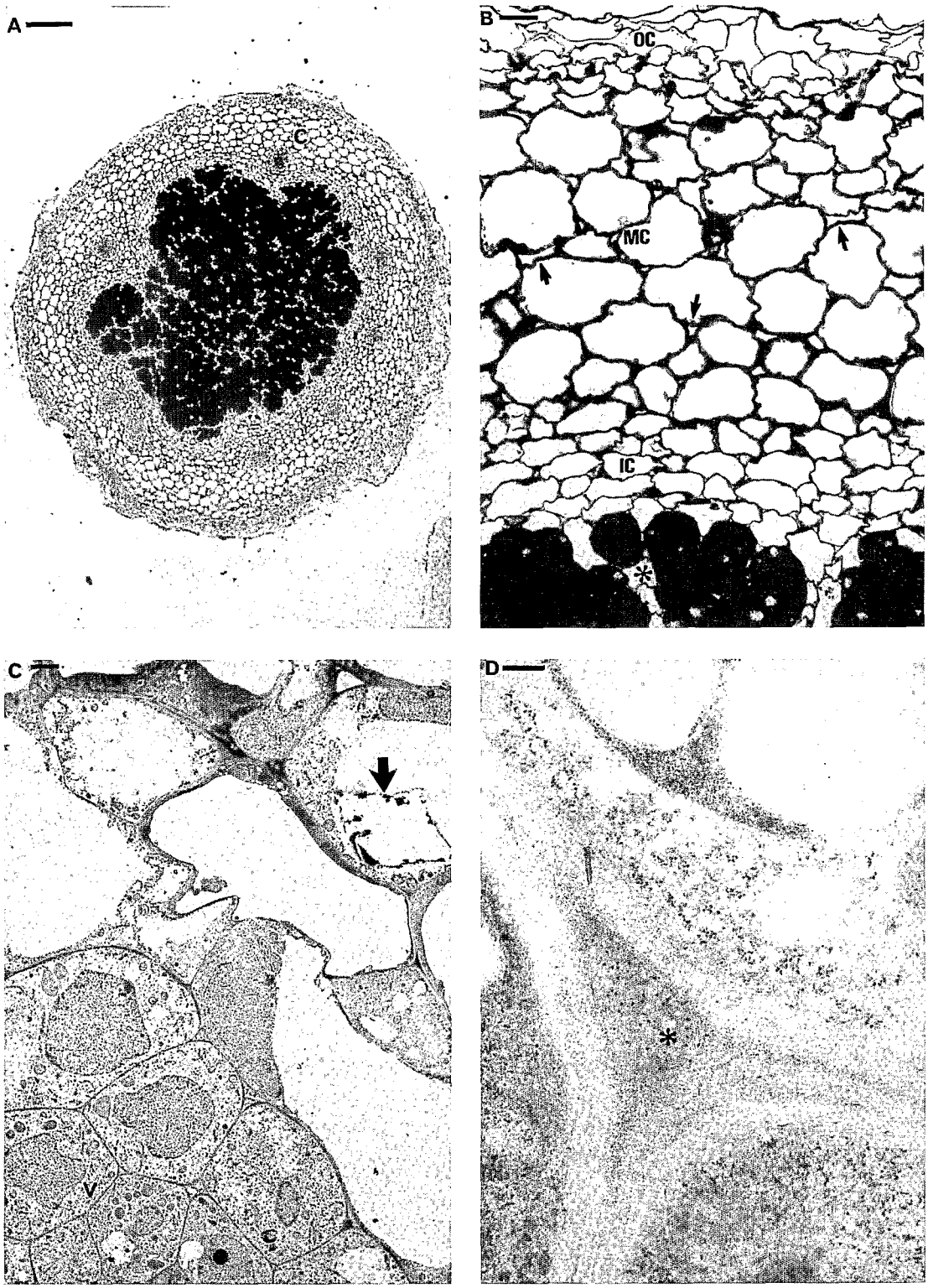


**Figure 4.** (A) Transverse section of a mature nodule showing the central, infected zone (dark-stained cells) and the uninfected cortex (C) (bar = 200 $\mu$m). (B) View of the nodule cortex showing the putative site of the oxygen diffusion barrier in the inner cortex (IC). The mid-cortex (MC) has larger cells and intercellular spaces (arrows). *, infected tissue; OC, outer cortex (bar = 20 $\mu$m). (C) TEM of a cortical cell, close to a vascular bundle (V), showing the remains of a calcium oxalate crystal (arrow) (bar = 2 $\mu$m). (D) Occluded intercellular space from the mid-cortex. The material occluding the space is immunogold labelled with the monoclonal antibody, MAC265 (*) (bar = 200 nm).

*V. subterranea*, in being generally shorter and with little or no matrix glycoprotein. Indeed, *V. subterranea* infection threads may be more similar to those in nodules of the temperate/subtropical legume, *Lotus*, in having large amounts of infection thread/infection droplet glycoprotein.

Mature *V. subterranea* nodules (Figure 4A) are spherical and similar in structure to other determinate nodules e.g. those on *V. radiata* and *L. corniculatus* (Newcomb & McIntyre 1981; Vance *et al.* 1982) in having no meristem and a central zone with infected cells interspersed with uninfected, interstitial cells, and a cortex containing oxalate crystals. The cortex of uninfected cells which surrounds the central $N_2$-fixing zone (Figure 4B) is similar to that described by Sutherland & Sprent (1984), Parsons & Day (1990), James *et al.* (1991), Dakora & Atkins (1990), Sen *et al.* (1986) and VandenBosch *et al.* (1994) for other determinate nodules such as those on soybean, cowpea and peanut. Typical features were calcium oxalate crystals in the outer cortex (Figure 4C, see also Sutherland & Sprent 1984), and glycoprotein occlusions of intercellular spaces in the mid and inner cortices. This is the first observation of intercellular glycoprotein in *V. subterranea*, but it has been observed previously in nodules from several other legume species such as pea, soybean, *Neptunia* and *Sesbania* (VandenBosch *et al.* 1989; James *et al.* 1991, 1992, 1994, 1996; Rae *et al.* 1991). Recent data from Iannetta *et al.* (1995) with *Lupinus albus* nodules suggests that the variable occlusion of intercellular spaces is an important part of the cortical $O_2$ diffusion barrier (Witty *et al.* 1986). It remains to be seen whether it has a similar role in the regulation of $O_2$ diffusion in *V. subterranea* nodules.

Although the growth habit of *V. subterranea* is similar to the peanut, there was no evidence from our study that the rhizobial infection processes were similar, even though both can be infected by the same strains (Kishinevsky *et al.* 1996). In contrast to that of *V. subterranea*, and most legumes so far studied [see reviews by Sprent (1989) and Brewin (1991)], the infection process of peanut does not involve root hairs or infection threads but appears to occur at natural wounds formed at lateral root junctions, where the bacteria penetrate the root intercellularly without infection threads (Chandler 1978). Indeed, the rhizobial strain appears to have little effect on the formation of legume nodules (unless it is ineffective), the host genotype being of more importance. For example, Sen *et al.* (1986) have shown that rhizobial strain 32H1 will form nodules on both peanut and cowpea (*V. unguiculata*), but that the nodules formed on the two species were quite different in structure: those of *V. unguiculata* were similar to the determinate nodules reported in the present study for *V. subterranea* (Figure 4A) and by Newcomb & McIntyre (1981) for *V. radiata*, whereas those on peanut were 'aeschynomenoid' i.e. spherical in morphology and lacking in interstitial cells and infection threads within the infected zone (Corby 1981; Sen *et al.* 1986; Sprent *et al.* 1989; VandenBosch *et al.* 1994).

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## References

Allen, O.N. & Allen, E.K. (eds) 1981 *The Leguminosae: A Source Book of Characteristics, Uses, and Nodulation.* Madison, Wisconsin: University of Wisconsin Press.

Brewin, N.J. 1991 Development of the legume root nodule. *Annual Review of Cell Biology* **7**, 191–226.

Chandler, M.R. 1978 Some observations on the infection of *Arachis hypogaea* L. by *Rhizobium*. *Journal of Experimental Botany* **29**, 749–755.

Corby, H.D.L. 1981 The systematic value of leguminous root nodules. In *Advances in Legume Systematics*, Part 2, eds Polhil, R.M. & Raven, P.H. pp. 657–669. Kew, London: Royal Botanic Gardens.

Dakora, F.D. & Atkins, C.A. 1990 Morphological and structural adaptations of nodules of cowpea to functioning under sub- and supra-ambient oxygen pressure. *Planta* **182**, 572–582.

Dixon, R.O.D. 1964 The structure of infection threads, bacteria and bacteroids in pea and clover root nodules. *Archiv für Mikrobiologie* **48**, 166–178.

Gibson, A.H. 1963 Physical environment and symbiotic nitrogen fixation. I. The effect of root temperature on recently nodulated *Trifolium subterraneum* L. plants. *Australian Journal of Biological Sciences* **16**, 28–42.

Goodchild, D.J. & Bergersen, F.J. 1966 Electron microscopy of the infection and subsequent development of soybean nodules cells. *Journal of Bacteriology* **92**, 204–213.

Gueye, M. & Bordeleau, L.M. 1988 Nitrogen fixation in bambara groundnut, *Voandzeia subterranea* (L.) *Thouars*. *Mircen Journal* **4**, 365–375.

Hepper, F.N. 1970 Bambara groundnut (*Voandzeia subterranea*). *Field Crop Abstracts (UK)* **23**, 1–6.

Iannetta, P.P.M., James, E.K., Sprent, J.I. & Minchin, F.R. 1995 Time-course of changes involved in the operation of the oxygen diffusion barrier in white lupin nodules. *Journal of Experimental Botany* **46**, 565–575.

James, E.K., Sprent, J.I., Minchin, F.R. & Brewin, N.J. 1991 Intercellular location of glycoprotein in soybean nodules: effect of altered rhizosphere oxygen concentration. *Plant, Cell and Environment* **14**, 467–476.

James, E.K., Sprent, J.I., Sutherland, J.M., McInroy, S.G. & Minchin, F.R. 1992 The structure of nitrogen fixing root nodules of the aquatic mimosoid legume *Neptunia plena*. *Annals of Botany* **69**, 173–180.

James, E.K., Iannetta, P.P.M., Naisbitt, T., Goi, S.R., Sutherland, J.M., Sprent, J.I., Minchin, F.R. & Brewin, N.J. 1994 A survey of $N_2$-fixing nodules in the Leguminosae with particular reference to intercellular glycoproteins and the control of oxygen diffusion. *Proceedings of the Royal Society of Edinburgh* **102B**, 429–432.

James, E.K., Iannetta, P.P.M., Nixon, P.J., Whiston, A.J., Peat, L., Crawford, R.M.M., Sprent, J.I. & Brewin, N.J. 1996 Photosystem II and oxygen regulation in *Sesbania rostrata* stem nodules. *Plant, Cell and Environment* **19**, 895–910.

Kishinevsky, B.D., Zur, M., Friedman, Y., Meromi, G., Ben-Moshe, E. & Nemas, C. 1996 Variation in nitrogen fixation and yield in landraces of bambara groundnut (*Vigna subterranea* L.). *Field Crops Research* **48**, 57–64.

Ndoye, I., de Billy, F., Vasse, J., Dreyfus, B. & Truchet, G. 1994 Root nodulation of *Sesbania rostrata*. *Journal of Bacteriology* **176**, 1060–1068.

Newcomb, W. 1976 A correlated light and electron microscope study of a symbiotic growth and differentiation in *Pisum sativum* root nodules. *Canadian Journal of Botany* **54**, 2163–2186.

Newcomb, W. & McIntyre, L. 1981 Development of root nodules of mung bean (*Vigna radiata*): a reinvestigation of endocytosis. *Canadian Journal of Botany* **59**, 2478–2499.

Newcomb, W., Sippel, D. & Peterson, R.L. 1979 The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. *Canadian Journal of Botany* **57**, 2603–2616.

Parsons, R. & Day, D.A. 1990 Mechanism of soybean nodule adaptation to different oxygen pressures. *Plant, Cell and Environment* **13**, 501–512.

Polhill, R.M. & Raven, P.H. (eds) 1981 *Advances in Legume Systematics* Part 1. Kew, London: Royal Botanic Gardens.

Rae, A.L., Perotto, S., Knox, J.P., Kannenberg, E.L. & Brewin, N.J. 1991 Expression of extracellular glycoproteins in the uninfected cells of developing pea nodule tissue. *Molecular Plant Microbe Interactions* **4**, 563–570.

Rae, A.L., Bonfante-Fasolo, P. & Brewin, N.J. 1992 Structure and growth of infection threads in the legume symbiosis with *Rhizobium leguminosarum*. *Plant Journal* **2**, 385–395.

Robertson, J.G., Lytleton, P., Bullivant, S. & Grayston, G.F. 1978 Membranes in lupin root nodules. I. The role of Golgi bodies in the biogenesis of infection threads and peribacteroid membranes. *Journal of Cell Science* **30**, 129–149.

Roughley, R.J., Dart, P.J. & Day, J.M. 1976 The structure and development of *Trifolium subterraneum* L. root nodules I in plants grown at optimal root temperatures. *Journal of Experimental Botany* **27**, 431–440.

Sen, D., Weaver, R.W. & Bal, A.K. 1986 Structure and organization of effective peanut and cowpea root nodules induced by rhizobial strain 32H1. *Journal of Experimental Botany* **37**, 356–363.

Sprent, J.I. 1989 Which steps are essential for the formation of functional root nodules. *New Phytologist* **111**, 129–153.

Sprent, J.I., Sutherland, J.M. & de Faria, S.M. 1989 Structure and function of root nodules from woody legumes. Advances in legume biology. *Monographs of Systematic Botany* **29**, 559–578.

Sutherland, J.M. & Sprent, J.I. 1984 Calcium-oxalate crystals and crystal cells in determinate root nodules of legumes. *Planta* **161**, 193–200.

Turgeon, B.G. & Bauer, W.D. 1982 Early events in the infection of soybean by *Rhizobium japonicum*. Time course and cytology of the initial infection process. *Canadian Journal of Botany* **60**, 152–161.

Vance, C.P., Johnson, L.E.B., Stade, S. & Groat, R.G. 1982 Birdsfoot trefoil (*Lotus corniculatus*) root nodules: morphogenesis and the effect of forage harvest on structure and function. *Canadian Journal of Botany* **60**, 505–518.

VandenBosch, K.A., Bradley, D.J., Knox, J.P., Perotto, S., Butcher, G.W. & Brewin, N.J. 1989 Common components of the infection thread matrix and the intercellular space identified by immunocytochemical of pea nodules and uninfected roots. *The EMBO Journal* **8**, 335–342.

VandenBosch, K.A., Rodgers, L.R., Sherrier, D.J. & Dov Kishinevsky, B. 1994 A peanut nodule lectin in infected cells and in vacuoles and the extracellular matrix of nodule parenchyma. *Plant Physiology* **104**, 327–337.

Verdcourt, B. 1980 The classification of *Dolichos* L. emend. Verdc., Lablab Adans., *Phaseolus* L., *Vigna Savi* and their allies. In *Legume Science*, eds. Summerfield, R.J. & Bunting, A.H. Kew, London: Royal Botanic Gardens.

Witty, J.F., Minchin, F.R., Skot, L. & Sheehy, J.E. 1986 Nitrogen fixation and oxygen in legume root nodules. *Oxford Surveys of Plant Molecular and Cell Biology* **3**, 275–314.

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