The morphogenesis of lupine root nodules during infection by *Rhizobium lupini*

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Abstract

The development of root nodules in *Lupinus luteus* infected by *Rhizobium lupini* was studied using cytological methods. The results obtained from examination of material sampled 6, 9, 13, 15, 20, 29 and 60 days after infection are given. The successive stages of development are described and the cytological characteristics of the tissue are presented. The mitotic divisions of the root cortex parenchyma cells, which initiated the formation of the nodule primordium, were accompanied by structural changes in the root hairs and divisions in the root pericycle. The development of the nodule was associated with the activity of the lateral meristems, which encompass both the infected cells and cells not containing bacteroids. Characteristics of bacteria found in the symplast and apoplast of the bacteroid tissue are given.

Key words: bacteroid, nodule, *Lupinus luteus*, *Rhizobium lupini*, bacteroid tissue

INTRODUCTION

The initiation of nodules, their development and morphogenesis are processes leading to the establishment of symbiosis between the host plant and bacteria from the genus *Rhizobium*, during which fixation of atmospheric nitrogen occurs. In spite of the fact that the general aspects of this symbiosis are commonly known, information on the morphogenesis of the nodule is only fragmentary. The process of nodule formation and the cytological characteristics of nodules have received the best light and electron microscopic

documentation in the pea (Libbenga and Harkes 1973, Newcomb 1976, Newcomb et al. 1979), soy (Newcomb et al. 1979) and clover (Mosse 1964). There is little information available in literature on the symbiosis between *Lupinus luteus* and *Rhizobium lupini*. Woźny and Młodzianowski (1979) described the anatomy and ultrastructure of month-old *Lupinus luteus* nodules, while the characteristics of the bacteroid tissue and description of bacteroids were presented by Jordan and Grinyer (1965), and Kidby and Goodchild (1966).

This study presents the successive stages of development of *Lupinus luteus* nodules resulting from infection by *Rhizobium lupini*.

MATERIAL AND METHODS

Lupinus luteus L. var. Ventus seeds were sterilized in a 3.5% solution of calcium hypochlorite and allowed to germinate at a temperature of 24°C on filter paper dampened with distilled water. When the radicles attained a length of about 1 cm, the plants were transferred to pots filled with previously sterilized Perlite. The pots containing the experimental plants were watered once with a suspension of *Rhizobium lupini* (line 3045). For seven days, both the experimental and control plants were watered with distilled water, after which the experimental plants were watered with a nitrogen-free medium, while control plants were given the same medium enriched with nitrogen in the form of ammonium nitrate at a concentration of 300 mg dm⁻³. The plants were cultivated at a temperature of 24°C, illuminated with light at an intensity of about 7000 lx in a photoperiod of 16 hr light and 8 hr darkness. The material was sampled from the experimental and control plants in the following manner: 3 and 6 days after infection — samples were taken from the root hair region of the root, 9 days after infection — samples from the lateral root region, 13 days after infection — samples were taken of the main root which already had visible nodules; from the control plants, samples were taken from the corresponding part of the root. In a similar way, samples were taken on the later dates, 15, 20, 29 and 60 days after infection. The root samples were fixed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2 (Karnovsky 1965) for 4 hours at room temperature. The material intended for light microscopy analysis was dehydrated in an ethyl alcohol series, mixtures of ethyl alcohol and xylene, xylene, and embedded in paraffin. Sections about 10 µm thick were made using a microtome and stained with fast green and safranin. The material intended for observations in the electron microscope was post-fixed in 1% OsO₄ for 2 hr at 4°C, dehydrated in ethyl alcohol, acetone and propylene oxide series and embedded in Epon 812 (Luft 1961).

The material was sectioned on an LKB microtome. Semi-thin sections, stained with methylene blue and azure A were viewed in a light microscope, and ultrathin sections, stained with uranyl acetate and lead citrate, were analyzed using a JEM 100C electron microscope.

Staining with floroglucine in HCl for lignified cell walls and with Sudan III for lipids in cell walls was carried out on fresh material.

RESULTS

The development of nodules in lupine roots started with the induction of divisions in the peripheral zone of the root cortex parenchyma (Fig. 1). These divisions were observed in the roots collected 6 days after infection with *Rhizobium lupini*. Deformation of root hairs, manifested as their twisting and adhering to the root epidermis surfaces, was observed in the plants. Mucigel, in which the deformed root hairs were submerged, was found in this region (Fig. 1). Bacteria were also found in this secretion (Figs. 2, 3). This secretion revealed an electron-transparent matrix and fibrillar and granular osmophilic structures (Figs. 2, 3). Large aggregations of the osmophilic structures were seen along the radial walls of the neighboring root epidermis cells (Fig. 3). Large groups of bacteria were also seen in these places. At the same time, below these cells, the first mitotic divisions in the root cortex, initiating the nodule primordium, were observed. The dividing cells in the peripheral zone of the root cortex constituted the mother tissue of the developing nodule (Fig. 4). Even in the earliest stages of development, differentiation of the cells into bacteroid tissue mother cells and cortical mother cells had already occurred. A characteristic of the bacteroid tissue mother cells was a dense protoplast containing a few bacteroids as well as tiny vacuoles (Fig. 6). The cortex tissue mother cells differed from them in their decidedly larger vacuoles containing an osmophilic, fibrillar material and by lack of bacteroids in their protoplasts (Fig. 5). In the peripheral zone of the developing nodule, root cortex cells were obliterated, their protoplasts underwent gradual degradation, and their thickened cell walls became vitreous, bent and compressed, forming strips having several layers. The first cell divisions initiating the nodule mother cells, were accompanied by cell divisions in the pericycle in the zone under the developing nodule, and by anticlinal divisions of cells in the inner layers of the root cortex parenchyma, positioned between the nodule initials and root stela (Figs. 1, 4). These divisions led to the differentiation of the conducting tissue linking the nodule with the root stela. In roots, 9 and 13 days after infection, the differentiation of this tissue was more advanced from the side of the root stela (Figs. 4, 7).

PLATE I

Fig. 1. A cross-section through a lupine root 6 days after infection. Divisions initiating the formation of a nodule (arrows) are visible in the peripheral layers of the root cortex. Deformed root hairs are seen in the layer of mucigel (between arrow heads). Due to their bending and twisting, multiple cross-sections of the same root hair are seen. The external layer of the secretion on the interface of the soil solution has a denser structure. The pericycle cells undergoing mitotic divisions are marked with an asterisk. RC — root cortex, ST — root stela. 87x. Fig. 2. The area outside of the root between two root hairs (RH). Bacterial cells are visible in the root secretion (mucigel). 20000x. Fig. 3. Bacteria and osmophilic structures of the mucigel in the neighborhood of the radial wall (Cw) between root epidermis cells (E). RH — root hairs. 20000x

PLATE II

Fig. 4. A cross-section through a root 9 days after infection, illustrating the next stage of nodule formation (arrow). The asterisk marks dividing pericycle cells. RC — root cortex, ST — root stela. 87x. Fig. 5. The ultrastructure of peripheral cells of a developing nodule. Vacuoles (V) contain an osmophilic material, arrow heads mark the points where cell wall areas are increased. N — cell nucleus. 3200x. Fig. 6. The ultrastructure of cells of the bacteroid zone. Few bacteroids (B) are visible in the cytoplasm, tiny vacuoles (V) contain an osmophilic material. The arrow head marks the increased apoplast area. 8500x

PLATE III

Fig. 7. A cross-section through a root 13 days after infection. Intensely stained bacteroid tissue (BT) is visible in the central part of the nodule. The process of differentiation of the nodule cortex (NC) is continuing in the peripheral region. The arrow marks the differentiating vascular bundle. RC — root cortex, ST — root stela. 87x. Fig. 8. An enlarged fragment of bacteroid tissue whose cells are undergoing mitotic divisions (arrows). Large nuclei with nucleoli are visible. 350x. Fig. 9. The ultrastructure of bacteroid tissue. Bacteroids (B), mitochondria (Mi), rough endoplasmic reticulum cisterns (ER) are marked in the cells. Fibrous osmophilic structures (arrow) are visible in the central zone of the bacteroid. V — vacuole, N — nucleus. 20000x

PLATE IV

Fig. 10. A section through a nodule 15 days after infection. Its central part is composed of bacteroid tissue (BT). On the border between the bacteroid tissue and nodule cortex, 3-4 layers of uninfected cells (arrow head) can be made out. RC — root cortex, ST — root stela. 87x. Fig. 11. The ultrastructure of bacteroid tissue. Bacteroids (B), mitochondria (Mi), endoplasmic reticulum cisterns (ER) are visible in the cytoplasm. Starch grains (S) are being accumulated in the amyloplasts. N — nucleus, V — vacuoles. 5600x. Fig. 12. Bacteroids in the cytoplasm of bacteroid tissue. The bacteroids are encased in an envelope (arrow), osmophilic granules (arrow head) and fibrous material (double arrow head) are visible in their cytoplasm. N — nucleus, Ga — Golgi body, ER — endoplasmic reticulum, Mi — mitochondrion. 17000x

PLATE V

Fig. 13. A fragment of the peripheral part of the bacteroid tissue. Bacteria are visible in the intercellular spaces (arrow). One of the cells of the bacteroid tissue (seen in its entirety on the electronogram) stands out due to its small number of bacteroids and lower cytoplasm density. NC — uninfected nodule cortex cells. 5200x. Fig. 14. An enlargement of the intercellular space seen on Fig. 13. Bacteria (B) are surrounded by an electron-transparent matrix (MA) and submerged in fibrillar material (asterisks). Cw — cell wall, S — starch grain in an amyloplast. 20000x

PLATE I

PLATE II

PLATE III

PLATE IV

PLATE V

PLATE VI

PLATE VII

PLATE VIII

PLATE VI

Fig. 15. A section through a nodule 20 days after infection. The differentiation of the bacteroid tissue into the intensely stained central part — differentiated (a), differentiating zone (b) and meristematic zone (c), is noticeable. NC — nodule cortex, NV — nodule vascular bundle, ST — root stela. 87x. Fig. 16. A fragment of a nodule 29 days after infection. The peripheral part of the nodule still retains its meristematic character (asterisk). Bacteroid tissue — BT, Nodule cortex — NC, protective tissue — P, vascular bundles — NV. The cell walls of nodule cortex cells are thickened (arrows). 87x

PLATE VII

Fig. 17. The ultrastructure of cells from the central, differentiated, part of the bacteroid tissue — 20 days after infection. Numerous bacteroids (B) are in the cytoplasm, in some of them, electron-transparent granules (arrow) are visible. Large starch grains (S) are in amyloplasts. N — nucleus, Nu — nucleolus, V — vacuole. 6600x. Fig. 18. The ultrastructure of cells from the meristematic part of the bacteroid tissue — 20 days after infection. A significantly smaller number of bacteroids (B) is visible in the cells as compared with Fig. 17. Amyloplasts with starch (S) are seen only sporadically. Cell walls (Cw) are very thin. N — prophase nucleus, Mi — mitochondria, Ga — Golgi bodies, ER — endoplasmic reticulum, V — vacuoles. 10000x

PLATE VIII

Fig. 19. The ultrastructure of bacteroid tissue cells from the degeneration zone 60 days after infection. The cytoplasmic structures of the host's cells are undergoing degeneration. B — bacteroids. 10000x. Fig. 20. A fragment of the bacteroid tissue in the degeneration zone, 60 days after infection. The area containing the remnants of degraded cellular structures is marked with an asterisk. N — nucleus, B — bacteroids. 8500x. Fig. 21. Fragments of 3 neighboring bacteroid tissue cells. Complete degradation of bacteroids (arrow) is seen in the cell marked by the asterisk. Bacteroids (B) are marked in the neighboring cells. 10000x

Further development of the nodule was the result of divisions of bacteroid tissue and cortical tissue mother cells (Figs. 7, 8). The continuing processes of differentiation led to the formation of bacteroid and cortical tissues. Bacteroid tissue cells were characterized by a dense, intensely staining cytoplasm, large nuclei with distinct nucleoli. In the cytoplasm, numerous free and bound ribosomes, mitochondria and a small number of amyloplasts with small starch grains were seen. The bacteroids were encased in an envelope — one bacteroid per envelope. A nucleoid zone, in which osmophilic, thread-like structures were visible (Fig. 9), was observed centrally in the bacteroid. In 15-day-old nodules, 3-4 layers of tiny, uninfected cells, dividing anticlinally (Fig. 10), arose on the border between the bacteroid and cortical tissues. The cells of the nodule core were large, highly vacuolized. In the external zones of the core, divisions leading to the formation of protective tissue (Fig. 10) were observed. In the bacteroid tissue, in which up to this

stage of development, all of the cells were undergoing divisions, differentiation into a peripheral zone, where the cells continued to divide, and a central zone, began to become apparent. In the latter zone, only sporadic divisions, or none at all were observed.

The bacteroid tissue was composed exclusively of infected cells having thin cellulose walls (Figs. 11, 12). Numerous cisterns of rough ER, Golgi bodies, mitochondria, which were often very elongated, were observed in the protoplasts of these cells. The vacuoles were filled with an osmophilic, fibrillose material. Starch was being accumulated in the amyloplasts positioned in the peripheral parts of the cells. An increased number of bacteroids was found in the cells of the bacteroid tissue. Some of them contained osmophilic granules (Fig. 12) in the nucleoid region in addition to the condensed fibrillar material. In the peripheral parts of the bacteroid tissue, structures resembling infection threads were observed in intercellular spaces (Figs. 13, 14). Present in these spaces were bacteria surrounded by an electron-transparent matrix and embedded in a fibrillar material resembling cell wall material. In contrast to the bacteria present in the cell protoplasts, these bacteria were not surrounded by an envelope (Fig. 14).

Further development of the nodule was the result of the activity of the peripherally located meristem, which encompassed several layers of the bacteroid tissue and 2-4 layers of uninfected cells (Figs. 15, 16). As the result of the functioning of two lateral meristems, the developing nodules encircled the roots. The following zones became discernible within the bacteroid tissue: central, differentiated, differentiating and meristematic zones (Fig. 15). In the central zone, the cells had a dense cytoplasm and contained a large number of bacteroids. Peripherally positioned amyloplasts containing large starch grains (Fig. 17) were characteristic here. The cells of the meristematic zone contained significantly fewer bacteroids, elongated mitochondria, proplastids and only a few amyloplasts (Fig. 18). In month old nodules, vascular bundles containing elements of phloem and xylem, several layers of pericycle and endodermis with Casparian strips, were found in the cortical parenchyma close to the root stela. In the cortical tissues of the nodule, a thickening of the cell walls of parenchyma cells was observed, mainly in their corners, although this was also sometimes seen over their whole diameter (Fig. 16). Staining with floroglucine and hydrochloric acid did not give a positive result indicating the presence of lignin in these walls. The protective tissue of nodule was composed of several layers of cells, in the walls of which the presence of lipids was determined by the Sudan III reaction.

In 8-week-old nodules, degradation of some of the bacteroid tissue cells was found. Also observed were degradation processes in membranous structures of the protoplasts of bacteroid tissue cells, after which the gradual degradation of the bacteroids occurred (Figs. 19, 20), as did degradation of bacteroids having intact protoplast structure (Fig. 21).

DISCUSSION

The first cell divisions initiating the formation of a nodule in lupine roots occur, similarly as in soybean (Newcomb et al. 1979) and bean (Dart 1975) in the peripheral layers of the root cortex. Bacteroid tissue mother cells and, positioned externally to them, cortex initials, can be discerned among the nodule mother cells. A few bacteroids, surrounded by an envelope, are found in the bacteroid tissue mother cells. Cortical cells are devoid of bacteroids but characterized by large vacuoles containing an osmophilic material. At the same time, very intense divisions of the pericycle cells under the nodule initials are observed. The root cortex cells between the mentioned mitotic centers also undergo divisions, mainly anticlinal, and it is here that strips of vascular bundles linking the nodule with the root stela differentiate. For a period of 7-9 days after initiation of the nodule, both the bacteroid and the cortical tissues retain their meristematic nature. During this period, the nodule grows in all directions. During the further development of the nodule, the meristematic activity remains only in its edges, and as the result of the activity of the two late al meristems located there, the initially spherical nodules grow laterally encircling the root. This type of nodule is known in literature as a "collar nodule" (Dart 1975). The meristematic zone of lupine nodules contains, in contrast with that of the pea (Libbenga and Harkes 1973, Newcomb et al. 1979), 2 types of cells: infected cells, in which cell divisions cause the growth of the bacteroid tissue, and 2-4 layers of uninfected cells producing the nodule core. The meristematic activity in lupine nodules, similarly as has been described in the pea (Newcomb et al. 1979), is still maintained 2 months after infection, when the central part of the bacteroid tissue is already undergoing degeneration. The nodules characterized by a long period of retention of meristematic activity and differentiation in the anatomical structure of the bacteroid tissue and degeneration zone, have been described in the pea (Libbenga and Harkes 1973, Newcomb et al. 1979), and clover (Mosse 1964) and are called "indeterminate nodules" (Morrison et al. in press). Our observations indicate that lupine nodules have the listed traits.

In the studied material, bacterial cells were found both in the symplast as well as in the apoplast of the bacteroid tissue. In the protoplast of the host, the bacteria were always encased by an envelope — one bacteroid per envelope (Jordan and Grinyer 1965, Kidby and Goodchild 1966, Woźny and Młodzianowski 1979). The bacteria in the intercellular spaces were lacking such an envelope. The presence of a fibrillar substance in these spaces and the occurrence of an electron-transparent matrix around the bacteria is suggestive of the ultrastructure of the infection thread described in *Lupinus angustifolius* (Robertson et al. 1978) and soybean (Turgeon and Bauer 1985). The question remains open about the significance of the

occurrence of bacteria in the intercellular spaces, since the observed process of morphogenesis of the nodule indicates that the bacteroid tissue is the product of several initially infected cells, whose mitotic divisions cause it to grow.

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REFERENCES

- Dart P. J., 1975. Legume root nodule initiation and development. In: The development and function of roots. Torrey J. G., Clarkson D. T. (eds.), Academic Press, London-New York-San Francisco, pp. 467-506.
- Jordan D. C., Grinyer I., 1965. Electron microscopy of the bacteroids and root nodules of *Lupinus luteus*. Can. J. Microbiol. 11: 721-725.
- Karnovsky M. J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 25: 137A.
- Kidby D. K., Goodchild D. J., 1966. Host influence on the ultrastructure of root nodules of *Lupinus luteus* and *Ornithopus sativus*. J. Gen. Microbiol. 45: 147-152.
- Libbenga K. R., Harkes P. A. A., 1973. Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. Planta 114: 17-28.
- Luft J. H., 1961. Improvements in epony resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409.
- Morrison N. A., Bisseling T., Verma D. P. S. Development and differentiation of the root nodules: involvement of plant and bacterial genes. In: Developmental biology: A comprehensive synthesis. Browder L. W. (ed.), Plenum Publishing Corp. (in press).
- Mosse B., 1964. Electron-microscope studies of nodule development in some clover species. J. Gen. Microbiol. 36: 49-66.
- Newcomb W., 1976. A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum* root nodules. Can. J. Bot. 54: 2163-2186.
- Newcomb W., Sippell D., Peterson R. L., 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. Can. J. Bot. 57: 2603-2616.
- Robertson J. G., Lyttleton 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. J. Cell Sci. 30: 129-149.
- Turgeon B. G., Bauer W. D., 1985. Ultrastructure of infection thread development during the infection of soybean by *Rhizobium japonicum*. Planta 163: 328-349.
- Woźny A., Młodzianowski F., 1979. Anatomy and ultrastructure of root nodules of *Lupinus luteus*. Acta Soc. Bot. Pol. 48: 119-124.

Morfogeneza brodawki lubinu w warunkach infekcji Rhizobium lupini

Streszczenie

Badano, przy użyciu metod cytologicznych, rozwój brodawek lubinu żółtego porażonego *Rhizobium lupini*. Przedstawiono wyniki uzyskane w oparciu o analizę materiału pobranego 6, 9, 13, 15, 20, 29 i 60 dni po infekcji. Opisano kolejne etapy rozwoju brodawki oraz przedstawiono cytologiczną charakterystykę jej tkanek. Podziałom mitotycznym komórek miękiszu kory pierwotnej korzenia, inicjującym powstanie primordium brodawki, towarzyszą zmiany strukturalne włósników oraz podziały perycyklu korzenia. Rozwój brodawki związany jest z funkcjonowaniem merystemów bocznych obejmujących zarówno komórki zainfekowane, jak i komórki nie zawierające bakteroidów. Przedstawiono charakterystykę bakterii występujących w symplasmie i apoplasmie tkanki bakteroidalnej.