MORPHOGENESIS OF ROOT NODULES IN WHITE CLOVER.
I. EFFECTIVE ROOT NODULES INDUCED BY THE WILD TYPE
RHIZOBIUM LEGUMINOSARUM BIOVAR. *TRIFOLII*

BARBARA ŁOTOCKA, JOANNA KOPCIŃSKA, WŁADYSŁAW GOLINOWSKI

Department of Botany, Faculty of Agriculture,
Warsaw Agricultural University,
Rakowiecka 26/30, 02-528 Warsaw, Poland
e-mail lotocka@delta.sggw.waw.pl

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ABSTRACT

The research aimed at investigating the morphogenesis of cylindrical root nodules in *Trifolium repens* L. induced by the wild type *Rhizobium leguminosarum* biovar. *trifolii* strain 24. It has been demonstrated that the ontogenesis of a nodule begins with a transverse division of cells of the pericycle followed by the dedifferentiation and divisions of cells of the endodermis and inner layers of the primary root cortex. Shifting of the nodule meristem from its initially lateral to the apical position characteristic for cylindrical nodules was observed. Bacteroidal, cortical and vascular tissues of the nodule are described up to 42 days after inoculation. At that time typical degraded zone had not yet appeared in the nodules.

KEY WORDS: pericycle; endodermis; root nodule primordium; root nodule structure; bacteroidal tissue; *Trifolium repens* L.

INTRODUCTION

Leguminous root nodules are highly specialized structures adapted to reduce and fix nitrogen for the benefit of the plant system as a whole, thus permitting excellent growth of nodulated leguminous plants under nitrogen deficient conditions. Although neither the leguminous host nor its microsymbiont can fix nitrogen under free-living conditions, the morphogenesis of root nodules locally creates the specific conditions required for the induction and functioning of the nitrogenase system. Therefore, in order to understand how symbiotic nitrogen fixation comes into being, the processes of root nodule morphogenesis have to be studied (Libbenga, Bogers, 1974). During the stage of mutual recognition of the symbiosis partners, rhizobial *nod* genes are induced *via* plant flavonoids to produce a Nod factor – lipo-oligosaccharide signal molecule, the effect of which is the deformation of root hairs and the dedifferentiation of root cortex cells (Hirsch, 1992). The localization of the newly formed meristem and the type of root nodule that develops, depends on the host-plant, not on the bacterial genome. There are two major types of nodules distinguished from one another mainly by the persistence of meristematic activity (Hirsch, 1992; Sprent, 1989). If the dedifferentiation is induced in outer cortex layers, the determinate spherical nodules arise, in which mitotic activity ceases completely after a determined period of time. If the root cortex dedifferentiation occurs in the inner layers of that tissue, indeterminate cylindrical nodules are formed with apical indeterminate growth meristem. This type of nodule is produced, for example, by alfalfa, pea, vetch and clover. The morp-

hogenesis of indeterminate root nodules was studied extensively on alfalfa and pea (Libbenga, Harkes, 1973; Newcomb, 1976; Newcomb et al., 1978; Dudley et al., 1987), but still there are many gaps in our knowledge in regard to the other species, especially in the understanding of the sequence of events occurring at the beginning of nodule formation. This work was aimed at the detailed examination of early stages of clover root nodule ontogenesis and comparison of mature nodule structure with the data concerning well-known indeterminate nodules of alfalfa and pea.

MATERIAL AND METHODS

Seeds of white clover 'Astra' were surface sterilized with 70% ethanol and 3.5% calcium hypochlorite, pregerminated at 24°C for 48 h (until the 3-5 mm long root hair zone arised) in sterile conditions and then, after the inoculation with *Rhizobium leguminosarum* biovar. *trifolii*, they were transferred to agar slopes containing modified Fahraeus nitrogen-free medium (Sahlman, Fahraeus, 1963). A wild strain 24 inducing effective nodules was used in the investigation. It was obtained from the Microbiology Department of Maria Skłodowska-Curie University in Lublin. Plants were cultivated at the temperature of 20-25°C, light intensity of 72 $\mu\text{Es}^{-1}\text{m}^{-2}$ (PhAR) and 16 h photoperiod. The 4÷14, 21, 28 and 42 day-old (days after inoculation) nodules were collected for the investigation. Only one nodule at a determined stage of development was collected from each plant. The nodules were fixed for 2 h in the mixture of 5% glutaraldehyde and

4% paraformaldehyde on 0.1 M cacodylate buffer pH 7.2±7.3 at room temperature and the air pressure of -0.4 kGcm⁻². Then they were rinsed a few times in the same buffer, post-fixed in 2% OsO₄ for 2 h at 4°C, dehydrated in the increasing concentrations of ethanol and acetone and embedded in Epon 812. The ultra-thin sections were cut on the Reichert microtome and contrasted with uranyl acetate and lead citrate. Observations were made in the JEM100C electron microscope. Series of semi-thin sections about 3 µm thick were cut on the microtome Jung RM2065 and stained with 2% methylene blue and 1% aqueous azure A on 2% borax and observed in the Axioskop microscope (Opton). In order to obtain material for observation at the early stages of the nodule development, at which it is not yet seen on the root surface, clover seedlings, prepared as above, were placed on the Petri dishes containing nitrogen-free medium of the above mentioned contents. The position of root tips was marked and 72 h after inoculation, the part of root which had grown since the inoculation was cut off the upper part and both parts were fixed in the way presented above. The upper part of the root was the source of 3 day-old primordia, whereas numerous younger primordia were found in the lower part. After rinsing in the buffer the material was examined under Nomarski optics. The positions of the root nodule primordia and lateral roots were marked on the cover glass and then they were cut out, dehydrated, saturated with resin and embedded in Epon blocks in the same a way, as the remaining material. Material was serial-sectioned in the transverse, radial and tangential planes.

RESULTS AND DISCUSSION

The first root nodules appeared 4 days after inoculation in the root zone which was the root hair forming region at the time of inoculation. On the average, in 28 days the plants produced 9.0 nodules with the number varying from 4 to 16 nodules per plant. The nodules appeared, with no exception, in the xylem poles of the triarch clover root. Such location is typical for cylindrical nodules (Libbenga, Harkes, 1973; Phillips, 1971).

1. The role of pericycle and endodermis in the root nodule primordium formation.

Initial divisions in pericycle

The most initial primordia collected from the lower part of the roots fixed 72 h after inoculation revealed only transverse divisions in the pericycle cells adjacent to the protoxylem (Fig. 1a). At such an initial stage it is not possible to distinguish between the lateral root and nodule primordia, however, the observations of lateral roots and nodules more advanced in their development, showed that in both cases the initial divisions took place in the pericycle. Thus, in clover, the initiation of cylindrical nodules takes different course than in *Pisum sativum* and *Medicago sativa* in which first divisions were claimed to occur in the innermost layer of the primary root cortex parenchyma (Libbenga, Harkes, 1973; Newcomb et al., 1978; Dudley et al., 1987; Joshi et al., 1991). However, the methods applied in the quoted investigations arouse doubts whether these authors described actually the youngest primordia. In addition, the lately published results on the reaction of alfalfa to the inoculation with rhizobia show that one of the earliest host response is connected with the pericycle and it consists in nodulin ENOD40 induction in its cells (Asad et al., 1994; Werner, 1992; Bhaskaran, Swaminathan, 1958).

Three day-old root nodule primordia could be already easily distinguished from much less numerous lateral root primordia. As in the observations by Truchet, Camut et al. (1989), lateral root primordia in our work – conical, compact, with dense cytoplasm – clearly differed from nodule primordia – of flattened, extended along the root axis shape, and with less dense cytoplasm (Fig. 1b, Fig. 2). In 3 day-old nodule primordia cell divisions were observed in the pericycle and endodermis and in the innermost layer of the primary root cortex parenchyma. The cells divided in all three planes: transverse, tangential and radial (less).

Endodermis

None of the papers, known to the authors, on the root nodule development discuss the problem of the role of endodermis in the formation of root nodules. In clover, in the root zone susceptible to infection (the region of root hair formation), the root endodermis in the xylem poles was at the stage of Casparian strips, while in the phloem poles at the same section it was more differentiated, being at the stage of the tertiary lignified cell wall formation. That wall was evenly deposited on the whole inner surface of endodermis cells. It was characterized by regular stratification and in the electron microscope examination the layers were electron translucent (similarly as in the secondary lignified wall). While differentiating, the endodermis cells gradually formed the tertiary lignified wall starting from the phloem poles towards the xylem poles, then filled up with a darkly staining substance, underwent protoplast degradation, lost turgor and collapsed (Fig. 3). In the region of the root with 4 day-old nodules, thus in a relatively small distance from the root hair forming region, a ring of such collapsed cells could encircle the whole root stele. The differentiation of the endodermis cells in the region of the root nodule primordium formation was inhibited. The endodermis cells in the primordium became dedifferentiated, as they grew larger and/or divided (Fig. 1b), mainly radially or obliquely, although the divisions were delayed in relation to the pericycle cells. Some enlarged endodermis cells with denser cytoplasm and the typical Casparian strips were observed. It was not possible to decide whether the dedifferentiation of the endodermis cells in the primordium region led to the removal of the strips. On the transverse root section the border between the collapsed endodermis cells and endodermis derivatives inside primordium was sharp – there were no cells at the transitory stage. On the basis of analysis of a serial cross-sections of 4 day-old nodules, which consisted in the tracking of files of the cells of the pericycle, endodermis and inner cortex, it was found out that the cell divisions in the endodermis did not keep up with the divisions in the pericycle. Thus, the pericycle derivatives pushed their way through the layer of endodermis derivatives and came into direct contact with the dividing cells of the primary root cortex (Fig. 4a-c, comp. with Fig. 1b). Then a vascular junction between the nodule and the root stele was formed out of the pericycle derivatives. At the later stages of nodule development the meristematic (apical) parts of the bundles were clearly connected with the nodule meristem (Fig. 7, Fig. 10b) – thus the successive parts of the nodule vascular bundles were formed not by the pericycle derivatives but by the nodule meristem originating from the cortex cells.

Fig. 1. Clover root nodule primordium: a) 24-48 hrs after inoculation, divided pericycle cells (shorter, with denser cytoplasm than in adjoining ones) visible, b) approx. 72 hrs after inoculation. PX – protoxylem strand, P – pericycle, Pd – pericycle derivatives, E – root endodermis, Ed – divided and dedifferentiated root endodermis cells, oE – obliterated root endodermis cells, PC – primary root cortex parenchyma, PCd – primary root cortex derivatives (divided inner layer cells only), Ep – epiblem. Bar 64.10 μ m.

Fig. 2. Lateral root primordium approx. 72 hrs after inoculation.
PX – protoxylem strand, Pd – pericycle derivatives, Ed – dedifferentiated root endodermis cells, PCd – primary root cortex derivatives (divided inner layer cells only), Ep – epiblem. Bar 64.10 μ m.

Fig. 3a, b. Ultrastructure of the root endodermis cell.
E – endodermis cell, PC – primary root cortex parenchyma, arrows – Casparian strip, PL – plasma membrane closely attached to the Casparian strip, T – tonoplast, IS – intercellular space, RER – rough endoplasmic reticulum, TCW – tertiary, layered cell wall of endodermis cell. Bar 3.79 μ m.

Fig. 4. Some transverse sections chosen from a series cut from 4 day-old root nodule: a) section close to the root meristem; endodermis cells still form a continuous ring.

Pd – pericycle derivatives within the nodule, asterisks – endodermis derivatives within the nodule, oE – obliterated root endodermis cells, PCd – primary root cortex derivatives, NC – nodule cortex, BT – infected cells of the bacteroidal tissue, arrows – infection thread, arrowhead – cell division. Bar 64.10 μ m.

2. The role of the primary root cortex in the root nodule primordium formation

The first divisions in the primary root cortex

At the time when changes connected with the infection and infection threads formation were observed in the root hairs, in the innermost layer of the primary cortex adjoining to the dedifferentiated endodermis, some divided cells were visible. No typical pre-infection structures characteristic for some plants, e. g. pea, in the form of thick cytoplasm strands marking the direction of the infection threads penetration in the outer cortex layer (Brussel van et al., 1992) were observed in clover. The wave of cell divisions gradually progressed towards the infected root hair, extending up to three layers of the cortex cells (the primary cortex in the clover is formed of 5-7 layers). The cells in one-two layers under the epiblem usually remained undivided and did not participate in the proper primordium formation. It was observed in alfalfa and pea that although these cells had started cycling, after passing the phase S of the cycle they become arrested at the phase G2 (Yang et al., 1995). The competence of some of these cells both in pea and alfalfa as well as in clover (this work), consisted in channeling of the infection thread elongation (the apoplast reaction only), thus enabling the infection of the deeper layers of the primordium. The cells of these two subepidermal layers adjacent to the nodule primordium (not necessarily only the cells which had formed the thread) grew significantly in the few successive days of the nodule development as compared to other cells of the same layers situated further from the nodule.

Infection thread penetration and bacteroidal tissue initiation

As seen on the radial root section, the dedifferentiation of the root cortex in the vertical direction usually included initially 3 storeys of cells (comp. Fig. 11). The analysis of a series of cross-sections of 4 day-old and younger root nodule primordia revealed that the direction of the infection thread elongation, after its penetration from the root-hair cell, changed in the cell of the subepidermal layer or the one next to it. The thread did not elongate along the shortest line towards the divided cells but towards the derivatives of the highest storey of the dedifferentiated cortex cell, a little above the infected hair. That group of competent cells was penetrated by the infection thread and/or its branches and infected by bacteria. In that part of primordium (comp. Fig. 11), after the release of rhizobia, cell divisions ceased and the infected cells gradually differentiated into the bacteroidal cell tissue: they grew significantly and symbiosomes appeared in them. However, some of the cells become necrotic and the location of such cells seemed to be accidental as they were observed in all parts of primordium.

The infection threads in the earliest infected zone of the nodule primordium, depending on the plant, showed various morphology. In some primordia the threads were very large, taking up considerable part of the cell volume (they were located intercellularly sometimes), with light matrix, distinctive thread wall and bacteria (Fig. 5a). In other primordia the threads had small diameter and could contain some strongly staining substance (Fig. 5b).

Fig. 4. Some transverse sections chosen from a series cut from 4 day-old root nodule: b) section behind the root meristem, c) section from the bacteroidal tissue region; discontinuous ring of endodermis cells.
Pd – pericycle derivatives within the nodule, asterisks – endodermis derivatives within the nodule, oE – obliterated root endodermis cells, PCd – primary root cortex derivatives, NC – nodule cortex, BT – infected cells of the bacteroidal tissue, arrows – infection thread, arrowhead – cell division. Bar 64.10 μ m.

Fig. 5. Transverse section of the infection thread penetration zone of 4 day-old root nodule:

a) nodule with light-staining infection threads,

b) nodule with dark-staining infection threads.

PX – protoxylem strand, Ph – phloem, C – cambium, oE – obliterated root endodermis cells, double arrow – necrotized cell, arrow – light-staining infection thread with thread wall and bacteria visible, double arrowhead – dark-staining infection thread. Bar 64.10 μ m.

Fig. 6. Radial section of the 4 day-old root nodule with laterally located meristem visible.

PX – protoxylem strand, oE – obliterated root endodermis cells, BC – nodule bundle cambium, M – nodule meristem, NC – nodule cortex, arrow – cell division, double arrowheads – dark-staining infection threads. Bar 64.10 μ m.

Fig. 7. Section of the 6 day-old root nodule with laterally located meristem.

PX – protoxylem strand, Ph – phloem, C – cambium, oE – obliterated root endodermis cells, NB – nodule bundle, M – nodule meristem, IZ – infection thread penetration zone (=infection zone), BT – bacteroidal tissue, IC – inner cortex of the nodule, NE – nodule endodermis (tannin cells), double arrowhead – intercellular space, arrowhead – cell division, double arrow – necrotized cell, arrow – infection thread, white arrow – amyloplast in the bacteroidal tissue cells, asterisk – uninfected bacteroidal tissue cell. Bar 64.10 μm .

The change of the meristem location

The primordium cells situated below the infected ones still divided and comprised the meristematic region of the primordium (Fig. 6, comp. with Fig. 11). In time, a gradual ceasing of divisions was observed in that region from the side of the stele, consequently the remaining meristematic part was directed towards the epiblem. Thus shifting, the meristematic activity caused the growing of the nodule primordium out of the root and downwards. The analysis of serial sections of the

8, 12, 14, 21 and 28 day-old nodules showed the not previously observed process of gradual shift of the nodule meristem from the lateral to apical position, the latter widely described as typical for cylindrical nodules. Such an apical meristem position could already be observed in the 28 day-old clover nodules and it was unquestionable in the 42 day-old nodules.

The described processes leading to the apical meristem formation in the white clover root nodules took different course

Fig. 8. Symptoms of the early degradation (widening of the peribacteroid space and myelin structures) in the cells of young bacteroidal tissue in 7 day-old root nodule: a) typical (top) and degenerating (bottom) cell, b) cell with myelin structures in symbiosomes. A – amyloplast, CW – cell wall, B – bacteroid, ER – endoplasmic reticulum, M – mitochondrion, arrow – widened peribacteroid space, double arrow – Golgi bodies, arrowhead – myelin structure. Bar 1.89 μ m.

Fig. 9. Infection zone cell with numerous profiles of cytoplasm in vacuolar sap, 7 day-old root nodule. CW – cell wall, N – cell nucleus, Pp – proplastid, S – symbiosom, M – mitochondrion, VS – vacuolar sap, arrow – profile of cytoplasm in vacuolar sap, surrounding tonoplast often damaged, double arrow – Golgi bodies, arrowhead – plasmodesm. Bar 2.50 μ m.

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: a) section of the nodule meristem. InC – initial cells zone, DC – differentiating cells zone, BC – nodule bundle cambium, NE – zone of the differentiated nodule endodermis cells. Bar 32.05 μm .

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: b) section of the nodule meristem.

InC – initial cells zone, BC – nodule bundle cambium, NE – zone of the differentiated nodule endodermis cells, IZ – infection zone, ES – early symbiosis zone in the bacteroidal tissue, IC – inner cortex, arrow – infection thread. Bar 32.05 μm .

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: c) section of the infection zone and early symbiosis zone. BC – nodule bundle cambium, NB – nodule bundle, NE – closing ring of differentiated nodule endodermis cells, double arrow – differentiating cells of nodule endodermis, IZ – infection zone, ES – early symbiosis zone in the bacteroidal tissue, IC – inner cortex, arrow – infection thread, arrowhead – necrotized cell, white arrow – large amyloplasts in the interzone II/III. Bar 32.05 μ m.

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: d) section of the infection zone, early symbiosis zone and active N_2 -fixation zone.

NB – nodule bundle, NE – closing ring of differentiated nodule endodermis cells, double arrow – differentiating cells of nodule endodermis, IZ – infection zone, ES – early symbiosis zone in the bacteroidal tissue, IC – inner cortex, BL – boundary layer, arrow – infection thread, arrow-head – necrotized cell, double arrowhead – uninfected cells with globular amyloplasts in differentiated bacteroidal tissue, white triangle – infected cells with plate-like amyloplasts located at the intercellular spaces. Bar 32.05 μ m.

Fig. 10. Ultrastructure of the infected cells of bacteroidal tissue, 42 day-old root nodule: e, f) early symbiosis zone, g) active N_2 -fixation zone. RER – rough endoplasmic reticulum, B or b – bacteroid, G – Golgi bodies, M – mitochondrion, A – amyloplast, CW – cell wall, IT – infection thread, arrow – peribacteroid membrane, arrowhead – intracellular membrane system in bacteroids. Bars: e) 1.89 μm , f) 0.78 μm , g) 2.50 μm .

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: h) section of active N_2 -fixation zone. NB – nodule bundle, NE – closed ring of nodule endodermis, IC – inner cortex, BL – boundary layer, arrowhead – uninfected cells with globular amyloplasts in differentiated bacteroidal tissue, white triangle – infected cells with plate-like amyloplasts located at the intercellular spaces. Bar 32.05 μ m.

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: i) section of senescing zone, insert: magnified fragment of the bacteroidal tissue, note globular bacteroids.

NB – nodule bundle, NE – nodule endodermis, IC – inner cortex, note disappearance of the boundary layer, arrowhead – uninfected cells with amyloplasts much smaller than in active N_2 -fixation zone, asterisk – infected cells with plate-like amyloplasts located at the intercellular spaces, note folding of the cell walls caused by the lack of turgor, arrow (insert) – dark-stained debris after symbiosome degradation appear first at the cell nucleus. Bar 32.05 μ m, insert: 64.10 μ m.

Fig. 10. Some transverse sections from a series cut from 42 day-old root nodule: j) section of the nodule base.

IC – nodule inner cortex, BT – bacteroidal tissue, NB – basal part of the nodule bundle close to its incorporation to the root vascular system, white arrow – nodule bundle cambium, C – root cambium, SPh – secondary phloem, NE – nodule endodermis joined with E – root endodermis, P – root pericycle. Bar 32.05 μm .

than in the cylindrical nodules of pea and alfalfa (Libbenga, Harkes, 1973; Newcomb et al., 1978) in which the whole primary meristem centre in the deep cortex layers was claimed to be infected with rhizobia and thus it was lost as its cells lost the ability to divide. At the same time in the middle cortex layers a new mitotic activity was initiated and it constituted the nodule meristem, which derivatives were penetrated by the infection threads from already infected cells (such a process would require the reversal of the thread growth direction). Thus the nodule meristem in these plants had apical position from the beginning and nodules grew perpendicularly to the root axis. Considering the fact that the structure of a mature nodule in white clover nearly does not differ from the anatomy of nodules in pea and alfalfa, it is surprising that the formation of the apical meristem in clover follows such a different course. It seems that the process of nodule formation in pea and alfalfa should be re-examined, especially in view of the data concerning the inhibition of the cell cycle in the outer cortex layers and their not-participating in the primordium formation (Yang et al., 1995).

It should be added that the first stages of the nodule primordium development in white clover (this work) were similar to the initiation of the lateral root primordium. In both cases the same root anatomical zones were stimulated, however, their mitotic activity varied. In case of the lateral root the vertical extent of the reaction of pericycle, endodermis and primary cortex cells was similar as in the nodule primordium (i.e. three storeys of cells), however, the divisions clearly

concentrated in the central part of the dedifferentiation region. In consequence, there was formed a compact meristem center of a conical shape in which the marginal zones were deprived of cell divisions (Fig. 2). On the cross-section of the root, the dedifferentiation included the same number of cell layers as in case of the nodule primordium but intensive divisions (in all three planes) were observed in the pericycle derivatives and not in the cortex. The endodermis cells divided several times transversally and radially and their derivatives did not enlarge. The divisions in the cell of inner cortex were sporadic.

The further stages of root nodule development

In the 4 day-old nodules three zones were visible: cortical region, region with infected cells and meristematic region. In the 6 day-old nodules (Fig. 7) all zones typical for cylindrical nodules were visible although they could be very narrow, limited to a few cells only. A prominent enlargement of the earliest infected cells in which strongly stained symbiosomes appeared was very characteristic. Some uninfected cells and widened intercellular spaces (wider than in the respective zone of the older nodules) could be observed between the infected cells. The nodule meristem was connected with the cambium strand of the nodule vascular bundle (comp. with Fig. 11). In its subapical part that strand produced first branching of the bundle. It was observed, that the basal part of the bundle, close to the protoxylem, already contained the differentiated cells: tracheary and sieve elements and nodule peri-

Fig. 11. A scheme of the morphogenesis of root nodule in white clover.

Px – protoxylem, P – pericycle, En – root endodermis, 1-5 – the subsequent files of the primary root cortex cells, E – epiblem.

a) initial divisions in pericycle approximately 24 h after inoculation. The next to dedifferentiate cells of endodermis and inner root cortex marked with asterisks;

b) further divisions in pericycle, endodermis and inner (5th) layer of root cortex, dedifferentiating cells in the adjoining (4th) layer, arrow – infection thread formation in the curled root hair;

c) approximately 3 days after inoculation: the infection thread (arrow) penetrates the derivatives of the highest storey of the initially dedifferentiated inner root cortex cells, the cell walls of original cells (comp. with a) are marked thicker;

d) approximately 4 days after inoculation: the cells in the zone I of primordium, after being penetrated with the infection thread (arrow) cease dividing and differentiate into bacteroidal cells. The derivatives in the zone III continue dividing and form the future nodule meristem. In this stage it is located laterally to the bacteroidal cells. oE – obliterated root endodermis, dE – dedifferentiated root endodermis derivatives within the nodule primordium, NC – initials of the nodule cortex, VB – initials of the nodule bundle;

e-f) gradual shift in the location of the nodule meristem with reference to the bacteroidal tissue. The differentiation of nodule cortex tissues and vascular bundle omitted. BT – bacteroidal tissue, TP – infection thread penetration zone, M – nodule meristem, VB – vascular bundle, NC – nodule cortex, arrow – infection thread.

cycle parenchyma. With the growth of the nodule there were visible changes in the proportion between particular zones of the bacteroidal tissue as the active nitrogen fixation zone increased.

In the 6 and 7 day-old nodules, in some of the earliest infected cells of the bacteroidal tissue the symptoms of symbiosome degradation could be noted in electron microscope (Fig. 8) and in the light microscope single necrotic cells were found (Fig. 7).

A zone of bacteroidal tissue with bacteroids showing vesicular invaginations of the inner membrane in the peripheral layer of their cytoplasm appeared in the 8 day-old nodules. Such an intracellular membrane system is a characteristic feature of bacteroids of *Rh. l. bv. trifolii* although its physiological function is not known. Similar system of membranes was also observed in many other species of Gram-negative and Gram-positive bacteria and in *Actinomyces* (Dart, Mercer, 1963a; Dart, Mercer, 1963b).

In the vacuoles of numerous cells of the infection thread penetration zone abundant cytoplasm profiles (Fig. 9) with frequent damages of surrounding membrane were seen. The presence of these cytoplasmic strands profiles and/or cytoplasm vesicles in the vacuolar sap could be a manifestation of an autolytic process connected with the reorganization of the vacuolar system characteristic for bacteroidal cell ontogenesis (Kijne, 1975).

The mature nodule

The 42 day-old nodules had all the developmental zones in the bacteroidal tissue. The apically situated **meristem** was built of dividing cells which were small, not vacuolated and without the intercellular spaces. Within the meristem, the initial cells of the vascular bundles were easily distinguishable because of their elongated shape and radial arrangement in relation to the nodule axis (Fig. 10a, b). A little lower, between the cambium strands of the vascular bundles the meristem derivatives were **penetrated by infection threads** and bacteria were released. The structure of the infection threads and the process of release of bacteria did not differ from the literature data (Dart, Mercer, 1964; Napoli, Hubbell, 1975; Mosse, 1964; Dixon, 1964; Callaham, Torrey, 1981). Cells penetrated by the thread started to differentiate which was manifested by their growth and vacuolation. The differentiation of the bacteroid tissue was quicker in its peripheral layers than in the central region (Fig. 10c).

Below the infection thread penetration zone one could observe cells containing very large amyloplasts characteristic for the, so called, **interzone II-III** (Fig. 10c, 10d; Vasse et al., 1990). The situated below, **intensively nitrogen-fixing regions** of bacteroidal tissue, contained infected cells with dense cytoplasm, numerous symbiosomes (Fig. 10d-i) and a little smaller, flattened amyloplasts placed parietally, most often close to the intercellular spaces (Fig. 10e). These cells were separated with uninfected cells, the number of which exceeded the number of cells with bacteroids. The uninfected cells were characterized by smaller size, small amount of cytoplasm and numerous globular amyloplasts, smaller from those in the infected cells. These amyloplasts were the trait allowing, with great certainty, the distinction of uninfected cells of the bacteroidal tissue from other uninfected cells, mainly cortical ones, the latter being deprived of plastids distinguishable in light microscope. It was observed that the boundary of bacteroidal

tissue in the regions neighbouring the bundles comprised the uninfected cells of the bacteroidal tissue while in the remaining regions the infected cells were often in the direct contact with the cells of the inner cortex (Fig. 10h).

In our work it was found that the white clover root nodules were connected with the root vascular system with only one bundle, contrary to similar alfalfa nodules having at least two bundles (Truchet et al., 1989). Since the 6th day of the nodule development that single clover vascular bundle branched first into two, and then many times, forming a system of bundles winding round the bacteroidal tissue. The bundles investigated here were built in a typical way (comp. Hirsch, 1992), i.e. they contained colaterally situated xylem and phloem with the phloem on the side of the bacteroidal tissue, surrounded with 1-2 layers of relatively large pericycle cells. The bundle was enveloped with endodermis containing Casparian strips. The endodermis of the nodule bundles never reached the tertiary cell wall stage, characteristic for the root endodermis. The oldest, basal bundle was very thick in the 42 day-old nodules. Presumably, that bundle grew during the whole period of nodule growth, due to the action of its cambium, continuous with the cambium of the root (Fig. 10j).

In this investigation the nodule cortex was three-layered, typical for cylindrical nodules (Hirsch, 1992; Lloret et al., 1989). The cortex cells were distinguished by dark granules situated on the inner side of the tonoplast. The outer cortex was built of large, loosely arranged cells which quickly peeled off. The inner cortex was 2-3 layered, widening only in the neighbourhood of vascular bundles. A layer of flattened, small cells without intercellular spaces separated the bundles from the bacteroidal tissue (Fig. 10e). These cells correspond to the, so called, boundary layer distinguished by Brown and Walsh (1994) and probably playing the role of one of the oxygen diffusion barriers. The outer cortex was separated from the inner by a single, strongly staining layer of the, so called, nodule endodermis, ultrastructurally not varying from the root endodermis with the tertiary cell wall formed. According to the anatomical classification of the nodule cortex presented by Brown and Walsh (1994), the nodule endodermis in white clover is built of tannin cells. The analysis of serial sections of the 42 day-old nodule revealed the uneven development of the nodule endodermis (Fig. 10a-d). In the regions where it adjoined differentiating bacteroidal tissue one could notice the aging, obliterated cells of the nodule endodermis while in the same cross-section in the regions facing the bundles and/or developmentally younger bacteroidal tissue the endodermis cells did not differ from cells of the cortical parenchyma.

In the senescing zone of the bacteroidal tissue some darkly staining granules appeared in the perinuclear cytoplasm (degrading symbiosomes) and the bacteroids assumed shapes close to spherical (Fig. 10f). The cells gradually lost turgor (Fig. 10f) and the intercellular spaces greatly increased. In the 42 day-old nodules the zone of bacteroidal tissue with fully degenerated protoplast had not been observed yet.

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MORFOGENEZA BRODAWEK KORZENIOWYCH KONICZNY BIAŁEJ.

I. EFEKTYWNE BRODAWKI KORZENIOWE INDUKOWANE PRZEZ DZIKI SZCZEP
RHIZOBIUM LEGUMINOSARUM BIOVAR. *TRIFOLII*

STRESZCZENIE

Badano morfogenezę cylindrycznych brodawek korzeniowych *Trifolium repens* L. indukowanych szczepem *Rhizobium leguminosarum* biovar. *trifolii* 24 (typ dziki). Wykazano, że ontogeneza brodawki rozpoczyna się poprzecznymi podziałami komórek perycyklu, po czym następuje odróżnicowanie i podziały komórek endodermy i wewnętrznych warstw kory pierwotnej korzenia. Stwierdzono przemieszczanie się merystemu brodawki z początkowego położenia lateralnego w typowe dla brodawek cylindrycznych położenie apikalne. Opisano tkanki brodawki: bakteroidalną, korowę i przewodzącą do 42 dnia po inokulacji. W tym terminie nie występowała jeszcze w brodawkach typowa strefa zdegradowana.

SŁOWA KLUCZOWE: perycykl; endoderma; zawiązek brodawki korzeniowej; budowa brodawki korzeniowej; tkanka bakteroidalna; *Trifolium repens* L.