MORPHOGENESIS OF ROOT NODULES IN WHITE CLOVER. II. THE EFFECT OF MUTATION IN GENES *nod IJ* OF THE MICROSymbionT UPON THE NODULE STRUCTURE

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ABSTRACT

Morphogenesis of ineffective root nodules initiated on the roots of white clover 'Astra' by the *Rhizobium leguminosarum* biovar. *trifolii* strains ANU261 (Tn5 insertion in *nod I* gene) and ANU262 (Tn5 insertion in *nod J* gene) was investigated. Following changes were observed, as compared to the wild-type nodulation: the exaggerated, not delayed reaction of root hairs; the delay in nodulation with the number of nodules the same as in plants inoculated with a wild strain; the formation and organization of the nodule primordium not changed in comparison with the wild-type nodules; infection threads abnormally branched and diffusing with bacteria deprived of light zone and enriched with storage material; infected cells of bacteroid tissue abnormally strongly osmiophilic and only slightly vacuolated; symbiosomes with very narrowed peribacteroid space, subject to premature degradation; abnormal accumulation of starch in the nodule tissues; nodule development blocked at the stage of laterally situated meristem and single nodule bundle; inhibition of divisions in the meristem and vacuolation of its cells; the appearance of single cells with colonies of saprophytic rhizobia embedded in the fibrillar matrix in the old, degraded regions of the bacteroid tissue.

KEY WORDS: Root hairs; infection thread; bacteroid tissue; *Trifolium repens*; *Rhizobium leguminosarum* biovar. *trifolii*; ineffective symbiosis.

INTRODUCTION

The programme of the root nodule morphogenesis is coded in the host-plant genome and can be carried through even without the presence of the symbiotic bacteria *Brady/Rhizobium*. Empty nodules which are formed in such case have anatomic structure typical for the plant species and typical nodule proteins, called nodulines, are expressed in them (Joshi et al., 1991; Hirsch, 1992). Empty nodules are ineffective – unable to fix atmospheric nitrogen. In other words, bacteria must participate in the process of effective nodule formation. The role of bacterial genome in symbiosis is confirmed by the fact that most of mutations in bacterial genes connected with symbiosis, lead to the decrease of effectiveness or even inability to nodulate of the host plant (Kijne et al., 1991; Gray et al., 1991; Finan et al., 1985; Truchet et al., 1980; Skorupska et al., 1991). The genes *nod ABC*, common for all rhizobia, which are induced by plant flavonoids (indirectly) and proteinous product of *nod D* gene, are responsible for the synthesis of lipo-oligosaccharide – the Nod factor precursor. Genes *hcn* modify its structure leading to the formation of the signal molecule – the Nod factor which induces the initial stages of the root nodule morphogenesis in the host plant (Lerouge et al., 1990; Spaink et al., 1993; Carlson et al., 1994). Also, genes *nod IJ* are a part of the operon *nod ABC* but their function is

less known than other *nod* genes. The aim of the present research was to demonstrate the effect of the mutation in genes *nod IJ* on the root nodule development in white clover.

MATERIAL AND METHODS

Seedlings of white clover 'Astra' prepared in a way described in part I of this paper (Łotocka et al., 1997) were inoculated with *Rhizobium leguminosarum* biovar. *trifolii* strain ANU261 and ANU262 inducing ineffective nodules (Djordjevic et al., 1985; Huang et al., 1988). The control for the results of the ineffective nodule observations comprised the nodules induced with a wild type strain 24 (Łotocka et al., 1997). The mutant strains kindly provided by Dr M. A. Djordjevic were obtained by the Tn5 mutagenesis, and mutations were mapped in the gene *nod I* in ANU261 and *nod J* in ANU262 (Djordjevic et al., 1985). The conditions for sterile plant cultivation and preparations for the light and electron microscope examination were the same as in part I (Łotocka et al., 1997).

The investigation material comprised of 4÷14, 21 and 28 day-old nodules. Older nodules were not taken because since the 7th week after inoculation the plants gradually desiccated. Not more than one nodule at a specified developmental stage

was collected from one plant. In order to obtain material for observation of the early stages of the nodule development, when nodule is not yet seen on the root surface, the clover seedlings, prepared in the above described way, were placed on the Petri dishes, 20 seedlings each. The position of the root tip was marked and, considering the delay in the plant nodulation inoculated with mutant strains, in 3, 4, 5, 6 and 7 days after inoculation the part of root which had grown after the inoculation was cut off the upper part, both parts were fixed and after rinsing in the buffer they were examined under Nomarski optics. No nodule primordia were found in the upper fragments while primordia of different age were observed in the lower fragments. The position of nodule primordia and lateral roots was marked on the cover-glass, then they were cut out and microscopic sections were prepared. Fragments of roots with primordia were cut in the transverse, radial and tangential planes.

RESULTS AND DISCUSSION

1. Root hairs

In our work plant inoculation with both ANU261 and ANU262 mutant strains resulted in the exaggerated and not delayed reaction of root hairs in comparison with plants inoculated with the wild type strain. Numerous deformations were observed consisting in branching, swelling and/or screw-like deformation of the root hair. Similar effect was observed earlier in *Trifolium subterraneum*, a different host belonging to the same cross-inoculation group, inoculated with the investigated strains (Djordjevic et al., 1985; Huang et al., 1988; Canter-Cremers et al., 1988). In *Trifolium repens* described in the present paper, after inoculation with the ANU261 strain, the root hairs curled in the shepherd's crook or in any other way allowing the infection (curling at the epiblem surface or curling of the root hair branches), amounted to 56% of the total number of deformed root hairs; only some of them (2%) contained the infection thread. After inoculation with the ANU262 strain the curled root hairs comprised 34% of the deformed hairs and 4% of the curled hairs contained the infection thread (for comparison, in plants inoculated with the wild-type strain the curled hairs comprised 77% of all the deformed hairs and 13% of the curled root hairs contained the infection thread).

2. Root nodule number

In the investigations by Djordjevic et al. (1985) and Huang et al. (1980) the strains ANU261 and ANU262 nodulated less *T. subterraneum* plants (5-10% and 40-20%, respectively) than in the described experiment. In *T. repens* both strains nodulated approximately 60% plants with a few to several day delay as compared to plants inoculated with wild strain. The average number of nodules per plant was similar in plants inoculated with both strains amounting to 6.5 and 6.8, respectively for the strains ANU261 and ANU262. In both cases the individual variation was high and the number of nodules per plant varied from 1 to 21. The average number of nodules per plant did not differ from the control, unlike in the experiment by Huang et al. (1988) in which the average number of nodules per plant after inoculation with the ANU261 strain amounted to 5.0 but the wild strain induced, on the average, 16 nodules per *T. subterraneum* plant.

3. Root nodule primordium

The formation of the nodule primordium and its organization did not differ from that observed after inoculation with wild strain (see Łotocka et al., 1997), however, both the nodule primordia and nodules accumulated much more starch (Fig. 1c).

In the 4 day-old *nod I* and *nod J* nodule primordia the following regions were observed: meristematic with dividing cells (Fig. 1a), region of cells with infection threads (Fig. 1b) and region of cells with large amyloplasts (Fig. 1c), the latter corresponding to the inner layers of nodule cortex.

Cells containing infection threads were characterized by a large vacuole and a very thin layer of parietal cytoplasm (which clearly differentiated these cells from poorly vacuolated cells of the infection zone of the wild type nodules; Łotocka et al., 1997). That picture suggests that the vacuolation of meristematic cells after their penetration by the thread was quicker than in the control. The infection threads were surrounded by a wall and contained matrix (Fig. 3), as typical threads in the effective nodules, however, either there was no light zone around bacteria or it was greatly narrowed. Bacteria in the threads divided but many of them were clearly deformed and contained more poly- β -hydroxybutyrate than usual. During the whole development of *nod I* and *nod J* nodules untypically strong branching and/or diffusion of threads was often observed (Fig. 3a). Some of the threads filled the whole strongly enlarged intercellular spaces and could contain fine granular osmiophilic material. Bacteria were released from the threads (Fig. 3b).

In the cells penetrated by the infection threads and in young infected cells, large vacuoles with abundant membranous structures – vesicles surrounded with two membranes and containing tubular structures – were observed (Fig. 4a, b). These structures could be remnants of autophagosomes incorporated to the vacuole, still surrounded with their own membrane and tonoplast. That picture can be connected with the sudden vacuolation of the meristem cells after their penetration by the thread observed in the present experiment.

In the 4 day-old nodule primordia single cells undergoing necrosis appeared, as well as necrotized cells. The necrotizing cells contained infection threads and were characterized with strongly osmiophilic cytoplasm, greatly changed endoplasmic reticulum and lobed nucleus (Fig. 5a, b). It is not clear, however, whether these necroses resulted from infecting the cells by mutated bacteria because such single necrotized cells were also observed in young bacteroidal tissue in the wild type nodules regardless of their age (Łotocka et al., 1997).

4. The further development of *nod I* and *nod J* nodules

Anatomical structure of the 6-14 day-old ineffective nodules corresponded to the anatomy of the wild type nodules of the same age, i.e. they had laterally situated meristem and a single vascular bundle (Łotocka et al., 1997). However, these nodules were smaller than wild nodules due to poorer expansion of the bacteroidal tissue region.

Reorganization of the vacuolar system

In the infected cells of the bacteroidal tissue of the 8-14 day-old nodules no vacuole appeared close to the nucleus as it happened in the bacteroidal cells in the respective zone of the wild type nodules. Thus the reorganization of the vacuolar system during the ontogenesis of the bacteroidal cells infected with mutants had similar direction but the degree of the cell

Fig. 1. Structure of 4 day-old *nod F* root nodule:

a) meristematic zone with dividing cells, bar 2.50 μm ,

b) infection zone, bar 4.80 μm ,

c) zone with the cells containing large amyloplasts (inner cortex), bar 7.80 μm .

CW – cell wall, V – vacuole, A – amyloplast, CR – chromosomes, IT – infection thread, arrow – rhizobia within the thread, MS – membraneous structures, N – cell nucleus.

Fig. 2. Infection thread in *nod J* root nodule, note lack of the light zone around bacteria.

ITM – infection thread matrix, arrow – infection thread wall, arrowhead – unwall lateral blob of the thread, ring – rhizobia within the thread, V – vacuole, CW – cell wall, A – amyloplast, triangle – symbiosome, double arrowhead – endoplasmic reticulum. Bar 3.79 μm .

Fig. 3a. Branched and "overflowed" infection thread in *nod J* root nodule.

CW – cell wall, V – vacuole, IT – infection thread, arrowhead – wall of the original infection thread, star – branches of the infection thread, arrow – rhizobia within the thread. Bar 2.50 μm .

Fig. 3b. Release of the bacteria from infection thread in *nod J* root nodule.

IT – infection thread, arrowhead – infection thread wall, double arrowhead – plasma membrane around the thread, arrow – rhizobia within the thread, star – branches of the thread, N – cell nucleus, M – mitochondrion, V – vacuole. Bar 1.25 μm .

Fig. 4. Incorporation of membraneous structures to the vacuoles in:

a) *nod J* root nodule, bar 1.51 μm ,

b) *nod J* root nodule, bar 0.96 μm .

MS – membraneous structures, arrowhead – tonoplast, double arrowhead – membrane surrounding the vesicle, CW – cell wall, V – vacuole, M – mitochondrium, A – amyloplast, arrow – endoplasmic reticulum.

Fig. 5a, b. Necrosis-undergoing cells of 4 day-old *nod J* root nodule. NC – cell undergoing necrosis, CW – cell wall, M – mitochondrium, arrowheads – location of lobed and homogenous cell nucleus, V – vacuole, IT – infection thread, arrow – bacteria released from the thread. Bar 1.25 μm .

Fig. 6. Infected cells of bacteroidal tissue in:

a) *nod J* root nodule, note osmiofilic cytoplasm, widened intercellular space (IS) and large amyloplasts (A), CW – cell wall, triangle – symbiosome, bar 3.79 μm .

b) *nod I* root nodule, SG – starch grains (break-up of amyloplasts), CW – cell wall, S – symbiosome. Bar 1.25 μm .

Fig. 6c. Infection thread (IT) in *nod I* root nodule, incorporation of osmiofilic material (arrow) to the thread, arrowhead – plasma membrane surrounding the thread, BA – bacterium degraded within the thread, S – symbiosome. Bar 0.78 μm .

Fig. 6d, e. Ultrastructure of symbiosomes in *nodJ* root nodule, arrowhead – peribacteroid membrane closely adjoining to the outer bacteroid membrane (double arrowhead), B – bacteroid, CW – cell wall, note plasmodesms, V – vacuole, M – mitochondrion, A – lobed amyloplast, dS – deformed symbiosome, arrow – endoplasmic reticulum. Bars: d) 0.63 μm , e) 0.96 μm .

reaction was greatly exaggerated as compared to the situation observed after infection with a wild strain (Łotocka et al., 1997). The vacuolation of the meristem cells, after the infection thread penetration, was quicker and more advanced and then it underwent nearly a total reduction with the increase of the number of symbiosomes. The next stage of the vacuolar system transformation (vacuolation increase in senescing cells) was strongly accelerated due to a quicker degradation of bacteroids.

Bacteroidal tissue

Protoplast of the differentiated bacteroidal cells was more osmiophilic than in the wild nodules, it contained numerous symbiosomes and much larger than in the wild nodules, partially situated amyloplasts with compound starch grains (Fig. 6a). The breakage of the amyloplast envelope and hydrolysis of the starch grains in contact with the cell cytoplasm (Fig. 6b) were often observed. That tissue had very large intercellular spaces (Fig. 6a), however, that was not a symptom connected with the ineffectiveness, rather, but with that developmental stage because such large intercellular spaces were also observed in the wild type nodules at a respective developmental stage (Łotocka et al., 1997).

In the *nodJ* nodules the infection threads of the bacteroidal tissue often contained some additional osmiophilic material in their walls. There was observed the incorporation of similar osmiophilic granules from the cytoplasm to the threads (Fig. 6c). Bacteria in such threads were changed – deformed with homogenous regions in their cytoplasm, without a clear nucleoid zone – which suggests that they were subject to degradation.

Numerous symbiosomes, also dividing ones, were observed in the infected cells of the bacteroidal tissue, however, among those normally-shaped there were some deformed (Fig. 6e). Symbiosomes always had a very narrowed peribacteroid space and in many cases the peribacteroid membrane adhered to the outer bacteroid membrane (Fig. 6d). Symbiosomes were subject to premature degradation, separately inside the peribacteroid membrane (Fig. 7a, b) or in groups in the lytic vacuoles (Fig. 7c, d) – common membrane-bounded spaces. Two pictures of bacteroid degradation were observed in the symbiosomes (Fig. 7e) – either it was a gradual dilution of their cytoplasm or an accumulation of osmiophilic precipitates (it is possible that they were two stages of degradation). In both cases the bacteroid was deformed. At the successive stage of the infected cell degeneration (Fig. 7f), simultaneous to symbiosomes, the protoplast of the host-cell also degenerated.

Fig. 7a, b. Bacteroids undergoing degradation individually within peribacteroid membrane in:

a) *nod J* root nodule,

b) *nod I* root nodule.

B – bacteroid under degradation, S – symbiosome, arrowhead – peribacteroid membrane, N – cell nucleus. Bars: a) 1.25 μ m b) 2.50 μ m.

The rate of degeneration in the bacteroid cell depended very strongly on the individual plant, however it appeared inevitably in all the infected cells and even in the youngest layers of the bacteroid tissue. Thus the nodules induced by the mutant strains did not contain a zone in the bacteroid tissue which, on the basis of its ultrastructural picture, could be accepted as the one which actively fixes nitrogen.

The uninfected cells of the bacteroid tissue in the 8÷14 day-old nodules were less numerous than in the wild type nodules, they contained less cytoplasm, and their amyloplasts were less numerous and stained less intensively.

The diversity of bacteroid tissue in nod J nodules

Among the population of the examined *nod J* nodules, a considerable diversity of the picture of bacteroid tissue was noted. One nodule was found which bacteroid tissue was devoid of intercellular spaces. Infected bacteroid cells did not contain amyloplasts but proplastids and had strongly proliferated endoplasmic reticulum merging into vacuoles with large amounts of floccular material in the vacuolar sap (Fig. 8). It is possible that this characteristic picture of endoplasmic reticulum was the early stage of the lysosome system formation.

In another examined nodule, contrary to most of material at the same developmental stage, most cells in the region of bacteroid tissue was strongly vacuolated with floccular material in the vacuole and light cytoplasm devoid of symbiosomes (Fig. 9a). Cortex cells varied from the described ones with clearly smaller contents of floccular material in the vacuolar sap and the presence of osmiophilic granules on the inner surface of the tonoplast (Fig. 9b). The intracellular spaces were completely filled with osmiophilic substance. Only a few infected cells in which the symbiosomes were subject to degradation were found in that whole nodule.

In some nodules one could observe cells with numerous cytoplasm profiles in the vacuolar sap, similar to the cells from the infection zone in wild nodules (Fig. 10a) in which such a picture was connected by the authors with the reorganization of the vacuolar system of the cells after infection (Łotocka et al., 1997). In ineffective nodules, these cells contained infection threads and a small number of symbiosomes, including the dividing ones, but many symbiosomes were abnormally enlarged and deformed (Fig. 10b).

The late stages of nod I and nod J nodules development

In the 21 day-old *nod I* and *nod J* nodules the processes of the bacteroid tissue degradation already encompassed whole

Fig. 7c, d. Bacteroids undergoing degradation within lytic vacuoles in:
c) *nod J* root nodule,
d) *nod I* root nodule.

B – bacteroids under degradation, arrow – membrane of the lytic vacuole, N- cell nucleus. Bars: c) 1.89 μm , d) 0.96 μm .

cells and not only individual symbiosomes (Fig. 11a). Anatomically those nodules did not differ from the previous stage, so their development was inhibited at the stage of laterally situated meristem and a single, not branched vascular bundle (Fig. 11b). It corresponded to the 6-8 day-old nodules of the wild type.

During the following week no further nodule development was observed – the 28 day-old nodules had the same anatomical structure as the 21 day-old ones. At that time a vacuolation of the nodule meristem cells and complete inhibition of its mitotic activity took place. In the region of inhibited meristem one could observe some cells with infection threads releasing bacteria (Fig. 12a), with the simultaneous host protoplast degradation – the breakdown of membranes and diffusion of the cytoplasm (Fig. 12b) as well as ultrastructural changes in the plastids (Fig. 12a). No symbiosomes were observed. Thus at the late stages of the development of the *nod I* and *nod J* nodules the processes of rejecting the microsymbiont by the host were accelerated and directed against bacteria being released from the newly penetrated thread. The anatomical effect of the host protoplast autolysis was the appearance of caverns with degraded and obliterated cells in the region of bacteroidal tissue.

In some of the 28 day-old *nod I* and *nod J* nodules, in the region of bacteroidal tissue with cells devoid of any remnants of the protoplast some single cells (from 1 to 7 per nodule) appeared, filled with fibrillar matrix and bacteria immersed in it, which were similar to those observed in the rhizosphere. Infection threads, empty or with bacteria, were also visible in the matrix (Fig. 13 a-d). In the cells populated by the saprophytic rhizobia, a zonal degradation of bacterial colonies together with surrounding matrix was observed (Fig. 14). It is obvious, that the infection threads observed in the matrix penetrated the cells at the time when they still contained the protoplast, since it is the protoplast that builds the thread. Thus a question arises why only single cells were invaded by bacteria from the thread since in that tissue there were many degraded cells containing the threads. Possible reason is, that most of the threads were wholly walled up by the bacteroidal cell protoplasts (even on the ends which usually have no wall and release bacteria) prior to the protoplast degeneration. Only in some, not numerous, cells in which the end of the thread remained unsealed during the protoplast degradation, the bacteria could leave the thread for the degraded cell and multiply in it. The picture of bacteria degrading in the threads (Fig. 6c) suggests another possibility – the population of alive bacteria in the threads could be drastically reduced during the nodule

Fig. 7e. Two pictures of symbiosome degradation in *nod I* root nodule.

Star – dispersion of bacteroid cytoplasm, white star – accumulation of osmiofilic precipitations in bacteroid. Bar 0.78 μm .

Fig. 7f. Final stage of the degeneration of bacteroidal tissue infected cell in *nod J* root nodule, note electron-transparent cytoplasm with the debris after degradation of host-cell protoplast and symbiosomes.

CW – cell wall, dC – degraded cell, V – vacuole, B – bacteroid. Bar 1.51 μm .

Fig. 8. Infected bacteroidal tissue cell with characteristically proliferated endoplasmic reticulum in *nod J* root nodule.

V – vacuole with fibrillar precipitations, CW – cell wall, S – symbiosome. Bar 2.50 μm .

Fig. 9. *nod J* root nodule with

a) abundant fibrillar precipitations in vacuolar sap of the cells in the bacteroidal tissue zone and
b) characteristic osmiofilic granules (arrow) at the tonoplast inner surface in nodule cortex cells.

CW – cell wall, IS – intercellular space, A – amyloplast, arrowheads – tonoplast, V – vacuole, N – cell nucleus. Bars: a) 1.25 μm , b) 3.79 μm .

development. Matrix filling the cells with saprophytic rhizobia could be produced by the rhizobia themselves at their rod-shape developmental stage. That supposition was made on the basis of observations of a similar structure surrounding the bacterial colony in the niche of curled root hair, outside its walls (Fig. 15).

Consideration on the disturbances in infection thread development

During the whole morphogenesis of nodules induced with both mutant strains, the disturbances were observed in the host-plant apoplast reaction to the infection which consisted in the improper growth of the infection threads, changes in their morphology and in the development of bacteria inside the thread. The observed degradations of bacteria in the threads testify to the lack of the host recognition of the symbiont already at that stage and thus might be an effect of the plant's defence reaction. Another manifestation of such reaction could be the impregnation of the thread wall with the osmiophilic material which was never observed in the threads populated by the compatible bacteria. Such an impregnation could preclude the apoplast contact of bacteria with the host cytoplasm and prevent the diffusion of nutrients. Morphological abnormalities, especially intensive branching of the thread

ends, were similar to the picture of infection threads in the abortive nodules of lupin (Łotocka, Golinowski, unpublished). The constant feature of infection threads in the nodules induced with the strains ANU261 and ANU262 was the lack of the light zone which surrounded the wild type bacteria in the threads. Such a picture suggests that the mutants, as compared to the wild strain, had altered surface structures. It is difficult to state whether the not-appearance of the light zone around the bacteria in the threads was connected with the lack of the peribacteroid space around the bacteroids in the symbiosomes. Both these phenomena could result from the same physiological disturbances of rhizobium. So far there are no precise data concerning the role of bacteria, and particularly its surface structures, in the formation of the light zone and peribacteroid space.

What was the mechanism of the ineffectiveness?

It should result from the function of the *nod IJ* genes. They belong to the operon *nod ABC* (and thus are expressed together with it). It is known that their function, or rather the function of the gene *nod I*, consists in coding the ATP-binding protein connected with the bacterial cytoplasmic membrane. It is a trans-membrane protein of the permease character probably transporting low molecular-weight compound (compo-

Fig. 10a, b. Infected bacteroidal tissue cell with characteristic profiles of cytoplasm (arrow) in vacuolar sap in *nod J* root nodule.

a – amyloplast, CW – cell wall, M – mitochondrion, S – symbiosome, ER – endoplasmic reticulum,

b – deformed bacteroid, arrowhead – peribacteroidal membrane only in a few places protruding from the bacteroid, asterisk – poly- β -hydroxybutyrate granules. Bars: a) 3.79 μ m, b) 1.51 μ m.

unds) through the membrane. It has been suggested that this transported molecule could be the Nod factor or its part (Gottfert et al., 1990; Kondorosi, 1991; Hirsch, 1992; Okker et al., 1992; Schlaman, 1992a; Schultze et al., 1994). There are data demonstrating various effects of *nod II* genes mutation on the activity of the Nod factor in the nodulation of plants in particular cross-inoculation groups. Thus, the mutation of these genes in biovar. *viciae* had only limited effect on the nodulation in vetch, while the mutation of the same genes in biovar. *trifolii* caused significant disturbances of the symbiotic system with clover (Canter-Cremers et al., 1988). In the supernatant from the culture of bv. *trifolii nod II* mutants the Nod factor was undetectable, as it was detained completely inside the bacterial cells, while in bv. *viciae* mutations in the same genes did not cause any changes in the amount and structure of Nod factor in supernatant, as compared to the wild strain (Schultze et al., 1994). The quoted authors suggested that the difference between biovars resulted from the existence of an alternative, independent of the genes *nod II*, system of the Nod factor transportation in bv. *viciae*. There is still controversy whether the *nod* genes function additionally in the modification of the rhizobial surface structures which have significant effect on the host recognition of the microsymbiont (Kijne et al., 1991; Gray et al., 1991). It is reported

in the paper of Werner (1992) that the *nod I* mutants of *Rh. l. bv. trifolii* had a decreased amount of the 3-hydroxybutyrate substitute in the exopolysaccharide.

As results from the above data, the mutation of *nod II* genes in the investigated *Rh. l. bv. trifolii* strains ANU261 and ANU262 could cause either the decrease of the Nod factor excretion or improper structure of the exopolysaccharides. If the first hypothesis is considered, it is important at which period of the symbiosis development the *nod II* genes are expressed. They are induced with the whole operon *com nod* (consisting of the genes *nod ABCIJ*) while rhizobia are still in the rhizosphere. In biovar. *viciae* their expression lasts for the whole period of the bacteria presence in the infection threads and the repression of the operon takes place immediately prior to the release of bacteria from the infection threads to the host cell. At the anatomical level, the *nod ABCIJ* transcripts were relatively abundant in the invasion zone of the 15 day-old pea root nodules (Schlaman, 1992b). The amount of these transcripts declined very rapidly in the early symbiotic zone, where the bacteria are released from the threads and the transcripts were not visible in the late symbiotic zone. In the investigation presented, the recognition of the microsymbiont by the host plant was evidently disturbed. Such prolonged period of the *nod II* genes expression does not answer the que-

Fig. 11a. Transverse section of 21 day-old *nod I* root nodule.

NE – nodule endodermis, IC – inner cortex, star – degraded cells of bacteroidal tissue, triangle – infected bacteroidal tissue cells, asterisk – uninfected bacteroidal tissue cells, RS – root stele. Bar 64.10 μm .

Fig. 11b. Longitudinal section of 21 day-old *nod I* root nodule arrested in the stage of laterally located meristeme and single nodule bundle.

NE – nodule endodermis, IC – inner cortex, M – meristematic zone, IZ – infection zone, arrow – infection thread, NB – nodule bundle, asterisk – uninfected bacteroidal tissue cells with globular amyloplasts, triangle – infected bacteroidal tissue cells with plate-like amyloplasts, E – root endodermis, RS – root stele. Bar 32.05 μm .

Fig. 12. Zone of arrested meristem in *nod J* root nodule, note release of bacteria from infection thread occurring simultaneously with degradation of bacteria and host protoplast.

a) IT – infection thread, BA – bacteria leaving the thread, P – plastid with accumulated plastoglobules, N – degenerating cell nucleus, V – vacuole, tonoplast (arrow) and plasma membrane (arrowhead) around infection thread apparently still intact, bar 1.25 µm;

b) CW – cell wall, V – vacuole, tonoplast broken, M – mitochondrion, BA – bacteria embedded in developing fibrillar matrix (MX), bar 0.63 µm.

stion whether the improper recognition of the microsymbiont is of a single character and takes place during the exchange of signals initiating symbiosis and the whole complex of the later effects results from improper reaction connected with the Nod factor. It is possible that, for the whole period of bacterium development in the threads (and the expression of *nod ABCIJ* operon), the plant receives the incorrect signal from the microsymbiont and, not recognizing it, treats it as an enemy and destroys it.

If the second hypothesis is considered which assumes that the *Rh. l. bv. trifolii nod II* genes affect the EPS structure, then it is difficult to explain why the plant reactions induced at a distance (e.g. before the direct contact of the symbionts takes place) through the mediation of Nod factor (the root hair reaction, meristem induction) are disturbed. The disturbances at later stages, i.e. at the time of infection thread formation and during bacteria release and symbiosome formation, when bacteria are checked on the basis of their surface struc-

tures (Brewin, 1991; Skorupska et al., 1991; Yang et al., 1992) would result from the changed EPS structure.

It is also possible that the particle (or particle family) transported by the products of *nod II* genes is a part of both the Nod factor and the EPS. In such a case mutation has a pleiotropic character and both the long time Nod factor effects and the processes of the *in planta* microsymbiont recognition are disturbed.

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Fig. 13a. Degraded cells of bacteroidal tissue in *nod I* root nodule occupied by saprophytic rhizobia (arrow) embedded in fibrillar matrix (MX).

IS – intercellular space, D – degraded cell, star – bacteria-containing cell which invaginated to the adjoining degraded cell. Bar 6.25 μ m.

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Fig. 13b. Magnified fragment of degraded cells of bacteroidal tissue in *nod J* root nodule occupied by saprophytic rhizobia (arrow) embedded in fibrillar matrix (MX). Bar 4.80 μ m.

Fig. 13c, d. Ultrastructure of the cells occupied by saprophytic rhizobia (arrow) in *nod I* root nodule.

MX – fibrillar matrix, IT – wall-lacking infection thread, asterisk – poly- β -hydroxybutyrate granules. Bars: c) 1.51 μ m, d) 1.25 μ m.

Fig. 14. Zonal degradation of saprophytic rhizobia (arrow) together with surrounding matrix (MX) in *nod J* root nodule. Star – degraded region, CW – cell wall. Bar 0.63 μm .

Fig. 15. Colony of rhizobia (arrow) in rhizosphere (RZ) surrounding curled root hair (RH). MX – fibrillar matrix, arrowhead – rhizobia inside the root hair. Bar 3.79 μm .

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MORFOGENEZA BRODAWEK KORZENIOWYCH KONICZYNY BIAŁEJ.
II. WPŁYW MUTACJI W GENACH *nod IJ* MIKROSymbionta NA BUDOWĘ BRODAWKI

STRESZCZENIE

Badano morfogenezę brodawek korzeniowych inicjowanych na korzeniach koniczyny białej 'Astra' przez szczepy *Rhizobium leguminosarum* biovar. *trifolii* ANU261 (Tn5 insert w genie *nod I*) i ANU262 (Tn5 insert w genie *nod J*). Stwierdzono następujące zmiany w porównaniu do brodawek typu dzikiego: wzmożoną, ale nie opóźnioną reakcję włośników; opóźnienie w brodawkowaniu przy liczbie wytworzonych brodawek takiej, jak po inokulacji szczepem dzikim; brak różnic w wytwarzaniu i organizacji zawiązka brodawki; nienormalnie rozgałęzione i rozlewające się nici infekcyjne, z bakteriami pozbawionymi jasnej strefy i bogatszymi w materiał zapasowy; zainfekowane komórki tkanki bakteroidalnej nietypowo silnie osmoofilne i słabo zwakuolizowane; symbiosomy o bardzo zwężonej przestrzeni peribakteroidalnej, podlegające szybkiej degradacji; nienormalne nagromadzenie skrobi w tkankach brodawek; zahamowanie rozwoju brodawki na etapie lateralnie położonego merystemu i pojedynczej wiązki, zahamowanie podziałów w merystemie i wakuolizacja jego komórek; pojawianie się pojedynczych komórek z koloniami saprofitycznych rizobiów pograżonymi we włóknistej matriks w starych, zdegradowanych rejonach tkanki bakteroidalnej.

SŁOWA KLUCZOWE: włośniki korzeniowe; nić infekcyjna; tkanka bakteroidalna; *Trifolium repens* L.; *Rhizobium leguminosarum* biovar. *trifolii*; nieefektywna symbioza.