SYMBIOTIC PROPERTIES OF *BRADYRHIZOBIUM* SP. (*LUPINUS*) ASSAYED ON SERRADELLA PLANTS

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(Received: July 25, 1995. Accepted: October 28, 1995)

ABSTRACT

Physiological and symbiotic properties of *Bradyrhizobium* sp. (*Lupinus*) nodule isolates were compared to the standard slow-growing *Bradyrhizobium* sp. (*Lupinus*) strain USDA 3045. Lupine nodules isolates showed typical characteristics for bradyrhizobial strains and nodulated small seed legume, serradella (*Ornithopus sativus*), in tube test. We observed a permanent physiological segregation of the effective (Fix⁺) and ineffective (Fix⁻) symbiotic phenotype for all tested bradyrhizobial strains during the growth of serradella in plant tube test. The ultrastructural differences between Fix⁺ and Fix⁻ serradella nodules were observed. Rapid and visible nodulation as well as easy assay of the reduction of acetylene make serradella a convenient system for studies of *Bradyrhizobium* sp. (*Lupinus*) strains in laboratory conditions.

KEY WORDS: *Bradyrhizobium* sp. (*Lupinus*, *Ornithopus*), serradella, plant test.

INTRODUCTION

Leguminous plants living in symbiosis with soil bacteria are able to form nodules, highly specialized organs in which nitrogen-fixation occurs. The legume-nodulating bacteria belonging to the family Rhizobiaceae have been recently divided into three genera: fast-growing *Rhizobium* and *Azorhizobium*, and slow-growing *Bradyrhizobium*. Within genus *Bradyrhizobium* the well characterized species, *B. japonicum* and *B. elkanii* nodulate *Glycine max*, *G. soja* and other legumes. *Bradyrhizobium* sp. (*Parasponia*) infects *Parasponia andersonii* (*Ulmaceae*), the only nonlegume plant genus able to form nitrogen-fixing root nodules (van Rhijn and Vanderleyden 1995). The slow-growing rhizobia were also isolated from nodules of lupine (*Lupinus*) and serradella (*Ornithopus sativus*) but they have not been classified into species (Elkan 1992). The large nodules of lupine have been commonly used as the source of RNA and proteins specific for symbiotic nitrogen fixation, including structure and function of leghemoglobin (Kretovich et al. 1972, Stróżycki et al. 1985, Sikorski et al. 1989). Little is known regarding the genetic control of nodule development and nitrogen fixation, partially due to difficulties with plant assay system (Robertson et al. 1978, Golinowski et al. 1987, Tang et al. 1992). The large seed plants do not form nodules in agar tube tests, in controlled conditions, so it is difficult to use lupine-*Bradyrhizobium* system in the genetic

experiments. These experiments require a simple and rapid plant test system for screening numerous clones of slow growers on symbiotic properties.

In this work, we developed a plant test for assaying the nodulation phenotype of lupine isolates on small seed plant, serradella (*Ornithopus sativus*). Using the bacteria isolated from lupine nodules we investigated the kinetics of formation of root nodules on serradella and the symbiotic nitrogen fixation. The morphology and ultrastructure of the serradella nodules were examined using light and transmission electron microscopy. Some physiological properties of the *Bradyrhizobium* sp. (*Lupinus*, *Ornithopus*) strains that nodulate lupine and serradella plants were also described.

MATERIALS AND METHODS

Bacteria

Bradyrhizobium sp. (*Lupinus*) strains USDA 3045 was obtained from US Department of Agriculture Collection; *Bradyrhizobium* sp. (*Lupinus*) strains L1, L2 and S3 were isolated by R. Staniewski, Department of General Microbiology, UMCS Lublin, Poland.

Media and growth conditions

Bacteria were cultivated aerobically at 28°C in yeast-extract mannitol medium (YEM) or minimal M1 medium (Vincent 1970). Indicator YEM agar plates containing 25 µg·cm⁻³ of

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Congo red were used for the assay of absorption of Congo red. The test for alkalization was performed on YEM agar medium supplemented with $150 \mu\text{g}\cdot\text{cm}^{-3}$ of bromothymol blue as a pH indicator. The ability to utilization of different compounds as sole carbon sources was tested by the growth of rhizobia in M1 medium supplemented with 0.2% (w/v) sugar or organic acids. Generation times of *Bradyrhizobium* spp. were determined in YEM liquid medium by measuring optical density of the cultures at 550 nm, using three replicate tubes for each strain.

Resistance to antibiotics was determined by spread plating saline dilutions of the stationary rhizobial cultures on YEM agar containing appropriate concentration of antibiotics. Plates were incubated at 28°C and examined for the appearance of bacterial growth after 10 days.

Plant tests. Seeds of serradella (*Ornithopus sativus* Brot. cv. Bydgoska) were surface-sterilized with 70% (vol/vol) ethanol for 5 min then in 30% (vol/vol) H_2O_2 for 5 min and washed several times in sterile distilled water. The sterilized seeds were germinated on 1% agaroid plates for 4 days at 4°C and then for 2 days at 28°C in darkness. Upon germination the seedlings were transferred onto sterile slants of the nitrogen-free R agar medium in test tubes (Vincent 1970). The five-day old plants were infected with dense water suspension of the bacteria. Plants were incubated under controlled standard condition: $24 \pm 2^\circ\text{C}$, RH 70-80%; day/night 12/12h; irradiance 40 Wm^{-2} (white fluorescent lamp, POLAM). Reisolation of bacteria from surface-sterilized nodules were performed as described by Vincent (1970). Nitrogenase activity was determined by standard acetylene reduction assay (Hardy et al. 1982).

Electron microscopy. Nodules of *Ornithopus sativus* harvested four weeks after inoculation were fixed for 24 h with

2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 postfixed in 1% OsO_4 in cacodylate buffer (1h, 22°C), dehydrated in an ethanol series and embedded in Araldite. Ultrathin sections (70 nm) were stained with uranyl acetate for 10 min and lead citrate for 10 min. The staining of ultrathin sections for glycogen was carried out according to Thiery (1967). Observations were carried out using the Tesla BS 500 transmission electron microscope operated at 80 kV.

RESULTS

Physiological characteristic of Bradyrhizobium sp. (Lupinus) strains.

The strains isolated from lupine nodules were examined for the characteristics typical for *Bradyrhizobium* species i.e. alkalization of growth medium, generation time, reaction with Congo red and utilization of carbon sources (Elkan 1992) and were compared to standard *Bradyrhizobium* sp. (*Lupinus*) strain USDA 3045 and fast-growing *Rhizobium* strains. The lupine isolated bacteria alkalized the YEM medium with bromothymol blue, did not give red colour with Congo red dye, the mean generation times were calculated about 9 h and the single colonies appeared after 7-8 days of the incubation on YEM agar plates (Table 1). The tested bradyrhizobial strains alkalized media supplemented with: glucose, fructose, galactose, mannitol, glycerol and succinate as sole carbon source. The same strains formed acid end product from arabinose, similarly to other slow-growing rhizobia. *Bradyrhizobium* sp. (*Lupinus*) strains were resistant to β -lactam antibiotics, moderately resistant to chloramphenicol, neomycin/kanamycin, tetracycline and sensitive to rifampicin (Table 2). We have observed some differences in antibiotic resistance between USDA 3045 and Polish isolates. On the contrary to the Polish

TABLE 1. Physiological properties of the bradyrhizobial strains.

Characteristic	<i>Bradyrhizobium</i> sp. (<i>Lupinus</i>)				<i>R. leg. bv.</i>	<i>R. meliloti</i>
	USDA 3045	L1	L2	S3	<i>trifolii</i> 24	L5.30
Alkali production on YEM	+	+	+	+	—	—
Reaction with Congo Red	—	—	—	—	+	+
Generation time in hours	10.8	10.4	10.8	9.4	3.5	2.5
Days needed to form 1mm colonies	8	8	8	7	3	2
Utilization of the compounds as sole carbon source:						
glucose	b	b	b	b	a	a
galactose	b	b	b	b	a	a
fructose	b	b	b	b	a	a
mannitol	b	b	b	b	a	a
arabinose	a	n	n	a	a	a
glycerol	b	b	b	b	a	a
succinate	b	b	b	b	a	a

a — utilization of the compound with acid production;
b — utilization of the compound with alkali production;
n — no growth.

TABLE 2. Resistance to antibiotics of *Bradyrhizobium* sp. (*Lupinus*) strains

Antibiotic	Concentration ($\mu\text{g}\cdot\text{cm}^{-3}$)	USDA 3045	L1	L2	S3
ampicillin	50	+	+	+	+
	100	+	+	+	+
	200	+	+	+	—
carbenicillin	50	+	+	+	+
	100	+	+	+	—
	200	+	+	—	—
chloramphenicol	20	+	+	+	—
	50	+	—	—	—
kanamycin	20	+	+	+	+
	50	—	—	—	—
neomycin	20	+	+	+	+
	50	—	—	—	—
streptomycin	20	—	+	+	+
	50	—	+	+	+
	100	—	+	+	+
tetracycline	5	—	+	+	+
	10	—	—	—	—
rifampicin	10	—	—	—	—

strains, *Bradyrhizobium* sp. (*Lupinus*) strain USDA 3045 was sensitive to streptomycin, tetracycline and resistant to 50 $\mu\text{g}\cdot\text{cm}^{-3}$ of chloramphenicol.

Symbiotic properties of *Bradyrhizobium* sp. (*Lupinus*) strains.

Bradyrhizobium strains isolated from lupine nodules were tested for the ability to nodulate serradella plants (Table 3). To test the kinetics of the infection and nitrogen-fixation ability, serradella plants were inoculated with *Bradyrhizobium* sp. (*Lupinus*) strains. The first nodules were visible within 7–10 days after inoculation and nearly 100% of the infected plants nodulated within 14 days. Most of the plants formed large, pink nodules on the main roots (Figs 1–2). 10–35% of the inoculated plants formed small, white nodules on the lateral roots which did not have characteristic pink pigment caused by the presence of leghemoglobin. The plants forming white nodules were small and yellow and differed significantly from the plants with pink nodules which were green and tall (Fig. 1). The plants with white nodules showed about 10% of nitrogenase activity in comparison to the plants forming pink nodules.

Four weeks after inoculation white and pink nodules induced on serradella by *Bradyrhizobium* sp. (*Lupinus*) strain USDA 3045 were harvested and assayed in the light and the electron microscopy. In the central part of the nodule tissue in Fix^+ and Fix^- nodules, infected and uninfected plant cells were observed (Figs 3–4). The infected plant cells of Fix^+ nodules were filled with numerous bacteroids (Figs 5–6). The Fix^- nodules in comparison to Fix^+ nodules contained a reduced number of invaded plant cells (Figs 3–4). The infected cells were surrounded by the plant cells that contained numerous

starch grains. Starch accumulation was more pronounced in Fix^- than Fix^+ nodules (Figs 3–4). The two or more bacteroids in the peribacteroid membranes in Fix^+ nodules were observed (Fig. 8). Inside bacteroids the dark granules of glycogen and transparent inclusions of PHB (p-hydroxybutyric acid) were visible (Figs 9–10). Either one or two bacteroids were observed within the peribacteroid membranes of Fix^- nodules (Fig. 7).

Bacteria were reisolated from surface sterilized nodules of nitrogen-fixing and non-fixing plants. The isolates were again assayed for the physiological characters as generation time, Congo red absorption, alkali production and resistance to antibiotics (ampicillin, carbenicillin, chloramphenicol, kanamycin, neomycin, streptomycin, tetracycline, rifampicin). The serradella nodules isolates did not differ in regard to tested properties from bradyrhizobial strains used to inoculation.

The single reisolated clones of USDA 3045 derived from Fix^- and Fix^+ serradella nodules were repeatedly used to inoculation of serradella seedlings. Both reisolates still induced effective and ineffective nodules with similar frequency (Table 4). Five successive passages through serradella failed to eliminate this physiological instability. The four *Bradyrhizobium* sp. (*Lupinus*, *Ornithopus*) strains USDA 3045, L1, L2, and S3 showed similar effect in plant test on serradella i.e., permanent segregation of Fix^+ and Fix^- nodules.

DISCUSSION

The aim of this work was physiological and symbiotic characterization of slow-growing strains isolated in Poland from lupine nodules and adaptation of the system serradella-*Bra-*

Fig. 1. *Ornithopus sativus* inoculated with *Bradyrhizobium* sp. (*Lupinus*) forming Fix^- (left) and Fix^+ (right) nodules.
Fig. 2. The roots of *Ornithopus sativus* with the effective, round nodules formed by *Bradyrhizobium* sp. (*Lupinus*).

bradyrhizobium sp. (*Lupinus*) to the genetic experiments. Main differences between fast and slow-growing rhizobia are connected with the growth rate and pH change in yeast-extract mannitol medium (YEM) (Jordan 1984). The fast-growing rhizobia have the generation times ranging from 2 to 6 h and they produce an acid on YEM agar. The slow-growing rhizobia show the generation time of 8 h or longer, and alkalize YEM medium. The lupine isolated strains tested in this work

exhibit the main characteristics of *Bradyrhizobium* sp. A wide variety of carbon compounds can be metabolized by all strains. Alkaline end products were produced from all carbon sources with the exception of arabinose. The similar carbon metabolism was described for the other slow-growing rhizobia (Stowers 1985).

In general, the slow-growing rhizobia are more resistant to antibiotics than the fast-growing strains (Pankhurst 1977). All

TABLE 3. Symbiotic properties of *Bradyrhizobium* sp. (*Lupinus*) strains on *Ornithopus sativus*.

Strain <i>Bradyrhizobium</i>	No of nodules/plant	No of Fix^- /no of Fix^+ plants (% of Fix^- plants)	Nitrogenase activity*
USDA 3045	3.0	3/17 (15%)	180
L1	3.2	2/18 (10%)	221
L2	2.8	4/13 (20%)	220
S3	3.4	7/13 (35%)	140

*Nitrogenase activity is expressed as mean value in nmol ethylene/h/ Fix^+ plant.

Figs 3-6. Light micrograph of semithin section of the nodules induced on serradella by *Bradyrhizobium* sp. (*Lupinus*),
 Fig. 3. Longitudinal section of Fix- nodule; the darkly stained infected cells are filled with bacteroids; x 80,
 Fig. 4. In the same section the numerous starch grains (s) are shown; x 150,
 Figs 5-6. Section of the Fix⁺ nodule; the dark infected plant cells are filled with bacteroids; x 80.

Fig. 7. Electron micrograph of the ultrathin section of the non-effective nodule induced on serradella by *Bradyrhizobium* sp. (*Lupinus*). The single bacteroids, with darkly stained cytoplasm in the peribacteroid membranes are visible; x 8 200,

Figs 8-10. Electron micrograph of ultrathin section of the effective nodule induced on serradella by *Bradyrhizobium* sp. (*Lupinus*).

Fig. 8. One to four bacteroids inside the peribacteroids membranes are seen; x 8 200,

Fig. 9. In the cytoplasm of the bacteroids darkly stained granules of glycogen (g) and transparent inclusions of PHB (h) are visible; x 18 000,

Fig. 10. The infection thread (it) with bacteroids (arrow) is visible; x 18 000.

tested strains were extremely resistant to β -lactam antibiotics, moderately to aminoglycosides, and they are sensitive to rifampicin.

Bradyrhizobium sp. (*Lupinus*) strains USDA 3045, L1, L2 and S3 induced nodules with high efficacy (up to 100%) under gnotobiotic conditions in test tubes. However, all tested strains produced permanently the effective and ineffective nodules on *Ornithopus sativus* what was evidenced by measurement of the nitrogenase activity. The nodules formed on serradella plants infected by *Bradyrhizobium* sp. (*Lupinus*) belong to a common type of cylindrical, indeterminate nodules containing the apical, persistent meristem (Kopcińska i Golinowski 1991, Vance 1991). The electron microscopy of Fix^+

and Fix^- serradella nodules showed on several differences between them. In the ineffective nodules we have observed less plant cells infected by bacteria, much more starch grains in cells surrounding the infected plant cells and only one or two bacteroids within the peribacteroid membranes. The appearance a few bacteroids inside one peribacteroid membrane in infected nodule cells of serradella is a result of fusion of membranes connected with aging of nodules (Kopcińska and Golinowski 1991). The bacteria isolated from the ineffective nodules have the same physiological properties as those from the effective nodules and they still exhibit the symbiotic differentiation with nearly stable frequency. The occurrence of the clonal polymorphism has been observed among various strains

TABLE 4. Symbiotic properties of of *Bradyrhizobium* sp. (*Lupinus*) strain USDA 3045 isolated from serradella Fix⁺ and Fix⁻ nodules.

Bacteria used for inoculation of serradella	no of plant with white nodules (% of total)	no of plant with pink nodules	nmol ethylene/h/plant
Reisolates from:			
Fix ⁺ plants	60 (25%)	172	132
Fix ⁻ plants	61 (27%)	165	121

of slow-growing rhizobia (Berry and Atherly 1984). Hahn and Hennecke (1987a,b) found repeated sequences (RS) in the genome of *B. japonicum*, which may be responsible for the instability and genetic rearrangements of this organism. The reason of the symbiotic instability observed in our work is not clear and the explanation of this phenomenon requires the further experiments on the genetic level. In conclusion, usefulness of serradella – *Bradyrhizobium* sp. (*Lupinus*) symbiotic system is restricted to the study the first steps in symbiosis. The later steps in the infection process i.e., nitrogen-fixation ability of *Bradyrhizobium* sp. (*Lupinus*) strains should be analyzed with a great care.

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WŁASNOŚCI SYMBIOTYCZNE *BRADYRHIZOBIUM* SP. (*LUPINUS*) BADANE NA SERADELI

STRESZCZENIE

Porównano fizjologiczne i symbiotyczne własności szczepów *Bradyrhizobium* sp. (*Lupinus*) izolowanych z brodawek łubinu, ze standardowym szczepem *Bradyrhizobium* sp. (*Lupinus*) USDA 3045. Bakterie izolowane z brodawek łubinu wykazywały własności typowe dla szczepów wolnorosnących i były zdolne do tworzenia brodawek na seradeli, w teście próbówkowym. Zaobserwowano na seradeli, w przypadku wszystkich badanych wolnorosnących szczepów, stałą segregację na efektywny i nieefektywny fenotyp symbiotyczny. Zaobserwowano różnice w ultrastrukturze brodawek efektywnych i nieefektywnych. Wczesne tworzenie brodawek i łatwość oznaczania redukcji acetyleny czyni seradelę dogodną rośliną w badaniu szczepów *Bradyrhizobium* sp. (*Lupinus*), w kontrolowanych warunkach.

SŁOWA KLUCZOWE: *Bradyrhizobium* sp. (*Lupinus*, *Ornithopus*), seradela, test próbówkowy.