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Diversity in symbiotic specificity of bacterial strains nodulating lupins in Poland

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Abstract. Bacteria isolated from root nodules of yellow lupin (*Lupinus luteus*), white lupin (*L. albus*), and blue lupin (*L. angustifolius*) were analyzed for their symbiotic specificity. Although these plant species belong to the same cross-inoculation group, they are not always nodulated by the same bacterial strains. There is a certain level of species and cultivar nodulation specificity among analyzed bacterial population. This characteristic is particularly expressed in bacterial populations isolated from white lupin. Serradella (*Ornithopus sativus)* can be nodulated by several analyzed strains of bacteria of both types (fast and slow growing) from root nodules of lupins, but many strains did not induce nodules on serradella roots, or nodules were not effective. Birdsfoot trefoil (*Lotus corniculatus*) is also classified in the same cross-inoculation group with lupins and serradella. Although the analyzed bacterial population has some potential for creating symbiotic systems with *L. corniculatus*, it is not a common phenomenon among lupin microsymbionts and is rather due to a large heterogeneity of the population.

key words: lupin, rhizobia, nodulation, symbiosis, serradella

INTRODUCTION

Lupins are legumes which have been cultivated in Europe for the last 2000 years, used in human and animal feeding, as green manure in agriculture (Rosolem et al*.,* 2002; Jensen et al*.,* 2004) and in soil stabilization. This plant is currently considered a good alternative as an animal foodstuff due to the high quality of its proteins (Erbas et al*.*, 2005; Faligowska et al*.*, 2007). Lupin seeds are a rich source of functional components that are found in modern food. They may be nutritional and non-nutritional compounds and have a positive effect on health, physical development and wellbeing (Lampart-Szczapa, Łoza, 2007).

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A substantial portion of the world's supply of organic nitrogen is fixed via the symbiosis between root nodulating rhizobial bacteria and leguminous host plants (Postgate, 1998). This association is generally assumed to be mutualistic, but rhizobial strains vary in effectiveness (Burdon et al*.*, 1999) and ineffective bacteria are widespread, indicating that cheating may occur. Therefore there is increasing interest in these plants to be used in sustainable agriculture due to its high potential to provide protein without nitrogen fertilization, estimated at 150–200 kg of nitrogen per ha in symbiosis with rhizobia (Robinson et al*.*, 2000). Using the optimal bacterial strain is critical to obtain the expected results of nitrogen fixation in agricultural practice.

Root-nodule bacteria comprise several distantly related proteobacterial lineages, most notably the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium* (Sawada et al*.*, 2003), that have acquired the ability to form nodules on legumes and symbiotically fix nitrogen. Lupins, like many other species belonging to the *Leguminosae*, are able to initiate a symbiotic relationship with bacteria of the family *Rhizobiaceae*. In the current taxonomy of this family*,* there is no separate entity comprising strains nodulating lupins. Different species of lupins (*Lupinus*) and serradella (*Ornithopus*) are effectively nodulated by both slow-growing strains classified within *Bradyrhizobium*, as well as the fast-growing strains of *Rhizobium* and *Mesorhizobium*. Lupin microsymbionts have been characterized to a much lesser extent than, for example, populations of *B. japonicum* or *R. leguminosarum*. Despite the interest of this symbiosis there are few studies about the identity of strains nodulating lupins (Barrera et al*.*, 1997; Stepkowski et al*.*, 2005; Andam, Parker, 2007). The strains isolated to date from effective nodules of lupins in different countries belong rather to the genus *Bradyrhizobium* (Barrera et al*.*, 1997; Rivas et al*.*, 2009). The results showed that the lupin endosymbionts belong to several chromosomal lineages within the genus *Bradyrhizobium* that could represent new species

of this genus. Among a group of these bacteria *L. albus* bradyrhizobia constitute a lineage that could represent an allelic group present up to date only in within microsymbionts of this species (Velázquez et al*.,* 2010)

The aim of this study was to analyze the nodulation specificity of lupin microsymbionts population isolated from three different species of lupins cultivated in Poland.

MATERIALS AND METHODS

***Lupinus* plants for sampling of rhizobial bacteria and strains isolation.** Field sites from 16 geographical locations in Poland were examined with the goal of isolating and characterizing the indigenous strains of *Rhizobiaceae* able to nodulate lupins plants. A total of 235 strains were isolated and 167 of them were taken for further biochemical characterization. The strains were isolated from 18 varieties of three species of *Lupinus*: 8 varieties of yellow lupin (*Lupinus luteus*), 6 varieties of narrow leaf lupin (*L. angustifolius*) and 4 varieties of white lupin (*L. albus*). The strains originated from places of different lupin cultivation history. At the locations of the plant breeding stations (Nowa Wieś Zbąska, Przebędowo, Wiatrowo) lupins were present often for several years or even yearly (Wiatrowo). At the other locations (Głębocko, Długa Goślina, Lipnica, Gurówko) lupins were not grown for at least 7 years before. On all the places no artificial inoculums were used and all isolated plants contained root nodules. Fifty-eight strains from the entire population were taken to the nodulation tests.

Isolation of rhizobia from nodules. Plant roots were washed with tap water until no soil particles were apparent. Plants were dried lightly with paper towels. Nodules were dissected from roots using scalpels and forceps, and dissected nodules were immediately placed in presterilized centrifuge tubes. Dissected nodules were surface sterilized in 0,1% $HgCl_2$ solution for 2 minutes and then rinsed three times in sterile distilled water. Nodules were then individually crushed with a flame-sterilized glass rod and each resultant slurry was streaked onto two replica plates containing 25 ml of solid AG medium (Somasegaran, Hoben, 1994).

Type of metabolism. One of the distinctive characteristics of the bacteria belonging to the different genera of *Rhizobiaceae* is their influence on culture medium pH (Brenner et al., 2005). Bacterial colonies were grown on Petri dishes on YM agar solid medium (Somasegaran, Hoben, 1994) with 0,5% (ethanol solution) of brome thymol blue (BTB) as pH indicator. Initially the YM medium was adjusted to pH 6.8 (green color). Bacterial growth caused changes of the medium pH as well as color of the pH indicator (yellow when lowering pH and blue when the pH was raised). Each strain of bacteria were inoculated on 3 separate Petri dishes by four-quadrant streaking method and placed in a dark incubator at a temperature of 28°C. After 5 days observations of the growing medium color were carried out. They were considered positive for cases in which a clear bacterial growth was observed, and if at least 2 of 3 repetitions for each strain showed an identical and distinct color change of culture medium.

Relative growth rate of isolates is another important distinctive characteristic of the bacteria belonging to the different genera of *Rhizobiaceae* (Brenner et al., 2005). Relative growth rate of isolates was analyzed in the solid medium. Three groups of isolates with different relative growth rate at a temperature of 28°C were distinguished. Strains classified as slow growing showed single colonies after 3 days, after 5 days colonies were 1–2 mm in diameter. Intermediate strains showed visible growth after 2 days and after 3 days colonies were well formed (ca. 2 mm). Fast growing bacteria showed well formed colonies after 2 days while after 3 days some colonies were joined and difficult to distinguish.

Analysis of nodulation specificity was performed in three independent experiments: on the perlite-vermiculite solid medium on Leonard jars (1st and 2nd experiment) and on an artificial agar slants (3rd experiment) (Somasegaran, Hoben, 1994). Nodulation analyses of bacterial isolates in the greenhouse experiments were carried out using sterilized Leonard jars with a liquid nitrogen free Dilworth medium at pH of 6.8 (setup of Leonard jar is presented on Fig. 1). As the culture medium a mixture of perlite and vermiculite in a 1:3 ratio by volume was used. Seeds were sown in three places in each Leonard jar at a depth of about 1.5 cm. For a single jar the seeds of one plant species analyzed were sown. In each of the three places two seeds of the species were sown. Seeds were infected with bacterial culture in an amount of 1.5 ml of culture in place (two seeds). Single jar was inoculated by the culture of one bacterial isolate. Seeds were covered with a layer of growing medium, and then the entire surface covered with 1.5 cm layer of paraffin coated sand, in order to avoid possible cross-infection during the growing season. At the stage of first leaves just visible one plant of every pair was cut off leaving single plant in every of three places per one Leonard jar. Four repetitions of each combination of plant species x bacterial isolate were used. Leonard jars with biological material were placed in the greenhouse for a period of 35 days. Nodulation specificity analysis of selected bacterial isolates to small-seed legume plants was carried out using the nitrogen free Dilworth medium solidified with agar in the form of slants in glass tubes. Test tubes used were 200 mm of length and 20 mm crosswise. Serradella (*Ornithopus sativus*) and birdsfoot trefoil (*Lotus corniculatus*) were grown on agar slants. Before sowing the seeds were sterilized and scarified as described bellow. Before

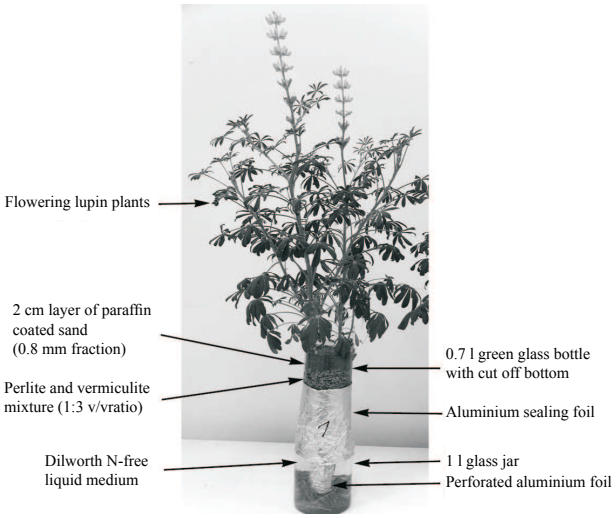


Fig.1. Setup of Leonard jar used for nodulation tests in the greenhouse experiments.

laying on slants the seeds were germinated. Germination was on Petri dishes with agar (in H_2O) in a dark incubator at a temperature of 28°C. Seeds were infected with bacterial culture of 1 ml of culture per tube. Ten repetitions of each combination of plant species x bacterial isolate were used. Photoperiod used: 16 hours artificially lighted and 8 hours darkness.

Seeds sterilization. In the greenhouse experiment three species were grown: yellow lupin (*Lupinus luteus*), serradella (*Ornithopus sativus*) and soybean (*Glycine max* (L.) Merr.). The surfaces of seeds were sterilized before sowing using different procedures, depending on plant species: a) sterilization of lupins and soybeans: Seeds were washed twice with distilled water and placed for 10 min. in a solution of mercuric chloride, and then washed five times with plenty of sterile distilled water; b) seeds of serradella were washed twice with distilled water and placed for 15 min. in concentrated sulfuric acid and then washed at least 10-fold with plenty of sterile distilled water.

Plant seeds used. *L. albus* cv. Wat, *L. luteus* cv. Lidar and cv. Ventus, *L. angustifolius* cv. Sonet and *O. sativus* cv. Igela and cv. Mazurska biała (all above from Poznan Plant Breeding Station in Tulce); *Lotus corniculatus* cv. Skrzeszowicka (from Plant Breeding Station Nieznanice); *Glycine max* (L.) Merr. Var. Nawiko (from Dept. of Genetics, Univ. of Life Sci., Poznan)

RESULTS AND DISCUSSION

There is no major problems with lupins nodulation in the field crops in Poland because many of microsymbiont strains are endogenous and they have the capacity to survive in soils for many years in the absence of their host-plants (Martyniuk et al., 2005). For all field collected plants, root nodules were observed. But their nodulation and nitrogen fixation effectiveness is still unknown. The studied strains were previously analysed and showed large variations in the structure of the population. The high diversity of the lupin microsymbionts were shown by both biochemical as well as molecular analyzes (Pudelko, 2010).

In the group of strains presented herein, two types of metabolism were observed: characteristic of slow-growing bacteria of the *Rhizobiaceae* family (mainly of the *Bradyrhizobium* genus) metabolism, resulting in alkalinization of culture medium, as well as typical for the rapidly growing *Rhizobium* coupled with the acidification of culture medium (Table 1). Interestingly while typical for *Rhizobium* are fast-growing strains, alkalizing *Bradyrhizobia* are usually slow-growing. In the analyzed population all the combinations of growth rate and metabolic type were represented.

The ability of the tested strains to nodulate selected legume species was analyzed. Nodulation of yellow lupin (var. Ventus), soybean and serradella were tested in the first greenhouse experiment in Leonard jars. Serradella has been classified in the same group of nodulation specificity (cross-inoculation group) as lupins, while soybean is nodulated by *Bradyrhizobia* closely phylogenetically related to some of the lupins microsymbionts (Table 2). Nodulation of small-seed leguminous plants – serradella and birdsfoot trefoil were analyzed also on agar slants in the growing chamber.

Table 1. Percentage of isolates representing different metabolism type and different relative growth rate within analyzed population.

Type of metabolism	Relative growth rate of isolates			Total
	slow	intermediate	fast	
Acidifying strains	17.24%	22.41%	**37.93%**[#]	77.59%
Alkalizing strains	**10.34%**[##]	8.62%	3.45%	22.41%

[#] typical characteristic for *Rhizobium*
[##] typical characteristic for *Bradyrhizobium*

Table 2. Nodulation ability of bacterial strains isolated from three species of lupins with yellow lupin, serradella and soybean as potential plant hosts.

Source host plant for inoculum strains	Yellow lupin (*L. luteus* cv. Ventus)	Serradella (*O. sativus* cv. Mazurska biała)	Soybean (*G. max.* cv. Nawiko)
L. luteus	81.82%	71.43%	0.00%
L. angustifolius	60.00%	21.43%	0.00%
L. albus	42.86%	16.67%	0.00%

Greenhouse experiment showed considerable variation among isolates in their nodulation capability.

Depending on the primary host plant species serving as a source for the bacterial strains we could observe important differences in the nodulation ability within tested population. As many as 81,82% of the bacteria originated from yellow lupin were able to initiate symbiosis with the plants of the same kind while only 42,86% of the strains isolated from white lupin nodulated yellow lupin. That confirms previous observations suggesting higher species specificity among *L. albus* microsymbionts (Rivas et al. 2009). The similar scheme, although at a lower level, we could observe when serradella was used as a host plant. The highest nodulation frequency was for strains originally isolated from yellow lupin, while the lowest from white lupin. We observed strains capable of simultaneous nodulation of yellow lupin and serradella, which confirms the possibility of classifying these two species in the same cross-inoculation group. But there are strains in the analyzed population (mainly isolated from roots of blue lupin) capable to nodulate yellow lupin, which do not induce nodules on the roots of tested serradella plants. Therefore, it seems that not in every experimental case can serradella replace lupin plants in nodulation and nitrogen fixation performance studies. That would suggest, that among all strains capable of lupins nodulation the most versatile are these infecting *L. luteus* plants in the field conditions.

Interestingly, in several cases lack of nodules on the roots of lupins was observed, despite the bacterial strains used as inoculum were isolated from the roots of plants of this kind. However, in the presented nodulation analysis only one variety (Ventus) of one species of lupin (*L. luteus*) was used. That would suggest some level of cultivar specificity among analyzed population. There were no nodulation cases in the analysed population, when soybean was used as the potential host plant. It seems that, despite the high phylogenetic relationship of *Bradyrhizobium japonicum* (soybean microsymbiont), and some bacteria nodulating lupins (Barrera et al. 1997), the latter do not have the capacity to initiate symbiosis with soybeans.

This suggested species specificity for nodulation was confirmed in the second experiment, when total number of nodules, as well as fresh weight of nodules were analyzed. For this experiment we have chosen (from the previous test) six strains capable to effectively nodulate yellow lupin cv. Ventus and representing all six rhizobia metabolic types. The following plants were tested: *L. albus* cv. Wat, *L. luteus* cv. Lidar and cv. Ventus, *L. angustifolius* cv. Sonet.

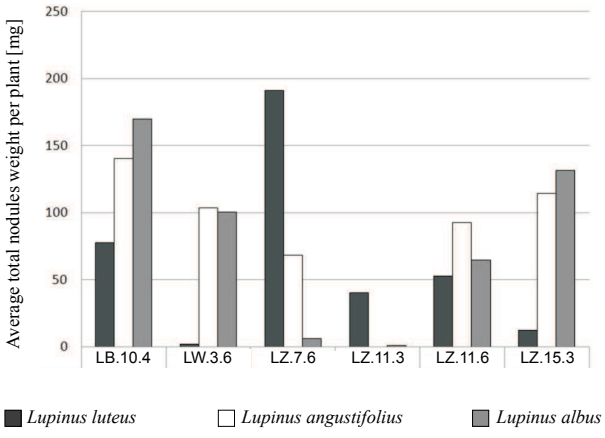
It is reported that total nodule weight related positively with the quantity of nitrogen fixed, even better than total number of bacteroids of the effective bacterial strain (Wadisirisuk, Weaver, 1985). Data presented in Fig. 2 shows high variability in total nodule weight and hence an estimated nitrogen fixation rate. There is also a certain level of bacterial strain specificity to the plant species.

Table 3. Metabolic characteristics of the stains used in 2nd and 3rd experiment.

Type of metabolism	Relative growth rate of isolates		
	slow	intermediate	fast
Acidifying strains	LZ.15.3	LW.3.4	LB.10.5
Alkalizing strains	LZ.7.6	LZ.11.3	LZ.11.6

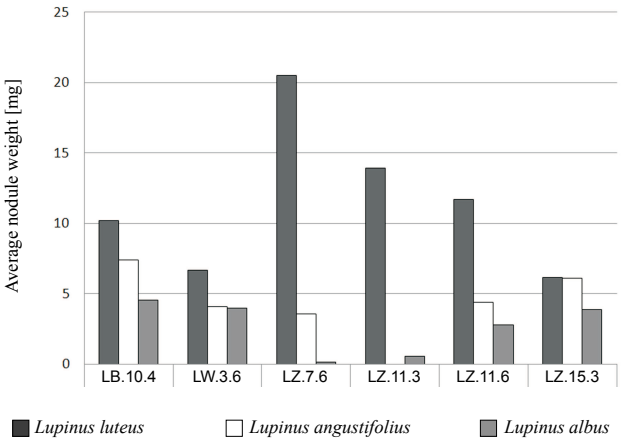
Strain LZ.7.6 (obtained from *L. luteus*) induced nodules weighing 191.22 mg when yellow lupin was inoculated during the experiment. It is almost three times more comparing to blue lupin and 30 times more comparing to white lupin used as a host plants in the nodulation tests with the same strain. The effect was opposite with strain LB.10.4 obtained from roots of white lupin.

After the initial infection, nodules grow and harbor increasing populations of bacteria until the nodules senesce and the rhizobia are released into the soil. However, rhizobial effectiveness in nodules is not always guaranteed. Host species differ in the type of nodules they form, and this can determine the degree to which differentiated bacteroids can repopulate the soil (Denison, 2000; Mergaert et al., 2006). Furthermore, some legumes can hinder the growth of nodules with ineffective rhizobia, thus punishing uncooperative symbionts (Simms, Taylor, 2002). Plants might modulate resource allocation or impose sanctions on individual nodules. Indeed, a recent model by West and colleagues (West et al., 2002) predicts that high rates of N-fixation by rhizobia can be maintained only if plants allocate resources to nodules on the basis of the N-fixation rate of their occupants. While many physiological and biochemical



The strains designation is based on their source host plant (LB – *L. albus*, LW – *L. angustifolius* and LZ – *L. luteus*)

Fig. 2. Average total nodules weight per plant of three lupin species induced by six different bacterial strains.



The strains designation is based on their source host plant (LB – *L. albus*, LW – *L. angustifolius* and LZ – *L. luteus*)

Fig. 3. Average nodule weight on roots of three lupin species induced by six different bacterial strains.

mechanisms might be involved in sanctions or differential allocation, these mechanisms should all produce a consistent phenotype in nodules occupied by ineffective bacteria: small size (Simms, Taylor, 2002).

Nodules smaller than normal are generally an indication of infection by an ineffective or less effective strain of rhizobia. In the analyzed group of strains we could also observe the meaningful differences in the nodule sizes in respect of plant species and strain used as inoculums. The most specialized strains (LZ.7.6, LZ.11.3 and LZ.11.6 in Fig. 3) induced the formation of the biggest nodules on their host plant, while on two other lupin species the nodules (if created) were of marginal size.

Third experiment of nodulation potential carried out on agarose slants generally confirms that in the studied population of bacteria isolated from lupin roots there exists an ability to initiate symbiosis with serradella (Table 4). To some extent, although with a significantly lower frequency, lupin microsymbionts are able to initiate symbiosis also with birdsfoot trefoil.

Attention can be put to the relatively high frequency of ineffective root nodules. The term „ineffective" describes the nodules which do not contain a noticeable amount of leghemoglobine. The emergence of these "white" nodules confirms the thesis of a limited "compatibility" of lupins nodulating bacteria to serradella. A similar situation to that, occurring with greater intensity, can be observed in systems with birdsfoot trefoil used as a potential host of the symbiosis.

It is worth to note that in this experiment we could observe the presence of noneffective nodules on the serradella roots in systems where strains effectively nodulating lupins rather than serradella were, in the pot experiment. We could suppose that regulated and optimal conditions of temperature and lighting can foster the adoption by the plant of even unauthorized bacterial symbiosis partners, on the other hand, continuous contact of a large number of bacterial cells to plant roots without the competitive impact of biotic and abiotic environment may increase the likelihood of this kind of biological artifact in the very artificial environment in the test tube with agar slants.

Understanding and measurement of the field population complexity can lead to obtaining a high proportion of nodule occupancy by applied inoculant strains under these conditions. As both, the strain richness and genetic diversity of rhizobial populations associated with a given host legume, is likely to vary between sites, selected host, strain and management practice combinations aimed at improving nodule occupancy by inoculant strains would need to be screened for effectiveness. For legume species that harbour inherently diverse populations of rhizobia in their nodules, it may be preferable to select crop varieties that nodulate effectively with the resident rhizobia (McInnes, Haq, 2003), rather than attempting to manipulate the competitive ability of introduced inoculant strains. Nodule induction at high frequency by introduced inoculant strains has been readily demonstrated in soils where indigenous rhizobia are deficient. However, most inoculated legume seed is sown into soils containing established rhizobium populations, and in these situations there are reports of inoculant strains inducing the majority of nodules in the first year and of their progressive disappearance and replacement by indigenous rhizobia in succeeding years. In other instances, indigenous rhizobia were a barrier to the successful introduction of inoculants, resulting in low levels of establishment of the applied strains in the year of inoculation. Therefore, an understanding of the nature of indigenous populations of rhizobium, of the factors that affect their distribution and dynamics, and of their role in

Table 4. Nodulation ability of bacterial strains isolated from three species of lupins with serradella and birdsfoot trefoil grown on agar slants. Percentage of nodulated and not nodulated plants.

Primary host plant for inoculum strains	Serradella			Birdsfoot trefoil		
	effective nodules	nonefective nodules	no nodules	effective nodules	nonefective nodules	no nodules
L. luteus	75.00%	16.67%	8.33%	33.33%	50.00%	16.67%
L. angustifolius	33.33%	41.67%	25.00%	8.33%	16.67%	75.00%
L. albus	20.00%	20.00%	60.00%	20.00%	0.00%	80.00%

inoculant strain competition and persistence is of considerable agricultural significance.

CONCLUSIONS

1. Lupins are nodulated by both fast-growing as well as by slow-growing strains. In the analyzed population majority (more than 77%) is constituted by isolates representing growth rate typical for *Rhizobium*.

2. Lupins (*L. luteus, L. angustifolius* and *L. albus*) are cross nodulated by strains of bacteria isolated from root nodules of yellow lupin, white lupin and blue lupin. However, they form a common cross-inoculation group, species nodulation specificity can be observed. This characteristic is particularly expressed in bacterial populations isolated from white lupin.

3. Serradella is nodulated by several analyzed strains of bacteria from root nodules of lupins of both types (fast and slow growing*)*, but as much as 50% of the strains studied did not induce effective nodules on the serradella roots in the glasshouse experiment. In test tubes nodulation analysis showed that 24% of strains do not produce root nodules, and a further 27% of the strains induced inefficient roots nodules on serradella. These observations confirm that lupins and serradella can be classified within the same cross-inoculation group but they also call into question the usefulness of serradella (no doubt more easily grown in the laboratory) as a substitute of lupins in the studies of nodulation and nitrogen fixation efficiency.

4. *Lotus corniculatus* is also classified in the same cross-inoculation group with lupins and serradella. Presented work shows however that classification should be regarded rather as conditional and not absolute. Although the analyzed bacterial population has some potential for creating symbiotic systems with birdsfoot trefoil, it is not a common phenomenon among lupin microsymbionts and is rather due to a large heterogeneity of the population.

5. Soybean, despite reports of high phylogenetic relationship between its microsymbiont (*Bradyrhizobium japonicum*) and bradyrhizobia nodulating lupins, is probably not nodulated by the same strains as lupins and other species belonging to the same group of nodulation specificity. There was not a single case of inoculation, resulting in the formation of symbiotic system involving strains belonging to the analyzed population.

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