FRAGM. AGRON. 27(1) 2010, 107–116

DIVERSITY AMONG FIELD POPULATIONS OF BACTERIAL STRAINS NODULATING LUPINS IN POLAND

KRZYSZTOF PUDEŁKO

Department of Biochemistry and Biotechnology, Poznań University of Life Sciences

bioline@home.pl

Abstract. 235 bacterial isolates were collected from 23 locations of lupine field crops, including 16 geographical locations, mainly from Wielkopolska. Bacteria were isolated from root nodules of yellow lupine (*Lupinus luteus*), white lupine (*Lupinus albus*), and blue lupine (*Lupinus angustifolius*). Observations presented in this paper confirmed the existence of large genetic diversity within the bacterial population nodulating lupines, but also allowed the separation of groups of bacteria with similar characteristics. It seems that the symbiotic processes involving the participation of lupines and bacteria belonging to at least two types of *Rhizobiaceae*. Interesting in this context is as the ratio of strains alkalizing substrate (characteristic for *Bradyrhizobium*) to acidifying strains (which is characteristic for *Rhizobium*) in the analyzed population. This ratio is 3:7 respectively, suggesting the domination of *Rhizobium* among lupine microsymbiont population Poland. The large diversity of populations and a wide range of bacteria *Rhizobium* and *Bradyrhizobium* confirmed by the observations of this work indeed appears to show a long history of bacteria belonging to these groups, and that bacterial populations capable of lupine nodulation are indeed endogenous, perfectly suited to local environmental conditions in Polish soils.

Key words: lupine*, Rhizobium, Bradyrhizobium,* nodulation

INTRODUCTION

Rhizobial bacteria comprise several distantly related proteobacterial lineages, most notably the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium* [Brenner *et al.* 2005], 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 lupines (*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*. Lupines microsymbionts have been characterized to a much lesser extent than, for example, populations of *B. japonicum* or *R. leguminosarum*.

Strain richness indices have shown that resident rhizobial populations and communities are often structurally and genetically diverse [McInnes *et al.* 2004]. 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 nodulates effectively with the resident rhizobia [McInnes and

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 have been 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 inoculant strain competition and persistence is of considerable agricultural significance.

The aim of this study was to analyze the diversity and structure of lupin microsymbionts population nodulating three different species of lupins cultivated in Poland.

MATERIALS AND METHODS

23 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 lupines plants. In the years 1996–2004 235 strains were isolated and 167 of them were further characterized. The strains were isolated from 18 varieties of three lupines species: 8 varieties of yellow lupine (*L. luteus*), 6 varieties of narrow leaf lupine (*L. angustifolius*) and 4 varieties of white lupine (*L. albus*). The strains originated from the places of different lupines cultivation history. On the locations of the plant breeding stations (Nowa Wieś Zbąska, Przebędowo, Wiatrowo) lupines were present often for several years or even yearly (Wiatrowo). On the other locations (Głębocko, Długa Goślina, Lipnica, Gurówko) lupines 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. Location, host plant and no. of isolates obtained are presented in Table 1.

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 and Hoben 1994].

One of the distinctive elements of the bacteria belonging to the different types of *Rhizobiaceae* is their influence on culture medium pH [Gerhardt *et al.* 1994]. Bacterial colonies were grown on Petri dishes on YM agar solid medium [Somasegaran and 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 and placed in a dark incubator at a temperature of 28°C. After 5 days observation of the growing medium color were carried out. 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.

All isolates were tested for intrinsic resistance to antibiotics as previously described [Mądrzak *et al.* 1995]. The following antibiotics were tested: streptomycin (20 $mg \cdot ml^{-1}$), spectinomycin

Table 1. Origins of bacterial isolates obtained from lupin production fields in Poland

Location	Host plant	No of isolates
Brody	*Lupinus albus*	6
	Lupinus luteus	6
Długa Goślina	*Lupinus angustifolius*	6
Głębocko	*Lupinus luteus*	6
Gorzyń	*Lupinus angustifolius*	6
	Lupinus luteus	9
Gurówko	*Lupinus angustifolius*	6
Lipnica	*Lupinus luteus*	8
Łopuchowo	*Lupinus angustifolius*	6
Nowa Wieś Zbąska	*Lupinus albus*	4
Olsztyn	*Lupinus luteus*	42
	Lupinus luteus	10
Potrzanowo	*Lupinus luteus*	12
Przebędowo	*Lupinus albus*	6
Rawicz	*Lupinus angustifolius*	12
	Lupinus luteus	12
	Lupinus luteus	8
Rozalinowo	*Lupinus luteus*	4
Wiatrowo	*Lupinus albus*	7
Wierzenica	*Lupinus angustifolius*	10
	Lupinus luteus	6
	Lupinus luteus	16
Złotniki	*Lupinus albus*	26

(10 mg·ml^{-1}), kanamycin (20 mg·ml^{-1}), tetracycline (30 mg·ml^{-1}), nalidixic acid (30 mg·ml^{-1}), erythromycin (30 mg·ml^{-1}), rifampin (1 mg·ml^{-1}).

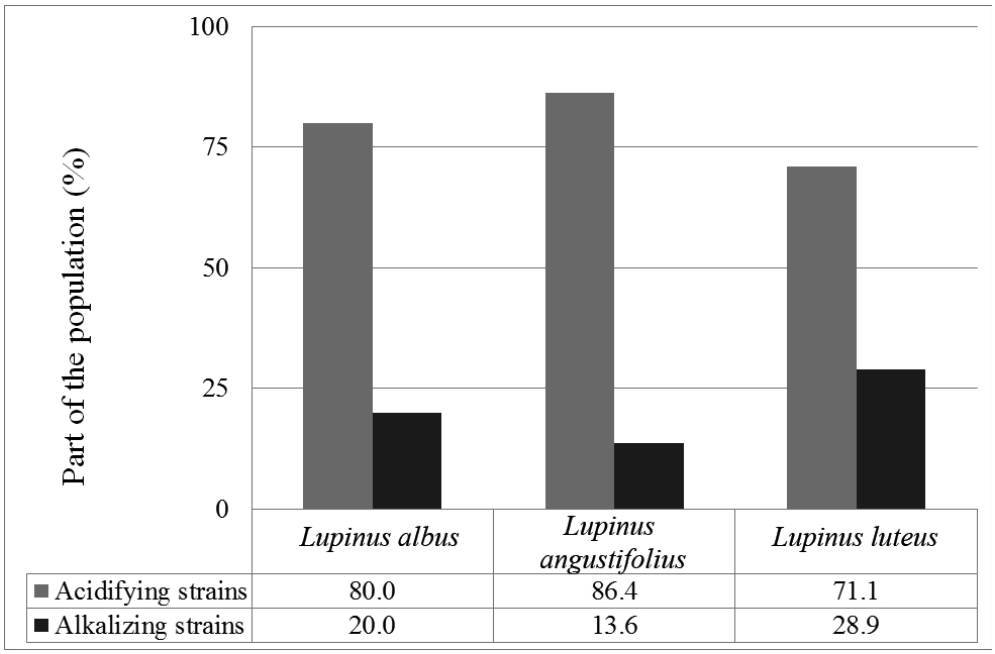
Relative growth rate of isolates analyzed in the solid medium. Three groups of isolates with different relative growth rate at a temperature of 28°C were distinguished. Strains classified as slow growing showed single colonies after 48 hours, after 72 hours colonies were clearly visible. Intermediate showed visible growth after 48 h and after 72 hours colonies were well formed. Fast growing showed well formed colonies after 48 h while after 72 hours, colonies were joined and difficult to distinguish.

The analysis of the genome structure was carried out using the method of rep-PCR. The PCR was performed with repetitive extragenic palindromic (REP) primers [Judd *et al.* 1993, Schneider and de Bruijn 1996], and the REP-PCR fingerprints were converted into a two-dimensional binary matrix and analyzed statistically.

The analysis of the structure of 16S RNA genes was performed based on the presence in the 16S RNA gene sequence fragment of about 350 bp length with a variable primary structure, located in the initial region of a gene [Van Rossum *et al.* 1995, Young *et al.* 1991].

RESULTS AND DISCUSION

Collected isolates were characterized in terms of represented type of metabolism. Generally it is assumed that organisms belonging to the genus *Rhizobium* acidify medium, while bacteria classified within *Bradyrhizobium* cause an increase in pH of culture medium. In this group of strains, two types of metabolism were observed: characteristic for 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 *Rhizobiaceae* coupled with the acidification of culture medium. Fig. 1 presents isolates divided into groups of two categories depending on their original host plant. Analysis of the frequency of two types of metabolism in the population has brought quite unexpected results. The vast majority of isolates acidified culture medium. These observations are in conflict with a previously assumed an overwhelming dominance of slow-growing strains classified as *Bradyrhizobium* sp. among microorganisms capable to initiate an efficient symbiotic system with lupins [Andam *et al.* 2007, Martinez-Romero and Caballero-Mellado 1996, Stępkowski *et al.* 2007]. However, when compared with other symbiotic systems of *Rhizobiaceae* and leguminous plants, lupine symbiosis with soil bacteria is relatively poorly known.



	Lupinus albus	*Lupinus angustifolius*	*Lupinus luteus*
Acidifying strains	80.0	86.4	71.1
Alkalizing strains	20.0	13.6	28.9

Fig. 1. Metabolic type distribution in the population by host plant

As shown in Fig. 1 the ratio of strains alkalizing to acidifying the medium in the population is similar regardless of the plant host species. Differentiation of the populations structure was observed while analyzed separately from the different geographical locations (Fig. 2).

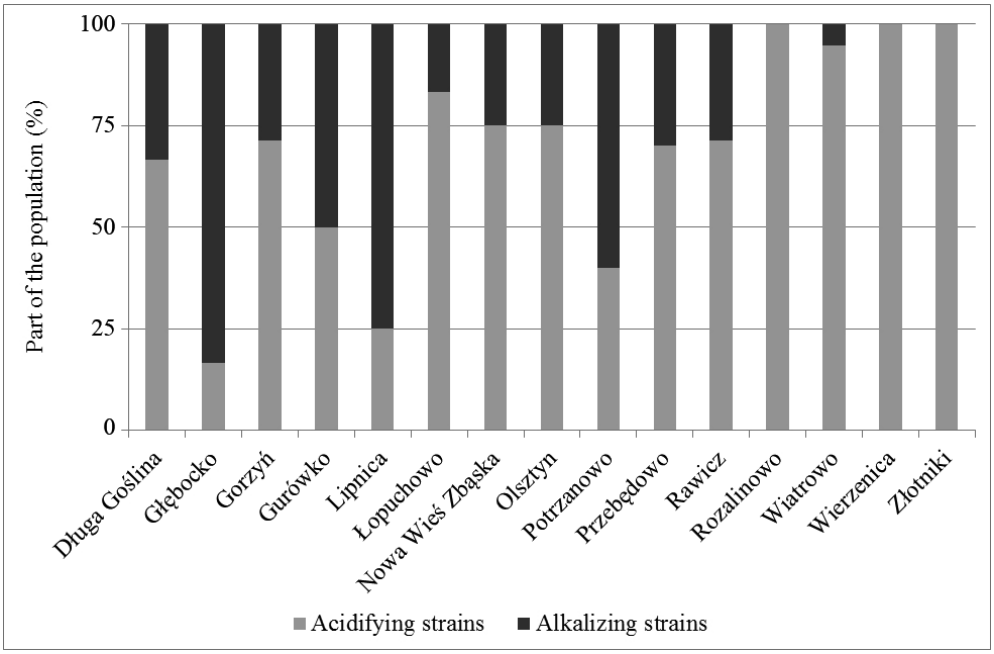


Fig. 2. Metabolic type distribution in the population by geographical location

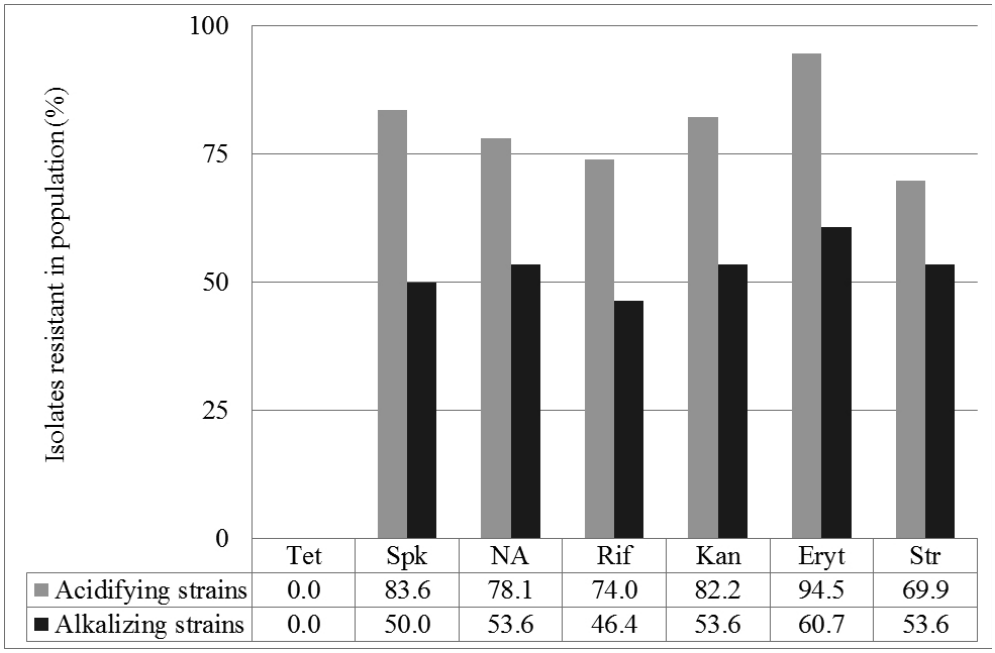
The entire range of population structures could be observed: from those where there were no alkalizing strains (Rozalinowo, Wierzenica, Złotniki), to the locations with a predominance of isolates with metabolism typical for *Bradyrhizobium* (Głębocko, Lipnica, Potrzanowo). Complex structure of the bacterial populations nodulating lupins in Poland had been also confirmed by the analyses of the relative growth rate of isolates. 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 (Tab. 2).

Table 2. Relative growth rate of isolates of different metabolism type within population (%)

Type of metabolism	Relative growth rate of isolates		
	Slow	Intermediate	Fast
Acidifying strains	24.7	29.9	45.5*
Alkalizing strains	53.9**	34.6	11.5

With asterisk the typical characteristic for *Rhizobium** and *Bradyrhizobium*** are indicated

Intrinsic antibiotic resistance is one of the important discriminating feature while studying the bacterial populations. Analyzed isolates of soil bacteria show a relatively high antibiotic resistance (Fig. 3.). Also the resistance profile is very interesting. Particular attention should be put on high resistance for kanamycin in the entire population. Overall, among the *Rhizobiaceae* resistance for kanamycin occurs at a much lower level than in the studied group. Equally interesting is a total lack of resistance to the applied concentration of tetracycline, to which most of *Rhizobiaceae* has a relatively high tolerance. In this group of strains different levels of antibiotics resistance can be observed, depending on the metabolic type represented. Strains alkalizing substrate have a lower level of natural resistance to antibiotics in comparison with the acidifying strains. Based on the analysis of antibiotics resistance, a comparison between the mutual similarities and differences between organisms within the populations had been studied. Alkalizing and acidifying strains were analyzed separately.



	Tet	Spk	NA	Rif	Kan	Eryt	Str
Acidifying strains	0.0	83.6	78.1	74.0	82.2	94.5	69.9
Alkalizing strains	0.0	50.0	53.6	46.4	53.6	60.7	53.6

Fig. 3. Intrinsic antibiotic resistance within population

Among the acidifying strains 20 groups of varying size and level of mutual similarity were identified. These groups are organized in three clusters of up to a parent different from each other. Inside two separate clusters differentiation reaches a level of about 43%, while the third group of internal diversity is greater and reaches more than 60%. The population of alkalizing strains shows greater internal diversity. 28 analyzed strains can be distinguished into 13 specific groups of varying size and level of mutual similarity. The largest of these include seven isolates. In the whole population two clusters of strains with a high level of mutual differentiation at the level of 75% can be identified.

The analysis of the genomes structure of bacteria was carried out using the method of REP-PCR. Total genomic DNA were used as templates for PCR with REP primers. Comparative analysis of obtained fingerprints show high repeatability. The coefficient of similarity (r value) ranged from 0.89–0.94 for samples amplified in the same PCR reaction and distributed on the same agarose gel, and the range of 0.84–0.90 for samples amplified in separate PCR reactions and distributed at different agarose gels. These results are consistent with other results [Rademaker and de Bruijn 1997]. To classify the strains by using this technique, the results from the stained agarose gels were converted into a two-dimensional binary matrix, and analyzed statistically. A dendrograms based on pairwise comparisons of PCR products were generated.

Hierarchical grouping of strains based on REP-PCR profiles were carried out by Ward method [Ward 1963], using the estimate of the distance between clusters variance analysis aimed at minimizing the sum of the squares of any two (hypothetical) clusters that can be formed at each stage of clustering. This analysis indicates the presence of significant genetic diversity in the population of studied isolates. Population of acidifying strains is much more diverse in comparison with isolates alkalizing substrate. Both populations can be divided internally into diverse groups with differentiation at the level of 0.3. But the linkage of these clusters, showing a total diversity of the population, is in the level 0.4 between the alkalinizing strains, while in group of acidifying strains is at a very high level of 0.6–0.7. This may suggest the merits of a separate classification of strains belonging to these groups within the *Rhizobium* type strains.

Analysis of 16S rRNA gene was based on the presence of highly variable region located in the initial part of the whole 16S rRNA gene. The result showed high diversity of the PCR product sizes between 240 and 420 bp. 7 groups of bacterial isolates distinguished by the size of PCR product were formed. Analysis of the size of the variable region in 16S rRNA gene, together with the distribution of repetitive elements in the bacterial genome helped to organize the tested isolates in groups forming separate clusters in the two-dimensional space. The image obtained by comparing strains of the k-average method is shown in Fig 4.

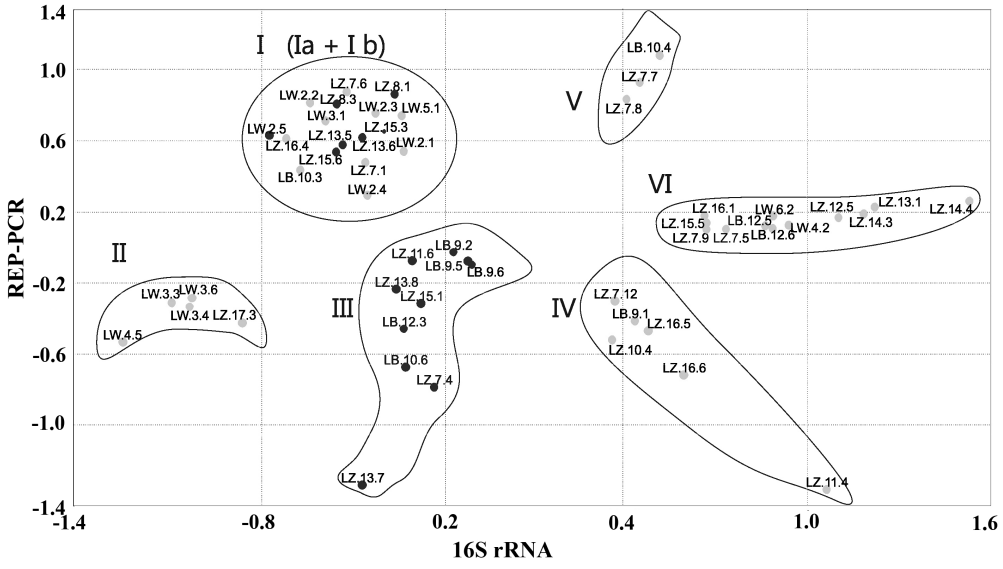


Fig. 4. Groups of isolates distinguished on the basis of REP-PCR and the variable region of 16S rRNA gene

Analysis of the mutual distances of group-forming suggests that the group marked as I in fact consists of two different clusters of isolates, including strains of alkalizing (Ia) and acidifying (Ib) substrate, superimposed on a two-dimensional space. It can be concluded that studied strains form seven distinct groups, showing different degrees of internal differentiation, but clearly differ among themselves. Also these statistical data highlight the differences between isolates alkalizing (groups Ia and III) and acidifying substrate (group Ib, II, IV, V, VI).

While it is suggested that nonsymbiotic rhizobia could play an important role in competition to colonize host root surfaces [Sachs *et al.* 2009] and conflicts occurring on legume roots can be more complex and potentially more intense than previously predicted, it seems that trap hosts (particularly multiple species) appears to be effective in recovering most rhizobial strain types from soil. The large diversity of populations and a wide range of *Rhizobium* and *Bradyrhizobium* bacteria, confirmed by the observations of this study, indeed appears to show a long history of bacteria belonging to these groups, and that bacteria capable to nodulate lupine plants are indeed endogenous, perfectly suited to local environmental conditions in the Polish soil.

CONCLUSIONS

1. There is no major problems with lupines nodulation in the field crops in Poland because many of these strains are endogenous. For all analyzed plants, root nodules were observed. There is no significant effect of the frequency of lupines cultivation on the diversity and composition of the population of symbiotic bacteria.
2. The large diversity of strains from the remote field stations and the lack of one dominant type isolates in the study population appears to confirm that the *Rhizobiaceae* populations are not the typical clonal populations.
3. Analysis of the bacterial collection nodulating lupins in field crops in Poland shows large variations in the structure of the population. It seems that the symbiotic processes can involve the participation of bacteria belonging to at least two genera of *Rhizobiaceae*. Interesting in this context is the ratio of strains alkalizing substrate (characteristic for *Bradyrhizobium*) to the strains of acidifying substrate (which is characteristic of the *Rhizobium* type bacteria) in the analyzed population. This ratio is 3:7, suggesting predominance of *Rhizobium* spp. in the group of lupines microsymbionts. These observations are in a conflict with the previous information suggesting the domination of *Bradyrhizobia* in this group of bacteria
4. The lack of one dominating strain within studied population suggest also a potentially successful introduction of the selected strain as inoculums in the lupines cultivation practice.

ACKNOWLEDGEMENTS

Author would like to thank prof. C. Mądrzak for his advices, supervision and support.

REFERENCES

Andam C.P., Parker M.A. 2007. Novel alphaproteobacterial root nodule symbiont associated with *Lupinus texensis*. Appl. Environ. Microbiol. 73: 5687–5691.

Brenner D.J., Krieg N.R., Staley J.T., Garrity G.M. (ed.) 2005. Bergey's manual of systematic bacteriology. The Proteobacteria. Part C, The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. Springer, New York, 2nd ed. II: pp. 1414.

Judd A.K., Schneider M., Sadowsky M.J., de Bruijn F.J. 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. 59: 1702–1708.

Mądrzak C.J., Golińska B., Króliczak J., Pudełko K., Łażewska D., Lampka B., Sadowsky M.J. 1995. Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl. Environ. Microbiol. 61: 1194–1200.

Martinez-Romero E., Caballero-Mellado J. 1996. Rhizobium phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. 15: 113–140.

McInnes A., Haq K. 2003. Contributions of rhizobia to soil nitrogen fertility. In: Abbott, L.K., Murphy, D.V. (Eds.), Soil Biological Fertility: a Key to Sustainable Land Use in Agriculture. Kluwer Academic Publishers, Dordrecht: pp. 99–108.

McInnes A., Thies J.E., Abbott J.G., Howieson J.G. 2004. Structure and diversity among rhizobial strains, populations and communities–a review. Soil Biol. Biochem. 36: 1295–1308.

Gerhardt P., Murray R.G.E., Wood W.A., Krieg N.R. (ed.) 1994. Methods for general and molecular bacteriology. American Society for Microbiology, Washington D.C.: pp. 791.

Rademaker, J.L.W., de Bruijn F.J. 1997. Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. DNA Markers: Protocols, Applications and Overviews. G. Caetano-Anollés and P.M. Gresshoff, (ed.). J. Wiley and Sons, Inc., New York. pp. 1–26.

Sachs J.L., Kembel S.W., Lau A.H., Simms E.L. 2009. In situ phylogenetic structure and diversity of wild *Bradyrhizobium* communities. Appl. Environ. Microbiol. 75: 4727–4735

Schneider M., de Bruijn F.J. 1996. Rep-PCR mediated genomic finger-printing of rhizobia and computer-assisted phylogenetic pattern analysis. World J. Microbiol. Biotechnol. 12: 163–174.

Somasegaran P., Hoben H.J. 1994. Handbook for Rhizobia. Methods in legume – Rhizobium technology. Springer-Verlag, New York: pp. 450.

Stępkowski T., Moulin L., Krzyżańska A., McInnes A., Law I.J., Howieson J. 2005. European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of Western Australia and South Africa. Appl. Environ. Microbiol. 71: 7041–7052.

Van Rossum D., Schuurmans F.P., Gillis M., Muyotcha A., Van Verseveld H.W., Stouthamer A.H., Boogerd F.C. 1995. Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. Appl. Environ. Microbiol. 61: 1599–1609.

Ward J.H. 1963. Hierarchical grouping to optimize an objective function. J. Am. Stat. Assoc. 58: 236–244.

Young J.P.W., Downer H.L., Eardly B.D. 1991. Phylogeny of the phototrophic *Rhizobium* strain BTail by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. 173: 2271–2277.

K. Pudełko

ZRÓŻNICOWANIE POPULACJI BAKTERII BRODAWKUJĄCYCH ŁUBINY W UPRAWACH POLOWYCH W POLSCE

Synopsis. Zebrano 235 izolatów bakterii z 23 stanowisk polowych upraw łubinów, obejmujących 16 lokalizacji geograficznych, głównie z terenu Wielkopolski. Bakterie izolowano z brodawek korzeniowych łubinu żółtego (*Lupinus luteus* L.), łubinu białego (*Lupinus albus* L.) oraz łubinu wąskolistnego (*Lupinus angustifolius* L.). Prezentowane w niniejszej pracy obserwacje potwierdziły istnienie dużego zróżnicowania genetycznego w obrębie populacji bakterii brodawkujących łubiny, umożliwiły również wyodrębnienie grup bakterii o podobnej charakterystyce. Z badań wynika, że w procesach symbiotycznych z udziałem łubinów udział biorą bakterie należące do co najmniej dwóch rodzajów *Rhizobiaceae*. Interesująco na tym tle przedstawia się stosunek ilościowy szczepów alkalizujących podłoże (cechy charakterystycznej dla *Bradyrhizobium*) do szczepów zakwaszających podłoże (co jest charakterystyczne dla bakterii rodzaju

Rhizobium) w analizowanej populacji. Stosunek ten odpowiednio 3:7 sugeruje przewagę *Rhizobium* w grupie mikrosymbiontów łubinów w Polsce. Duże zróżnicowanie populacji oraz szeroki zasięg występowania bakterii *Rhizobium* i *Bradyrhizobium* potwierdzone obserwacjami niniejszej pracy świadczą o długiej historii bakterii należących do tych grup, a także o tym, że bakterie zdolne do brodawkowania łubinów stanowią populacje istotnie endogenne, doskonale przystosowane do lokalnych warunków środowiska w polskich glebach.

Słowa kluczowe: łubin, *Rhizobium, Bradyrhizobium,* brodawkowanie