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The Structure and Metabolic Diversity of Population of Pea Microsymbionts Isolated from Root Nodules

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Research Article

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ABSTRACT

Aims: The study of strains belonging to local rhizobial population, concerning their diversity in the genetic, metabolic and symbiotic properties, and their prevalence in the microsymbiont population.
Methodology: 257 rhizobial isolates recovered from nodules of five pea (*Pisum sativum* cv. Ramrod) plants grown at one site were classified using PCR-RFLP analysis of 16-23S rRNA ITS. After that, for representative group of 55 strains, 16-23S rRNA ITS region was sequenced, *nodA-F* region was analyzed by PCR-RFLP and sequencing, metabolic capabilities were studied using Biolog`s and growth tests and symbiotic performance in plant tests were assayed.
Results: Individual plants were infected by numerous and diverse strains, however, in the entire sampled population of microsymbionts, only three large clusters of strains could be distinguished on the basis of PCR-RFLP and sequence analyses of 16S-23S rRNA ITS region. *Rhizobium* strains belonging to different groups varied in plasmid number and the amount of plasmid DNA, utilization of carbon and energy sources, growth on soil extract-based media and the ability for symbiotic plant growth promotion. The most numerous group of the isolates was characterized by the high plasmid DNA content, low number of utilized sugar substrates, and comprised numerous strains with low symbiotic efficiency.

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Conclusion: Sampled population of pea microsymbionts had its own characteristic structure with clearly distinguishable sub-populations, composed of numerous strains - probably descendants of a few old lineages, which diversified in the lapse of time. These strains are still competing during root nodule colonization, resulting in the symbiosis of individual pea plants with broad spectrum of different *Rhizobium* strains.

Keywords: Rhizobium; pea microsymbionts; population structure; diversity;

1. INTRODUCTION

The *Rhizobium*-legume symbioses are widespread all along the world and are very important for numerous ecosystems, since they provide the plants with an additional nitrogen source derived from bacterial atmospheric dinitrogen fixation (Peoples et al., 1995; Vance, 2001). The symbiosis requires an exchange of specific molecular signals, such as plant flavonoids and bacterial chitolipooligosaccharides (Nod factors), which in turn triggers the expression of plant genes and initiates root nodule formation (Jones et al., 2007; Perret et al., 2000). Rhizobia invade nodules via infection threads, are released from them into nodule cells and differentiate into bacteroids, which convert atmospheric dinitrogen into ammonia transferred to plants in exchange for photosynthates (Schulze, 2004; Simms and Taylor, 2002; White et al., 2007). As a consequence of precise molecular signaling, rhizobial-legume symbioses are in most cases species-specific, so defined plant hosts can be nodulated only by one or a few closely related rhizobial species. Nodules of pea (*Pisum*) as well as vetch or bean (*Vicia*) are mostly colonized by *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) (Mutch and Young, 2004; Willems, 2006). Recently, some other *Rlv*-related rhizobial species nodulating pea and bean, like *Rhizobium fabae* and *Rhizobium pisi* were described (Ramirez-Bahena et al., 2008; Tian et al., 2008).

Rhizobium genomes are large (>6 Mb) and composed of the chromosome and the plasmids, one of which usually contains genes responsible for nodulation and nitrogen fixation, thus called pSym (Gonzalez et al., 2006; Young et al., 2006). These genomes are characterized by dynamic architecture due to frequent recombination events between replicons and plasmid transfers (Brom et al., 1991; Broughton et al., 1987; Guo et al., 2007; Souza et al., 1992). The genomes can also be subject to permanent point mutation acquisition, further affecting the adaptation to soil and endosymbiotic environment (Depret and Laguerre, 2008). All these factors cause substantial genetic diversity of rhizobial populations (Andrade, 2002; Fagerli and Svenning, 2005; Louvrier et al., 1996; Mutch and Young, 2004; Silva et al., 2007).

In some soils the rhizobial populations are numerous, reaching up to 10^5 cells/g of soil (Andrade, 2002; Martyniuk et al., 2005). Thus, large quantities of rhizosphere rhizobia compete with each other initially in plant root colonization and then inside plant tissues (Duodu et al., 2009; Stuurman et al., 2000; Wielbo et al., 2010a). The plant host could favor particular bacterial genotypes from the pool of compatible rhizobial strains present in the rhizosphere (Depret and Laguerre, 2008; Mutch and Young, 2004; Rangin et al., 2008) but there are many bacterial traits affecting rhizobial competitiveness (Hynes and O`Connel, 1990; Oresnik et al., 1998; Oresnik et al., 1999; Robleto et al., 1998; Vinuesa et al., 2003; Wielbo et al., 2007). It seems plausibly, that there are several efficient alternative

competition strategies for rhizobia allowing them for multi-strain colonization of individual plants in the field conditions, which was signaled in our previous work (Wielbo et al., 2010b). In this study, genetic and physiological diversity of pea nodule isolates originating from five pea plants grown at one site was examined. We demonstrated that the sampled rhizobial isolates form distinct clusters that differ significantly in their genetic, metabolic and symbiotic properties.

2. MATERIAL AND METHODS

2.1 Pea Nodule Isolates

257 *Rhizobium* isolates were obtained from nodules of pea (*Pisum sativum* cv. Ramrod) growing in sandy loam (N:P:K 0.157:0.014:0.013%). The soil contained a relatively high number of *R. leguminosarum* bv. *viciae*, bv. *trifolii* and bv. *phaseoli* cells i.e., 9.2 x 10^3, 4.2 x 10^3 and 1.5 x 10^3 bacteria/g of soil, respectively, as determined by the most probable number (MPN) method (Martyniuk et al., 2000). Plants were grown on 1 m^2 plot for six weeks between May and June, 2008. Five randomly chosen pea plants growing in each other's vicinity were harvested, the nodules were collected, surface-sterilized, crushed and their content plated on 79CA medium (Vincent, 1970). Pure cultures were used in further experiments.

2.2 Bacteria Growth Media with Soil Extracts

To prepare different soil extracts, the samples of soil were taken from six 1 m^2 plots in which clover, pea, vetch, bean or grass were cultivated, and the last one was herbicide fallow. After ten weeks, plants were removed and soil extracts were prepared from soil samples according to method described by Lorch et al. (Lorch et al., 1995). Soil extract-based media used for bacterial growth assays contained 10 ml appropriate soil extract, 0.01 g glucose and water to final volume of 100 ml.

2.3 Plasmid Profile Analyses

Screening of plasmid content of the 257 isolates was performed according to Eckhardt (Eckhardt, 1978) procedure. Then, 55 isolates with different plasmid profiles were chosen and their high molecular weight (HMW) genomic DNA was separated using pulse-field gel electrophoresis (PFGE) as described previously (Król et al., 2007). Estimation of plasmids sizes was performed using BIO-PROFIL V11.01 (Vilber-Lourmat, France) and *R. leguminosarum* bv. *viciae* strain 3841 (Young et al., 2006) as plasmids standard. Symbiotic plasmids were identified by Southern hybridization in which *nodA* was probed against blotted PFGE separated HMW DNA of isolates. The *nodA* gene fragment was amplified from *R. leguminosarum* bv. *viciae* strain 3841 genomic DNA as template using primers: nodA-1 5`-TGCRGTGGAARNTRNNCTGGGAAA-3` and nodA-2 5`-GGNCCGTCRTCRAAWGTCARGTA-3` (Haukka et al., 1998) and labeled with DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.4 PCR Fingerprinting and Sequencing

PCR-RFLP assays of 16S-23S rRNA internal transcribed spacer (ITS) were carried out using genomic DNA isolated from 257 isolates as templates and primers: FGPS1490-5'-TGCGGCTGGATCACCTCCTT-3' and FGPL132-5'-CCGGGTTTCCCCATTCGG-3' (Laguerre et al. 1996). PCR amplicons were digested with *Bsu*RI and *Taq*I restriction

enzymes and separated by 3% agarose gel electrophoresis. The 16-23S ITS amplicons of 55 *Rlv* isolates with different plasmid profiles were sequenced using BigDye™ Terminator Cycle Sequencing Kit and ABI PRISM 310 sequencer or ABI PRISM 3730 XL (Applied Biosystem) and submitted to GenBank. Accession numbers GQ358016 to GQ358071 were given to them.

PCR-RFLP assays of *nodD* gene were conducted using genomic DNA of 55 *Rlv* isolates as templates and primers: NBA12-5'-GGATSGCAATCATCTAYRGMRTGG-3' and NBF12-5'-GGATCRAAAGCATCCRCASTATGG-3' (Laguerre et al., 1996). PCR amplicons were digested with *Bsu*RI and separated by 3% agarose gel electrophoresis.

Sequence data were analyzed with Lasergene analysis software (DNASTAR, Inc). For the DNA sequences multiple alignments Clustal-W algorithm was used (Thompson et al., 1994). UPGMA algorithm was applied for the phylogenetic trees construction. The phylogenetic trees were visualized with TreeView (Page, 1996).

2.5 Physiological Tests

The utilization of different carbon and energy sources by 55 selected isolates was assessed using BIOLOG GN2MicroPlate™ (Gram Negative Identification Test Panel) (BIOLOG, Hayward, USA) containing 95 carbon sources, including sugars, amino acids, and organic acids, as described earlier (Wielbo et al., 2007).

2.6 Bacterial Growth Assays

For bacterial growth assays, the isolates were grown overnight at 28 ºC in 5 ml TY liquid medium. The cultures were then diluted to OD_{550} of 0.2, and used for inoculation (1:40 v/v) of a set of media based on soil extracts, each in 200 μl aliquots. The cultures were grown in 96-well microplates for 24h in Microtec AK120 shaker (Infors AG, Switzerland), at 28 ºC, 800 rpm. After 24 h, OD_{550} of cultures was measured. Each experiment was conducted in six replicas.

2.7 Vetch (*Vicia villosa*) - *Rhizobium* Symbiosis

Vetch seeds (*Vicia villosa* cv. Wista) were surface sterilized with 0.1% $HgCl_2$ and 70% ethanol and sown in plastic pots with sterile sand (600 g of sand/pot). Each pot was inoculated with 5 ml suspensions of individual strain (~$7x10^7$ CFU/ml). The pots, each containing six plants, were irrigated once with 50 ml nitrogen-free Fåhraeus liquid medium (Vincent, 1970) and watered every two days. The growth chamber was set at a 16 h/8 h day/night and at 22 °C/15 °C. After 6 weeks plants were harvested, number of nodules was counted and fresh mass of shoots and roots was estimated. Each treatment was carried out at 3 replicates.

2.8 Data Statistical Analysis

The relationship between genetic classification of isolates and their capability to use metabolic substrates (quantity of different substrates) was analyzed using median test with the Yates correction (Ferguson and Takane, 1989), which is a non-parametric alternative to the one-way ANOVA. The studied feature was the number of different metabolic substrates used by strains, while genetic classification based on PCR-RFLP and sequencing of 16S-23S ITS region (at three levels) was the grouping factors.

For principal component analysis (PCA), used to study bacterial capability to utilize particular substrates or groups of substrates, the results of BIOLOG test were coded in the binary system. Factor loadings of PCA were the correlation coefficients between the original variables (metabolic substrates) and the new obtained factors named PC1, PC2 and PC3. In this manner, PCA method allowed us to transform the numerous variables (utilization of individual substrates) into small groups of uncorrelated factors (utilization of groups of substrates), and to interpret the defined PCA factors.

To compare the growth of bacteria belonging to three different clades (A, B, C) ANOVA test was used.

The results of plant tests were subjected to two types of tests applied to estimate the differences in the symbiotic capabilities between three groups of strains. Median test was used to analyze nodule number as non-continuous trait, and the Kruskal-Wallis ANOVA by ranks to analyze continuous traits (shoot and root wet and dry mass). The second test is highly recommended (Freidlin and Gastwirth, 2000), but in the case of non-continuous traits it may be substituted by median test. All the described analyses were performed with STATISTICA software.

3. RESULTS AND DISCUSSION

3.1 Genetic Diversity of Isolates Recovered from Pea Nodules

257 isolates originating from nodules of pea plants growing in each other's vicinity were characterized by plasmid patterns and RFLP of 16S-23S rRNA ITS region, which was PCR amplified and digested with *Bsu*RI and *Taq*I restriction enzymes. Six distinct PCR-RFLP profiles were indentified and named: *a, a`, b, c, c`* and *d*. Each group contained different number of isolates (103, 2, 53, 90, 5 and 4, respectively). Profiles *a`* - *a*, and *c`* - *c* differed only in *Taq*I fragments length (details not shown).

From the initial 257 isolates, a group of 55 strains harboring most differentiated plasmids in size and number, belonging to all 16S-23S rRNA PCR-RFLP groups was chosen and subjected to further genetic and physiological analyses. The phylogenetic tree constructed on the basis of multiple alignment of 16-23S rRNA sequences allowed grouping the strains into three major clades (Fig. 1): clade A (PCR-RFLP profiles *a*/*a`*), clade B (profile *b*) and clade C (profiles *c*/*c`*), which included 98.4% of sampled population. The remaining strains displayed profile *d*, and formed the fourth group D.

The frequency of appearance of strains belonging to A, B, C and D groups in the nodules of individual pea plants varied (Fig. 2). The most abundant nodule inhabitants were strains of A (42 ± 11% of colonized nodules) and C groups (35 ± 16%), whereas strains from B group were found less frequently (20 ± 17%). The strains of A, B and C groups were recovered from all the sampled plants, while the strains of D group were found in nodules of only one of the plants (Fig. 2).

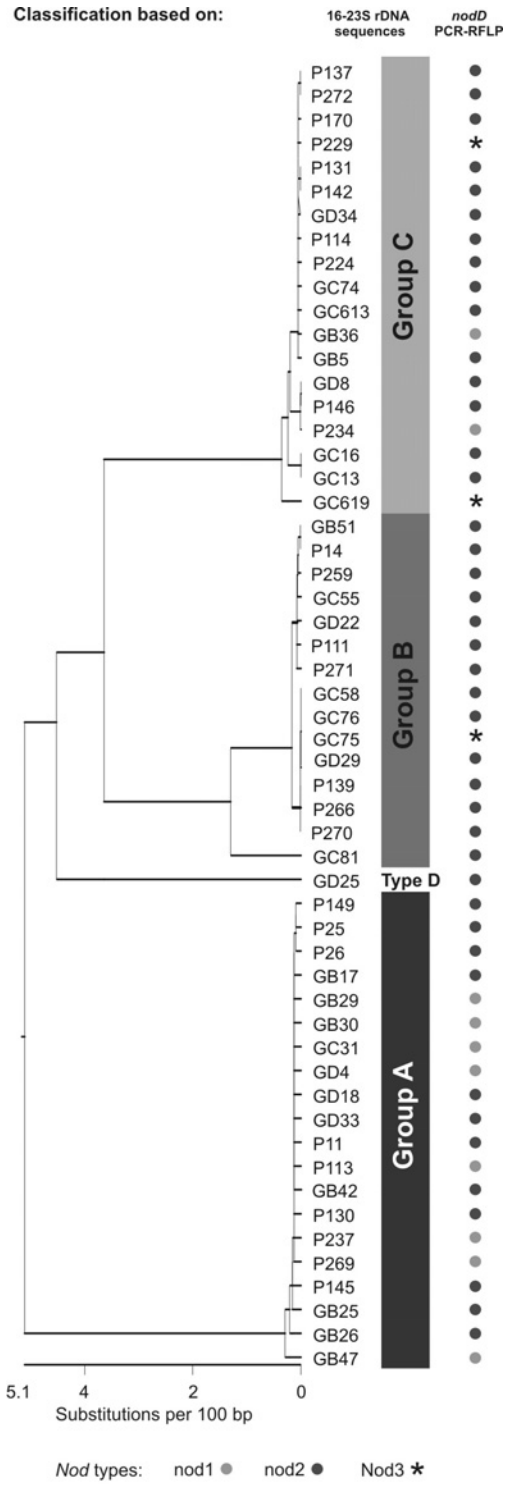

Fig. 1. UPGMA based dendrogram constructed with sequences of 16S-23S rRNA ITS for selected 55 pea nodule isolates

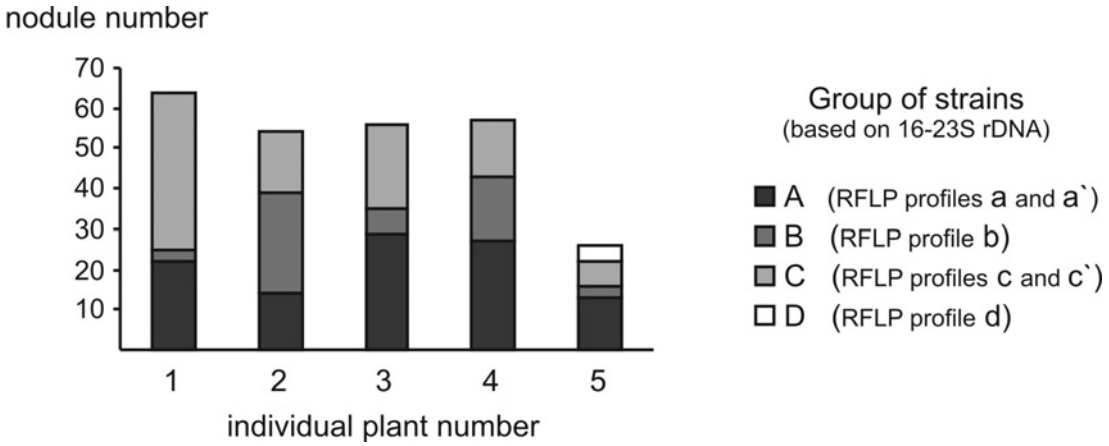

Fig. 2. Colonization of nodules of individual pea plants by rhizobial isolates classified into different 16-23S rRNA groups

Grouping of the 55 representative isolates based on 16-23S ITS rDNA sequences was further supplemented with PCR-RFLP of *nodD* gene. Three distinct PCR-RFLP types were obtained after *Bsu*RI digestion of *nodD* amplicons, named nod1, nod2 and nod3 (details not shown), which were found at different frequencies in the studied population (20%, 75% and 5%, respectively). Some correlation between the occurrence of 16-23S rDNA types and *nod* type was shown, i.e., nod1 type was preferentially coupled with 16-23S rDNA type A (Fig. 1). According to the classification of strains based both on 16-23 ITS and *nodD* gene PCR-RFLP, four most numerous groups could be distinguished within the 55 sampled strains: A-nod1, A-nod2, B-nod2 and C-nod2 (16%, 20%, 25% and 27% of population, respectively).

The comparison of our sequences with the reference ones derived from *Rhizobium leguminosarum* bv*. viciae* 3841, *R. fabae* CCBAU33202 and *R. pisi* (GenBank accession numbers AM236080, FJ392873 and DQ200174, respectively) (Ramirez-Bahena et al., 2008; Tian et al., 2008; Young et al., 2006) revealed that isolates of our cluster A are the closest relatives of *Rlv,* whereas those of cluster B are the closest relatives of *R. fabae*. A relative of group C isolates was not found, and *R. pisi* was the most divergent form all used sequences (details not shown). The reliable defining of taxonomic status of described isolates would require an extensive genetic, biochemical and physiological studies (see Tian et al., 2008), and might be an interesting future task. However, irrespective of precise taxonomic status of our isolates, their appreciable genetic and physiologic diversity illustrates the level of heterogeneity of single rhizobial population, as well as it demonstrates the complexity of symbiotic interactions between individual plants and particular rhizobial strains.

3.2 Diversity of Plasmid Profiles

The PFGE analysis of HMW DNA of 55 selected strains revealed the presence of 2 to 5 plasmids. In each of the isolates one of the plasmids was identified as pSym by Southern hybridization with *nodA* probe (Fig. 3).

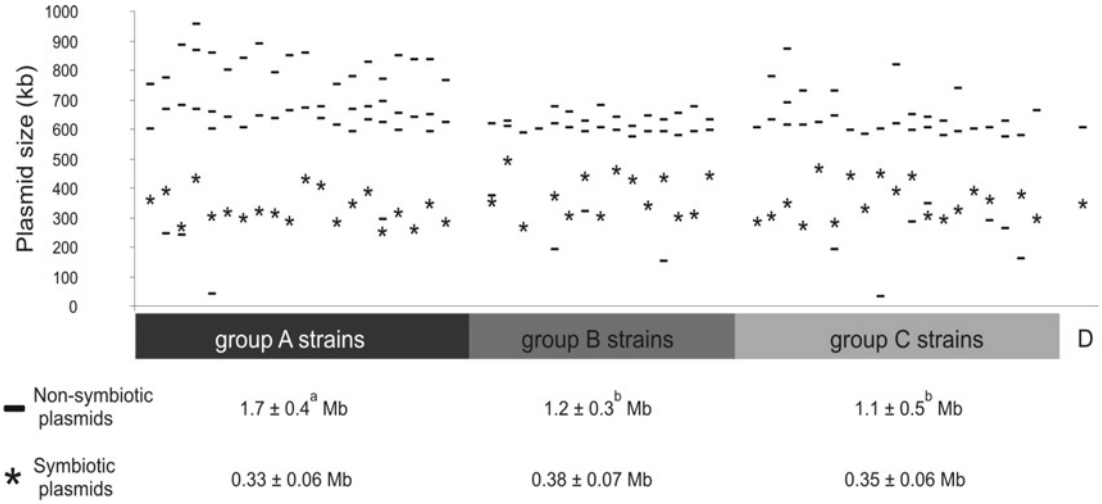

Fig. 3. Schematic representation of the PFGE separated plasmid profiles of 55 selected strains

Values below the picture are mean (± SD) of the summarized size of non-symbiotic plasmids and mean (± SD) size of pSym for three identified classes of pea microsymbionts. Values marked with different letters differ significantly at p<0.05.

The molecular weights of symbiotic plasmids were similar (349 ± 64 kb on average), whereas the size of the other plasmids varied over a wide range, from 40 kb to 954 kb. Strains belonging to group A contained significantly more DNA in the plasmid pool than groups B and C (2.1 ± 0.4 Mb vs. 1.5 ± 0.4 Mb and 1.5 ± 0.4 Mb, respectively; $p<0.05$), as a result of greater plasmid size as well as higher number of other plasmids (Fig. 3).

The considerable variability of plasmid profiles found in the sampled *Rhizobium* population (Fig. 3) is a good illustration of the commonly observed dynamic state of rhizobial genomes resulting in their diversification. Detailed analysis of the variability of plasmid DNA content showed that the strains of group A contained significantly more DNA in a plasmid pool, having from 3 to 5 plasmids. The pSym plasmids were similar in size regardless of genetic class, whereas the DNA content and number of other plasmids was significantly greater in *Rlv* group A than in other groups (Fig. 3). Upon investigation of the divergence of the symbiotic part of the genome by PCR-RFLP analyses and sequencing of *nodD*, three classes were found, with nod2 predominating. The nod1 class was very similar to nod2 (with number of nucleotide substitutions not exceeding 2 per 100 bp), whereas the rare nod3 class was most divergent (details not shown). The dominance of nod2 and nod1 in all 16-23S rRNA groups might support the hypothesis of a common origin of pSym in most of tested strains. Considering the fact that the sampled strains originated from one site, it is plausible that they might be descendants of three (A, B, C) lineages, which long ago acquired a single type of transferable pSym (Broughton et al., 1987; Souza et al., 1992). Accumulation of point mutations may have caused differentiation of nod1 and nod2 in the cluster A background, and group C-nod1 strains might originate from a relatively recent pSym transfer. To confirm this hypothesis sequencing of large number of pSym plasmids or extensive PCR-based analyses for tracking plasmid transfer in such rhizobial populations is needed. However it is plausible, considering the pangenomic-type approach to study rhizobial genomes, which showed very conserved sequence of symbiotic genes despite divergence of the remaining parts of the genomes (Gonzalez et al., 2010).

3.3 Correlation between Genotypes and Metabolic Capabilities of Isolates

It was assumed that isolates classified into different genetic groups and extremely different with respect to plasmid profiles might also vary in their metabolic properties. Subsequently, the metabolic variation might further reflect adaptive and competitive abilities of strains. The metabolic potential of 55 genetically distinct representative isolates was assayed by using commercial Biolog GN2 MicroPlate™ test. The obtained results revealed that the number of different carbon and energy sources metabolized by individual isolates varied from 24 (strain GB47) to 52 (strain P139) (details not shown). Moreover, strains from group A metabolized significantly ($p<0.05$) less substrates (33 ± 8) than strains from group B and C (41 ± 7 and 35 ± 9, respectively) (Table 1).

Table 1. Biolog`s substrates utilization by clusters of pea microsymbionts

Group of strains	Number of utilized substrates	Percentage of utilized substrates in PCA groups		
		PC1	PC2	PC3
A	33 ± 8 a	17 ± 10 a	53 ± 12 a	28 ± 7 a
B	41 ± 7 b	25 ± 13 a	66 ± 7 b	29 ± 9 a
C	35 ± 9 ab	21 ± 12 a	57 ± 10 ab	32 ± 7 a

a, b – values marked with the same letter did not differ at p<0.05

The results of Biolog test were subjected to principal component analysis (PCA) to identify which group (or groups) of nutrients is responsible for most differences in substrate utilization by groups of strains. A total 95 carbon and energy sources used in the test were arbitrarily divided into nine groups: monosaccharides [S], complex saccharides [cS], modified saccharides [mS], non-modified acids [A], modified acids [mA], sugar acids [sA], non-modified aminoacids [AA], modified aminoacids [mAA] and others (with amines predominating) [O]. The PCA analysis enabled grouping all the mentioned carbon and energy sources into three factors explaining most of the total variance: principal component 1 (PC1) composed of A, sA and AA (total number of substrates equal to 40); principal component 2 (PC2) composed of S, cS, mS and mAA (41 substrates); and principal component 3 (PC3) including mA and O (14 substrates). Considering the PC1, PC2 and PC3 groups of carbon/energy substrates, the percentage of utilization of substrates belonging to these groups were estimated for the sampled strains (Table 1).

According to the obtained data, two large groups of substrates differentially utilized by the sampled rhizobia were distinguished. PC1 was interpreted as “non-sugar compounds” (as it included A, sA and AA) or “seldom utilized substrates” (due to mean utilization value equal to 21%), and PC2, which was interpreted as “sugar compounds” (due to it including mainly sugars: S, cS and mS) or “commonly utilized substrates” (mean utilization value equal to 59%). Significant differences in metabolic potential were found between strains belonging to groups A and B with respect to utilization of sugar compounds (PC2) (Table 1).

In addition to Biolog tests, the growth of the studied strains was examined on media containing soil extracts prepared from the soil after cultivation of vetch, pea, red clover, bean, grass and herbicide fallow. There were no differences in the growth of strains on media based on soil extracts from herbicide fallow and ones prepared from soil after cultivation of red clover, bean or grass. Unlike this, group A strains grew slightly better than group C strains on media with the extract of soil after pea cultivation (OD_{550} of 0.23 ± 0.04 and 0.20 ± 0.05, respectively), and slightly better than group B strains on the soil extract

from vetch cultivation (OD_{550} of 0.22 ± 0.04 and 0.20 ± 0.04, respectively). Despite the low magnitude of differences, they were significant at $p<0.05$.

In our previous work we demonstrated that competitiveness of rhizobia might be positively correlated with the number of different carbon substrates utilized by rhizobial strains (Wielbo et al., 2007). Strains of group A utilized smaller number of the substrates than strains from B and C groups, however, significant differences concerned only sugar substrates, which seem to be less important in competition (Wielbo et al., 2007). Moreover, cluster A strains grew slightly better on plant-derived soil extracts, and this might compensate for the lower metabolic potential measured in Biolog test. Plant exudates contain numerous and very diverse components, such as sugars, acids, aminoacids, amines and many other low molecular weight compounds, which may affect the growth and metabolism of microorganisms (Bertin et al., 2003; Gaworzewska and Carlile, 1982; Knee et al., 2001; Philips and Kapulnik, 1995; Teplitski et al., 2000) but it is not possible to distinguish between the effects of particular compounds on the microsymbionts. Moreover, it should be mentioned that OD measurements are not direct measurements of the number of bacterial cells, however, such approach is often applied to demonstrate the differences in growth of rhizobia (Daniels et al., 2002; Krishnan et al., 2007). Regardless of this, both types of metabolic tests (Biolog or growth) revealed metabolic differences between identified clusters of pea microsymbionts, dissecting group A from two others.

3.4 Correlation between Genotypes and Symbiotic Performance of Strains

Plant tests examining the symbiotic performance of the isolates in symbiosis with vetch were conducted under sterile conditions (Fig. 4).

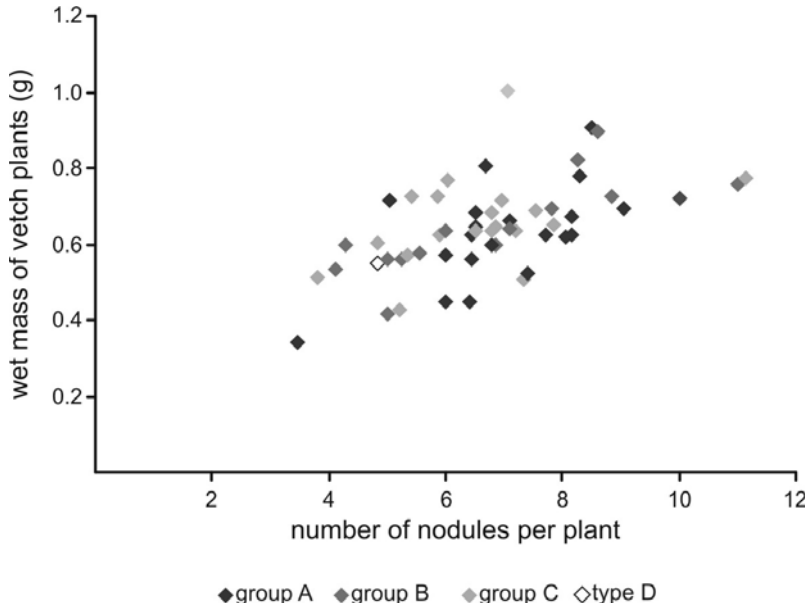

Fig. 4. The number of nodules and wet mass of vetch plants inoculated with selected strains

Each point on the plot represents one experimental group – plants inoculated with one strain.

Plants inoculated with group A-nod1 strains revealed significantly lower wet shoot masses than plants inoculated with strains of group C-nod2 (412 ± 86 mg and 483 ± 95 mg, respectively, $p<0.05$). The two other frequent groups, A-nod2 and B-nod2, demonstrated intermediate values of symbiotic parameters (details not shown).

The assay of symbiotic efficiency of rhizobia revealed the prevalence of low-effective strains in rhizobial nodule isolates, which was observed both in our previous work (Wielbo et al., 2010b) and in the currently studied pea population, in which numerous strains of group A-nod1 demonstrated the smallest beneficial effect on plant growth. This may be not without reason, because the final result of a symbiotic infection depends on several factors. The plant host controls the level of nutrients supplied to the nodules (Denison and Kiers, 2004; Prell and Poole, 2006) and this may be the means for supporting the effective rhizobial strains more than the ineffective ones. On the other hand, there is some evidence that rhizobial strains compete with each other not only in the rhizosphere but also in the infection threads and in the nodules, and the individual nodules can be inhabited by more than one strain (Duodu et al., 2009; Stuurman et al., 2000; Wielbo et al., 2010a). Since the plant host is not able to suppress the growth of low-effective strains in the case of a multi-strain infection of the nodules, the presence of numerous low-effective strains in plant nodules is not surprising.

4. CONCLUSION

In our previous work (Wielbo et al., 2010b), a substantial genetic and metabolic diversity of a local population of *R. leguminosarum* bv. *trifolii* (*Rlt)* was demonstrated. This was stipulated to be an effect of bacterial adaptation to the complex rhizosphere environment, which is influenced by microbial and plant activities, as well as management practices (Girvan et al., 2003; Martinez-Romero, 2009; Mutch and Young, 2004; Palmer and Young, 2000; Rangin et al., 2008). In this study, genetic and metabolic diversification of *Rhizobium* isolates originating from pea plants growing in the same site in cultivated soil has been described.

Concluding, the substantial genetic and physiological diversity of the individual isolates was recorded in the sampled pea nodule isolates, and it is noteworthy that individual pea plants are able to establish symbiosis with so numerous and diverse microsymbionts. Simultaneously, this differentiated rhizobial population formed only three (A, B, C) well-separated each other clusters. Group A differed from two others on the level of 16-23S rRNA sequences, the plasmid DNA content and frequency of *nodD* PCR-RFLP profiles. The genetic distinction of strains included to group A was also followed by their different metabolic properties, i.e., lower number of utilized sugars, different growth on pea- and vetch-derived soil extracts, and lower symbiotic efficiency. Taken together, our present work shows that sampled pea nodule isolates do not seem mixture of random, unrelated strains. Despite large diversity of the individual strains, plausibly resulting from the genomes' plasticity, cohabitation and competition between numerous strains in the changeable soil environment, this population has their own characteristic structure with clearly distinguishable sub-populations. As demonstrated by 16-23S ITS region sequencing and plasmid profiles, strains belonging to distinct sub-populations might be a diversified descendants of a few old lineages, which diversified in the lapse of time.

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