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Nodulation Competitiveness of *Ensifer meliloti* Alfalfa Nodule Isolates and Their Potential for Application as Inoculants

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Abstract

Alfalfa (*Medicago sativa*) is a widely cultivated legume, which enters into nitrogen-fixing symbiosis with *Ensifer* (*Sinorhizobium*) spp. In this study, an autochthonous rhizobial population of *Ensifer* sp. occupying alfalfa nodules grown in arable soil was used as the basis for selection of potential inoculants. Alfalfa nodule isolates were identified as *Ensifer meliloti* by partial 16S rDNA, *recA*, *atpD* and *nodC* nucleotide sequencing. The sampled isolates displayed different symbiotic performance and diversity in the number of plasmids and molecular weight. Isolates that were the most efficient in symbiotic nitrogen fixation were tagged with a constitutively expressed *gusA* gene carried by a stable plasmid vector pJBA21Tc and used in competition experiments in soil under greenhouse conditions. Two *E. meliloti* strains LU09 and LU12, which effectively competed with indigenous soil rhizobia, were selected. The metabolic profiles of these selected strains showed differences in the use of carbon and energy sources. In addition, the LU09 strain exhibited bacteriocin production and LU12 mineral phosphate solubilization, which are valuable traits for soil survival. These strains may be considered as potential biofertilizers for alfalfa cultivation.

K e y   w o r d s: biofertilizers, biological nitrogen fixation, competition, *Ensifer*/*Sinorhizobium*, symbiotic efficiency

Introduction

Biological nitrogen fixation is a global process that enriches soil in reduced nitrogen available for plants. The most productive in nitrogen fixation are symbiotic systems i.e., legume crops with their bacterial microsymbionts, which may fix up to a few hundred kg N per $10^4\,m^2$ per year, so they are very important elements of modern sustainable agriculture (Brockwell *et al*., 1995; Herridge *et al*., 2008). Early steps in the establishment of symbiosis include attachment of bacteria to root hairs, exchange of molecular signals (usually plant flavonoids and bacterial Nod factors) between rhizobia and the plant host, and induction of morphological changes in plant root tissues, which lead to formation of nodules on plant roots (Perret *et al.,* 2000; Jones *et al*., 2007). In these organs, physiologically distinct bacteroids surrounded by plant-derived peribacteroid membranes fix dinitrogen into forms useable for plants. The sequential stages of symbiosis are controlled by a set of bacterial *nod* genes responsible for nodulation and *nif-fix* genes involved in nitrogen fixation, and several plant host specific genes (Masson-Boivin *et al*., 2009). The bacterial genes are clustered on the symbiotic plasmid (pSym) or the chromosome in a region called a symbiotic island (Palacios and Newton, 2005).

A hallmark of *rhizobium*-plant symbioses is their high specificity, possibly being the evolutionary consequence of the high level of diversity of both rhizobia and legume species; e.g. *Rhizobium leguminosarum* bv. *trifolii* nodulates only clover (*Trifolium*), *R. leguminosarum* bv. *viciae* – peas (*Pisum*) and vetch (*Vicia*), *R. etli* – bean (*Phaseolus*) and *Ensifer meliloti* – alfalfa (*Medicago*)*,* and sweet clover (*Melilotus*) (Martinez-Romero and Caballero-Mellado, 1996). In some cases, the specificity of symbiosis may be disadvantageous, because legume plants become non-nodulated and nitrogen source-deprived when there are no specific microsymbionts in the rhizosphere. In such cases, the application of an efficient and competitive rhizobial biofertilizer is recommended for achieving satisfactory cultivation results (Brockwell *et al*., 1995).

The high level of genetic and physiological diversity of soil rhizobia has been described even within the same species or biovar (Laguerre *et al*., 1996; Wielbo *et al*., 2010a). In addition, rhizobial populations displaying genetic polymorphism differ in nitrogen fixation efficiencies, sensitivity to detrimental environmental

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factors, or competitive nodulation ability (Depret *et al.*, 2008). The population of soil rhizobia specific to the plant host is usually a mixture of genetically different strains. On the other hand, legume roots have several nodulation zones susceptible to rhizobial invasion (Calvert *et al.*, 1984; Mathesius *et al.*, 2000), so one plant can be nodulated by more than one strain (Wielbo *et al.*, 2010b). The genetically diverse strains occupying one nodule may significantly differ in their ability to metabolize carbon and energy sources and N_2 fixation efficiency (Simms and Taylor, 2002). Competition for nodulation of the host legume by resident rhizobial populations in soil represents a serious problem affecting the establishment of efficient nitrogen-fixing inoculants (Brockwell *et al.*, 1995). In many cases, selected, highly effective rhizobial strains used as plant inoculants are less competitive than autochthonous strains, and are outnumbered by the soil population (Streeter, 1994). Taken together, there is a need for further screening of soil population to identify highly efficient and competitive rhizobia that might be used as inoculants for leguminous plants.

Medicago sativa is infected mostly and efficiently with *E. meliloti* and less frequently but efficiently, with *E. medicae* or *Phyllobacterium* sp. and *Rhizobium* sp. (Silva *et al.*, 2007; Bromfield *et al.*, 2010). According to published data, alfalfa (*M. sativa*) microsymbionts have been found as less numerous compared with other rhizobial species in Polish soils (Martyniuk *et al.*, 2000; Martyniuk *et al.*, 2005). Therefore, there is a demand for selection of *Ensifer* spp. from a particular environment to use them as potential inoculants to increase alfalfa crop productivity.

The aim of this work was to select *E. meliloti* strains exhibiting high efficiency in nitrogen fixation with a competitive ability for nodule occupancy in relation to autochthonous rhizobia under greenhouse conditions. Metabolic profiles of the sampled *E. meliloti* strains were characterized. Two selected *E. meliloti* strains may be considered as inoculants in alfalfa field cultivation in Poland.

Experimental

Materials and Methods

Bacterial strains. *Ensifer* spp. isolates derived from root nodules of alfalfa (*Medicago sativa* cv. Radius) grown in arable soil in the region of Lublin, Poland. Nodules were surface sterilized by immersion in 0.1% $HgCl_2$ for 1 min, rinsed with sterile distilled water, then treated with 70% ethanol for 1 min, followed by extensive washing with sterile water. Suspensions from crushed nodules were grown and maintained in 79CA medium (Vincent, 1970). Rhizobia isolated from nodules were purified by successive isolation of single colonies grown on the same medium and used in further experiments.

The number of alfalfa microsymbionts in the soil was estimated by a most probable number plant infectivity test similar to described by Martyniuk *et al.*, (2000). Briefly, seeds were surface sterilized and sown in plastic pots containing 600 g of washed and sterilized sand, once watered with 150 ml N-free Fahraeus medium. Soil sample (10 g) was mixed with 90 ml of sterile water, and after sedimentation of solid phase tenfold dilutions of liquid phase (10^{-2}–10^{-6}) were prepared. 1 ml aliquots of these dilutions were used for inoculation of pots with alfalfa seeds, and for each dilution three pots were prepared. After emergence of seedlings, plants were randomly thinned and five alfalfa plants were left in each pot. Plants were grown for six weeks, and then the presence of root nodules was examined. A pot was scored as "positive" when root nodule/nodules were present on any root in the pot and as "negative" when none of plants grown in one pot were nodulated. The number of alfalfa microsymbionts present in soil was estimated according to the number of pots containing nodulated plants for each dilution of soil extract and using special tables (Brockwell, 1982).

DNA analyses. Plasmid content of the isolates was analyzed by the method of Eckhardt (1978). Plasmid size estimation was performed using Bio-Profile V11.01 (Vilber-Lourmat, France) by comparison with *R. leguminosarum* bv. *viciae* 3841 standard plasmids (Young *et al.*, 2006).

Genomic DNA of seven selected most efficient isolates was extracted from 5 ml of 2-day cultures in liquid 79CA medium using method of Pitcher *et al.*, 1989. The primers used for BOX-PCR, ERIC-PCR, amplification and sequencing the genes encoding 16S rRNA, *recA*, *atpD* and *nodC* are described in Table I. BOXA-PCR and ERIC-PCR genomic fingerprints were obtained as described by Louws *et al.*, 1994 and Versalovic *et al.*, 1991. The sequences were aligned with those from GenBank using the MEGA5.05 software package (Tamura *et al.*, 2011). The distances were calculated according to the Kimura's 2-parameters (Kimura, 1980). Phylogenetic trees were inferred using neighbor-joining method (NJ). Bootstrap analyses were calculated based on 1000 replications (Felsenstein, 1985). Chromosomal 16S rRNA, *recA*, *atpD* and *nodC* gene sequences determined in this study have been deposited in the EMBL database, and the GenBank under following accession numbers: KJ652948 – KJ652952, EU182657 (16S rRNA), KJ847190 – KJ847195 (*nodC*), KM096990 – KM096993, KJ847188, KJ847189 (*recA*), KJ847196 – KJ847200, KJ825827 (*atpD*).

Symbiotic performance test of *Ensifer meliloti* nodule isolates in sterilized sand. Alfalfa seeds were

Table I
Primers used in this study

Gene	Primer	Sequence 5'–3'	Annealing temperature	Amplicon length (bp)	References
atpD	atpD294	ATCGGCGAGCCGGTCGACGA	58°C	460	Gaunt *et al.* 2001
	atpD771	GCCGACACTTCCGAACCNGCCTG			
16S rRNA	fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	55°C	1299	Weisburg *et al.* 1991
	rD1	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC			
recA	recA6f	CGKCTSGTAGAGGAYAAATCGGTGGA	56°C	533	Aoki *et al.* 2010
	recA640r	ACATSACRCCGATCTTCATGC			
nodC	nodCmelF	TTCAATGAGGACCCAGGCATCCTCTC	56°C	915	This study
	nodCmelR	GCTTTCCCCCGCTGACTGGTAC			
	BOX 1AR	CTCCGGCAAGGCGACGCTGAC	53ºC		Louws *et al.* 1994
	ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	52ºC		Versalovic *et al.* 1991
	ERIC 2	AAGTAAGTGACTGGGGTGAGCG			

surface sterilized and sown in pots filled with 400 g washed, sterilized sand and supplemented with 100 ml N-free Fahreus medium (Vincent, 1970). Individual 18 isolates were grown with shaking in 79CA medium for 48 h at 28°C, centrifuged and the bacterial pellet was suspended in sterile water. 3 ml of cell suspension (OD_{550} 0.1, approx. 10^8 cells ml^{-1}) was added to each pot. Plants (10 seedlings per pot, in triplicate) were grown in a greenhouse under natural light supplemented with artificial light (14 h day/10 h night, at 24/19°C) and were periodically watered with sterile water. After 6 weeks, the plants were harvested, weighted and root nodules

were counted. All plants from one experimental group were collected, dried at 65°C for 48 h, weighted and mean dry mass of shoots was calculated after dividing the whole mass of dried shoots by a number of plants. The six most efficient growth-promoting isolates (as assayed by weighing the green parts of plants) were chosen for further experiments (Table II).

Tagging of rhizobia with *gusA* gene. To study an ability to nodule occupancy and competitiveness, six most efficient in nitrogen fixation nodule isolates were tagged with a stable plasmid vector pJBA21Tc carrying a constitutively expressed *gusA* (Wielbo *et al.*, 2007).

Table II
Symbiotic performance of alfalfa nodule isolates grown in sterile sand

Isolate	Fresh mass of shoots* (mg)	Dry mass of shoots* (mg)	Relative fresh mass of shoots (uninoculated control plant = 1)	Number of nodules per plant*
LU02	186 ± 26	33	4.13	6.8 ± 2.9
LU04	202 ± 39	36	4.5	8.3 ± 8.3
LU05	165 ± 27	30	3.75	13.5 ± 6.2
LU06	182 ± 24	32	4.0	11.9 ± 4.8
LU07	161 ± 36	26	3.25	9.7 ± 5.6
LU08	180 ± 28	28	3.5	12.0 ± 6.1
LU09	217 ± 18	39	4.88	12.6 ± 5.8
LU10	216 ± 62	38	4.75	9.6 ± 4.9
LU11	200 ± 27	36	4.5	10.7 ± 3.2
LU12	194 ± 35	36	4.5	12.0 ± 5.8
LU15	116 ± 45	23	2.88	6.8 ± 2.4
LU17	134 ± 21	23	2.88	6.4 ± 1.0
Uninoculated alfalfa grown on sand with Fahraeus medium supplemented with 20 mM NH$_4$Cl	175 ± 30	29	3.63	–
Uninoculated alfalfa grown on sand with N-free Fahraeus medium	57 ± 19	8	1.0	–

* Values are mean of 30 plants. ± standard deviation

The pJBA21Tc plasmid was introduced into rhizobia by electroporation (1800V, 200Ω, 50μF) as described previously. After electroporation, rhizobia were selected on 79CA agar medium supplemented with 10 μg ml⁻¹ tetracycline and 50 μg ml⁻¹ X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide) substrate for β-glucuronidase. Stability of plasmid pJBA21Tc in rhizobia was confirmed earlier (Wielbo and Skorupska, 2001).

E. meliloti competition assay in sterile N-free medium. Alfalfa seeds were surface sterilized, germinated, and grown on Fahraeus agar medium slants (1 seedling/tube) (Vincent, 1970). 5-day-old seedlings were inoculated with a mixture (1:1 v/v) of *gusA* marked and unmarked *E. meliloti* isolates. A 200 μl cell suspension at an OD_{550} 0.1 was added to each slant. Plants were cultured for 6 weeks, harvested and weighted. Alfalfa roots were stained for β-glucuronidase (Gus) activity in 50 mM sodium phosphate buffer (pH 7.2) with 50 μg ml⁻¹ 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlcA), 0.1 mM EDTA, 0.38 mM $K_3Fe(CN)_6$ and 0.38 mM $K_4Fe(CN)_6$ for about 1 h at room temperature (Wilson *et al.*, 1995). The number of stained (blue) and unstained (white) nodules colonized by tagged and non-tagged strains were counted.

In experiment 1, non-sterilized alfalfa seeds were soaked 15 min in a water suspension of respective *E. meliloti* isolate marked with pJBA21Tc*gusA* (OD_{550} 0.1) and sown in the pots. Twenty alfalfa plants were grown per pot filled with 1500 g of non-sterile soil (N:P:K ratio: 0.16% w/w N: 14.2 mg P_2O_5: 12.8 mg K_2O).

In experiment 2, non-sterilized alfalfa seeds were sown in the pots with the same soil and inoculated with suspension of respective *E. meliloti* isolate tagged with pJBA21Tc *gusA* with defined increasing number of rhizobia, *i.e.* 10⁸, 10⁹ or 10¹⁰ per pot. Alfalfa plants were grown in a greenhouse under natural light supplemented with artificial light (14 h day/10 h night, at 24/19°C) and were periodically watered with water. After 6 weeks, whole roots of alfalfa plants were harvested, washed with sterile water and stained for β-glucuronidase (Gus) activity. Competitive potential of the tested inoculants was estimated by comparing the number of blue nodules colonized by the introduced *gusA*-tagged strains with white nodules colonized by autochthonous strains.

Biolog phenotypic assay. To study metabolic activity of *E. meliloti* isolates Biolog GN2 MicroPlate™ (Gram Negative Identification Test Panel) (Biolog, Hayward, U.S.A.) was used. Briefly, rhizobia grown overnight at 28°C on 79CA agar medium were collected and washed twice with sterile water. Next, the pellet was diluted in water to an initial OD_{550} of 0.1 (approximately 10⁸ cells ml⁻¹) and 150 μl of the rhizobial suspension was inoculated into each of 95 wells of the GN2 microplate. The plates were incubated for 72 h at 28°C and colour development (absorbance at 590 and 750 nm) in the wells was recorded using a Benchmark Plus microplate reader (Bio-Rad Laboratories, U.S.A.). The conversion of colourless tetrazolium violet to a purple-coloured compound meant a normal process of respiration (positive phenotype), whereas, when the phenotype was negative, the wells remained colourless. The optical density values of the Biolog microplate wells were corrected using background colour developed in the control well.

Solubilization of phosphates assays and bacteriocin production assay. *E. meliloti* strains were grown on 79 CA medium supplemented with 5 g l⁻¹ $Ca_3(PO_4)_2$ for 48 h at 28°C and diameter of cleared zones around bacterial colonies were measured. The width of cleared zones was assumed as proportional to the ability of rhizobia to solubilization of $Ca_3(PO_4)_2$. The ability to bacteriocin production was tested on two layer plates in 79 CA medium and *Ensifer meliloti* L5-30 was used as bacteriocin-sensitive indicator strain.

Data statistical analysis. Statistical analysis was carried out by the analysis of variance and Tukey test (significance level of $P < 0.05$) with STATISTICA for Windows software (StatSoft Inc., Tulsa, U.S.A.).

Results

Characteristics of the *Ensifer* sp. alfalfa nodule isolates. The number of microsymbiont population in soil originating from the local site of alfalfa (*M. sativa*) cultivation was estimated by a most probable number plant infectivity test in the growth pouches and calculated as 4.4×10^4 cells g⁻¹, which allowed us to classify this soil as rich in these bacteria. Alfalfa microsymbionts were isolated from nodules of alfalfa plants grown in the soil. Eighteen randomly chosen *Ensifer* sp. nodule isolates were assayed for the content of plasmids in order to eliminate identical strains. On the basis of plasmid content screening, a very high level of diversity among the isolates was observed. Twelve of the 18 isolates had distinct profiles of plasmids and these were considered as different strains. Each of the twelve isolates possessed from 1 to 4 plasmids with molecular weight in the range from ca. 40 kb to above 1 Mb: six of the twelve strains possessed a very large megaplasmid (approx. 1 Mb) (Fig. 1).

To select potential *Ensifer* sp. inoculants, twelve strains differing in the pattern of plasmids were tested for nodulation and capability of plant growth promotion on *M. sativa* grown in sterile sand supplemented with N-free medium. The sampled isolates varied in the symbiotic activity, *i.e.* fresh and dry shoot mass and the number of nodules per plant (Table II). They promoted alfalfa growth measured by fresh mass of shoots in the range from 2.03 to 3.8 times, and dry mass from
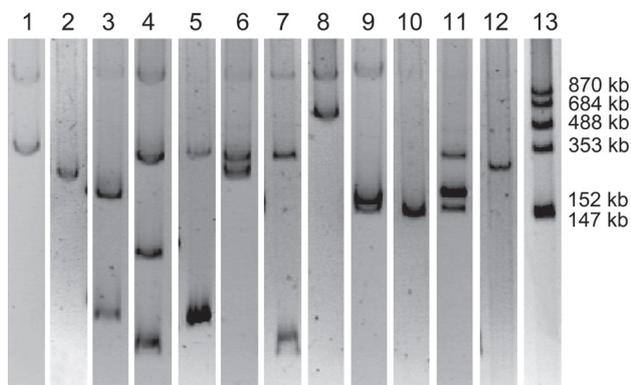
Fig. 1. Plasmid profile of the selected rhizobial strains isolated from alfalfa nodules. The plasmids were visualized by the Eckhardt method. Lanes 1 – 12, *E. meliloti* strains: LU02, LU04, LU05, LU06, LU07, LU08, LU09, LU10, LU11, LU12, LU15, LU17, respectively; lane 13 – plasmids of *R. leguminosarum* bv. *viciae* strain 3841 used as a molecular weight standard.

2.88 to 4.88 in relation to uninoculated control plants grown on sterile sand with N-free medium. The growth of alfalfa inoculated with the selected isolates was also better than that of non-inoculated plants growing on sand with Fahreus medium supplemented with 20 mM ammonium chloride. Six symbiotically most efficient strains (LU04, LU06, LU09, LU10, LU11, LU12) were chosen for further competition experiments.

Another parameter used to evaluate rhizobial efficiency is the number of nodules per plant; however, this value usually does not correlate with the level of nitrogen fixation. In our experiment, the most efficient isolates selected formed from 8.3 to 12.6 nodules per plant, which is almost two times more than the least efficient isolates (LU02, LU15, LU17) (Table II).

The genetic relationships between the six selected isolates were studied by generating genomic fingerprints using BOX-PCR and ERIC-PCR. Visual analyses of BOX- and ERIC- fingerprints showed similar patterns in more than a dozen bands of different intensities and indicated apparent relatedness among the isolates. In general, low differentiation of the isolates was observed with the use of these methods, despite substantial plasmid diversity in the various strains. Dendrogram obtained on the basis of two combined profiles showed LU10 and LU12 as most similar (90%) and LU09 as the most diverse (~68% similarity) strains (Fig. 2).

Since *M. sativa* enters into symbiotic relationships with species from the *Ensifer*, *Phyllobacterium* and *Rhizobium* genera (Bromfield *et al*., 2010), the phylogenetic position of the six selected isolates was examined by partial sequencing of the 16S rRNA gene (1299 bp). The *rrn* sequences of the six isolates were identical to each other (100%) and identical to several *E. meliloti* strains. To confirm the species identification, the *atpD* (460 bp), and *recA* (533 bp) housekeeping genes were sequenced and found to be identical (100%) to *E. meliloti* type strains (data not shown).

The *nod* genes involved in Nod factor synthesis are localized in *E. meliloti* on the pSymA symbiotic plasmid (Barnett *et al*., 2001) and generally have different phylogenetic history than chromosomal core genes (Laguerre *et al*., 2003; Young *et al*., 2006). The *nodC*, a key gene for Nod factor synthesis, encoding N-acetyl-glucosaminyl transferase is located in *E. meliloti* on the pSymA plasmid. The *nodC* genes of 6 alfalfa isolates were PCR amplified, sequenced, and used for construction of the phylogram (Fig. 3). In *nodC* tree, 5 of the 6 isolates were located in a separate, closely related cluster together with *E. kummerowiae* CCBAU71714 and *E. meliloti* AK83, 2011, 1021. The LU11*nodC* was more divergent but still 99% identical to *E. meliloti* SM11 and 96% identical to *E. meliloti* AK83 and other strains of this cluster. In conclusion, based on chromosomal *rrn, recA, atpD* and *nodC* plasmid gene sequences, the six alfalfa nodule isolates selected were identified as *E. meliloti*.
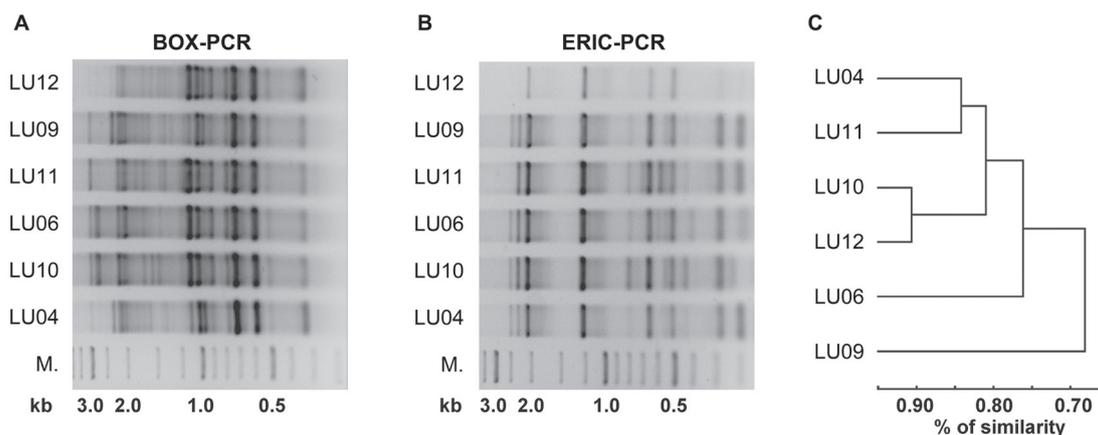

Fig. 2. A) BOX-PCR and B) ERIC-PCR fingerprint patterns from the genomic DNA of the microsymbionts isolates. The names of the isolates are shown on the left of the figures. M – the DNA molecular size marker in kb.
C) Cluster analysis-based dendrogram (UPGMA method) constructed on the basis of combined BOX- and ERIC-PCR-generated genomic fingerprints showing diversity of the selected *Ensifer* sp.
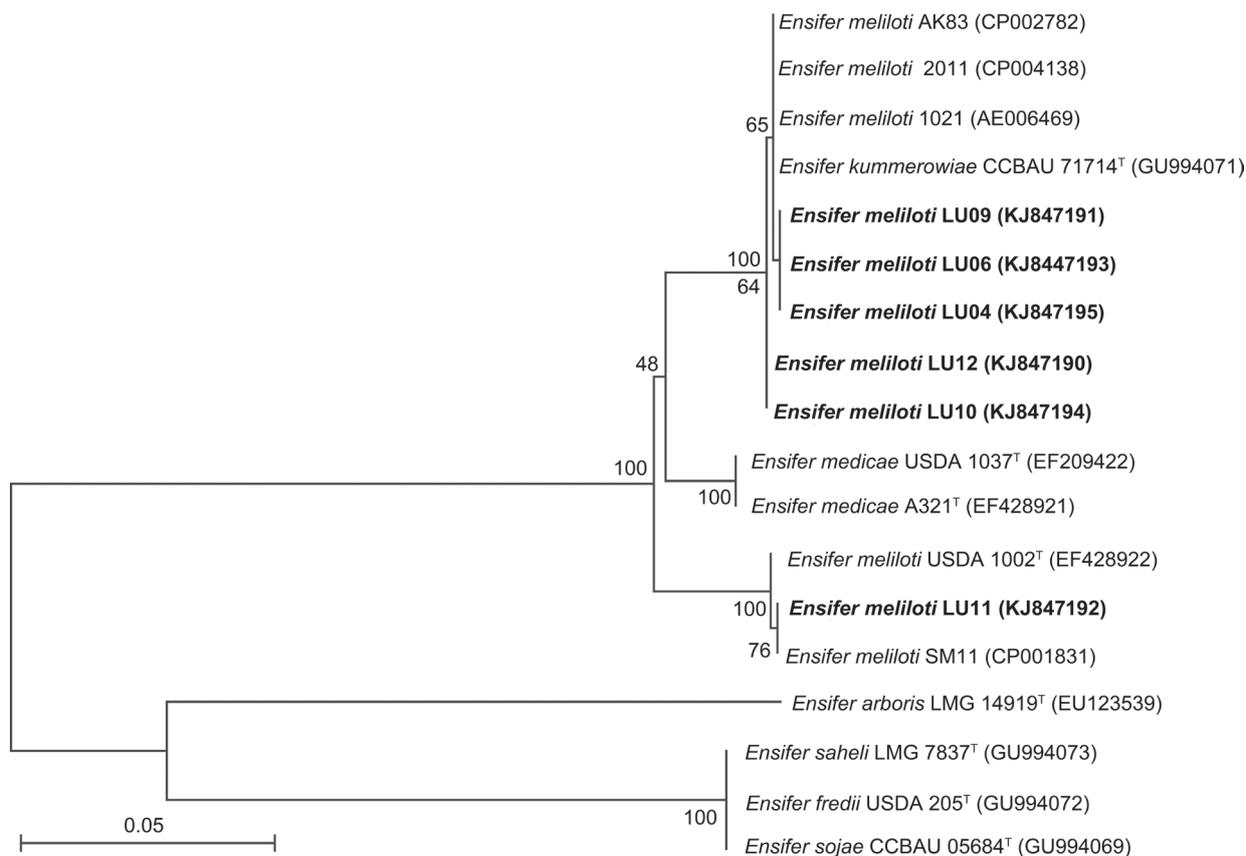
Fig. 3. Neighbor –joining (NJ) tree showing the phylogenetic relationship between *Ensifer* sp. isolates and related *Ensifer* species based on *nodC* gene sequences. Bootstrap values are given at the nodes. GenBank accession numbers are given in parentheses. Bar, nucleotide substitutions per nucleotide position.

Nodulation competitiveness of *E. meliloti* isolates. The ability to compete for host plant nodulation is a complex phenomenon and depends on genetic traits of microsymbionts and on soil and environmental factors (Triplett and Sadowsky, 1992). To study the nodulation competitiveness of the isolates and differentiate the sampled isolates from autochthonous rhizobia, the most efficient *E. meliloti* isolates (6 strains) were marked with *gusA* carried on a stable pJBA21Tc plasmid. First, a potential impact of *gusA*-tagging on the strain ability of nodule colonization was studied. Alfalfa seedlings grown on N-free agar slants were inoculated with a mixture of *gusA*-tagged LU04, LU09, and LU12

and the isogenic parental strains (Table III). As results, an equal number of Gus⁺/Gus⁻ nodules was found only in the case of LU09gusA/LU09 inoculation. The LU04gusA and LU12gusA-tagged strains were less competitive in relation to the parental strains but their ability of plant growth promotion was confirmed (Table III). In conclusion, *gusA*-tagging does not seem to affect substantially the nodule occupation ability under laboratory conditions.

In the next experiment, competitiveness of the six isolates in relation to resident soil rhizobia were investigated under competitive conditions in the soil (experiment 1). The *gusA*-tagged *E. meliloti* strains were

Table III
Symbiotic performance and alfalfa nodule occupancy of *E. meliloti* Gus⁺ versus Gus⁻ strains assayed under laboratory conditions. Alfalfa plants were inoculated with 1:1 mixture of *gusA*-tagged and parental, isogenic rhizobia and grown on N-free agar medium slants.

Strains	Green mass of shoots* (mg)	Mass of whole plant* (mg)	Number of nodules*	Percentage of GUS⁺/ GUS⁻ nodules
LU04gusA/ LU04	76.6 ± 23.3	117.6 ± 26.9	12.3 ± 7.6	35/65
LU09gusA/ LU09	81.6 ± 27.5	129.5 ± 27.4	12.7 ± 6.2	53/47
LU12gusA/ LU12	87.3 ± 21.3	126.2 ± 33.8	10.5 ± 5.3	39/61
Uninoculated control	25.6 ± 9.9	63.6 ± 20.4	0	–

* Values are mean of 20 plants. ± standard deviation.

Ensifer meliloti gusA – tagged strains	Gus⁺/Gus⁻ nodules* (%)			
	Experiment 1	Experiment 2		
	Seeds soaking with ~10^8 cells/ml/pot	number of rhizobia / pot		
		10^8	10^9	10^{10}
LU04*gusA*	60/40	25/75	36/64	46/54
LU06*gusA*	53/47	6/94	24/76	29/71
LU09*gusA*	56/44	23/77	27/73	55/45
LU10*gusA*	67/33	14/86	21/79	42/58
LU11*gusA*	38/62	25/75	60/40	87/13
LU012*gusA*	78/22	15/85	57/43	87/13

* In experiment 1 – alfalfa seeds were soaked with respective *E. meliloti gusA*-tagged suspension (OD$_{550}$ 0.1, approx. 10^8 cells ml⁻¹). In experiment 2 – defined numbers of particular rhizobia were added to pot with the soil. Total number of white and blue nodules in each of 20 plants from one pot was estimated and percentages of blue nodules were counted. Values are mean of GUS⁺ nodules of 20 plants.

introduced into soil through alfalfa seeds imbibed with rhizobia at a concentration of ~10^8 ml⁻¹ and 6 weeks after inoculation, colonization of nodules by marked and autochthonous rhizobia was examined (Table IV, experiment 1). In experiment 1, the Gus⁺ nodules were detected in the range from 38% (LU11*gusA*) to 78% (LU12*gusA*) of the total number of nodules. LU12gusA, LU10gusA, and LU04gusA were considered the most competitive in relation to soil resident rhizobia.

In experiment 2, the competitiveness of individual *E. meliloti* strains was studied as an effect of an increasing number of rhizobia added to the pots (Table IV, experiment 2). The percentage of Gus⁺ nodules in alfalfa was correlated with the increasing number of rhizobia added to the soil. LU11*gusA* and LU12*gusA* were shown to be the most competitive occupying up to 87% of nodules after inoculation with 10^{10} rhizobia per pot. Moreover, LU12 colonized the nodules efficiently in both experiments, giving a high percentage of GUS⁺ nodules irrespective of the method used for seeds inoculation. Similarly to LU12, the competitiveness of LU09gusA (about 55%) was very similar in both types of seed inoculation; however, it was lower than that of LU12. It was shown that alfalfa inoculation with a high number of bacteria (10^{10} cells/pot) gave comparable results to those obtained with soaking the seeds with a rhizobial suspension containing *ca.* 10^8 cells; however, from the practical point of view, seed imbibition may be more convenient because it requires a lower total amount of the inoculant (Table IV). Rhizobial competition for nodule occupation studied under competitive conditions once again occurred to be very complex and ambiguous. Overall, among the sampled *E. meliloti* alfalfa nodule isolates, two strains (LU09 and LU12) proved to be repeatedly effective competitors efficient in symbiotic nitrogen fixation.

Metabolic profiles of the sampled *E. meliloti* strains. The metabolic properties of rhizobia have enabled them to adapt to the specific, soil and plant tissue environments. The metabolic profiles of the six *E. meliloti* isolates were studied employing Biolog GN2 microplates containing 96 carbon and energy sources. To simplify the analysis, the substrates in the GN2 microplates were divided into sugars (polysaccharides, oligosaccharides, monosaccharides – comprising 34.4% of the tested compounds), modified sugar acids, carboxylic acids (29.2%), amino acids (21.9%), nitrogen bases and other compounds (15.5%) (Fig. 4).
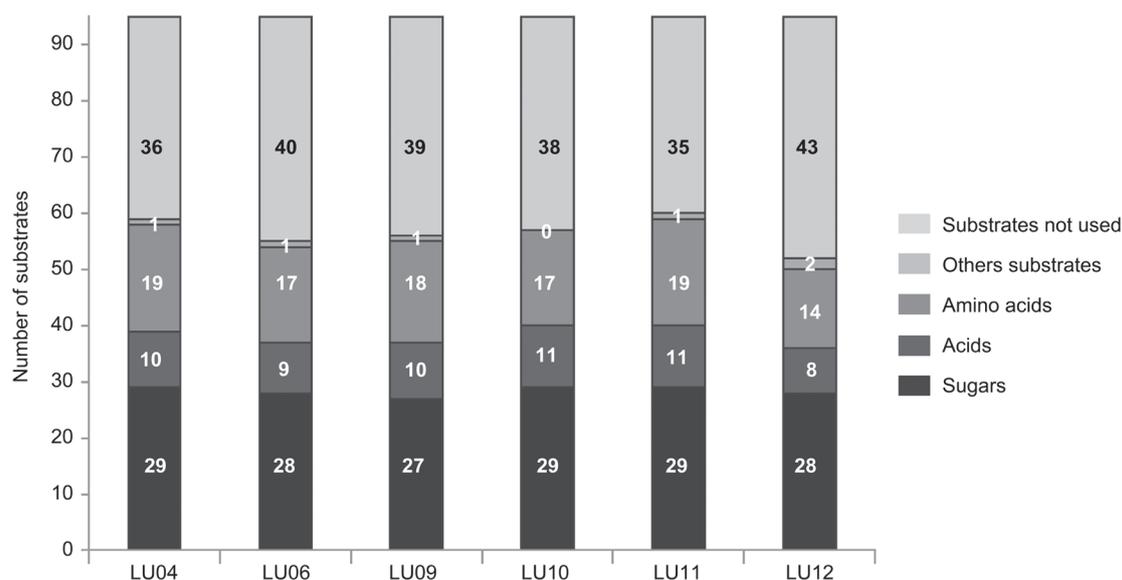

Fig. 4. Metabolic profiles of *Ensifer meliloti* isolates assayed on Biolog GN2 microplates. The metabolic substrates were divided into 4 groups – sugars, acids, amino acids and other substrates. The number of substrates utilized in each of the groups and number not used substrates

The assay revealed that the sampled *E. meliloti* strains have similar capability to use various carbon and energy sources, and the total number of substrates metabolized by individual strains varied from 52 (LU12) to 60 (LU11) (Fig. 4). The differences in using "sugars" refer mainly to glycogen and N-acetyl-D-galactosamine metabolism. In the group of "acids", LU12 did not use α- and β-hydroxybutyric acids, α-keto-butyric, and α-keto-valeric acid. Similarly, LU06, LU09, and LU10 did not use some of the organic acids (Table V). The most noticeable changes in the metabolic profile of the sampled isolates were observed in the group of "amino acid" substrates; in particular, LU12 was defective in using D-alanine, glycyl-L-aspartic acid, L-histidine, and L-threonine. On the other hand, this strain used thymidine, phenylethyl-amine, and D, L-α-glycerol phosphate, which were not metabolized by the other strains. Unexpectedly, the most competitive LU12 strain was able to use the lowest number of compounds compared to other strains.

The selected rhizobia were also investigated for mineral phosphate solubilization and bacteriocin production. These capabilities of rhizobia may be considered as factors affecting survival in the soil and competitiveness. LU10 and LU12, producing 12–13 mm clear zones around the bacterial colonies, were found to be the most efficient strains in mobilization of inorganic phosphates. The LU11 strain did not solubilize mineral phosphates at all. LU04, LU06, and LU09 formed zones of 6–7 mm in diameter. Among the six sampled strains, only LU09 was able to produce antimicrobial compounds, which inhibited the growth of bacteriocin-sensitive *E. meliloti* L5.30.

Discussion

Nodulation competitiveness and efficient nitrogen fixation are two main traits determining the agricultural value of rhizobial strains used as plant inoculants. These traits are independent of each other and remain under the influence of many genetic and environmental factors (Laguerre *et al.*, 2003). Probably, highly efficient and simultaneously competitive strains constitute only a small fraction among numerous rhizobia strains in soil. Nitrogen fixation in agriculture can be improved by inoculation of legume crops with selected more efficient rhizobia, which can outcompete indigenous less effective bacteria. Effective legume inoculants have been sought for years, because most selected strains are not fit for agricultural application due to their low competition abilities or low symbiotic efficiency (Streeter, 1994; Rodríguez-Blanco *et al.*, 2010). Some effort has been made for construction of genetically engineered rhizobia with improved competition capabilities (van

Dillewijn *et al.*, 2001), but the real use of such strains remains controversial. In addition, lowered competitiveness could be a consequence of the presence of other (non-rhizobial) strains affecting the growth of legume microsymbionts or obstacles in proper plant-bacteria signal exchange (Li and Alexander, 1986; Zhan and Smith, 2002; Laguerre *et al.*, 2012).

In this study, we selected and characterized *E. meliloti* nodule isolates, which have potential to be used as efficient and competitive inoculants for alfalfa. The autochthonous soil rhizobia, taxonomically identified as closely related to *E. meliloti,* proved to be considerably diverse in respect to the content of plasmids, which was markedly higher than that reported earlier (Broughton *et al.,* 1987; Hynes and O'Connel, 1990). Despite the diversity in the plasmid content, the BOX- and ERIC-PCR genomic DNA fingerprints of the selected six isolates were similar in 68–90%. Besides the genomic similarity, in the study of the competitiveness of *E. meliloti gusA*-tagged strains, considerable diversity in the ability to occupy nodules was noticed. In the two methods used for seed inoculation *i.e.*, seed soaking and seed watering with a suspension of the inoculant, the levels of nodule occupation measured by the number of nodules occupied by the *gusA*-tagged strains were comparable when large doses of bacteria were applied (Table III). LU12*gusA* and LU09*gusA* were reproducibly good competitors in respect to the indigenous rhizobial soil population regardless of the inoculation method and, concomitantly, efficient in N_2 fixation.

There is some evidence that the ability to utilize various carbon and energy sources is one of the factors that may influence competitiveness of rhizobia (Baldani *et al.*, 1992; Bringhurst *et al.* 2001; Ding *et al.*, 2012; Hynes and O'Connel, 1990; Oresnik *et al.*, 1998; Vanderlinde *et al.*, 2014; Wielbo *et al.*, 2007; 2010a; Yost *et al.*, 2004). In the study of the selected *E. meliloti* strains, we noticed low diversity in the abilities of utilization of carbon and energy sources. The strains utilized on average 56 of 94 (59.6%) of the tested compounds and the differences between the isolates concerned mainly the utilization of individual substrates from the groups of acids and amino acids (Fig. 4, Table V). These nutrients are the major components of leguminous root exudates and may act as strong chemoattractants for rhizobia (Gaworzewska and Carlile, 1982; Miller *et al.*, 2007).

Amino acids are important compounds in the plant-bacteroid exchange of carbon and energy (Lodwig and Poole, 2003). The most metabolically distinct LU12 used the lowest number of acids and amino acids; however, it used thymidine and phenylethyl-amine, and D, L-α-glycerol phosphate, which were not used by the other strains. In addition, LU12 was distinguished by its capability to mobilize inorganic phosphates, which

Table V
Metabolic profiles of *Ensifer* sp. isolates assayed on Biolog GN2 microplates.

nr		LU04	LU06	LU09	LU10	LU11	LU12
1	Water	0	0	0	0	0	0
2	α-Cyclodextrin	0	0	0	0	0	0
3	Dextrin	0	0	0	0	0	0
4	Glycogen	1	0	0	1	1	0
5	Tween 40	0	0	0	0	0	0
6	Tween 80	0	0	0	0	0	0
7	N-Acetyl-D-Galactosamine	1	1	0	0	1	0
8	N-Acetyl-D-Glucosamine	1	1	1	1	1	1
9	Adonitol	1	1	1	1	1	1
10	L-Arabinose	1	1	1	1	1	1
11	D-Arabitol	1	1	1	1	1	1
12	D-Cellobiose	1	1	1	1	1	1
13	i-Erythritol	1	1	1	1	1	1
14	D-Fructose	1	1	1	1	1	1
15	L-Fucose	1	1	1	1	1	1
16	D-Galactose	1	1	1	1	1	1
17	Gentiobiose	1	1	1	1	1	1
18	α-D-Glucose	1	1	1	1	1	1
19	m-Inositol	1	1	1	1	1	1
20	α-D-Lactose	1	1	1	1	1	1
21	Lactulose	1	1	1	1	1	1
22	Maltose	1	1	1	1	1	1
23	D-Mannitol	1	1	1	1	1	1
24	D-Mannose	1	1	1	1	1	1
25	D-Melibiose	1	1	1	1	1	1
26	β-Methyl-D-Glucoside	1	1	1	1	1	1
27	D-Psicose	1	1	1	1	1	1
28	D-Raffinose	1	1	1	1	1	1
29	L-Rhamnose	1	1	1	1	1	1
30	D-Sorbitol	1	1	1	1	1	1
31	Sucrose	1	1	1	1	1	1
32	D-Trehalose	1	1	1	1	1	1
33	Turanose	1	1	1	1	1	1
34	Xylitol	0	0	0	0	0	0
35	Pyruvic Acid Methyl Ester	1	1	1	1	1	1
36	Succinic Acid Mono-Methyl-Ester	1	1	1	1	1	1
37	Acetic Acid	1	1	1	1	1	1
38	Cis-Aconitic Acid	0	0	0	0	0	0
39	Citric Acid	0	0	0	0	0	0
40	Formic Acid	1	1	1	1	1	1
41	D-Galactonic Acid Lactone	0	0	0	0	0	0
42	D-Galacturonic Acid	0	0	0	0	0	0
43	D-Gluconic Acid	0	0	0	0	0	0
44	D-Glucosaminic Acid	0	0	0	0	0	0
45	D-Glucuronic Acid	0	0	0	0	0	0
46	α-Hydroxybutyric Acid	1	1	1	1	1	0
47	β-Hydroxybutyric Acid	1	1	1	1	1	0
48	γ-Hydroxybutyric Acid	0	0	0	0	0	0

Table V. Continued

nr		LU04	LU06	LU09	LU10	LU11	LU12
49	p-Hydroxy Phenylacetic Acid	0	0	0	0	0	0
50	Itaconic Acid	0	0	0	0	0	0
51	α-Keto Butyric Acid	1	0	1	0	1	0
52	α-Keto Glutaric Acid	0	0	0	0	0	0
53	α-Keto Valeric Acid	0	0	0	1	1	0
54	D,L-Lactic Acid	1	1	1	1	1	1
55	Malonic Acid	0	0	0	0	0	0
56	Propionic Acid	1	1	1	1	1	1
57	Quinic Acid	0	0	0	0	0	0
58	D-Saccharic Acid	0	0	0	0	0	1
59	Sebacic Acid	0	0	0	0	0	0
60	Succinic Acid	1	1	1	1	1	1
61	Bromosuccinic Acid	1	1	1	1	1	1
62	Succinamic Acid	0	0	0	0	0	0
63	Glucuronamide	0	0	0	1	0	0
64	L-Alaninamide	1	1	1	1	1	1
65	D-Alanine	1	1	1	1	1	0
66	L-Alanine	1	1	1	1	1	1
67	L-Alanyl-glycine	1	1	1	1	1	1
68	L-Asparagine	1	1	1	1	1	1
69	L-Aspartic Acid	1	1	1	1	1	1
70	L-Glutamic Acid	1	1	1	1	1	1
71	Glycyl-L-Aspartic Acid	1	0	0	0	1	0
72	Glycyl-L-glutamic Acid	1	1	1	1	1	1
73	L-Histidine	1	1	1	0	1	0
74	Hydroxy-L-Proline	1	1	1	1	1	1
75	L-Leucine	1	1	1	1	1	1
76	L-Ornithine	1	1	1	1	1	1
77	L-Phenylalanine	0	0	0	0	0	0
78	L-Proline	1	1	1	1	1	1
79	L-Pyroglutamic Acid	0	0	0	0	0	0
80	D-Serine	0	0	0	0	0	0
81	L-Serine	1	1	1	1	1	1
82	L-Threonine	1	1	1	0	1	0
83	D,L-Carnitine	1	0	1	1	1	1
84	γ-Amino Butyric Acid	1	1	1	1	1	0
85	Urocanic Acid	1	1	1	1	1	1
86	Inosine	0	0	1	0	0	0
87	Uridine	1	1	0	0	1	0
88	Thymidine	0	0	0	0	0	1
89	Phenylethyl-amine	0	0	0	0	0	1
90	Putrescine	0	0	0	0	0	0
91	2-Aminoethanol	0	0	0	0	0	0
92	2,3-Butanediol	0	0	0	0	0	0
93	Glycerol	1	1	1	1	1	1
94	D,L-α-Glycerol Phosphate	0	0	0	0	0	1
95	α-D-Glucose-1-Phosphate	0	0	0	0	0	0
96	D-Glucose-6-Phosphate	0	0	0	0	0	0

0 – negative result; 1 – positive result.

is an important trait influencing bacterial persistence in soil and plant growth promotion (Rodriguez and Fraga, 1999). Also, LU09 was the only one of the six sampled strains that was able to produce antimicrobial compounds inhibiting the growth of the bacteriocin-sensitive *E. meliloti* L5.30. Both capabilities could enhance their persistence in soil. In the previous study of *Rhizobium leguminosarum* bv. *trifolii* population, it was evidenced that rhizobia with specialized metabolism rarely occurred in the soil but they were more effective in symbiosis (Wielbo *et al.*, 2010a). It may be speculated that the capability to utilize substrates that are more difficult to metabolize is the discriminatory trait for competitive and uncompetitive strains, and specific adjustment of rhizobial metabolism to the plant exudate content is one of the most important elements of competitiveness. Overall, by gradual selection of the *E. meliloti* isolates, LU12 and LU09 were selected as efficient and reproducibly competitive strains in symbiosis with *M. sativa*, which will be further examined in order to develop alfalfa inoculants.

Literature

Aoki S., S. Nakata, T. Kajita and M. Ito. 2010. Genotypic and phenotypic diversity of rhizobia isolated from *Lathyrus japonicus* indigenous to Japan. *Syst. Appl. Microbiol.* 33: 383–397.

Baldani J.I., R.W. Weaver, M.F. Hynes and B.D. Eardly. 1992. Utilization of carbon substrates, electrophoretic enzyme patterns, and symbiotic performance of plasmid-cured clover rhizobia. *Appl. Environ. Microbiol.* 58: 2308–2314.

Barnett M.J., R.F. Fisher, T. Jones, C. Komp, A.P. Abola, F. Barloy-Hubler, L. Bowser, D. Capela, F. Galibert, J. Gouzy and others. 2001. Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* pSymA megaplasmid. *Proc. Natl. Acad. Sci. U.S.A.* 98: 9883–9888.

Bringhurst R.M., Z.G. Cardon and D.J. Gage. 2001. Galactosides in the rhizosphere: utilization by *Sinorhizobium meliloti* and development of a biosensor. *Proc. Natl. Acad. Sci. USA* 98: 4540–4545.

Brockwell J. 1982. Plant-infection counts of rhizobia in soils, pp. 41–58. *In*: Vincent JM (ed.), *Nitrogen fixation in legumes*. Academic Press Australia, Sydney 1982.

Brockwell J., P.J. Bottomley and J.E. Thies. 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant Soil* 174: 143–180.

Bromfield E.S.P., J.T. Tambong, S. Cloutier, D. Prévost, G. Laguerre, P. van Berkum, T.V. Tran Thi, R. Assabgui and L.R. Barran. 2010. *Ensifer*, *Phyllobacterium* and *Rhizobium* species occupy nodules of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover) grown at a Canadian site without a history of cultivation. *Microbiology* 156: 505–520.

Broughton W.J., N. Heycke, U. Priefer, G.M. Schneider and J. Stanley. 1987. Ecological genetics of *Rhizobium meliloti*: diversity and competitive dominance. *FEMS Microbiol. Lett.* 40: 245–249.

Calvert H.E., M.K. Pence, M. Pierce, N.S.A. Malik and W.D. Bauer. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. *Can. J. Bot.* 62: 2375–2384.

Depret G. and G. Laguerre. 2008. Plant phenology and genetic variability on root and nodule development strongly influence genetic structuring of *Rhizobium leguminosarum* biovar *viciae* populations nodulating pea. *New Phytol.* 179: 224–235.

Ding H., C.B. Yip, B.A. Geddes, I.J. Oresnik and M.F. Hynes. 2012. Glycerol utilization by *Rhizobium leguminosarum* requires an ABC transporter and affects competition for nodulation. *Microbiology* 58: 1369–1378.

Eckhardt T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* 1: 584–588.

Felsenstein J. 1985. Phylogenies and the comparative method. *American Naturalist* 125: 1–15.

Gaunt M.W., S.L. Turner, L. Rigottier-Gois, S.A. Lloyd-Macgilps and J.P.W. Young. 2001. Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Intern. J. Syst. Evol. Microbiol.* 51: 2037–2048.

Gaworzewska E.T. and M.J. Carlile. 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* 128: 1179–1188.

Herridge D.F., M.B. Peoples and R.M. Boddey. 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311: 1–18.

Hynes M.F. and M.P. O'Connel. 1990. Host plant effect on competition among strains of *Rhizobium leguminosarum*. *Can. J. Microbiol.* 36: 864–869.

Jones K.M., H. Kobayashi, B.W. Davies, M.E. Tagaand, G.C. Walker. 2007. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat. Rev. Microbiol.* 5: 619–633.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111–120.

Laguerre G., K. Heulin-Gotty, B. Brunel, A. Klonowska, A. Le Quéré, P. Tillard, Y. Prin, J. Cleyet-Marel and M. Lepetit. 2012. Local and systemic N signaling are involved in *Medicago truncatula* preference for the most efficient *Sinorhizobium* symbiotic partners. *New Phytologist* 195: 437–449.

Laguerre G., P. Louvrier, M.R. Allard and N. Amarger. 2003. Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulation of host legumes. *Appl. Environ. Microbiol.* 69: 2276–2283.

Laguerre G., P. Mavingui, M.R. Allard, M.P. Charnay, P. Louvrier, S.I. Mazurier, L. Rigottier-Gois and N. Amarger. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* 62: 2029–2036.

Li D.M. and M. Alexander. 1986. Bacterial growth rates and competition affect nodulation and root colonization by *Rhizobium meliloti*. *Appl. Environ. Microbiol.* 52: 807–811.

Lodwig E. and P. Poole. 2003. Metabolism of *Rhizobium* bacteroids. *Critic. Rev. Plant Sci.* 22, 37–78.

Louws F.J., D.W. Fulbright and C.T. Stephens. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.* 60: 2286–2295.

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

Martyniuk S., J. Oroń and M. Martyniuk. 2005. Diversity and numbers of root-nodule bacteria (rhizobia) in Polish soils. *Acta Soc. Bot. Polon.* 74: 83–86.

Martyniuk S., A. Woźniakowska, M. Martyniuk and J. Oroń. 2000. A new sand pouch-plant infection technique for enumeration of rhizobia in soil. *Acta Soc. Bot. Polon.* 69: 257–261.

Masson-Boivin C., E. Giraud, X. Perret and J. Batut. 2009. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? *Trends Microbiol.* 17: 458–466.

Mathesius U., J.J. Weinmann, B.G. Rolfe and M.A. Djordjevic. 2000. Rhizobia can induce nodules in white clover by "hijacking" mature cortical cells activated during lateral root development. *Mol. Plant Microbe Interact.* 13: 170–182.

Miller L.D., C.K. Yost, M.F. Hynes and G. Alexandre. 2007. The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol. Microbiol.* 63: 348–362.

Oresnik I.J., L.A. Pacarynuk, S.H.P. O'Brien, C. Yost and M.F. Hynes. 1998. Plasmid-encoded catabolic genes *in Rhizobium leguminosarum* bv. *trifolii*: evidence for a plant-inducible rhamnose locus involved in competition for nodulation. *Mol. Plant Microbe Interact.* 11: 1175–1185.

Palacios R. and W.E. Newton. 2005. *Genomes and genomics of nitrogen-fixing organisms*. Springer, Dordrecht.

Perret X., C. Staehelin and W. Broughton. 2000. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64: 180–201.

Pitcher D.G., N.A. Saunders and R.J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidine thiocyanate. *Lett Appl. Microbiol.* 8: 151–156.

Rodriguez H. and R. Fraga. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotech. Adv.* 17: 319–339.

Rodríguez-Blanco A., M. Sicardi and L. Frioni. 2010. Competition for nodule occupancy between introduced and native strains of *Rhizobium leguminosarum* biovar *trifolii*. *Biol. Fertil. Soils* 46: 419–425

Silva C., F.L. Kan and E. Martinez-Romero. 2007. Population genetic structure of *Sinorhizobium meliloti* and *S. medicae* isolated from nodules of *Medicago* spp. in Mexico. *FEMS Microbiol. Ecol.* 60: 477–489.

Simms E.L. and D.L. Taylor. 2002. Partner choice in nitrogen-fixation mutualism of legumes and rhizobia. *Integ. Comp. Biol.* 42: 369–380.

Streeter J.G. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Can. J. Microbiol.* 40: 513–522.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.

Triplett E.W. and M.J. Sadowsky. 1992. Genetics of competition for nodulation of legumes. *Ann. Rev. Microbiol.* 40: 399–428.

Van Dillewijn P., M.J. Soto, P.J. Villadas and N. Toro. 2001. Construction and environmental release of a *Sinorhizobium meliloti* strain genetically modified to be more competitive for alfalfa nodulation. *Appl. Environ. Microbiol.* 67: 3860–3865.

Vanderlinde E.M., M.F. Hynes and C.K. Yost. 2014. Homoserine catabolism by *Rhizobium leguminosarum* bv. *viciae* 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation. *Environ. Microbiol.* 16: 205–217.

Versalovic J., T. Koeuth and R.J. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 9: 6823–6831.

Vincent J.M. 1970. *A manual for the practical study of the root-nodule bacteria. IBP handbook no.15.* Blackwell Scientific Publishers, Oxford, England.

Weisburg W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697–703.

Wielbo J., M. Marek-Kozaczuk, A. Kubik-Komar and A. Skorupska. 2007. Increased metabolic potential of *Rhizobium* spp. is associated with bacterial competitiveness. *Can. J. Microbiol.* 53: 957–967.

Wielbo J., M. Marek-Kozaczuk, A. Mazur, A. Kubik-Komar and A. Skorupska. 2010a. Genetic and metabolic divergence within a *Rhizobium leguminosarum* bv. *trifolii* population recovered from clover nodules. *Appl. Environ. Microbiol.* 76: 4593–4600.

Wielbo J., J. Kuske, M. Marek-Kozaczuk and A. Skorupska. 2010b. The competition between *Rhizobium leguminosarum* bv. *viciae* strains progresses until late stages of symbiosis. *Plant Soil* 337: 125–135.

Wielbo J. and A. Skorupska. 2001. Construction of improved vectors and cassettes containing *gusA* and antibiotic resistance genes for studies of transcriptional activity and bacterial localization. *J. Microbiol. Methods* 45: 197–205.

Wilson K.J., A. Sessitsch, J.C. Corbo, K.E. Giller, A.D. Akkermans and R.A. Jefferson. 1995. β-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other gram-negative bacteria. *Microbiology* 141: 1691–705.

Young J.P., L.C. Crossman, A.W. Johnston, N.R. Thomson, Z.F. Ghazoui, K.H. Hull, M. Wexler, A.R. Curson, J.D. Todd, P.S. Poole and others. 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components, *Genome Biol.* 7: R34.

Yost C.K., K.L. Del Bel, J. Quandt and M.F. Hynes. 2004. *Rhizobium leguminosarum* methyl-accepting chemotaxis protein genes are down-regulated in the pea nodule. *Arch. Microbiol.* 182: 505–513.

Zhan F. and D.L. Smith. 2002. Interorganismal signaling in suboptimum environments: the legume-rhizobia symbiosis. *Adv. Agronom.* 76: 125–161.