New *Rhizobium leguminosarum* bv. *trifolii* isolates: Evaluation of competitiveness for clover nodule occupancy

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ABSTRACT

An interrelationship between introduced and indigenous rhizobia focused on their competitiveness in nodulation was evaluated in a soil pot experiment. Clover seeds were inoculated by six different gradual concentrations of inoculum of two effective *Rhizobium leguminosarum* bv. *trifolii* isolates (inoculation strains 1/2 and 14/2). At the beginning of flowering, clover plants were removed from the pots, and nodules from each pot representing different degrees of inoculum level were taken for reisolate cultivation. The PCR technique was used for the identification of rhizobial reisolates, random amplified polymorphic DNA product patterns were acquired and analysed. Nodule occupation by inoculation strains or indigenous *Rhizobium leguminosarum* bv. *trifolii* strains was assessed by comparing the number of nodules formed by inoculation or indigenous strains and inoculation strains competitiveness was calculated. Nodule occupancy by the inoculation strain 1/2 increased sharply with the increase in inoculum dose, whereas in inoculation strain 14/2 more nodules were formed gradually starting from low inoculum level. Competitiveness of inoculation strain 1/2 was calculated as low and was documented by an absence of nodule occupancy in four inoculation levels. On the other hand, competitiveness of the inoculation strain 14/2 was considerably higher, and even in low inoculum dose this strain was more competitive than native rhizobia. Although both the inoculation strains 1/2 and 14/2 were found highly efficient in nitrogen fixation, only the strain 14/2 was able to manifest this characteristic due to the higher competitiveness when applied in lower doses.

Keywords: *Rhizobium leguminosarum* bv. *trifolii* strains; inoculation; indigenous rhizobia; PCR identification; nodule occupancy; competitiveness

Rhizobium strains selected for use as inoculants have to possess two important characteristics: (i) they should show high nitrogen-fixing ability with their target host legume (Howieson et al. 2000), but (ii) the inoculation strains should also be able to compete with indigenous rhizobia present in soils and capable of nodule formation on a plant host (Mårtensson 1989). Triplett (1990) indicated that a high competitiveness of inoculation strains in comparison with native rhizobia strains is as important as the effectiveness of symbiotic N_2 fixation itself. An assessment of rhizobial competitiveness is frequently used in *Rhizobium* spp. studies and it became one of the criteria in the respective selection programmes (Date 2000). Various inoculation methods have been used in experiments determining *Rhizobium leguminosarum* bv. *trifolii* strains competitiveness: (i) repeated and increased inoculation with single strain culture (Mårtensson 1989), (ii) preparation of single or mixed strain peat-based inoculants (Svenning et al. 2001) or (iii) inoculation with slurry containing different concentrations of marked rhizobia (Denton et al. 2003). Amarger and Lobreu (1982) and Amarger (1984) presented an excellent model for studying the competition problem based on the fact that the percentage of nodules formed by the inoculation strain is related to the logarithm of the number of bacteria in the inoculum. Rhizobial strain is thus more competitive than others if the number of nodules it has occupied is higher than its proportional representation in the inoculum. Later, this model was modified (Beattie et al. 1989) and experimentally verified for various rhizobia (Šimon et al. 1996, Sessitsch et al. 1997, Denton et al. 2003). Rapid and precise

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identification methods are presently required for studying competition between microorganisms in soil however. Molecular tools including PCR techniques are now available and used almost routinely for identification of the rhizobia isolated from root nodules (Hebb et al. 1998). The objective of our study was to evaluate the competitiveness of two new *Rhizobium leguminosarum* bv. *trifolii* isolates in soil containing indigenous rhizobia. Both new isolates were identified and found as highly efficient in N_2 fixation (Šimon 2006).

MATERIAL AND METHODS

The pot experiment with soil was carried out for evaluation of the interrelationship between introduced and indigenous rhizobia focusing on their competitiveness in nodulation. *Trifolium pratense*, tetraploid cultivar Amos (Plant Breeding Station, Hladké Životice, Czech Republic) was the host plant. Different gradual concentrations of inoculum by the two effective isolates (inoculation strains) of *Rhizobium leguminosarum* bv. *trifolii* were used in the experiment. These two strains, 1/2 and 14/2, were previously isolated, identified and screened for the nitrogenase activity (Šimon 2006). The number of colony forming units (CFU) was adjusted to 10^9 per 1 ml culture suspension of the respective strains before the experiment. Simultaneously, the CFU counts of native rhizobia at 5×10^5 cells in 1 g soil were determined by disk dilution method. The scheme of inoculations used in the experiment, and the respective ratios between inoculum and native rhizobia are shown in Table 1. A control non-inoculated variant was also run in the experiment. The individual pots with holes in bottom were filled with 10 kg of sieved and mixed soil taken from an experimental field of the Research Institute of Crop Production in Prague. Luvic chernozem, clay-loamy, $pH_{KCl} = 6.4$, with a medium nutrient supply is characteristic for the soil where no inoculants have been applied for years. Seeds of experimental plants were sterilised with 0.2% $HgCl_2$ solution (Vincent 1970) and sown into pots in April 2005. Soil in the pots was moistened and immediately inoculated with relevant volumes of suspension of inoculation strains. Plant density was reduced to three plants per pot after seedlings emerged. The pots with plants were placed in a greenhouse and soil humidity was maintained at 60% by regular moistening. The individual pots were kept at a distance from each other. After the beginning of flowering in July 2005, the plants were removed from the pots, roots were thoroughly washed with water and ten nodules from each pot were taken for reisolate cultivation (Vincent 1970). Some reisolates had to be excluded from collection however, due to their poor growth or some uncertainty as to the culture purity. Finally, the total number of 71 *Rhizobium leguminosarum* bv. *trifolii* reisolates was maintained and inoculated into test tubes with a slant pea agar.

Molecular identification. A random amplified polymorphic DNA (RAPD) technique was used for the identification of rhizobial reisolates. Individual reisolates were cultivated in 15 ml yeast extract mannitol liquid medium (Vincent 1970) at 28°C overnight. Genomic DNA was extracted using the DNeasy Tissue Kit (QIAGEN, Hiden, Germany) following the manufacturer's instruction. Amplifications were performed in an automated thermal cycler (model PTC-200, MJ Research) programmed for one cycle of 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, and finally one cycle of 72°C for 5 min, and then kept at 4°C till distinquishing by electrophoresis. Amplified products were size-fractioned by electrophoresis in 1.5% agarose gels and DNA bands were visualized by ethidium bromide staining. A total of 61 decamer primers from Operon (Cologne, Germany) were screened with the DNA samples from *Rhizobium leguminosarum* bv. *trifolii* isolates. Two primers (OPI03 and OPI07) were selected for a reliable identification of the inoculation strains 1/2 and 14/2. RAPD amplifications were repeated twice, and only reproducible DNA fragments ranging from 500 to 2000 bp were further considered. Negative controls, i.e., without DNA, were included in each experiment in order to verify that neither self-amplification, nor DNA contamination occurred. Molecular sizes of the

Table 1. The scheme of inoculations and ratios (number of CFU) between inoculum and native rhizobia

Pot No.	Inoculated with (ml)	Ratio inoculation strain: native rhizobia	Log of the ratio
1	0.001	10^6:10^9	–3
2	0.01	10^7:10^9	–2
3	0.1	10^8:10^9	–1
4	1	10^9:10^9	0
5	10	10^{10}:10^9	1
6	100	10^{11}:10^9	2

amplification products were estimated by utilizing an O´GeneRulerTM 100 bp DNA Ladder Plus (Fermentas Life Sciences, Lithuania).

Nodulation analysis. Nodule occupation by inoculation strains or indigenous *Rhizobium leguminosarum* bv. *trifolii* strains was assessed by comparing the number of nodules formed by inoculation or indigenous strains. The competitive abilities of inoculation strains in forming nodules was evaluated using the relationship described by Amarger and Lobreu (1982): $\log N_A/N_B = \log C_{AB} + k \log (I_A/I_B)$ where A is the competitor strain; B are indigenous soil strains of rhizobia; N_A and N_B are the numbers of nodules formed by strain A and B, respectively; I_A and I_B are the numbers of bacteria A and B, respectively, at the moment of inoculation; C_{AB} is equal to N_A/N_B when I_A is equal to I_B and k is the slope of the regression line.

RESULTS AND DISCUSSION

DNA of each *Rhizobium leguminosarum* bv. *trifolii* reisolates was amplified with two primers and RAPD product patterns were recorded. The patterns of reisolates with primer OPI07 are shown in Figures 1 and 2. RAPD patterns were completely analysed after electrophoreograms recording, and patterns of individual reisolates were compared with a profile of the respective inoculation strain to find matchings. If a matching was found, it was evident that the reisolate is identical with the original inoculation strain, which means it is the original inoculation strain. Patterns of reisolates that showed dissimilarities from a pattern of inoculation strain and were not identical either were apparently composed of rhizobial populations from soil used for the experiment. On the basis of pattern comparison, the presence of inoculation strains in tested nodules of experimental plants was deduced (Table 2) and percentage representation of inoculation strains in the dependence on the inoculum volume was calculated.

The inoculation strain 1/2 was identified in all nodules when the plants were treated with 100 ml of inoculum (approximately 100 times higher concentration of the inoculation strain in comparison to native rhizobia) (Figure 1, Table 2). By reduction of inoculum volume, the appearance of inoculation strain in nodules strongly decreased (25% for the ratio 10^{10}:10^9). Starting from an inoculation level of 1 ml (i.e., concentration of inoculum as corresponding to that of native rhizobia) no inoculation strain was isolated from nodules of plant

Table 2. Presence of inoculation strains in nodules of experimental plants when different inoculum volumes were used

Inoculum (ml)	Nodule No.										Occupancy of nodules (%)
	1	2	3	4	5	6	7	8	9	10	
Inoculation strain 1/2											
100	A	A	**A**								100
10	A	B	C	D							25
1	E	E	E	E	E	F	E				0
0.1	E	E	G	G	H	I	J				0
0.01	K	L	K	M	N	O	P				0
0.001	Q	R	S	T	U	V	E				0
Inoculation strain 14/2											
100	A	**A**	B	A	A	**A**					83
10	A	A	A	**A**	C						80
1	D	A	E	A	**A**						60
0.1	A	A	A	F	G	H					50
0.01	A	I	J	K	L	M					17
0.001	N	O	P	Q	I	I	I	R			0

A = inoculation strain, B–V = native rhizobial populations different from inoculation strain

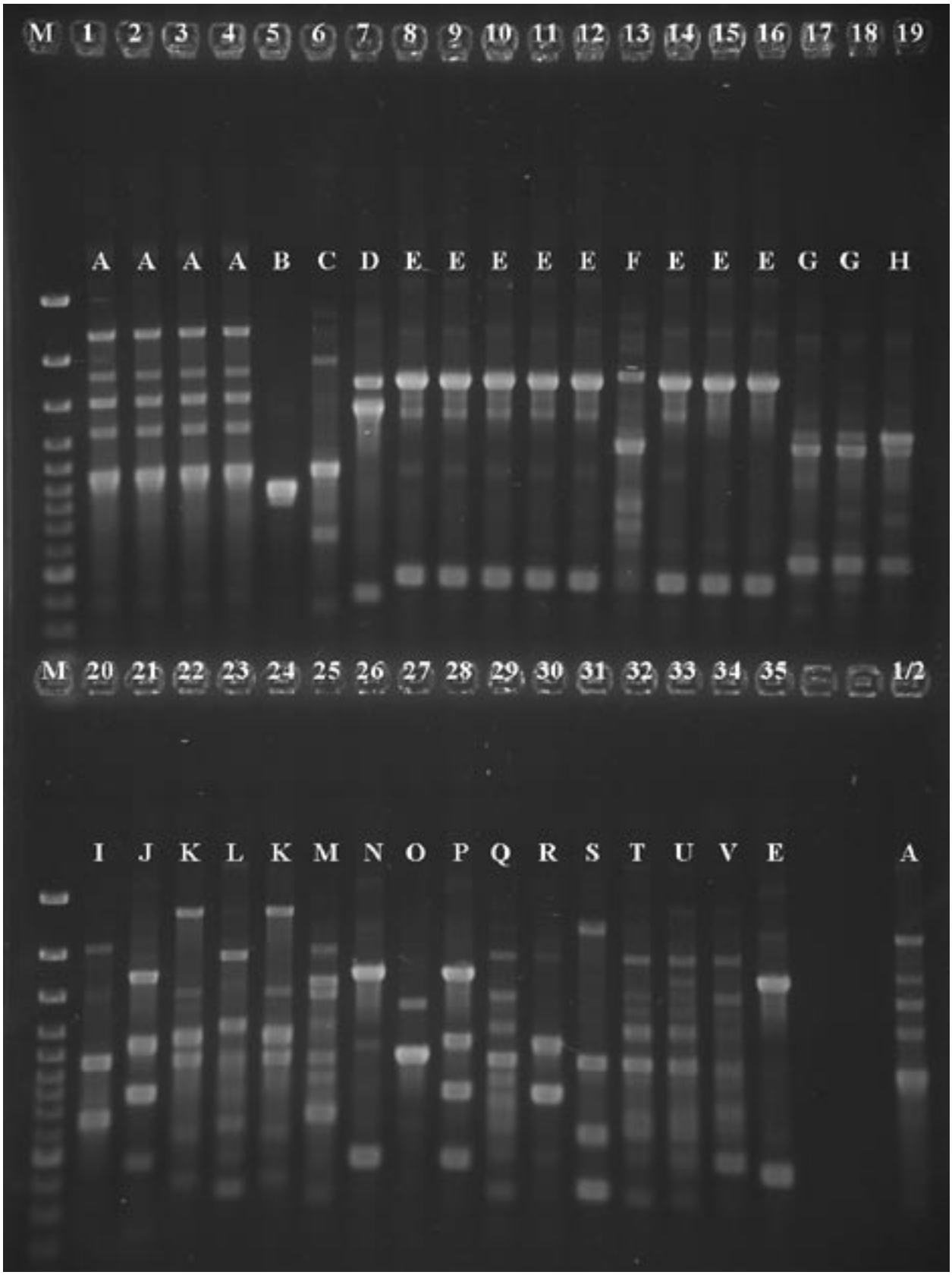

Figure 1. Random amplification polymorphic DNA patterns of *Rhizobium* reisolates generated with primer OPI07; the number corresponding to each reisolate is at the top of each lane; below the reisolate number, there is a letter corresponding to a particular RAPD pattern; lane M is the molecular weight marker (Fermentas Life Sciences, Lithuania), lane 1/2 is the inoculation strain

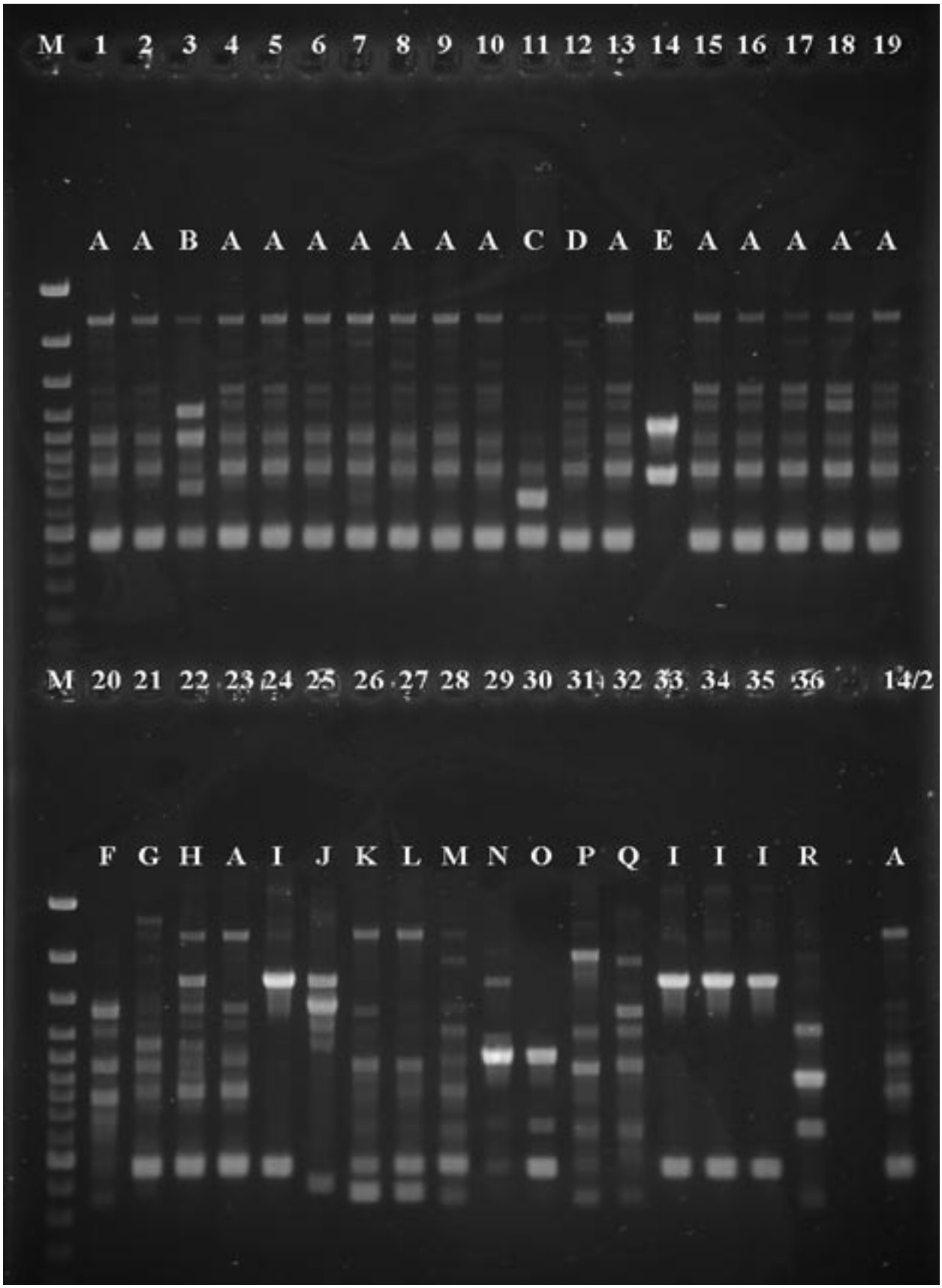

Figure 2. Random amplification polymorphic DNA patterns of *Rhizobium* reisolates generated with primer OPI07; the number corresponding to each reisolate is at the top of each lane; below the reisolate number, there is a letter corresponding to a particular RAPD pattern; lane M is the molecular weight marker (Fermentas Life Sciences, Lithuania), lane 14/2 is the inoculation strain

Table 3. Correlation coefficient r and k values at linear regression analysis where log N_A/N_B depends on log I_A/I_B

Inoculation strain	r	k
1/2	1[a]	1.477
14/2	0.961[a]	0.336

[a]statistically significant ($P < 0.05$)

Table 4. Competitiveness of two *Rhizobium leguminosarum* bv. *trifolii* strains compared to native rhizobia at different proportional representation in inoculum

A = 1/2		A = 14/2	
B = native rhizobia		B = native rhizobia	
A:B	C_{AB}	A:B	C_{AB}
10^6:10^9	–	10^6:10^9	–
10^7:10^9	–	10^7:10^9	0.96
10^8:10^9	–	10^8:10^9	2.17
10^9:10^9	–	10^9:10^9	1.50
10^{10}:10^9	0.011	10^{10}:10^9	1.85
10^{11}:10^9	0.011	10^{11}:10^9	1.04

undertesting. The inoculation strain 14/2 was more frequently identified in host root nodules than the 1/2 strain (Figure 2, Table 2). The presence of the 14/2 strain in nodules when 100 ml inoculation was applied was 83% (only one reisolate failed to be identified as 14/2), and along with a decrease of inoculation level the percentage occupation of nodules by inoculation strain subsequently decreased. Nevertheless, inoculation strain 14/2 was still identified in one nodule of six in an inoculation level as low as 0.01 ml, i.e., when native rhizobia 100 times outnumbered the inoculation strain 14/2 (Table 2). These results evidenced the dependence of the nodule occupancy by inoculation strains on the volume of inoculum applied and the ratio of inoculum to indigenous rhizobia at the time of inoculation. For a more detailed calculation of competitiveness of the inoculation strains, the ratios of individual inoculum levels to native rhizobia were changed to their logarithms (Table 1).

According to the relationship described by Amarger and Lobreu (1982) the log N_A/N_B was calculated to proceed to regression analysis and to calculate slope of regression line, k, an important parameter for further calculation of competitiveness of inoculation strains. The values of k were found to vary according to the experiment (Amarger 1984); the extreme values were 0.15 and 1.22 but most of the values were between 0.3 and 0.5. Denton et al. (2003) calculated k for four *Rhizobium leguminosarum* bv. *trifolii* inoculation strains in a competition experiment, and the values varied between 0.693–1.551. Correlation coefficient r and k values for both inoculation strains used in our experiment are shown in Table 3, inoculation strains 1/2 and 14/2 were designed as a competitor strain A and indigenous rhizobia as a reference strain B. High values of r and k calculated for inoculation strain 1/2 were caused by the lack of log N_A/N_B values for r and k calcula-

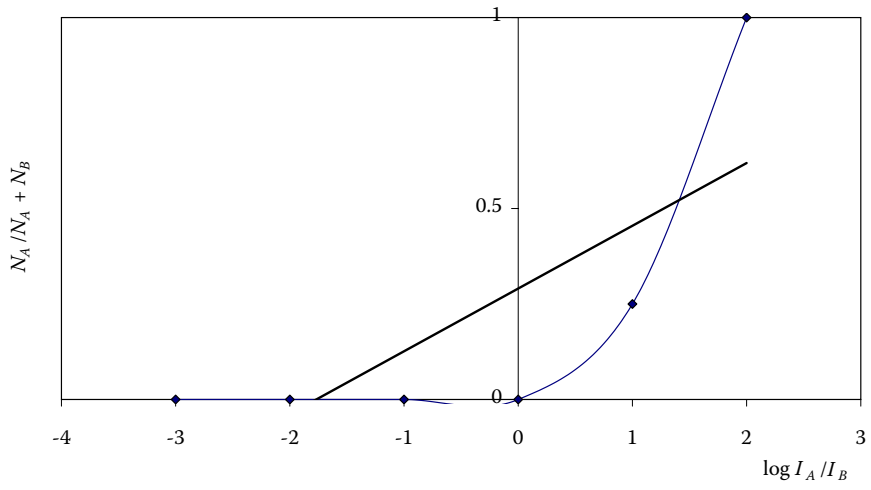

Figure 3. Proportion of nodules formed by *R. leguminosarum* bv. *trifolii* strain 1/2 as function of proportion of inoculation strain in soil after inoculation

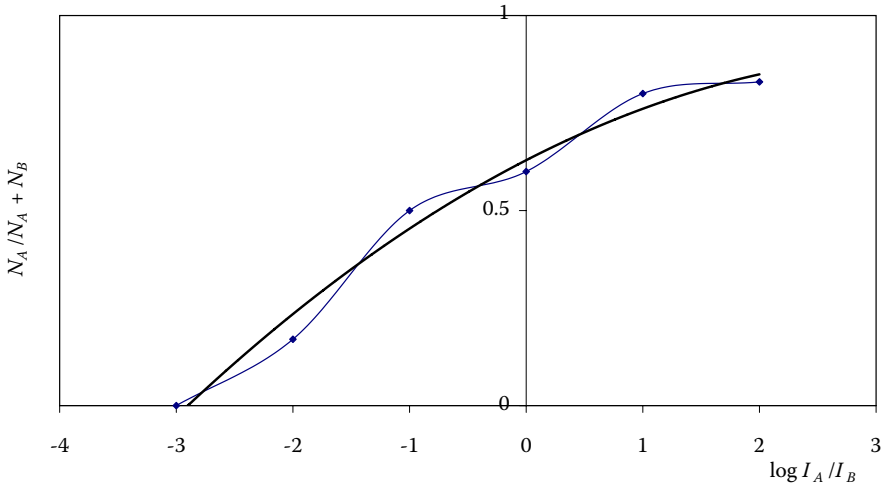

Figure 4. Proportion of nodules formed by *R. leguminosarum* bv. *trifolii* strain 14/2 as function of proportion of inoculation strain in soil after inoculation

tion due to no nodule occupancy of host plants starting from inoculation level 1 ml. Using the calculated k, the values of C_{AB} were estimated as the y intercept of the line. The individual values of C_{AB} define the competitiveness of the competitor strain A versus the reference strain(s) B at different proportional representation at the moment of inoculation (Amarger 1984). If $C_{AB} > 1$, strain A is more competitive, and if $C_{AB} < 1$, strain A is less competitive than strain(s) B. Table 4 indicates the values of C_{AB} for both inoculation strains used in our experiment. According to the above-mentioned definition, competitiveness of inoculation strain 1/2 was very low, which was also documented by the absence of nodule occupancy in four inoculation levels lower than 10 ml (Table 2). On the other hand, competitiveness of inoculation strain 14/2 was considerably higher; this strain was, even in low inoculum dose, more competitive than native rhizobia. To be more illustrative, the results were transferred to Figure 3 and 4 that characterize the proportion of nodules formed by inoculation strains ($N_A/N_A + N_B$) as function of log I_A/I_B. Nodule occupancy by the inoculation strain 1/2 increased sharply with increasing inoculum doses (Figure 3), whereas the inoculation strain 14/2 formed more nodules gradually starting from low inoculum levels (Figure 4). With further increasing the number of introduced rhizobia, native rhizobia were substantially eliminated from nodule occupancy.

Based on these results it is possible to predict the chance of success with a particular inoculum. While both inoculation strains 1/2 and 14/2 are highly efficient in nitrogen fixation (Šimon 2006), only the strain 14/2 is able to manifest this clearly even when applied in lower doses. On the other hand, when the inoculation strain 1/2 outnumbers native rhizobia by a factor 100 it is able to occupy all root nodules and fully exert its N_2 fixation ability. In conclusion, the results demonstrate that a direct selection for both high nitrogen fixation and high competitiveness is a precondition of a successful inoculation of a plant seeds by *Rhizobium* strains. This is the way how to add thoroughly selected elite strains to legume seeds effectively and consequently prevent the formation of nodules by low effective indigenous rhizobia.

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