

Competitiveness of *Rhizobium leguminosarum* bv. *trifolii* Strains in Mixed Inoculation of Clover (*Trifolium pratense*)

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Abstract

Rhizobium leguminosarum bv. *trifolii* (*Rlt*) establishes beneficial root nodule symbiosis with clover. Twenty *Rlt* strains differentially marked with antibiotic-resistance markers were investigated in terms of their competitiveness and plant growth promotion in mixed inoculation of clover in laboratory experiments. The results showed that the studied strains essentially differed in competition ability. These differences seem not to be dependent on bacterial multiplication in the vicinity of roots, but rather on complex physiological traits that affect competitiveness. The most remarkable result of this study is that almost half of the total number of the sampled nodules was colonized by more than one strain. The data suggest that multi-strain model of nodule colonization is common in *Rhizobium*-legume symbiosis and reflects the diversity of rhizobial population living in the rhizosphere

Key words: clover growth promotion, competitiveness, mixed inoculation, rhizobia

Introduction

Biological nitrogen fixation (BNF) is a process in which atmospheric dinitrogen is reduced to forms available to plants and is provided by free-living bacteria or bacteria symbiotically associated with leguminous plants, commonly known as rhizobia. Rhizobia belong to different taxonomic families of α -proteobacteria such as *Rhizobiaceae*, *Phylobacteriaceae* and *Bradyrhizobiaceae*, and to β -proteobacteria such as the recently described *Burkholderia* spp. (reviewed by Perret *et al.*, 2000; Masson-Boivin *et al.*, 2009). Significance of rhizobial symbioses in agriculture stems from providing an extra source of nitrogen for plant growth, supplementing low nitrogen level in the soil and replacing nitrogenous fertilizer (Peoples *et al.*, 1995; Ramos *et al.*, 2001).

Rhizobium leguminosarum bv. *trifolii* (*Rlt*) induces and colonizes nodules elicited on roots of clover (*Trifolium* spp.). The symbiosis between rhizobia and legumes is a multi-step process including the exchange of molecular signals between the microsymbiont and

its host (reviewed by, Perret *et al.*, 2000; Spalink, 2000; Jones *et al.*, 2007). Initially, flavonoids released by plants attract bacteria to the roots and induce synthesis of bacterial Nod factors that are lipochitooligosaccharide signals that trigger several plant responses, such as root hair curling and differentiation of plant meristems leading to the formation of root nodules. Nodules are colonized by rhizobia *via* tubular structures known as infection threads in which rhizobia multiply. After releasing from infection threads bacteria surrounded by peribacteroid membranes colonize nodule tissue forming symbiosomes in which bacteria are converted into bacteroids. Bacteroids localized in the symbiotic zone of mature nodules reduce atmospheric dinitrogen into forms, which are easily assimilated by plants (Vasse *et al.*, 1990; Timmers *et al.*, 2000).

Competition for nodulation is a quantitative phenotype, which determines the ability of *Rhizobium* strains to dominate in the nodules of a given legume host in competition with other strains present in the root rhizosphere (Dowling and Broughton, 1986). Soil populations of *Rhizobium* usually consist of numerous strains,

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which compete with each other during the phase of vegetative growth in the soil, as well as during several stages of plant colonization and nodule formation (Wilson *et al.*, 1998; Stuurman *et al.*, 2000; Duodu *et al.*, 2009). Rhizobial strains are genetically and metabolically diverse, reveal significant differentiation in competitive fitness to infect and occupy nodule of the host plant and differ in the symbiotic activities (Maier and Triplett, 1996; Wielbo *et al.*, 2007; Depret and Laguerre, 2008). To investigate the competition between rhizobial strains, numerous molecular markers were developed and employed. Spontaneous or acquired antibiotic resistance markers are commonly used due to feasibility of detection and a broad differentiation allowing simultaneous identification a number of strains (Sharma *et al.*, 1991; Cresswell *et al.*, 1994; Sessitsch *et al.*, 1998; Wilson *et al.*, 1998; Stuurman *et al.*, 2000).

In this work, we investigated the competition ability of 20 *Rlt* strains originating from clover root nodules under laboratory conditions. The results showed that in most cases simultaneous inoculation of plants with a mixture of rhizobial strains carrying different antibiotic resistance markers resulted in nodule colonization by more than one strain. The commonly observed nodule colonization by multiple strains that differ in respect to the efficiency of plant growth promotion may have significant impact on overall symbiosis in the soil.

Experimental

Materials and Methods

Rhizobium leguminosarum *bv.* *trifolii* strains. 20 *Rlt* strains used in this study were obtained from nodules of clover (*Trifolium pratense*) cultivated in arable sandy loam soil in the region of Lublin, Poland. Rifampicin, streptomycin and trimethoprim-resistant spontaneous mutants were obtained as follow: from overnight cultures of *Rlt* isolates grown in liquid TY medium (Sambrook *et al.*, 1989) with constant shaking, about 5×10^9 cfu/ml of the given culture was plated on TY agar medium supplemented with rifampicin, streptomycin or trimethoprim (40 µg/ml, 200 µg/ml, 200 µg/ml, respectively). Single colonies resistant to appropriate antibiotic were isolated. Nalidixic acid for selection of naturally resistant strains was used in the concentration of 150 µg/ml. Tetracycline resistant *Rlt* strains were obtained by introducing pJBA21Tc plasmid *via* triparental conjugation (Wielbo and Skorupska, 2001). Each of 20 strains was resistant to one of the following antibiotics: rifampicin, streptomycin, tetracycline, trimethoprim and nalidixic acid (Table I).

Table I
Relevant characteristics of *Rhizobium leguminosarum* *bv.* *trifolii* strains used in this study

Group I	Group II	Group III	Group IV	Group V
K416 Tm ^R	K417 Rf ^R	K38 Rf ^R	K29 Tc ^R	K313 Tc ^R
K411 Rf ^R	K108 Tm ^R	K413 Nal ^R	K87 Str ^R	K312 Str ^R
K36 Str ^R	KO17 Tc ^R	KO18 Tc ^R	K322 Nal ^R	K415 Rf ^R
KO27 Tc ^R	K71 Str ^R	K91 Str ^R	K810 Rf ^R	K107 Tm ^R

The spontaneous mutants were isolated in the case of Rf^R – rifampicin resistance, Str^R – streptomycin resistance and Tm^R – trimethoprim resistance. Tc^R – tetracycline resistant strains were obtained by introducing pJBA21Tc plasmid. Nal^R – natural nalidixic acid resistance.

Plant tests. Red clover seeds (*Trifolium pratense* L. cv. Dajana) were surface-sterilized, germinated on TY agar medium, and then transferred onto agar slants with nitrogen-free Fåhraeus medium (Vincent, 1970). Clover seedlings were inoculated with 200 µl single strain suspension in water, which was scratched from TY agar medium plate, or with 200 µl mixture of four strains differently tagged with antibiotic-resistance marker (1:1:1:1 v/v). In both cases the bacterial suspensions contained about 1.0×10^9 cfu/ml. Plants were grown in a greenhouse under natural light supplemented with artificial light (14 h day/10 h night, at 24/19°C). After 5 weeks, clover plants were harvested and nodules were counted. The efficiency of symbiotic nitrogen fixation was estimated by weighing fresh mass of shoots and roots. For each experimental group 20 plants were used and two independent experiments were conducted.

Estimation of rhizobial competitiveness. Ten clover plants were randomly chosen from each experimental group inoculated with a mixture of four strains and wet masses of shoots and roots were estimated. Root nodules were surface-sterilized and the content of individual nodules was plated on TY agar medium. Colonies derived from individual nodules were used for preparation of water suspension an OD₅₅₀ of 0.05 ($\sim 5 \times 10^7$ cfu/ml). 20 µl of each suspension was spotted on a set of TY agar medium plates containing one of four antibiotics, depending on antibiotic resistance markers carried by strains in a particular mixture. Bacteria were grown for three days at 28°C. Detectable growth on only one plate from a set was interpreted as the presence of pure culture in the nodule, whereas the growth on more than one plate from a set was interpreted as mixed colonization of nodule.

Rhizobial growth assay. 5 ml Fåhraeus medium supplemented with appropriate amount of TY medium was inoculated with 50 µl of overnight culture of *Rlt* strains growing in TY medium, and incubated for 2 days at 28°C. Bacterial growth was measured by monitoring OD₅₅₀. For all tested strains the experiment was conducted in triplicate.

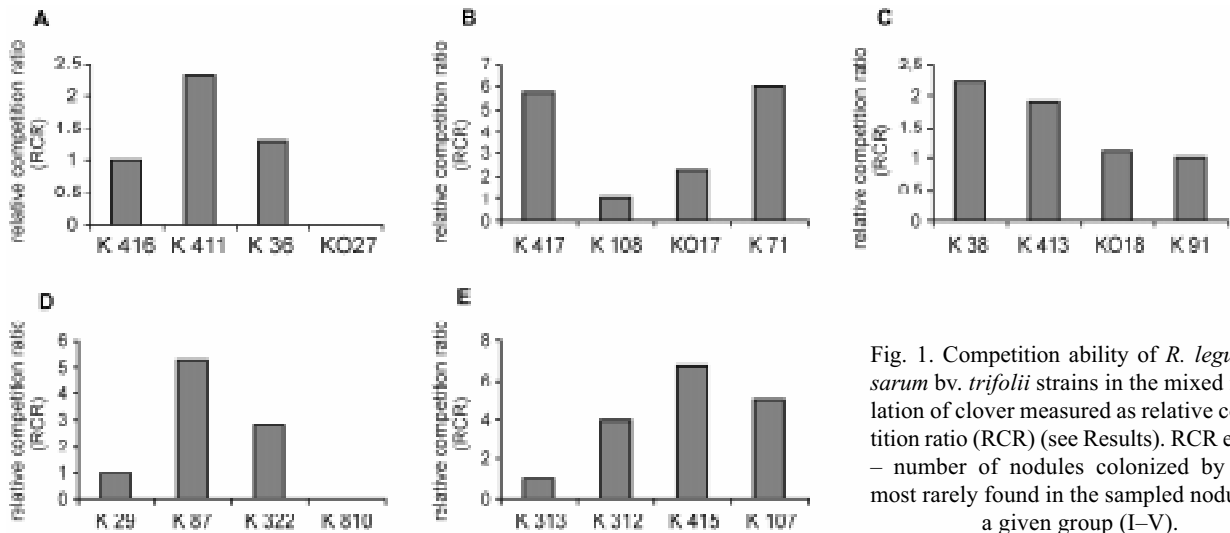


Fig. 1. Competition ability of *R. leguminosarum* bv. *trifolii* strains in the mixed inoculation of clover measured as relative competition ratio (RCR) (see Results). RCR equal 1 – number of nodules colonized by strain most rarely found in the sampled nodules of a given group (I–V).

Results

To study nodulation competitiveness, twenty *Rlt* strains were divided into five groups (I–V). Strains were assigned to groups randomly, and four strains belonging to the same group differed from each other with respect to the antibiotic resistance markers they were carrying (Table I). Clover seedlings were inoculated with individual strains or with a mixture of four strains belonging to a given group. After 5 weeks, samples of nodules were taken and rhizobia were recovered from nodules by selection on TY medium supplemented with an appropriate antibiotic. In the case of groups II, III and V, all four strains used for clover inoculation were recovered from the nodules, whereas in the case of groups I and IV, three of four strains used for inoculation were found in the sampled nodules. For *Rlt* strains assigned to a given group the “relative competition ratio” (RCR) was calculated as follow: (a) the number of nodules occupied by the

least frequently found strain was normalized to 1; (b) RCR for the three other strains were calculated by dividing the number of nodules colonized by a particular strain by the number of nodules occupied by the least frequent strain; (c) if a strain was absent in all tested nodules, the RCR was 0 (Fig. 1A, B, C, D, E). As regards their high “relative competition value”, K411 (group I), K417, K71 (group II), K38, K413 (group III), K87 (group IV) and K415 (group V) strains can be considered competitive. On the other hand, KO27 and K810 strains were not recovered from the sampled nodules and they were considered uncompetitive.

Based on the antibiotic resistance selection of *Rlt* strains recovered from clover nodules, it has been found that nodules occupied by only one strain comprised at average $56 \pm 7\%$ (Fig. 2 A, B, C, D, E). The others nodules were occupied by two or three strains that were distinguished by different antibiotic markers. Two-strain nodule occupancy ranged from 28% to

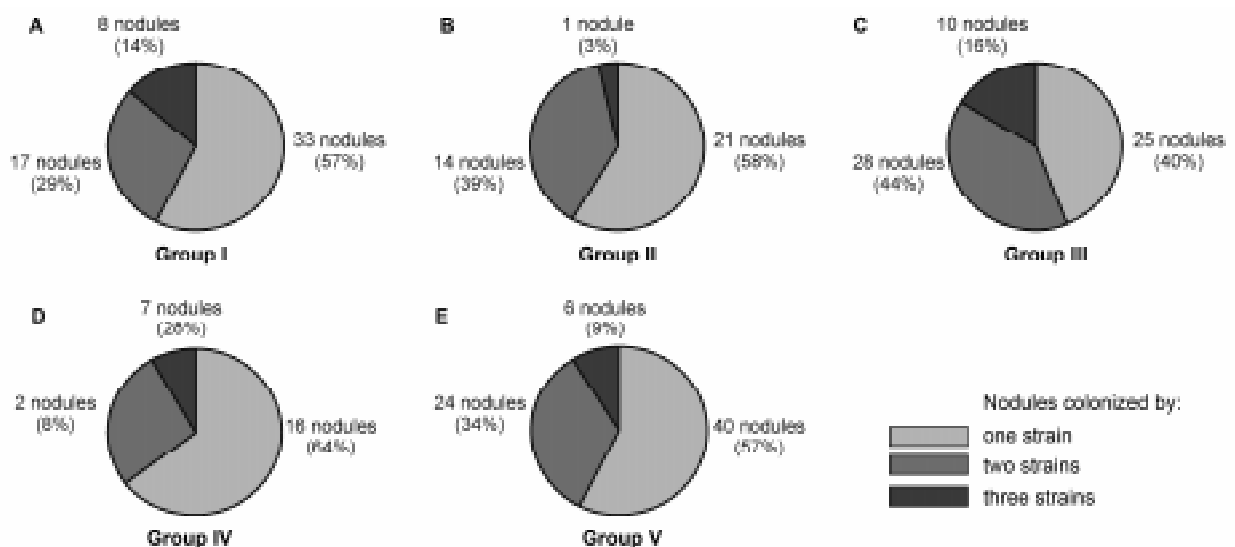


Fig. 2. Distribution of nodules infected by one, two or three strains in particular groups of *R. leguminosarum* bv. *trifolii* strains.

Table II
Symbiotic activity of *R. leguminosarum* bv. *trifolii* strains

<i>Rlt</i> strain number	No of nodules/plant	Fresh mass of shoots/plant (mg)	Fresh mass of roots/plant (mg)	Fresh mass of plant/nodule (mg)
Group I of <i>Rlt</i> strains				
K 416	5.4 ± 1.5 a	26.3 ± 3.5 a	22.7 ± 1.3 a	9.0
K 411	6.8 ± 0.7 a	31.3 ± 9.4 a	29.7 ± 10.2 a	8.9
K 36	4.3 ± 1.0 a	36.8 ± 2.1 a	18.5 ± 3.6 a	12.8
KO27	6.6 ± 2.3 a	28.6 ± 6.6 a	32.3 ± 5.3 a	9.2
Mixed inoculation (I)	7.4 ± 2.2 a	36.2 ± 8.3 a	23.7 ± 7.4 a	8.1
Group II of <i>Rlt</i> strains				
K 417	7.0 ± 0.7 b	45.8 ± 9.3 ab	25.6 ± 11.6 a	10.2
K 108	5.4 ± 0.7 ab	50.9 ± 8.5 b	26.5 ± 10.0 a	14.3
KO17	3.6 ± 0.4 a	33.4 ± 1.9 a	12.6 ± 4.2 a	12.7
K 71	3.7 ± 0.6 a	34.2 ± 8.6 a	13.7 ± 3.8 a	13.0
Mixed inoculation (II)	4.7 ± 1.9 ab	30.5 ± 10.0 a	10.5 ± 6.3 a	8.8
Group III of <i>Rlt</i> strains				
K 38	5.2 ± 2.0 a	36.6 ± 4.1 a	16.0 ± 0.9 a	10.1
K 413	8.6 ± 2.1 a	44.3 ± 2.5 a	39.4 ± 7.0 b	9.8
KO18	10.2 ± 0.9 a	68.5 ± 7.3 b	43.3 ± 7.1 b	11.0
K 91	5.8 ± 2.7 a	56.6 ± 3.0 c	40.0 ± 12.5 b	16.6
Mixed inoculation (III)	9.0 ± 2.8 a	33.9 ± 3.4 a	24.9 ± 6.3 ab	6.5
Group IV of <i>Rlt</i> strains				
K 29	3.6 ± 1.2 a	31.2 ± 7.0 a	12.1 ± 2.0 a	12.2
K 87	2.8 ± 0.7 a	24.6 ± 9.4 a	12.4 ± 4.1 a	13.4
K 322	4.2 ± 1.8 a	34.6 ± 9.2 a	18.8 ± 8.2 ab	12.7
K 810	6.8 ± 1.6 b	29.5 ± 4.5 a	21.8 ± 5.5 b	7.5
Mixed inoculation (IV)	3.3 ± 0.9 a	25.2 ± 7.1 a	10.4 ± 2.5 a	10.9
Group V of <i>Rlt</i> strains				
K 313	3.8 ± 1.5 a	44.1 ± 12.9 a	23.7 ± 10.5 ab	17.7
K 312	3.4 ± 1.0 a	35.0 ± 7.0 a	13.3 ± 2.1 a	14.4
K 415	6.2 ± 1.2 ab	41.3 ± 0.9 a	22.8 ± 5.0 b	10.4
K 107	5.1 ± 1.6 ab	55.3 ± 10.4 a	27.4 ± 8.1 b	16.4
Mixed inoculation (V)	6.8 ± 0.4 b	44.7 ± 7.0 a	25.7 ± 6.1 b	10.3
Average values				
Inoculation by individual strains	6.2 ± 2.3 a	34.1 ± 7.2 a	19.0 ± 7.9 a	12.1 ± 2.8 a
Inoculation by a mixture of strains	5.4 ± 1.9 a	39.4 ± 11.4 a	23 ± 9.6 a	8.9 ± 1.8 b

Values are the mean ± standard deviation of 20 clover plants in experiment. Means within the same column and followed by the same letter are not significantly different ($P > 0.05$).

40%, and three-strain nodule occupancy from 3% to 16% of nodules, depending on the group of strains (I–V) used for clover inoculation (Fig. 2 A, B, C, D, E). Nodules simultaneously colonized by all four strains were not detected. The obtained results showed that nodule colonization by more than one strain was prevalent, at least in competition experiments conducted under laboratory conditions.

Rlt strains used in competition experiments were characterized with regard to the symbiotic properties such as nodulation ability and plant growth promotion (Table II). The tested strains varied both in the number of nodules elicited and in the impact on fresh mass of clovers. In the case of some strains the differences in nodule number and shoot and root mass were statistically significant. It is worth noting that plants

inoculated with a mixture of four strains showed intermediary values of symbiotic parameters, which can reflect averaged effects of individual strains on symbiosis. In almost all cases, fresh mass of total plant per nodule was lower in the mixed inoculation in comparison to the same parameter in clover inoculation by individual strains (Table II).

The differences observed in the nodule colonization ability of *Rlt* strains might result from some physiological differences between the strains, for instance, in the growth rate. In the competition experiment, the mixtures of strains used as inocula were prepared by mixing equal volumes of four bacterial water suspensions with the same OD_{550} , which should minimize the effect of possible differences in the initial cell density. In addition, growth of rhizobia was

assayed in the medium composed of nitrogen-free Fåhraeus medium used in plant tests supplemented with 1%, 2%, 5%, 10% or 20% TY medium. It was assumed that addition of some amount of TY to the plant medium allows for the growth of bacteria while simultaneously resembling the plant growth medium. Rhizobia were grown in these media for 2 days and OD₅₅₀ was measured. The results showed that *Rlt* strains used in the competition experiments grow weakly and only in Fåhraeus medium supplemented with 10% or 20% of TY, reaching OD₅₅₀ values from 0.13 to 0.2 (data not shown). Differences in growth of strains on Fåhraeus medium supplemented with 10% TY, as well as on medium supplemented with 20% TY were observed, however, the analysis of variance indicated that these differences were statistically insignificant (data not shown). In conclusion, the possible differences in the initial growth of *Rlt* strains on plant medium at the time of inoculation could not essentially affect their competitiveness.

Discussion

Local population of *R. leguminosarum* specific for a given legume host is composed of numerous strains characterized by great variability in genetic and metabolic traits (Lakzian *et al.*, 2002; Wielbo *et al.*, 2010). Rhizobial competitiveness is a complex process dependent on genetic and overall metabolic status of bacteria (Wielbo *et al.*, 2007), susceptibility to plant molecular signals (Mabood *et al.*, 2008; Maj *et al.*, 2010), motility of bacteria (Mellor *et al.*, 1987), production of resistance to bacteriocins (Wilson *et al.*, 1998) or even distribution of bacteria in the soil (Lopez-Garcia *et al.*, 2002). Several studies indicate that both the legume hosts and microsymbionts affect the outcome of rhizobial competition (Kiers *et al.*, 2006; Depret and Laguerre, 2008; Rangin *et al.*, 2008).

All these and other variables characterizing individual strains influence their overall competition abilities. As a result, appreciable variability in rhizobial competitiveness is observed, both in highly controlled experiments under laboratory conditions, and in experiments carried out in the soil (Maier and Triplett, 1996; Svenning *et al.*, 2001; Wielbo *et al.*, 2007; Duodu *et al.*, 2009; Sachs *et al.*, 2009).

The strains compete with each other on the level of root adsorption, root hair infection, growth inside the infection threads, nodule tissue colonization and survival after release into the soil, where they constitute part of population capable of symbiotic interactions (Duodu *et al.*, 2005; Silva *et al.*, 2007; Rangin *et al.*, 2008; Sachs *et al.*, 2009). Less competitive rhizobia comprising another part of the population may be eliminated from nodule infection (Streeter,

1994). Finally, nonsymbiotic rhizobia constitute the fraction that lost the nodulation genes but is still able to colonize root surfaces and can play an important role in competition (Sachs *et al.*, 2009).

In several studies, the competition between rhizobia was commonly investigated in a two-strain competition assay under controlled conditions. In this assay both strains were tagged with different markers such as antibiotic resistance genes (Bromfield, 1984), *lux* (Cresswell *et al.*, 1994) or *gfp* reporter genes (Stuurman *et al.*, 2000) and occupancy of both strains was easily determined in the nodule. The drawback of this method is the extreme simplification of the model, which does not reflect native conditions. The second most frequently used method for rhizobial competition analysis was to use a single marked strain vs. the entire unmarked soil population composed of numerous strains. Major drawback of this approach is lack of any information about the unmarked strains possibly present in the nodules. Recently, the competitiveness of unmarked rhizobial strains was studied by molecular identification of particular genotypes occupying the nodules (Svenning *et al.*, 2001).

In the present work, the two methods have been combined, and clover plants were inoculated by four differently marked strains. It allowed determining the differences in competitive ability of the individual strains, and the predominance of nodules occupied by more than one strain, in the case of a mixed inoculation. It is worth noting that infection by several strains negatively affected plant growth promotion when compared to single-strain infection.

Past studies showed mixed colonization of infection threads and nodules (Bromfield, 1984) in a two-strain competition assay (Stuurman *et al.*, 2000; Gage, 2004). Expanding the competition test to clover inoculation by four strains, we demonstrated the possibility of single clover nodule colonization by three different strains. The percent of nodules colonized by three strains was relatively low and ranged from 3 to 16% of the sampled nodules. Nodules colonized by four strains were not found. It is possible that the number of the sampled nodules should have been greater to allow detection of nodules occupied by all four strains. On the other hand, the limited space available in the infection threads and/or in young nodules might restrict the growth of rhizobia, and this might also explain why four strain nodule colonization was not observed. The most important finding from the presented study is that almost half of the total number of the sampled nodules was colonized by more than one strain. It suggests that multi-strain model of nodule colonization is predominant also in the soil environment, and reflects the complexity and diversity of rhizobial population in the rhizosphere.

The comparison of symbiotic efficiencies of clover inoculation by individual strains vs. a mixture of strains was not conclusive. Whereas, significant differences between the averaged data of shoots and roots masses in single strain inoculation and in the mixed inoculation have not been found, significant difference in ratios of fresh mass of the entire plant to nodule number was evident when these two types of inoculations were compared (Table II). A possible explanation for this result might be that the concentration of several Nod factors produced by particular strains in the mixed inoculation is substantially greater than in a single strain inoculation. Nod factors stimulate nodule organogenesis (Perret *et al.*, 2000; Spaink, 2000) and that nodule number increase was not accompanied by increased plant growth probably due to the relatively short timeframe of the experiment. This observation is consistent with our previous studies, in which treatment of clover seeds with Nod factors under controlled conditions enhanced the growth of clover only when a Nod factor was applied in appropriate concentration (Maj *et al.*, 2009).

In summary, our data revealed that *R. leguminosarum* strains investigated in the experimental competition model were greatly differentiated with respect to competitiveness for nodule occupancy. In the mixed inoculation of clover plants, nodule coinfection by two or three strains was commonly observed. The prevalence of nodule coinfection by native soil population is yet to be determined.

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