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The effect of biotic and physical factors on the competitive ability of *Rhizobium leguminosarum*

Research Article

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Abstract: **Background**: *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) is a soil bacterium which can form nitrogen-fixing symbiotic relationships with leguminous plants. Numerous rhizobial strains found in soils compete with each other. Competition can occur both during the saprophytic growth phase in the rhizosphere and inside plant tissues, during the symbiotic phase. Competition is important as it may affect the composition of rhizobial populations present in the soil and in the root nodules of plants. **Methodology**: We examined the link between physiological traits and bacterial competitive ability in eighteen *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) isolates during root nodule colonization using laboratory and field experiments. The competitive ability of *R/v* strains was measured as the percentage of root nodules colonized by *gusA*-tagged rhizobia in two types of host plants, peas and vetch. **Results**: The competitiveness of *Rlv* strains was significantly affected by soil type and the identity of the host plant. Of the eighteen bacterial traits examined in this study, the metabolic potential (number of utilized carbon and energy sources) and the responsiveness of nod genes to flavonoid activation were most important in affecting the competitive ability of *Rlv* strains. The amount of acylated homoserine lactones (AHL) produced by the strains was less important in influencing competitiveness. Finally, the preactivation of strains with flavonoids or the addition of AHL to *gus*-tagged *Rlv* strains did not significantly enhance competitiveness: of the gus-tagged inoculants in comparison to indigenous soil populations of vetch microsymbionts. **Conclusions**: The competitiveness of *Rlv* strains is dependent upon numerous physiological traits. However, environmental factors such as soil type and the type of host plant may be even more important in affecting rhizobial competitiveness.

Keywords: *Rhizobium leguminosarum* • *Rhizobium-legume symbiosis* • *Strain competitiveness*

1. Introduction

Rhizobium leguminosarum bv. *viciae* (*Rlv*) is a soil bacterium, which is able to establish nitrogen-fixing symbiotic relationships with leguminous plants of the tribe Vicieae (*Pisum Vicia, Lens* and *Lathyrus*). Symbiosis is a multi-stage process which involves co-recognition of the symbiotic partners *via* an exchange of plant and bacterial molecular signals. Flavonoids secreted by plant roots activate a rhizobial regulatory Nod protein resulting in the induction of nodulation genes that encode enzymes for the biosynthesis of lipochitin oligosaccharides. These oligosaccharides are referred to as the nodulation factors (Nod factors), which trigger early symbiotic responses in roots and initiate the developmental program of nodule formation. Infection threads filled with growing bacterial cells are then formed and release rhizobia into plant cells in the nodule primordia. Rhizobia colonize the developing nodules and differentiate into dinitrogen fixing bacteroids inside them. Bacteroids provide the host plant with ammonium from nitrogen fixation. In return, the plant supplies the bacteria with carbohydrates derived from photosynthesis [1-3]. Vegetative forms of bacteria multiply in the infection threads and saprophytic zones of indeterminate type nodules, and are released into the soil during the decay of nodules [4,5]. Amongst the heterogeneous microbial communities in the soil, rhizobia constitute numerous and very differentiated populations

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[6-10]. Several factors can influence the number and diversity of indigenous rhizobia, including nutrient availability, soil type, acidity and agricultural management regimes [6,11,12]. However, despite the numerous factors limiting their survival in the soil [13], these bacteria are common soil inhabitants in all climatic zones of the world [6,14,15].

Numerous biological traits affecting rhizobial nodulation competitiveness have been described in the literature [16]. They include the ability of rhizobia to utilize specific carbon and energy sources [17,18], the overall metabolic potential of bacteria [19], bacteriocin production, resistance to bacteriocin [20], the susceptibility to plant molecular signals [21,22] and bacterial motility [23]. In addition, the legume hosts themselves [24-26] and the distribution of rhizobia in the soil [27] may also influence the outcome of competitive interactions between rhizobia. Furthermore, it is possible that compounds other than plant-derived flavonoids and rhizobial Nod factors may influence strain competitiveness for nodule occupancy in *Rhizobium*-legume interactions [28,29]. As a result of competition between rhizobia during nodule colonization, strain composition inside root nodules may be diverse [30]. Therefore, nitrogen fixation efficiencies of these strains may also vary, which is important in the context of the development of commercial rhizobial inoculants [13].

Furthermore, the competition and proliferation of vegetative forms of bacteria inside the nodules may affect the total yield of rhizobia, which return to the soil after a vegetative period in the host plants [31-33]. Each of these factors affects the potential success of individual strains to persist in local populations. In the case of rhizobial inoculants, introduced strains may quickly disperse in the native population in rhizobia-rich soils [34] or remain as a significant proportion of a population for years in rhizobia-depleted soils [35].

Here, we investigated the contribution of important bacterial physiological and metabolic traits to the competitiveness of *Rlv* strains used to inoculate vetch and pea plants grown in different types of soil under noncompetitive and competitive conditions. We specifically examined the role of the rhizobial metabolic potential, the responsiveness of *nod* genes to flavonoid treatment, AHL production, and chemotaxis in influencing the competitive ability of *Rlv* strains.

2. Experimental Procedures

2.1 Rhizobial strains

Eighteen *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) strains used in this study were isolated from root nodules of pea (*Pisum sativum* cv. Ramrod) grown on arable sandy loam soil in Lublin, Poland [36].

2.2 Chemotaxis assay

Rhizobia were cultured in TY liquid medium [37] to an optical density of 0.3-0.4 at l=550 nm (OD_{550}) and centrifuged at 1000×*g* for 20 min at room temperature. Pellets were suspended in chemotaxis buffer (10 mM potassium phosphate, 0.1 mM sodium EDTA, pH 7.0) [38] to ~$5x10^7$ CFU/ml. A chemotaxis assay was conducted in capillary tubes placed into chemotaxis chambers [39,40]. Capillary tubes filled with chemotaxis buffer (control) or flavonoid extract (10μM) and the bacterial suspension (~$5x10^7$ CFU/ml) were placed inside the chambers for 4 h at 28ºC. After incubation, the content of capillary tubes was suspended in 100 μl of chemotaxis buffer, plated in dilutions onto 79CA medium and incubated for 72 h at 28ºC. The chemotaxis ratio was calculated by dividing the number of bacteria recovered from capillary tubes filled with flavonoid extract by the number of bacteria recovered from capillary tubes containing chemotaxis buffer. The assay was replicated four times for each strain.

2.3 Assay of P_{nodA}-*lacZ* promoter fusion activity

The derivatives of the *Rlv* strains harbouring pMPA221 plasmid with P_{nodA}-*lacZ* fusion [22] were used to study the effect of flavonoid induction of rhizobial *nod* genes. Overnight cultures of rhizobia in TY medium supplemented with tetracycline (10 μg/ml) were centrifuged, washed twice with M1 medium supplemented with 1 μg/ml thiamine, 0.5 μg/ml biotin and 1 μg/ml panthotenate [22], and resuspended in M1 to OD_{550} of 0.05. Then, flavonoid extract was added to the culture at a final concentration of 10 μM. The cultures were grown for an additional 24 h at 28°C. The level of *nod* gene expression was determined in Miller units by assaying β-galactosidase activity with the o-nitrophenyl-β-D-galactoside (ONPG) cleavage assay [41].

2.4 Isolation and detection of rhizobial AHLs

AHLs were isolated by ethyl acetate extraction from supernatants of 5 ml overnight cultures of *Rlv* grown in TY medium [42]. The samples were separated by thin-layer chromatography on C_{18} reversed-phase TLC plates (Merck), and overlaid with *Chromobacterium violaceum* CV026 [43]. Synthetic AHLs (Sigma-Aldrich) *N*-butyryl-dl-homoserine lactone (C_4-AHL), *N*-hexanoyl-dl-homoserine lactone (C_6-AHL), *N*-octanoyl-dl-homoserine lactone (C_8-AHL) and *N*-decanoyl-dl-homoserine lactone C_{10}-AHL) (solutions of 1 mg/ml in ethyl acetate) were used as standards.

AHLs for plant tests were extracted from GB36, GC1.6 and P1.13 *Rlv* strains, which produced relatively high levels of these compounds. Strains grown overnight in 5 ml TY medium were centrifuged and AHLs were extracted from supernatants of each culture with ethyl acetate. The extracts were dried and the pellets formed were dissolved in 100 µl of ethyl acetate. The quality of AHL preparations was verified using *Chromobacterium violaceum* CV026 biotest. Following this procedure, the preparations were pooled and used in plant tests.

2.5 Physiological tests

The utilization of different carbon and energy sources by the *Rlv* strains examined was assessed using GN2 Microplate™ (Biolog, Hayward, CA, USA). Bacterial cells were grown overnight at 28°C on TY agar medium, collected in sterile water and then washed twice in water. The pellets were subsequently diluted in water to an OD_{550} of 0.1 (approx. 10^8 CFU/ml). 150 µl of suspension of rhizobia was added to each well in the Biolog microplate. The microplates were incubated for 48h at 28°C, and the color development in the wells was monitored every 12h using Benchmark PlusTM microplate reader (Bio-Rad Laboratories, USA). Color development was measured as OD_{550} and OD_{750} - dual wavelength data as recommended by the microplate manufacturer.

2.6 Flavonoid induction in *Rlv*-vetch symbiosis: laboratory experiment under sterile conditions

Sterile flavonoid exudates from sprouted pea seeds at a final concentration of 10 mM were added to logarithmic *Rlv* strain cultures in M1 medium and incubated with shaking at 28°C for 16 h. Surface sterilized seeds of vetch (*Vicia villosa* cv. Wista) were sown in plastic pots with sterile sand (400 g of sand per pot) and inoculated with 1 ml flavonoid induced *Rlv* cultures containing ~10^9 CFU/ml. Each pot containing 6 plants was irrigated once with 50 ml nitrogen-free Fåhraeus medium [44] and watered every 2 days. The growth chamber was set at a 16/8 h light/dark and 22/15°C regime. After 6 weeks, we harvested the plants, counted the nodules and estimated the fresh mass of shoots and roots. Each treatment was carried out in 2 replicates and the experiment was repeated twice.

2.7 Flavonoid induction in *Rlv*-vetch symbiosis: laboratory experiment under competitive conditions

To study competitiveness, logarithmic cultures of *gusA*-marked *Rlv* strain derivatives were flavonoid induced as described above. The cultures were used to inoculate vetch seeds (*Vicia villosa* cv. Wista) sown in plastic pots with unsterile soil (600 g of soil per pot), containing ~$4.6x10^5$ of autochthonous rhizobia as estimated by the most probable number (MPN) method. Pots, Each pot, which contained 6 plants, was handled as described above. After 6 weeks, the plants were harvested, the roots were stained for β-glucuronidase activity, and the number of GUS-positive and GUS-negative nodules were counted. Each treatment was carried out in 2 replicates and the experiment was repeated twice.

2.8 AHLs in *Rlv*-vetch symbiosis: laboratory experiment under sterile conditions

Vetch seeds (*Vicia villosa* cv. Wista) were sown in plastic pots with sterile sand (600 g of sand/pot) and inoculated with 1 ml cultures of individual *Rlv* strains (~10^9 CFU/ml) with and without 20 µl AHL preparations. The approximate concentration of AHLs in the preparations was about 1.3 µg/ml, as determined by densitometry using Bio-Profil BioGene Windows Application V11.01 (Vilber-Lourmat, France); the densitometric assay was performed after a biotest with *C.violaceum* on C_{18} reversed-phase TLC plates (Merck) with *N*-hexanoyl-dl-homoserine lactone (C_6-AHL) (Sigma-Aldrich) as a standard. Each containing 6 plants was irrigated once with 100 ml nitrogen-free Fåhraeus medium and watered every 2 days. The growth chamber was set at a 16/8 h light/dark and 22/15°C temperature regime. After 6 weeks, the plants were harvested, the nodules counted and the fresh mass of shoots and roots determined. Each treatment was carried out in 2 replicates and the experiment was repeated twice.

2.9 AHLs in *Rlv*-vetch symbiosis: laboratory experiment under competitive conditions

Vetch seeds (*Vicia villosa* cv. Wista) were sown in plastic pots with nonsterile soil (600 g of soil/pot) containing ~$4.6x10^5$ of autochthonous rhizobia, and inoculated with 1 ml cultures of individual *gusA*-marked derivatives of the appropriate *Rlv* strains (~10^9 CFU/ml) with and without 20 µl AHL preparations. Each pot containing 6 plants was handled as described above. After 6 weeks, the plants were harvested, the roots were stained for β-glucuronidase activity, and GUS-positive and GUS-negative nodules were counted. Each treatment was carried out in 2 replicates and the experiment was repeated twice.

2.10 Assay for competitiveness of *Rlv* strains in outdoor experiment

The experiment was conducted at the Institute of Soil Science and Plant Cultivation, Puławy, Poland. Plants were

cultivated in Mitcherlich pots filled with (i) 7 kg of sandy soil containing ~1.5x10^5 of autochthonous rhizobia or (ii) 7 kg of brown soil containing ~9.3x10^5 of autochthonous rhizobia. Vetch (*Vicia villosa* cv. Wista) and pea seas (*Pisum sativum* cv. Ramrod) were sown into each type of soil. Seeds were inoculated with 10 ml of water suspensions with *gusA*-marked *Rlv* strains (~10^8 CFU/ml). The pots (6 plants per pot) were kept under a rainout shelter for 6 weeks in May and June 2010. After 6 weeks of growth, the plants were removed, the roots were gently washed in tap water and stained for β-glucuronidase activity; GUS-positive and GUS-negative nodules were counted. Each treatment was replicated twice.

2.11 Assay testing the competitiveness of *Rlv* strains in a two-step outdoor experiment

The experiment was conducted at the Institute of Soil Science and Plant Cultivation, Puławy, Poland. Plants were cultivated in Mitcherlich pots filled with 7 kg of sandy soil containing ~1.5x10^5 of autochthonous rhizobia. Vetch seeds were sown in pots and inoculated with 10 ml of water suspensions with *gusA*-marked *Rlv* strains (~10^8 CFU/ml). The pots (6 plants per pot) were kept for 8 weeks in May and June 2010. At the end of the 8 weeks, the parts of the plants which stood aboveground were removed. The roots were left in the pots for 5 weeks to decay and allow rhizobia to be released from the nodules. Additional vetch seeds were then sown into these pots (no rhizobia were added) and the plants were grown for 6 weeks (August and September 2010). Throughout the duration of the experiment, all pots were kept under a rainout shelter. After 6 weeks of growth, the plants were removed, the roots were washed in tap water and stained for β-glucuronidase activity; GUS-positive and GUS-negative nodules were counted. Each treatment was replicated twice.

2.12 Statistical analyses

The results of plant experiments were subjected to two types of tests: median test used to analyze differences in nodule number, and ANOVA employed to analyze the differences in shoot and root wet mass.

We used a two-way ANOVA to examine differences in strain competitiveness as a function of soil type and host plant identity.

To examine the relationship between the physiological traits of a strain and its competitive ability, strains were ranked on the basis of their success in a particular experiment. Since we examined eighteen strains, the ranks assigned in each experiment ranged from 1 to 18, from the least competitive to the most competitive strain, respectively. For each strain, we calculated the correlation coefficient between a given physiological trait and the strain's success using Spearman correlation. We used STATISTICA to conduct all analyses.

3. Results

3.1 Physiological characteristics of strains

Eighteen *Rlv* strains were characterized with respect to several physiological features that may have impacted their competitiveness in the symbiotic interaction with vetch and pea plants. We examined three metabolic properties of these *Rlv* strains: utilization of carbon and energy sources in a Biolog GN2 MicroPlate™ test, the ability to produce AHL production and the chemotactic response to flavonoids. In addition, we estimated the transcriptional response of *nodA* to the flavonoid inducer using P_{nodA}-*lacZ* fusion introduced into *Rlv* strains. This response can be advantageous for competitive interactions during nodulation, (Table 1).

The average number of Biolog`s substrates utilized by *Rlv* strains was 37±7 compounds. However, individual strains differed to a great extent in the number of utilized substrates, which varied from 25 (strain P229) to 52 (strain P139) compounds. Chemotactic coefficients (see Experimental Procedures section) ranged from 0.67 to 5.93. The transcriptional activity of P_{nodA}-*lacZ* fusion in response to the flavonoid treatment varied broadly from 18 to 282 Miller units. The production of AHL was not quantitatively assayed, but strains were divided into three groups on the basis of *Ch. violaceum* CV026 biotest results: "high-AHL" strains produced relatively high amounts of AHL, "low-AHL" strains producing low amounts of AHL, and "no-AHL" strains either did not produce AHL or AHL production was not detected by the assay (Table 1, Figure 1). Strains from the "high-AHL" group produced mainly C_6-AHL and some amounts of other AHLs (probably with an acyl residue longer than C_6). Strains from "low-AHL" group produced only C_6-AHL (Figure 1).

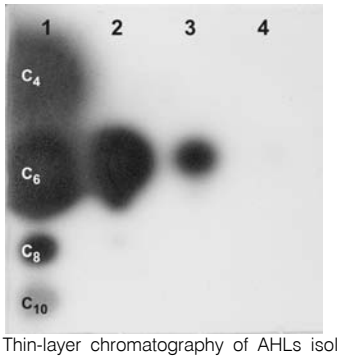

Figure 1. Thin-layer chromatography of AHLs isolated from tree groups of strains differentiated in AHL production. Lane 1 – marker (mix of synthetic AHLs), lanes 2 to 4 - representatives of three groups: lane 2 – P266 ("high-AHL"); lane 3 – P270 ("low-AHL"); lane 4 – P272 ("no-AHL").

Strain	Number of utilized substrates in Biolog GN2 test	β-galactosidase activity after flavonoid-induction of P_{nodA}-*lacZ* (Miller units)[a]	Chemotactic coefficient after flavonoid treatment[b]	AHL production[c]
GB25	31	282 ± 14	3.16 ± 0.20	+
GB36	34	19 ± 8	3.39 ± 0.10	+++
GB51	35	18 ± 9	4.09 ± 0.31	+
GC16	34	48 ± 8	3.35 ± 0.09	+++
GC31	49	25 ± 5	1.83 ± 0.03	+
GC613	46	192 ± 19	5.93 ± 1.02	-
GD22	34	162 ± 19	1.98 ± 0.15	+++
GD33	40	44 ± 13	1.83 ± 0.15	+++
P11	28	29 ± 11	4.55 ± 1.02	-
P111	38	131 ± 16	1.05 ± 0.12	+++
P113	39	238 ± 33	2.82 ± 0.22	+++
P139	52	42 ± 6	2.18 ± 0.81	+++
P146	35	115 ± 26	3.57 ± 0.95	-
P229	25	159 ± 12	1.30 ± 0.38	+++
P259	31	276 ± 36	0.67 ± 0.12	-
P266	49	81 ± 5	1.09 ± 0.30	+++
P270	40	198 ± 21	2.55 ± 0.22	+
P272	35	105 ± 21	1.24 ± 0.30	-

Table 1. Physiological properties of *Rlv* strains

[a] *values represent means of three β-galactosidase assays (41) after subtraction of background activity*
[b] *values represent means of three replicates*
[c] *strains with relatively high production of AHLs (+++), low production of AHLs (+) and no detectable production of AHLs (-) are referred as "high-AHL", "low-AHL" and "no-AHL" groups, respectively*

The metabolic traits examined were not correlated with each other. Correlation coefficients between (i) the number of utilized substrates and P_{nodA}-*lacZ* fusion activity, (ii) the number of utilized substrates and chemotactic ratio, and (iii) P_{nodA}-*lacZ* fusion activity and the chemotactic ratio, were -0,191, -0,103 and -0,229, respectively.

3.2 The effect of flavonoid activation on *Rlv*-vetch symbiosis and strain competitiveness - laboratory experiments

Additional experiments were performed to estimate the symbiotic efficiency and competitiveness of *Rlv* strains after a flavonoid pretreatment. Ten *gusA*-tagged strains were chosen (GB25, GB51, GC16, GC31, GC613, GD22, P11, P229, P259 and P266), either activated or not activated by the flavonoid treatment (experimental and control groups, respectively) and used for inoculation of vetch in two different (noncompetitive or competitive) pot experiments (detailed data not shown).

Vetch plants were grown in pots with sterile sand in the experiment conducted under noncompetitive conditions, We measured the effect of flavonoid pretreatment of rhizobia on the growth of plants colonized by single strains by counting nodules and weighting shoots and roots. The response of plants was strain-dependent: in most cases, the flavonoid treatment of microsymbionts promoted nodulation and the growth of the host plant, with the exception of strains GB51, P259 and P266. Despite this, the mean nodule number per plant elicited by flavonoid-treated strains was significantly higher comparing with nodule number elicited by untreated strains (6.78±1.95 and 4.23±1.3, respectively, $P<0.01$). We found a similar pattern for the wet mass of roots (336±114 mg and 248±52 mg, respectively, $P<0.05$). On the other hand, the wet mass of plant shoots was only insignificantly higher for plants inoculated with flavonoid-treated strains comparing with untreated strains (637±127 mg and 614±127 mg, respectively).

In a similar experiment conducted under competitive conditions, vetch plants were grown in pots with nonsterile soil. We determined the effect of flavonoid treatment of rhizobia on the growth of plants as well as the presence of GUS^+ nodules. The effect of flavonoid induction of bacteria on plant growth and nodule

abundance was not substantial: the mean number of root nodules was slightly higher in flavonoid-induced than in noninduced (control) strains (22.6±3.9 and 20.2±3.5 nodules/plant, respectively). In contrast, the percentage of GUS$^+$ nodules was the same in both cases (31%). However, the correlation coefficient calculated between the competitiveness of the strains (determined as the percentage of nodules colonized by *gusA*-tagged rhizobia) and the activity of P_{nodA}-*lacZ* fusion (Table 1) was 0.398, suggesting that strain competitiveness is dependent on the activation of *nod* genes.

3.3 The effect of exogenous AHLs on *Rlv*-vetch symbiosis and strain competitiveness - laboratory experiments

To estimate the symbiotic efficiency and competitiveness of *Rlv* strains in the presence of exogenous AHLs, *gusA*-tagged strains supplemented with AHLs were used in vetch inoculation. Plants were grown on sterile sand or nonsterile soil (detailed data not shown). Under both conditions, the effect of AHL supplementation on plant growth was non-significant. The most affected parameter was the mean nodule number, which increased from 96±40 to 118±54 (sterile sand) and from 102±37 to 113±46 (unsterile soil) after treatment with AHL. The competitiveness of the strains decreased slightly: GUS$^+$ nodules averaged 36.8±16.6% in control plants without AHLs and 34.8±15.1% in plants with AHL supplementation. When the data were analyzed by grouping the strains according to AHL production (*i.e.* "high AHL", "low AHL" and "no AHL"), the mean percentages of GUS$^+$ nodules were 42.5±13.7%, 33.1±16.6% and 25.9±13.0%, respectively. A significant difference ($P<0.05$) was observed between "high AHL" and "no AHL" groups, suggesting the possibility that this trait contributes to the competitiveness of a strain.

3.4 *Rlv* competition during the nodulation of vetch and pea plants - outdoor experiments

The competitiveness of *Rlv* strains during nodulation was examined using *gusA*-marked derivatives in outdoor experiments under competitive conditions, with two soils (sandy and brown) differing in the number of autochthonous rhizobia (~1.5x10^5 and ~9.3x10^5, respectively). The percentage of GUS$^+$ nodules emerging from pea roots varied depending on the soil type: it ranged from 28.6% to 66.5% in sandy soil *vs*. 17% to 57% in brown soil. In a similar experiment, vetch plants inoculated with the same *Rlv* strains formed from 32.7% to 72.4% GUS$^+$ nodules in sandy soil, and from 13.4% to 48.7% in brown soil (Table 2). Despite the

Strain	Sandy soil				Brown soil			
	vetch		pea		vetch		pea	
	Nodule no. per pot	Gus$^+$ nodules (%)	Nodule no. per pot	Gus$^+$ nodules (%)	Nodule no. per pot	Gus$^+$ nodules (%)	nodule no. per pot	Gus$^+$ nodules (%)
GB25	155	57.1	189	52.3	219	38.0	266	37.8
GB36	113	55.7	152	43.4	197	24.7	241	28.9
GB51	211	37.9	245	42.4	216	25.0	267	29.8
GC16	164	65.2	235	41.5	230	44.5	364	25.1
GC31	97	66.0	169	48.7	198	28.0	234	33.2
GC613	158	44.7	164	28.6	280	48.7	268	57.0
GD22	183	66.8	178	66.5	199	47.4	374	43.1
GD33	175	58.3	291	60.6	229	35.6	309	24.9
P11	106	60.6	133	43.4	208	22.3	254	23.0
P111	178	66.5	168	39.2	256	48.3	281	30.1
P113	179	63.4	240	45.5	320	44.4	375	31.4
P139	73	65.9	182	46.8	204	29.2	288	23.6
P146	170	65.0	226	60.1	307	21.7	379	22.2
P229	188	59.0	160	33.7	243	32.6	372	34.5
P259	58	32.7	37	34.2	197	13.4	159	20.2
P266	243	72.4	211	34.2	225	37.7	304	23.1
P270	259	46.1	174	46.2	235	45.6	338	26.3
P272	151	57.1	153	53.4	211	25.1	211	17.0

Table 2. Nodulation competition of *Rlv gusA*-marked strains in one-step outdoor pot experiments.

All values are means from two replicates (two pots).

different percentage of GUS$^+$ nodules observed in these two experiments, the mean percentage of GUS$^+$ nodules was significantly higher in sandy soil than in brown soil (51.7±1.7% and 31.7±1.7%, respectively, P<0.001). The mean percentage of GUS$^+$ nodules was also significantly higher in vetch plants than in pea plants (45.9±1.7% and 37.6±1.7%, respectively, P<0.01).

A two-step experiment was performed in the same sandy and brown soils with vetch as the host plant inoculated with *gus*-tagged *Rlv* strains (Table 3). In this experiment, the colonization of roots by *gusA*-marked strains was assayed after the second cycle of plant growth (see Materials and Methods). We assumed that bacteria would multiply inside the nodules during the first growth cycle of the vetch plants and would then be released into the soil, enriching the soil with more competitive microsymbionts. We observed that GUS$^+$ rhizobia colonized 9.7% to 74.0% of the nodules of vetch plants in sandy soil *vs*. 2.0% to 34.0% of the nodules in brown soil (Table 3), indicating larger differences in competition abilities of *Rlv* strains than in one-step assay. The mean value of GUS$^+$ nodules was lower here (41.0%) than in the one-step experiments (57.8%) with vetch grown on sandy soil. Similarly, lower numbers of GUS$^+$ nodules (17.6%) were found in vetch plants grown on brown soil in the two–step experiment than in the one-step experiment (34%).

3.5 Correlation between the physiological traits of *Rlv* strains and their competitiveness

As described above, the competitiveness of *Rlv* strains (estimated as the percentage of GUS$^+$ nodules) was affected by the type of soil and the identity of the host plant (vetch or pea) used in the pot experiments. Since strain competitiveness differed largely in each of the experiments, a simple comparison of *Rlv* strain competitiveness was not possible. However, we ranked the strains from most to least competitive in each experiment. The correlation between a given physiological trait and strain competitiveness was estimated with a Spearman correlation (see Experimental Procedures section, Table 4).

In general, the correlation coefficients were low, suggesting that differences in the competitiveness *Rlv* strains could not be attributed to any single trait examined. However, the number of utilized carbon/energy substrates was always positively correlated with competitiveness, especially in the two-step experiment. Moreover, a significant difference in correlation coefficients corresponding to P_{nodA}-*lacZ* fusion activity after flavonoid pretreatment was observed (*i.e.*, low positive correlation in one-step experiments and a negative correlation in the two-step experiment). This results suggest that the flavonoid responsiveness of the *nod* genes for competition in the soil over short time scales is important. Similar relationships were

Strain	Vetch grown on sandy soil		Vetch grown on brown soil	
	Nodule no. per pot	Gus$^+$ nodules (%)	Nodule no. per pot	Gus$^+$ nodules (%)
GB25	139	31.7	152	2.0
GB36	142	40.1	150	34.0
GB51	133	43.6	223	7.5
GC16	169	45.0	193	10.4
GC31	156	63.4	214	25.7
GC613	190	31.1	181	16.6
GD22	184	22.8	138	18.8
GD33	134	53.7	228	3.9
P11	113	9.7	183	31.1
P111	131	74.0	183	32.2
P113	212	61.8	211	3.3
P139	265	40.0	179	25.7
P146	120	27.5	189	4.2
P229	181	51.9	169	3.0
P259	103	17.5	176	4.5
P266	122	46.7	210	26.7
P270	267	33.0	125	33.6
P272	119	59.7	174	32.8

Table 3. Nodulation competition of *Rlv gusA*-marked strains in two-steps outdoor pot experiments.

All values are means from two replicates (two pots).

also demonstrated when the competitive abilities of the individual strains were compared in one-step *vs*. two-step experiments (Figure 2, Table 1). The strains that were more competitive in one-step experiments (e.g. GB25 or GD22) displayed a relatively high level of flavonoid-induced P_{nodA}-*lacZ* activity and a low number of utilized carbon sources. In contrast, GB36, GB51 and GC31 strains utilized a relatively high number of substrates, were weakly activated by flavonoids, and their competitiveness was higher in the two-step experiment. Strains with a higher metabolic potential as well as a strong *nod* gene activation by flavonoids (e.g. P111 or P266) belonged to the most competitive group in both experiments. It appears that a single physiological trait, even if it is most advantageous, does not guarantee high competitiveness. For instance, despite a high P_{nodA}-*lacZ* transcriptional activity in response to flavonoids, the P259 strain was the poorest competitor of all assayed strains. This may be due to a low number of utilized metabolic substrates or an unusually low chemotactic coefficient (0.67), indicating that flavonoids are poor chemoatractants for P259.

The relationship between AHL production and competitiveness was analyzed using mean competitiveness values calculated for groups of strains ("high AHL", low AHL" and "no AHL") in outdoors pot experiments. Considerable differences between the groups were found only in some experiments where vetch was used as a host plant (detailed data not shown). In these experiments, "high AHL" producer strains were more competitive than strains from the "no AHL" group – mean competitiveness values were 63.7±5.2% and 52.0±13.2%, respectively, in one-step experiment in sandy soil; 38.3±8.4% and 26.2±13.3% (one-step experiment in brown soil); and 48.6±14.5% and 29.2±19.2% (two-step experiment in sandy soil).

4. Discussion

The importance of bacterial traits and environmental factors affecting rhizobial competitiveness is the subject of extensive research in the laboratory, in greenhouses and in field experiments [16]. Most studies examine the effect of a single trait and use a simplified approach, which may not necessarily reflect the complexity of this phenomenon. In this study, we investigated the relationship between common features of bacterial *Rlv* strains (metabolic potential, response to plant signals, ability to synthesize microbial signal compounds) and their competitiveness under different soil conditions.

In general, the competitiveness of individual *Rlv* strains measured as the ability to colonize nodules differed to a large extent (from 2% to 74%), which was most evident in the two-step outdoor experiments. Moreover, the competitiveness of individual *Rlv* strains varied considerably from one experiment to the other. Both the type of soil (sandy or brown) and the host plant (pea or vetch) used in the experiments significantly influenced the competitive ability of rhizobia. The effect of soil type can be explained by the varying number of vetch autochthons (~1.5x10^5 in sandy and ~9.3x10^5/g in brown soil), resulting in a less competitive environment for the

Competitiveness of strains assayed in:	Physiological traits		
	number of utilized substrates	P_{nodA}-*lacZ* activity	chemotactic coefficient
one-step experiments	0,323	0,268	-0,162
two-step experiments	0,484*	-0,436	-0,333

Table 4. Correlation coefficients between competitiveness in symbiosis and selected physiological traits in *Rlv* strains

**significant at P<0.05*

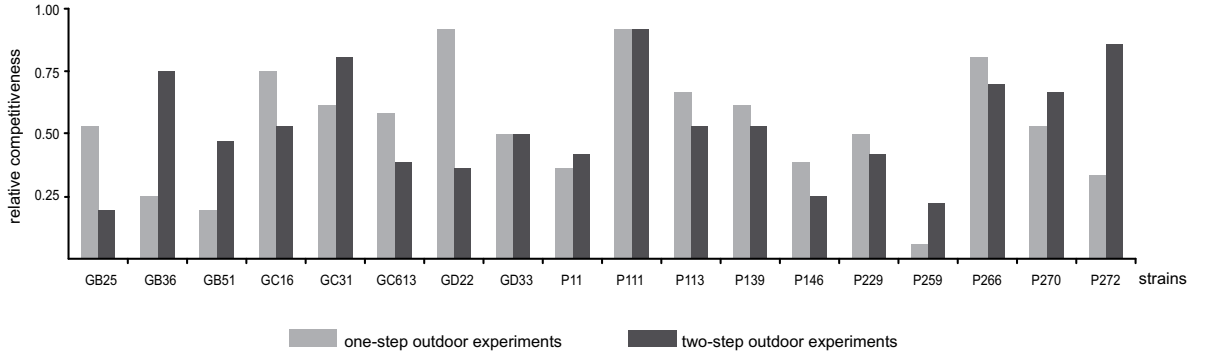

Figure 2. The comparison of the relative competitiveness of Rlv strains studied in outdoor experiments.*For one-step outdoor experiments, the relative competitiveness value was the ratio of the sum of ranks from four experiments divided by 72 (the sum of four maximal ranks). For two-step pot experiments, the relative competitiveness values were the ratio of the sum of ranks from two experiments, which was divided by 36 (the sum of two maximal ranks).

inoculants in the sandy soil. The effect of the abundance of indigenous rhizobia on the competition of the inoculant strains has been described previously [34,35]. The influence of the host plant on rhizobia competitiveness may be explained by the host favoring particular bacterial genotypes during the recruitment of microsymbionts from the rhizosphere [9,25,26]. However this may only explain differences in the competitiveness of certain strains and not others. In our experiments, significant differences in mean strain competitiveness were observed between vetch and pea. Perhaps the difference in the root system architecture of these plants can influence bacterial competition. In comparison with pea plants, the root system of vetch plants is more fibrous, which may facilitate contact with *gusA*-tagged inoculants present in the upper layers of the soil. Regardless of the differences observed, the competitiveness of individual *Rlv* strains was characterized under different environmental conditions (Figure 2).

The highest positive correlation coefficients were found for strain competitiveness and metabolic potential measured by Biolog`s tests. These data are compatible with our previously reported findings [19]. However, the correlation coefficients were not high, which may indicate that the metabolic potential was not the exclusive competition-affecting factor. The metabolic potential of rhizobia may be important for soil dwelling strains, since they can utilize the various nutrients (sugars, acids, amino acids, amines *etc*.) present in the rhizosphere [45-47]. Moreover, some of these substrates are important for bacterial nutrition inside the nodules [48], so the ability to utilize them may be important for saprophytic forms of the rhizobial microsymbionts that multiply in the nodules before being released into the soil [4,32,33]. Taking into consideration the importance of the different substrate groups, the highest correlation coefficients between strain competitiveness and the number of substrates utilized were found for modified aminoacids in the one-step outdoor experiment (0.499, $P<0.05$) and for complex saccharides (mainly oligosaccharides) (0.542, $P<0.05$) in the two-step outdoor experiment. Therefore, it could be speculated that different groups of carbon/energy substrates might be important for promoting strain competitiveness in different environments.

A weak positive correlation was found between strain competitiveness and the activation of *nod* genes by flavonoids in the one-step outdoor experiments. The importance of *nod* gene flavonoid responsiveness in rhizobial competitiveness has previously been postulated [21,22]. Interestingly, such a positive correlation was not observed in the two-step outdoor experiments, which, together with an increased positive correlation between metabolic potential and competitiveness, might be interpreted as the replacement of one bacterial feature (responsiveness to flavonoid induction) by another (metabolic potential). The flavonoid treatment of rhizobia was reported to increase symbiotic parameters of the host plants [49-51]. The higher expression of *nod* genes resulted in an increase of the Nod factor production. Thus, flavonoid pretreatment of individual bacterial strains or legume seeds may enhance the concentration of the Nod factor in the rhizosphere, and, consequently, may lead to a more frequent induction of nodule primordia. In contrast, under such conditions, the nodules may be colonized by any other compatible rhizobia (whether soil-borne or introduced as inoculants). The effect of flavonoid or Nod factors pretreatment of legume seeds [52] on rhizobial competitiveness may therefore yield similar, rather poor outcomes.

Chemotaxis is another factor that can be linked to flavonoid signaling. Despite reports suggesting the importance of chemotaxis for rhizobial competitiveness [23,45], we did not find any positive correlation between chemotactic coefficient and competitiveness of studied *Rlv* strains probably because all but one of our *Rlv* strains were positive in chemotaxis. However, the very low competitiveness of strain P259 and the low chemotactic ratio of 0.67 can suggest an involvement of chemotaxis in *Rlv* competition.

The production of AHL signals and bacterial communication *via* quorum sensing could be linked, to some extent, with *Rhizobium*-legume symbiosis [53]. This is due to the effect of bacterial AHLs on plant gene expression [54] and the effect of plant-derived compounds on rhizobial AHL production [55]. Dong *et al*. [56] suggested that AHL production by rhizobia is required for efficient plant infection, similarly to what was shown for plant infection by pathogenic bacteria. In our experiments, the high AHL producers were more competitive only in some experiments. Moreover, AHL supplementation of rhizobial inoculants did not stimulate strain competitiveness and only slightly increased plant host nodulation. Nevertheless, taking into consideration that (i) numerous traits are controlled by quorum sensing regulation [28,29], (ii) the expression of rhizobial *nod* genes may be influenced by the metabolic status of the cells [57], and (iii) *nod* genes as well as quorum sensing signaling may be involved in biofilm formation [58], a connection between the presence of AHL and Nod factor synthesis cannot be excluded. If that were the case, the effect of AHL supplementation of rhizobial inoculants would be similar but weaker than the flavonoid induction and would affect plant growth (but no rhizobial competitiveness) *via* Nod factor synthesis. Our data supports this hypothesis.

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