

Competitiveness and communication for effective inoculation by *Rhizobium*, *Bradyrhizobium* and vesicular-arbuscular mycorrhiza fungi

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Abstract. After a short summary on the ecology and rhizosphere biology of symbiotic bacteria and vesicular-arbuscular (VA) mycorrhiza fungi and their application as microbial inocula, results on competitiveness and communication are summarized. Stress factors such as high temperature, low soil pH, aluminium concentrations and phytoalexins produced by the host plants were studied with *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* on *Phaseolus* beans. Quantitative data for competitiveness were obtained by using *gus*⁺ (glucuronidase) labelled strains, which produce blue-coloured nodules. For *Phaseolus*-nodulating rhizobia, a group specific DNA probe was also developed, which did not hybridize with more than 20 other common soil and rhizosphere bacteria. Results from several laboratories contributing to knowledge of signal exchange and communication in the *Rhizobium/Bradyrhizobium* legume system are summarized in a new scheme, including also defense reactions at the early stages of legume nodule initiation. Stimulating effects of flavonoids on germination and growth of VA mycorrhiza fungi were also found. A constitutive antifungal compound in pea roots, β -isoxazolinonyl-alanine, was characterized.

Key words. *Bradyrhizobium*; communication; competitiveness; *Rhizobium*; vesicular-arbuscular (VA) mycorrhiza fungi.

Introduction

The ecology of symbiotic microorganisms, and their use for inoculation of soils to increase the yield of crop plants by improving their nutrient supply, have recently been reviewed in references 1, 3, 4, 7, 18, 23, 31 and 43. The genetic basis and biochemical characters which control the competitiveness of single strains within one infection group of *Rhizobium* or *Bradyrhizobium* are not understood in detail. Often, very efficient nitrogen-fixing and nodulating laboratory strains are not competitive against endogenous strains of the same species under field conditions. On the other hand, there is an increasing understanding of the molecular interactions involved in symbiotic communication^{6,10}. In the following we will summarize and discuss new experimental data from our work to characterize competitiveness and communication in rhizobia, and some results with vesicular arbuscular (VA) mycorrhiza fungi.

Competitiveness and stress factors

In *Phaseolus* beans under field conditions, a response to inoculation with specific strains of *Rhizobium leguminosarum* bv. *phaseoli*, *R. tropici* or *R. etli*, which can all form nodules in these host plants, is more the exception than the rule⁴¹. Studying the resistance of *R. leguminosarum* bv. *phaseoli* and *R. tropici* strains towards different stress factors, it was evident that the most competitive strain (KIM5) was rather tolerant against high temperature and aluminium concentrations, but

more sensitive than other strains to lower soil pH, and to tannins and phytoalexins produced by the host plants⁴⁵. The conclusion is that it is very difficult to combine the highest resistance against all relevant soil and stress factors into one competitive strain. Sensitivity of the *Rhizobium* strains towards tannins was greater at acidic pH⁴⁶. A large proportion of the arable land in the tropics has acidic soils. Soil pH there is a major factor limiting nodulation and nitrogen fixation in *Phaseolus* beans⁴². Co-inoculation with different strains at the same time proved not to be a successful strategy to overcome this problem, since the number of nodules formed by specific inoculant strains is affected by the competition of other strains^{11,37}.

Quantification of competitiveness: *gus* technology and gene probes

To improve the methods for quantification of competitiveness a *gus*⁺ (glucuronidase) strain of *R. leguminosarum* bv. *phaseoli* KIM5s was constructed and used in competition experiments against 17 other strains of *R. leguminosarum* bv. *phaseoli* and three strains of *R. tropici*. The results of these competition experiments at two pH levels (5.2 and 6.4) are summarized in table 1. The *gus* technology is very useful for direct identification of a single strain, because the nodules produced by the *gus*⁺ strain are blue in colour, in contrast to the red or pink nodules produced by the other inoculant strains. For better visibility of the root system, including the nodules,

Table 1. Nodule occupancy in different *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* strains when coinoculated with the *Rhizobium phaseoli* strain KIM5s *gus*⁺

<i>Rhizobium</i> spp. strain	Nodule occupancy/KIM5s (%)	
	pH 6.4	pH 5.2
CIAT 2510	4 ± 2	ND
H2C	4 ± 4	ND
CIAT 323	9 ± 5	10 ± 3
CFN 1500	11 ± 11	67 ± 20*
CE3	23 ± 2	ND
C-05-II	25 ± 17	82 ± 20*
CIAT 7004	30 ± 4	23 ± 8
CIAT 899	41 ± 4	76 ± 18*
CIAT 895	54 ± 10	33 ± 10*
Tal 182	68 ± 4	72 ± 15
CIAT 652	79 ± 4	73 ± 22
Costa 6	79 ± 11	ND
CIAT 7014	82 ± 7	67 ± 12
CFN 227	85 ± 11	ND
CIAT 151	89 ± 10	90 ± 10
CIAT 904	91 ± 4	86 ± 4
CIAT 163	91 ± 8	95 ± 5
CIAT 956	93 ± 6	93 ± 6
CFN 1600	94 ± 8	86 ± 13
CIAT 611	96 ± 4	96 ± 2

Means ± SD of two replicates of five plants each. *R. tropici* strains are underlined. Assays were performed in growth pouches at two pH levels. **p* < 0.05, versus nodule occupancy at pH 6.4. From: Streit et al., Biol Fertil. Soils 14 (1992) 140–144.

so-called growth pouches made of plastic material are used³⁹. With this technology, nodule occupancy between 4% for the least competitive strain and up to 96% for the most competitive strain could be observed (see table 1). The three strains of *R. tropici* studied had a very low nodule occupancy at pH 6.4, but a much better competitiveness at pH 5.2. The competitiveness of *R. leguminosarum* bv. *phaseoli* strains was also affected by pH. The utilization of 43 different carbon sources by these strains was studied. Most of the less competitive strains of *R. leguminosarum* bv. *phaseoli* were unable to metabolize ferulate or coumarate, whereas, with a few exceptions, the highly competitive strains had those catalytic capacities³⁹. Growth of *R. leguminosarum* bv. *phaseoli* and *R. tropici* strains on aromatic compounds was also shown^{19,47}, but no correlation between competitiveness and this capacity was demonstrated.

To improve the rapid identification of specific *Rhizobium* species and infection groups, DNA probes were developed, using the subtraction hybridization method⁴⁰. This method was developed in the laboratory of J. Cooper in Belfast, using subtraction hybridization and polymerase chain reaction amplification for the isolation of strain-specific *Rhizobium* DNA sequences². For the *R. leguminosarum* bv. *phaseoli* and *Rhizobium tropici* specific DNA probe, total DNA preparations from 8 different other *Rhizobium* species and soil bacteria were pooled and used as a subtracter DNA against total genomic DNA from

the *R. leguminosarum* bv. *phaseoli* strain KIM5s. Only one round of subtraction hybridization was necessary for removing all cross-hybridizing sequences. The specificity of this DNA probe is summarized in table 2. The probe hybridizes completely with all 17 strains tested from the *Phaseolus* infection group and is negative against 34 other soil bacteria such as *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Azotobacter vinelandii*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Xanthomonas campestris* and various other *Rhizobium* and *Bradyrhizobium* species. This DNA technology allows the rapid identification of the *R. leguminosarum* bv. *phaseoli* and *R. tropici* strains within a single day, whereas a 1–2 week test period is needed when plant inoculation is used. Requests from more than 30 different countries regarding this DNA probe and probe technique indicate the usefulness of this technology. With this DNA probe, a soil titer as low as 3×10^4 homologous indigenous rhizobia per g soil could be detected⁴⁰.

Microsymbiont-legume host plant communication during the early stages of nodule initiation

Contributions from a large number of laboratories have made it evident that the signal exchange between microsymbionts and host plants is much more detailed than simply the induction of the nodulation genes (*nod*) by flavonoids (fig. 1). The patterns of flavonoids present

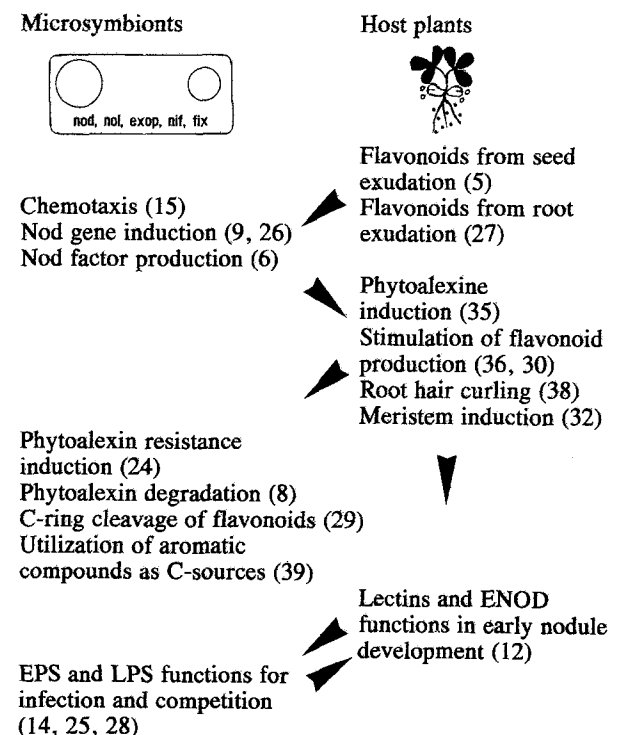


Figure 1. Microsymbiont-legume host plant communication during the early stages of nodule initiation, modified from Werner et al.⁴⁴. References are given in brackets.

Table 2. Cross hybridization of the developed DNA probe in dot blot hybridizations with total genomic DNA from different bacteria

Bacteria	Hybridizing with
<i>R. leguminosarum</i> biovar <i>phaseoli</i> KIM5s (3)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 7033 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 2510 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 904 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 895 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 652 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 613 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 611 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 323 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 163 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CIAT 141 (2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> CFN 1600 (1)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> TAL 182(2)	+
<i>R. leguminosarum</i> biovar <i>phaseoli</i> COSTA 6 (2)	+
<i>R. tropici</i> CFN 1500 (1)	+
<i>R. tropici</i> CIAT 899 (2)	+
<i>R. tropici</i> C-OS-II (2)	+
<i>Agrobacterium rhizogenes</i> DSM 30148 (6)	-
<i>Agrobacterium tumefaciens</i> DSM 30150 (6)	-
<i>Agrobacterium tumefaciens</i> C6-6 (5)	-
<i>Alcaligenes eutrophus</i> DSM 517 (6)	-
<i>Arthrobacter globiformis</i> (5)	-
<i>Azotobacter vinelandii</i> DSM 431 (6)	-
<i>Bacillus subtilis</i> DSM 1970 (6)	-
<i>Citrobacter freundii</i> DSM 30039T (6)	-
<i>E. coli</i> K12 DSM 498 (6)	-
<i>Enterobacter agglomerans</i> (5)	-
<i>Klebsiella pneumoniae</i> K11 (new isolate)	-
<i>Pseudomonas stutzeri</i> ATCC 14405 (6)	-
<i>Pseudomonas carboxydovorans</i> O.M.5 (6)	-
<i>Pseudomonas fluorescens</i> DSM 1694 (6)	-
<i>Pseudomonas facilis</i> DSM 620 (6)	-
<i>Pseudomonas putida</i> DSM 3226 (6)	-
<i>Paracoccus denitrificans</i> DSM 1404 (6)	-
<i>Proteus mirabilis</i> DSM 4479T (6)	-
<i>Shevanelia putrefaciens</i> NCIMB 10471 (6)	-
<i>Xanthomonas campestris</i> DSM 50859 (6)	-
<i>Rhizobium leguminosarum</i> biovar <i>trifolii</i> K9 (5)	-
<i>Rhizobium leguminosarum</i> biovar <i>viciae</i> F28 (4)	-
<i>Rhizobium leguminosarum</i> biovar <i>viciae</i> 248 (4)	-
<i>Rhizobium fredii</i> HH 103 (7)	-
<i>Rhizobium meliloti</i> 2011 (7)	-
<i>Rhizobium</i> sp. (<i>Robinia pseudoacacia</i>) R I (5)	-
<i>Rhizobium</i> sp. (<i>Robinia pseudoacacia</i>) R III (5)	-
<i>Bradyrhizobium</i> sp. (<i>lupinus</i>) USDA 3055 (8)	-
<i>Azorhizobium caulinodans</i> ORS 571	-
<i>Bradyrhizobium japonicum</i> USDA 123 (8)	-
<i>Bradyrhizobium</i> sp. (<i>Pueraria phaseolide</i>) CIAT 3918 (2)	-
<i>Bradyrhizobium</i> sp. (<i>Centrosema plumieri</i>) CIAT 3101 (2)	-
<i>Bradyrhizobium</i> sp. (<i>Centrosema macrocarpum</i>) CIAT 3111 (2)	-
<i>Bradyrhizobium</i> sp. (<i>Centrosema macrocarpum</i>) CIAT 3011 (2)	-

+ crosshybridization; - no crosshybridization;

Bacteria were obtained from: 1, Centro de Investigacion sobre Fijacion de Nitrogeno, Cuernavaca (Mexico); 2, Centro Internacional de Agricultura Tropical, Cali (Colombia); 3, Dr. J. Handelsman, Madison, Wisconsin (USA); 4, Prof. Dr. Lotz, Erlangen (Germany); 5, Dr. M. Röhm, Marburg (Germany); 6, Deutsche Sammlung von Mikroorganismen, Braunschweig (Germany); 7, Dr. B. Bohloul, NifTal, Hawaii (USA); 8, US Dept. of Agriculture, Beltsville (USA).

DNA from *R. leguminosarum* biovar *phaseoli* KIM5s was used as probe-DNA, subtracter strains are underlined.

From: Streit et al., FEMS Microbiol. Ecol. 13 (1993) 59-68; Springer-Verlag, Berlin.

in seeds⁵ and in roots are very different in terms of quality and quantity²⁷. Flavonoids, or the precursor cinnamic acid, can have chemotactic activity^{15,16}. Induction of *nod* and *nol* genes is the centre of interest in a large number of publications (e.g. refs 9, 26). *Nod* factor production⁶ leads to root hair curling³⁸, activation of cell

cycle machinery and meristem induction³², and also to a stimulation of flavonoid production³⁶. The microsymbionts themselves induce phytoalexin production in the host plants, but only during the first 6 to 12 hours of inoculation³⁵. Flavonoids can be enriched in the outer membrane of *R. meliloti*; this is perhaps a protection

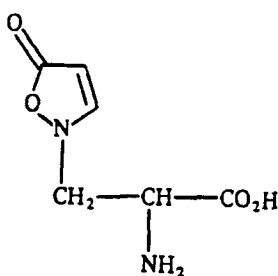
mechanism for the inner membrane¹³. There are two strategies against phytoalexins in the host rhizosphere. *Bradyrhizobium* can react by an induced resistance²⁴ and *R. leguminosarum* with a phytoalexin degradation of the phytoalexin wayerone⁸. C-ring cleavage of flavonoids²⁹ is a strategy of the microsymbionts to change the concentration of signalling compounds. More competitive strains of *R. leguminosarum* bv. *phaseoli* have also been shown to utilize different aromatic compounds as carbon sources to a larger extent than less competitive strains³⁹. Not much is known about how lectins and early nodulines produced by the host plants¹² are affected by microsymbiont strains with different competitiveness. Exopolysaccharides (EPS) and lipopolysaccharides (LPS) also have important functions for infection and competition; however, again the strategies in *Bradyrhizobium* and *Rhizobium* appear to be different^{14,25,28}. There are many variations from a generalized scheme such as that in figure 1. For example, in alfalfa, betaines such as trigonellin and stachydrin also have a gene-inducing activity, though at a much higher concentration than most flavonoids²⁷. Cyclic glucans, which can make up between 5 and 20% of the dry weight of cells of *Bradyrhizobium japonicum*²⁰, may play an important role in osmotic adaptation and thereby also in competition in the soil under varying water conditions.

Flavonoids and VA mycorrhiza development

Compared to our understanding of the molecular mechanisms of communication between *Rhizobium*, *Bradyrhizobium* and legume host plants, knowledge of the VA mycorrhizal symbiosis is far behind. However, in principle we can assume that there are some similar mechanisms, as indicated by the effect of various flavonoids on spore germination and hyphal growth of *Glomus* species (Kape et al.¹⁷). Significant increase of both growth parameters was observed by application of myricetin and quercetin. Also, a 2- and 6-fold increase of the phytoalexin concentration in roots of *Vicia faba* infected with mycorrhiza (*G. aggregatum*, *G. macrocarpum* and *G. mosseae*) was found in different host cultivars, compared to uninfected plants¹⁷. This confirms previous results by Morandi²¹ and Morandi et al.²² with other host plants. However, a major result of these data is that there is a large difference between a phytopathogenic interaction and a response to VA mycorrhiza, with the latter triggering phytoalexin concentrations that are lower by two orders of magnitude.

Constitutive antifungal components in roots

A major role in the competitiveness of symbiotic microorganisms and their communication with the host



Species	Strain	Inhibition ^a
<i>Saccharomyces cerevisiae</i>	DSM 70449 (typestrain)	++
<i>Saccharomyces uvarum</i>	MUCL 27835	++
<i>Saccharomyces exiguus</i>	MUCL 27835	+
<i>Candida utilis</i>	MUCL 30058	++
<i>Candida lipolytica</i>	MUCL 29853	++
<i>Kluyveromyces polysporus</i>	MUCL30238	++
<i>Pichia membranaefaciens</i>	MUCL 30004	++
<i>Botrytis cinerea</i>	New isolate	++
<i>Pythium ultimum</i>	New isolate	++
<i>Rhodosporidium toruloides</i>	MUCL 30249	-
<i>Leucosporidium scottii</i>	DSM 4636	-
<i>Rhodotorula glutinis</i>	DSM 70398	-
<i>Rhodotorula rubra</i>	DSM 70825	--

^a ++, Strong (inhibition zone 20–30 mm); +, moderate (inhibition zone 5–10 mm); -, not significant (inhibition zone <1 mm)

Figure 2. Structure of β -isoxazolinonyl-alanine (BIA) and susceptibility of fungi against this heterocyclic non-protein amino acid.

plants is related to induced components as described in the previous paragraphs. Moreover, we should not overlook the importance of constitutive components in the roots of the host plants which may affect invading microorganisms. A broad antifungal activity of β -isoxazolinonyl-alanine, a non-protein amino acid from roots of pea plants, has been described³⁴ (fig. 2). This compound inhibited a large number of fungi such as *Saccharomyces* species, *Candida* species, *Kluyveromyces polysporus* and *Pichia membranaefaciens*; however, it was ineffective against a number of other fungi such as *Rhodotorula* species and *Leucosporidium* species. This compound also had a significant antimycotic effect against phytopathogenic fungi such as *Phythium ultimum*, *Botrytis cinerea* and *Rhizoctonia solani*³³. The effect of these compounds against VA mycorrhizal infection has not been analyzed so far and deserves further work.

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