Biol Fertil Soils (2005) 42: 83–88
DOI 10.1007/s00374-005-0012-2

ORIGINAL PAPER

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Diversity of rhizobia isolated from an agricultural soil in Argentina based on carbon utilization and effects of herbicides on growth

Received: 19 November 2004 / Revised: 2 May 2005 / Accepted: 2 May 2005 / Published online: 28 June 2005

Abstract Seventy-six rhizobial isolates belonging to four different genera were obtained from the root nodules of several legumes (*Vicia sativa*, *Vicia faba*, *Medicago sativa*, *Melilotus* sp., *Glycine max* and *Lotus corniculatus*). The action of five commonly used herbicides [2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate (GF), dicamba, atrazine and metsulfuron-methyl] on the growth of rhizobial strains was assessed. Subsequently, GF and 2,4-D were tested in a minimum broth as C and energy sources for 20 tolerant strains. The ability of these strains to metabolize different carbon sources was studied in order to detect further differences among them. Tolerance of the bacteria to agrochemicals varied; 2,4-D and GF in solid medium inhibited and diminished growth, respectively, in slow-growing rhizobial strains. Among slow-growing strains we detected *Bradyrhizobium* sp. SJ140 that grew well in broth + GF as the sole C and energy source. No strain was found which could use 2,4-D as sole C source. The 20 strains studied exhibited different patterns of C sources utilization. Cluster analysis revealed three groups, corresponding to four genera of rhizobia: *Rhizobium* (group I), *Sinorhizobium* (group II) and *Mesorhizobium–Bradyrhizobium* (group III). On the basis of the results obtained on responses to herbicides and C sources utilization by the isolates investigated, it was possible to differentiate them at the level of strains. These results evidenced a considerable diversity in rhizobial populations that had not been previously described for Argentinean soils, and suggested a physiological potential to use natural and xenobiotic C sources.

Keywords Rhizobial diversity · Herbicides · Carbon sources · Argentinean soils

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Introduction

Genera of rhizobia able to nodulate different legumes species are *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Allorhizobium* (Velázquez et al. 2001).

A major limitation in the study of rhizobial populations is the difficulty to identify strains in their natural habitat. However, several techniques for characterization and identification of strains have been developed, such as C sources utilization analysis by traditional methods (Kennedy 1994) or by means of Biolog system (Swelim et al. 1997) and fatty acids and lipopolysaccharides profiles (Kennedy 1994; Zahran 2001), which reflect bacterial diversity present in soils.

It has been suggested that agriculture creates homogeneous and highly selective environments that reduce bacterial diversity (Martínez-Romero and Caballero-Mellado 1996). Conversely, it has been argued that cultivation results in more diverse populations and greater diversity of substrate utilization by total microbial communities (Kennedy and Smith 1995; Palmer and Young 2000). Arable soils are frequently disturbed by plowing, monoculture rotation and denudation by harvest, and they are subject to high levels of fertilizers, herbicides and other pesticides (Palmer and Young 2000). Intensification of agricultural practices in Argentina has resulted in a continuous increment of agrochemicals consumption and 60% of pesticides commercialized are herbicides (Bertonatti and Corcuera 2000). Despite the beneficial impact of herbicides on agricultural productivity, soil exposure to herbicides represents a considerable side effect of agricultural practices. As a consequence, interaction between soil microorganisms and herbicides may influence soil quality and fertility, because these biologically active chemicals may have deleterious effects on beneficial species; otherwise, microbes utilize and degrade these compounds (Alexander 1980; Dinelli et al. 1998). During their free-living heterotrophic phase, rhizobia can degrade pesticides such as atrazine (Bouquard et al. 1997) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Kamagata et al. 1997). Furthermore, it

has been shown that catabolic pathways exist for protocatechuate in *Rhizobium* and *Bradyrhizobium*, and for catechol in *Rhizobium leguminosarum* (Sadowsky and Graham 1998).

In the present research we studied the phenotypic diversity of rhizobia belonging to the main genera of rhizobia that colonize the same soil, by assessing the growth of different strains in the presence of five herbicides and the ability of the strains to utilize different energy and C sources.

Materials and methods

Isolation of strains

Vetch (*Vicia sativa* L.), horsebean (*Vicia faba* L.), birdsfoot-trefoil (*Lotus corniculatus* L.), alfalfa (*Medicago sativa* L.), soybean (*Glycine max* L. Merrill) and sweetclover (*Melilotus* sp. L.) were grown in pots containing soil from a farm (located in the area of Coronel Dorrego, Buenos Aires province, Argentina), where inoculated soybean had been grown earlier. Nodules were collected 30–45 days later, and isolates were obtained from sterilized and crushed nodules on yeast extract–mannitol agar (YEMA) (Vincent 1970). After incubation at 28°C for 2–5 days (fast-growing strains) to 7–10 days (slow-growing strains), a well-separated colony from each nodule was transferred to YEMA slants and stored at 4°C for subsequent study. Isolates were coded according to their host, as follows: vetch (VC), alfalfa (AA), horsebean (HB), soybean (SJ), sweetclover (ML) and birdsfoot-trefoil (LT).

Agrochemicals

Herbicides used in the experiments were (2,4-dichlorophenoxy)acetic acid (2,4-D); (2-methoxy-3,6-dichloro)benzoic acid (dicamba); 6-chloro-*N*-ethyl-*N*-isopropyl-1,3-5-triazin-2,4-diamine (atrazine); *N*-(phosphonomethyl)glycine (glyphosate); methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate (metsulfuron-methyl). The chemicals used were in the following commercial formulations: 2,4-D, Zamba soluble concentrate [100% active ingredient (a.i.)]; dicamba, Banvel 480 soluble concentrate (48% a.i.); atrazine, Gesaprim 90 granules (90% a.i.); glyphosate, Zamba soluble concentrate (48% a.i.); metsulfuron-methyl, Trimet wettable powder (60% a.i.).

Rhizobial resistance to herbicides

Several strains within each genus were screened for their sensitivity to agrochemicals when incorporated in a solid medium. Liquid cultures of the strains (10 µl strain^{-1}) were inoculated onto replicated Petri dishes containing YEMA mixed with herbicides at the following concentrations of a.i.: 2,4-D 200 mg l^{-1}, dicamba 200 mg l^{-1}, glyphosate 250 mg l^{-1}, metsulfuron-methyl 25 µg l^{-1}, and atrazine 500 mg l^{-1}. After 2–5 days (fast-growing strains) to 7–10 days (slow-growing strains) at 28°C, growth inhibition compared with growth on YEMA without herbicides (control) was evaluated.

Herbicides as nutrient sources in liquid medium

Twenty rhizobial strains that showed herbicide resistance were selected. The ability of these isolates to utilize 2,4-D and glyphosate was assessed in liquid medium (MB) described by Bergersen (1961) and modified in the following way: $Na_2HPO_4 \cdot 12\ H_2O$ 0.45 g l^{-1}; $MgSO_4 \cdot 7H_2O$ 0.1 g l^{-1}; $FeCl_3$ 20 mg l^{-1}; $CaCl_2$ 40 mg l^{-1}; sodium glutaminate 50 mg l^{-1}; mannitol 50 mg l^{-1}; thiamine 0.1 mg l^{-1}; biotin 0.125 mg l^{-1}. Herbicides were added to media at a concentration of a.i. of 50 mg l^{-1}.

Erlenmeyer flasks (125 ml) were prepared with 30 ml medium and bacterial strains were added as 1 ml inoculum (ca. 10^6 CFU ml^{-1}). A control for each strain (MB without herbicide) was included. Erlenmeyer flasks were maintained at 28°C, and periodically the optical density at 610 nm (OD_{610}) was determined via a spectrophotometer (Zeiss mod. PM2K). Positive growth was indicated if the $OD_{610} \geq 0.1$ (Wagner et al. 1995). Optical density of slow-growing strains was measured at 7, 14, 21, 28 and 43 days, and for fast-growing strains O.D was determined at 7, 14, 23 and 30 days.

Carbon sources utilization

Test tubes (10×100 mm) were prepared with 3 ml minimal medium (MM) (modified from medium CM; Kennedy 1994), with the following composition: $NH_4H_2PO_4$ 1.25 g l^{-1}; KCl 0.25 g l^{-1}; $MgSO_4 \cdot 7H_2O$ 0.25 g l^{-1}; K_2HPO_4 0.50 g l^{-1} (pH 7.0); in addition, these tubes contained the redox dye triphenyl trezazolium chloride (TTC) and 37 different C sources, at final concentrations of 0.005 and 0.1% (w/v), respectively. Tubes were inoculated with 95 µl of broth-grown cultures of rhizobial strains (OD_{610}=0.6), and maintained at 28°C for 4 days.

Substrates that tested as possible C and energy sources for rhizobial strains were: carbohydrates (L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-trehalose, sorbose, D-xylose, sucrose); carboxylic acids (acetate, citrate, fumarate, succinate, malate); polyols (glycerol); lipids (glycerol tributyrate); polymers (dextrine, Tween 80, inuline); aminoacids (arginine, threonine, lysine, tryptophane, cysteine); aromatic compounds (sodium benzoate, catechol, pyrocatechol); hydrosoluble vitamins (thiamine, inositol, L-ascorbic acid, calcium pantothenate).

Carbon sources utilization was assessed by spectrophotometric measurements at 590 nm on the fourth day. Previously, growth of one strain within each group was evaluated in MM with mannitol as C source and TTC at the concentration stated above, and a 4-day incubation time

was selected as yielding the most reliable results for both fast- and slow-growing strains (data not shown).

The C sources utilization patterns (the absorbance recorded at 590 nm for each tube) were used for cluster analysis using NCSS software trial (Hintze 2001). Distance matrices were produced using the Unweighted Pair-Group with Arithmetic Average (UPGMA) and represented in the form of a dendrogram.

Plant inoculation test

Surface-sterilized birdsfoot-trefoil and alfalfa seeds were placed in Hungate tubes (25×200 mm), containing agarified Jensen N-free medium (Vincent 1970). Aseptically germinated seedlings were inoculated with 0.5 ml bacterial suspension of *Sinorhizobium* sp. strains (ML22, ML26, AA324, AA235, AA340) and *Mesorhizobium* sp. strains (LT111, LT112, LT122, LT233), respectively, and transferred to the growth chamber. Aseptically pregerminated vetch and soybean seedlings were transferred to Hungate tubes containing liquid Jensen N-free medium and inoculated with 1 ml of *Rhizobium* sp. strains (VC130, VC157, VC347, HB316) and *Bradyrhizobium* sp. strains (SJ128, SJ138, SJ140, SJ324, SJ325), respectively. Non-inoculated plants were included as controls. Four replicate plants for each strain were made and tubes were kept in growth chamber at 24°C for 30–40 days, until nodulation was observed.

Results and discussion

Seventy-six strains belonging to four genera of rhizobia (*Sinorhizobium*, *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*) were obtained from the same soil. Indigenous and naturalized legumes grow spontaneously in the Pampas region, including *Vicia*, *Medicago*, *Melilotus*, *Lotus* and *Trifolium* (Burkart 1952). In contrast, soybean and *Bradyrhizobium* inoculants strains have been only recently introduced. Therefore, the four genera of rhizobia coexist in the same soil.

Confirmation of the isolates as rhizobia belonging to four different genera was performed based on their ability to nodulate their host legumes. The action of five herbicides, commonly used in the region, on the growth (tolerance/inhibition) of 76 rhizobial strains was assessed. Subsequently, 20 herbicide-resistant strains (five of each genera) were characterized for their ability to use glyphosate and 2,4-D as C and energy sources, and to metabolize 37 C substrates.

Bacterial sensitivity to herbicides

Most strains were tolerant to dicamba, metsulfuron-methyl and atrazine in YEMA at tested doses (Table 1). Glyphosate and 2,4-D showed different effects on the growth of slow-growing strains in YEMA. 2,4-D inhibited the growth of most *Bradyrhizobium* and *Mesorhizobium* strains (94.4 and 52.6%, respectively), whereas glyphosate reduced the growth of strains of these genera (61.1 and 57.9%, respectively). Conversely, 2,4-D and glyphosate had no considerable effects on strains of *Sinorhizobium* and *Rhizobium* (Table 1).

Glyphosate adversely affects the growth of rhizobia and symbiotic processes. Faizah et al. (1980) reported that the behavior of tropical rhizobia (cowpea group) in the presence of glyphosate depends on the strain. Previous works have shown that this agrochemical not only inhibits the growth of *Bradyrhizobium* and *Rhizobium*, but also nodulation and/or nitrogen fixation in glyphosate-resistant soybean (Zablotowicz and Reddy 2004) and subterranean clover (Eberbach and Douglas 1983).

Herbicide utilization in broth culture

Growth curves of two rhizobial strains studied are shown in Fig. 1.

Genera of rhizobia differed in their growth under low-nutrient conditions (MB); only *Bradyrhizobium* strains SJ324 (Fig. 1b), SJ128, SJ138 and SJ325 (data not shown) grew in this medium, probably due to a better adaptation to oligotrophic conditions, as reported by Saito et al. (1998).

Table 1 Effect of agrochemicals on the growth of rhizobial strains from 4 different genera determined by grading of growth appearance on yeast extract–mannitol agar (YEMA)

Genera	Total no. of isolates	Observed growth pattern on agrochemicals[a]				
		GF	2,4-D	AT	DC	MET
Sinorhizobium	19	+	+	+	+	+
Rhizobium	14	+	+	+	+	+
	3	+	±	+	+	+
	1	+	±	±	+	+
	2	+	+	±	+	+
Mesorhizobium	8	+	+	+	+	+
	3	±	−	−	+	±
	4	±	−	−	+	+
	1	±	−	±	+	+
	1	±	−	±	+	+
	1	±	−	+	+	±
	1	±	±	±	+	+
Bradyrhizobium	1	+	+	+	+	+
	10	±	−	+	+	+
	1	±	−	±	+	+
	2	−	−	+	+	+
	3	+	−	+	+	+
	1	+	−	+	−	+

GF Glyphosate, *2,4-D*, 2,4-dichlorophenoxyacetic acid, *AT* atrazine, *DC* dicamba, *MET* metsulfuron-methyl

[a]Effects were registered as − inhibition, ± reduced growth, + and normal growth when compared to the growth of the control (untreated)

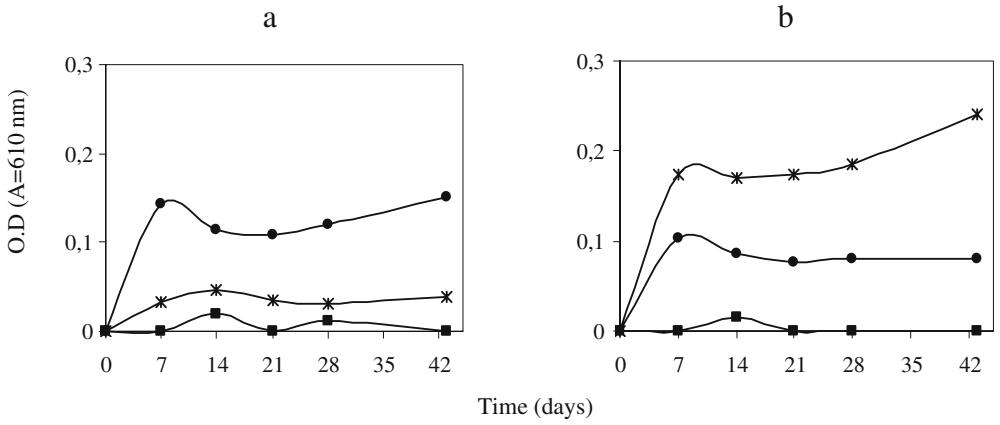

Fig. 1 Growth curves of rhizobial strains in MB, containing glyphosate (GF) or 2,4-D as carbon sources. *Curves* shown here correspond to SJ140 (**a**) and SJ324 (**b**). Symbols correspond to Control (*), GF (•) and 2,4-D (▪)

Examination of growth curves of the strain SJ324 (Fig. 1b), and the others collected (data not shown), shows there is no evident herbicide utilization.

Neither fast- nor slow-growing strains utilized 2,4-D (OD_{610}<0.1) as a sole source of C and energy. However, slow-growing 2,4-D-degrading strains, phylogenetically related to *Bradyrhizobium*, have been isolated from pristine soils (Kamagata et al. 1997).

Only *Bradyrhizobium* sp. SJ140 (Fig. 1a) grew (OD_{610}>0.1) on glyphosate as sole source of C and energy. Utilization of a given substance seems to be a characteristic at the strain rather than at species or genera levels (e.g., Pipke and Amrhein 1988; Liu et al. 1991). Liu et al. (1991) demonstrated that glyphosate degrading ability is widespread in the family *Rhizobiaceae* with the use of herbicide as the sole source of P, by initial cleavage of C–P bond with release of sarcosine as the immediate breakdown product. However, Liu et al. (1991) did not find any organism which could use glyphosate as C or N source. Concerning *Bradyrhizobium* sp. SJ140, our results may suggest that this strain is able to metabolize glyphosate via sarcosine and subsequent utilization of sarcosine as a C source—a hypothesis that deserves further research. Alternatively, bacteria could be consuming other compounds (surfactant or inert product) present in a commercial product.

In view of their widespread distribution in soils, rhizobia may participate in some steps of glyphosate breakdown pathway.

Carbon sources utilization

Relationships among the 20 strains studied based on 37 C sources utilization are represented in a dendrogram (Fig. 2), which revealed that 18 of the 20 isolates were distributed in three distinct groups at Euclidean distance of 1.00. Groups corresponded to the four genera of rhizobia studied: *Rhizobium* (group I), *Sinorhizobium* (group II), *Mesorhizobium* and *Bradyrhizobium* (group III).

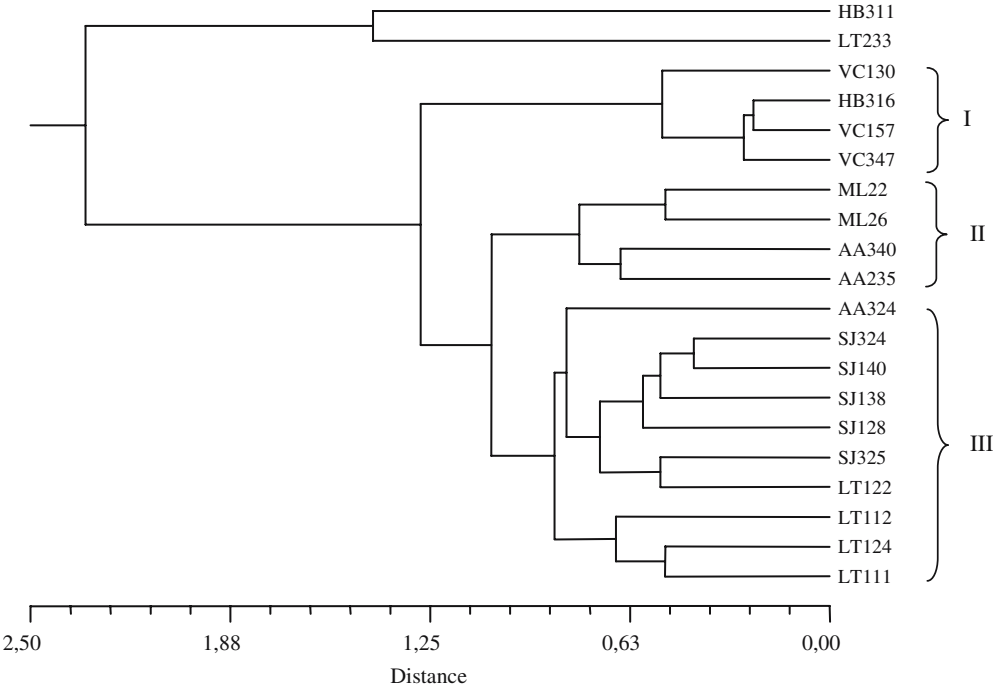

Fig. 2 Dendrogram showing phenotypic relatedness, based on utilization of 37 carbon sources and Euclidean distance, among 20 rhizobial strains. Cluster analysis was performed using the Unweighted Pair-Group with Arithmetic Average method in the NCSS program. The correlation cophenetic value (*r*) was 0.895

Most strains exhibited growth on diverse carbohydrates (raffinose, xylose, arabinose, mannitol, lactose, and trehalose), vitamins (inositol, thiamine and panthothenate) and various other compounds (dextrine, arginine, glycerol, glycerol tributyrate and succinate). Strains of groups II and III grew on hexoses (fructose, glucose, mannose, rhamnose, sorbose and galactose), disaccharides (maltose, cellobiose and sucrose), carboxylic acids (malate and fumarate) and three other substrates (inuline, Tween 80 and lysine).

Strains in group I (*Rhizobium*) constituted the most homogeneous group (within the group distances ranged from 0.24 to 0.52) and had low activity in most of the tested C sources. Our results differ partially from what Jordan (1984) established as differential characteristics of the species *R. leguminosarum*, since our strains grew poorly on several carbohydrates (fructose, mannose, maltose, sucrose) and organic acids (fumarate and malate), and are dissimilar to those obtained by Chakrabarti et al. (1981) for glucose utilization by *R. leguminosarum* biovar *trifolii*. One possibility for these dissimilarities is that Chakrabarti et al. (1981) and Jordan (1984) tested different species and biovars. Alternatively, composition of MM used to test C sources utilization or incubation time may have caused the observed differences (Wagner et al. 1995).

Strains in group II (*Sinorhizobium*) grew intensely in a wide range of C sources that are used to differentiate the species *Sinorhizobium meliloti* (Chakrabarti et al. 1981; Jordan 1984; Holt et al. 1994), with the only exception of citrate. This is probably due to an inhibitory effect of citrate on fast-growing rhizobial strains (Werner 1992; Fulchieri et al. 1999).

Group III (*Mesorhizobium* and *Bradyrhizobium*) comprised strains of moderate growth on many C sources tested. This group was also the most diverse, with distances within the group ranging from 0.42 to 0.86. Possible explanations for the observed heterogeneity can be that slow-growing strains may show variability in C sources utilization (Chakrabarti et al. 1981), and/or that rhizobia nodulating *Lotus* may include both fast- and slow-growing strains (Fulchieri et al. 1999). With respect to *Bradyrhizobium* sp. strains, results obtained with our method were consistent with those observed by Swelim et al. (1997) using the commercial system Biolog for strains of *Bradyrhizobium* sp. (*Leucaena*). As regards *Mesorhizobium* strains, our results are similar to those obtained with strains of *Mesorhizobium loti* (Fulchieri et al. 1999) and *Mesorhizobium chacoense* (Velázquez et al. 2001) isolated from Argentinean soils.

Dendrogram analysis shows that there are remarkable differences among the strains isolated from the same soil. Despite the fact that it may be necessary to examine the genotypic diversity of these isolates, it can be expected that results based on C sources utilization show congruence with genotypic fingerprinting, since rhizobia classification based on functional attributes (C sources utilization) closely reflects their genetic differences (McInroy et al. 1999).

As the strains analyzed were obtained only from nodules, this research is limited to a fraction of the soil population that is not representative of the whole population (Paffetti et al. 1996). The methodology we used in this study allowed us to discriminate the 20 isolates as different rhizobial strains, revealing the existence of a remarkable phenotypic diversity (still within genera). The presence of neophytic and widely distributed legumes (Ulrich and Zaspel 2000) and agricultural practices (Palmer and Young 2000) could have created conditions that favor the introduction of diverse rhizobial types or their diversification.

The heterogeneity observed in C sources utilization among rhizobial strains may have ecological relevance because it could influence organism's ability to live saprophitically in soil, to colonize plant roots and to live symbiotically (Chakrabarti et al. 1981; McInroy et al. 1999). Those strains with a broad range of C sources utilization may have an ecological advantage in colonizing the soil or the rizosphere, when compared with strains having a degree of specificity in their requirements (Chakrabarti et al. 1981).

Conclusions

Rhizobial responses to herbicides, as well as C sources utilization, allow us discriminate the metabolically diverse isolates at the level of strains. This differentiation evidences a remarkable rhizobial diversity that has not been previously described for Argentinean soils, and suggests a physiological potential to use natural and xenobiotic C sources. This diversity has a particular importance, for example, when evaluating the feasibility to use an inoculant for a certain legume. It is probable that indigenous and naturalized strains colonize a plant's rizosphere at the same time and compete between them for C sources of root exudates, thus affecting the persistence and/or competitiveness of the desirable inoculant strain.

Acknowledgements The authors gratefully acknowledge Dr. Norman Peinemann for giving permission to use laboratory equipment, and the Secretaría General de Ciencia y Tecnología of Universidad Nacional del Sur (SGCyT-UNS) for the scholarship for post-graduate study granted to M.C. Zabaloy.

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