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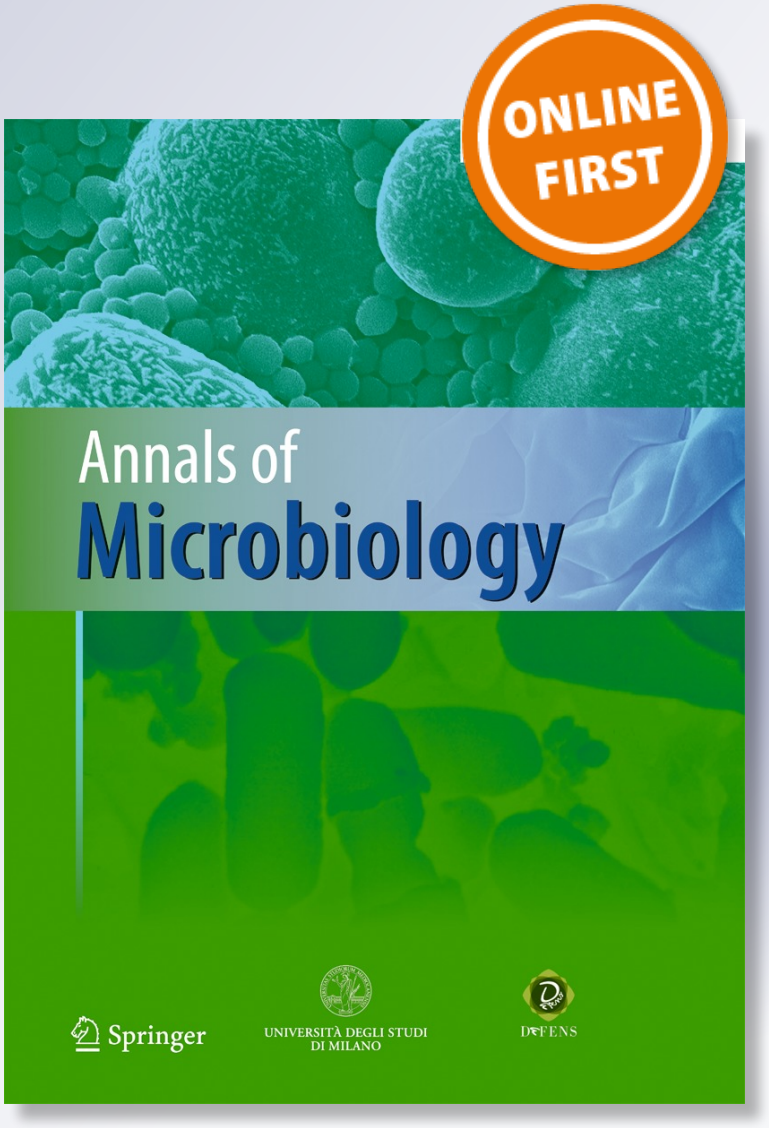
Annals of Microbiology

ISSN 1590-4261

Ann Microbiol
DOI 10.1007/s13213-013-0619-8



Springer

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DOI 10.1007/s13213-013-0619-8

ORIGINAL ARTICLE

Denitrifying capacity of rhizobial strains of Argentine soils and herbicide sensitivity

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Received: 14 September 2012 / Accepted: 11 February 2013

Abstract Agrochemical application in soils is a matter of environmental concern, and among soil microorganisms, rhizobia and their action before different pesticides are interesting to study, due to their taxonomic and functional diversity. The objectives of the present work were to assess the capacity of rhizobial populations to use herbicides as source of nutrients, as well as their ability to reduce nitrates and / or denitrify. Eighty-one strains belonging to four populations of different genera of rhizobia (*Rhizobium*, *Mesorhizobium*, *Ensifer* and *Bradyrhizobium*) were assessed. The effect of glyphosate, 2,4-dichlorophenoxyacetic acid, and atrazine on growth of the strains, as well as the ability of the strains to act on herbicide transformation to reduce nitrate and denitrify, were evaluated. The genera studied showed different responses to pesticides. *Bradyrhizobium* had the greater capacity to utilize the herbicides and among the compounds evaluated, atrazine was the most used as a source of energy. To conclude, some *Bradyrhizobium* strains were able both to denitrify and to use the atrazine herbicide. The results obtained in this study increase expectations of the use of rhizobia as inoculants, causing changes at the agricultural and environmental level and allowing an appropriate management of agricultural soil fertilization, efficiency in nitrogen fixation and a faster biodegradation of pesticides in soil.

Keywords Rhizobial populations · Herbicides utilization · Denitrification · Soil

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Introduction

The amount of pesticide used worldwide in the last years was approximately 2.4 billion kilograms. Herbicides accounted for the largest portion of total use, followed by other pesticides, insecticides, and fungicides (Grube et al. 2011). Herbicides are beneficial in terms of agricultural productivity, but soils are the sites that are exposed to these compounds and it is important to consider this side effect in agricultural practices (Zabaloy and Gómez 2005). Pesticides with a chemical structure similar to natural products are biodegradable; therefore, their use seems a reasonable compromise for the protection of crops and the environment (Boivin et al. 2005). Microorganisms are important in improving the environment and in the degradation of herbicides (Marecik et al. 2008). The adaptation of soil microorganisms found on sites treated repeatedly with herbicides and the resulting degradation of the herbicide is faster than on untreated soil sites (Freemark and Boutin 1995). The positive effect of xenobiotics degradation by microorganisms can be explained in terms of great biodiversity of decomposers and the broad spectrum of enzyme production (Marecik et al. 2008). Often, degradation of herbicides is carried out by the cooperation of several microbes excreting metabolites that are exchanged and processed sequentially, resulting in the gradual transformation of the molecule (Barriuso et al. 1996).

Also, excessive use of nitrogenous fertilizers can be harmful to ecosystems (Fernández 2007), resulting in a significant source of nitrate (NO_3^-) pollution in soil and water (Ortega et al. 2002). The behavior of agrochemicals in the soil affects its dispersal in the environment; hence, soil occupies a central position in the regulation of pollution from agricultural production (Barriuso et al. 1996). Denitrification is the main biological mechanism by which fixed nitrogen returns to the atmosphere from land or water,

Published online: 09 March 2013

completing the cycle. It is a gradual reduction, an alternative respiration pathway used in the absence of oxygen by phylogenetically heterogeneous microorganisms capable of many different functions of importance in ecosystems (Philippot and Hallin 2005). Many studies have reported that nitrate-reducing bacteria and denitrifiers are affected in the rhizosphere. The concentrations of the major factors that regulate denitrification, i.e., carbon, oxygen and nitrate, are known to be modified in the soil surrounding plant roots (Henry et al. 2008).

Rhizobia, typical soil nitrogen-fixing bacteria, contribute significantly to the denitrification process of soils, but by comparison with other strains, their denitrification potential is low (Fernández 2007). Studies in *Bradyrhizobium* sp. isolated from nodules of soybeans in Argentine soils have been reported with different denitrifiers capacities. Values (nmol N_2O μg^{-1} cell protein) are between 0.22 and 4.20 for isolates and at about 7 for collection strains such as USDA 110 (Fernández et al. 2008). Addition of pesticides can reduce bacterial denitrification, probably due to cell death or cell inactivation, but it can also stimulate this process due to (1) the use of the pesticide as an electron donor by the denitrifiers; (2) death of organisms caused by the pesticide, which results in an easily available source of carbon for denitrification; or (3) an unspecific stress response. The reaction patterns of denitrifiers in response to pesticide application are not as clear, which may be related to the vast number of taxonomically, distantly related genera of denitrifiers in soils (Philippot et al. 2007).

Xenobiotic biodegradation under anaerobic conditions such as in groundwater, sediment, landfill, sludge digesters and bioreactors has gained increasing attention over the last two decades. The enzymatic reactions common to many pesticides include dechlorination, hydrolysis, nitro reduction, and dealkylation. A bacterium may be partially responsible for these metabolic activities, and in some cases the bacteria may have a metabolic shift from one pathway to another (Zhang and Bennett 2005).

Rhizobia have a wide variety of functions associated with the agricultural environment where they thrive. These can degrade pesticides such as glyphosate (Liu et al. 1991), 2,4-dichlorophenoxyacetic acid (2,4-D) (Itoh et al. 2002) and atrazine (Bouquard et al. 1997). It has been shown that some rhizobia can solubilize phosphates (Rodriguez and Fraga 1999), use organic phosphate (Abd-Alla 1994) and be denitrifiers (Mesa et al. 2004).

In the present study, we evaluated the rhizobial potential in the transformation of likely polluting compounds of agricultural land and in the contribution of essential nutrients to crops. Specifically, we studied four different genera rhizobial population responses to the action of three herbicides. Furthermore, we examined the physiological capabilities of rhizobia to transform compounds, in particular herbicides utilization and nitrate reduction and / or denitrification.

Materials and methods

Bacterial strains

A total of 81 strains of Rhizobia isolated from Argentine agricultural soils were studied. The strains were deposited in the Microorganisms of Agro-Environmental Interest collection of the Unité Mixte de Recherche (UMR) de Microbiologie du Sol et de l'Environnement of INRA, Dijon, France. These correspond to different genotypes analyzed by RFLP-PCR ITS 16S-23S and 16S sequencing (Gómez 2001). Eight strains of the genus *Rhizobium*, five of *Mesorhizobium*, five of *Ensifer* (formerly *Sinorhizobium*) and 63 of *Bradyrhizobium* were assessed. Among the 81 strains tested, a genotype matched up to *Bradyrhizobium japonicum* USDA 110; therefore, it was used as control in denitrification assays for its denitrifying ability (Fernández 2007).

Rhizobial resistance to and utilization of herbicides

Herbicides used in the experiments were glyphosate, 2,4-D, and atrazine. The concentrations of active ingredient (a.i.) used were 0.5 %, 2.5 %, and 10 %, respectively. The chemicals used were in the following commercial formulations: glyphosate, Roundup soluble concentrate (48 % a.i.); 2,4-D, Zamba soluble concentrate (100 % a.i.); atrazine, Gesaprim 90 granules (90 % a.i.).

In this study, the 81 strains were evaluated according to their sensitivity to agrochemicals and the utilization of herbicides as a source of nutrients. Liquid cultures of the strains (10 μl $strain^{-1}$) were inoculated onto duplicated Petri dishes containing yeast extract mannitol agar (YEMA) (Vincent 1970) for rhizobial resistance study, and solid Bergersen Medium (BM) modified (Zabaloy and Gómez 2005) for herbicides utilization study. In both experiments for each strain, growth medium without herbicides was used as control, and growth medium supplemented with different herbicides was used as treatment. The Petri dishes were incubated at 28 °C in a moist chamber and were controlled weekly in the course of a month. The herbicides effect on strains growth was evaluated by comparison to development on control. Herbicides usage was assessed according to the development of each strain in BM with or without herbicides. When the development of a strain in the treatment exceeded the development in the control, it was considered that the strain had the potential to use the herbicide as a source of nutrients. The utilization of atrazine was identified by the appearance of a transparent halo, since this herbicide is insoluble in the medium used.

YEMA is the most commonly used medium for the culture of rhizobia, as it provides the nutrients needed for proper growth. In this study, YEMA supplemented with the

different herbicides was used to evaluate the sensitivity of rhizobia to these compounds. It was considered that strains with no growth were affected negatively by the herbicide, since there are no limits of nutrients.

However, BM is a mineral base that was used to study the capacity of rhizobia to use herbicides as a source of nutrients. Strains that had a good development were able to use the herbicide for growth, since the medium does not have the nutrients needed for development.

Concentration and type of nutrients in each medium determined different development for a same strain. Results obtained were compared with their respective control (medium without herbicide). The use of each medium had different objectives; therefore, the results are independent.

Denitrification potential of rhizobia

To study denitrification and nitrate reduction capacity of the 81 strains, optical density of a 500 µl liquid culture of each strains was adjusted to 0.5 ($A_{620\ nm}$). Inverted Durham tubes used to visualize gas production were filled with 5 ml of liquid BM supplemented with potassium nitrate (KNO_3) (10 mM), inoculated with each strain, and incubated at 28 °C for 14 days in jars with the GasPak Anaerobic System (BBL). After this period, the presence or absence of gas was registered (Tiedje 1994) and nitrate and nitrite reduction was tested by Griess Ilosvay's Nitrite Reagent (Merck), which reacts with nitrate to form a red diazo dye, technique described by MacFaddin (2003). Strains were considered positive for denitrification activity if the cultures had gas bubbles and if nitrate and nitrite disappeared. When the microorganism was able to reduce nitrate and nitrite, but not to produce gas, an assimilative reduction took place (Fernández et al. 2008).

Utilization of atrazine in liquid medium and molecular analysis of denitrifiers genes

Three strains of the genus *Bradyrhizobium* that demonstrated the ability to use atrazine in solid medium and denitrify were selected and called "Strains Double Capacity" (SDC). The ability of these strains to grow in liquid medium BM supplemented with atrazine was assessed. The herbicide was added to the medium at a concentration of a.i. of 50 mgl^{-1}. Erlenmeyer flasks were prepared with 50 ml medium supplemented with atrazine and bacterial strains were added as 1 ml inoculum (ca $1 \times 10^6 CFU\,ml^{-1}$). Erlenmeyer flasks were maintained at 22 °C and optical density ($A_{620\ nm}$) via a clinical analyzer (Metrolab 1600 DR) was determined weekly for 6 weeks. When the culture optical density increased in time, it was considered that the strain grew due to herbicide atrazine usage.

At the same time, DNA was extracted from pure cultures of SDC using UltraClean® Microbial DNA isolation Kit (MOBio, Solana Beah, CA) according to manufacturer's instructions. Primers used and PCR conditions were performed according to Fernández et al. (2008). Briefly, touchdown PCR was used to amplify *nap*A, *nir*K, *nor*C and *nos*Z genes in strains using gene-specific oligonucleotide primers (Bedmar et al. 2005) (Table 1). The reaction mixture contained: genomic DNA (80–100 ng), 2 mM of each dNTP, 15 pmol of each of the oligonucleotide primers, 1 U of Taq DNA polymerase (Promega, WI) and 1 x green buffer (Promega, WI). The program consisted of the following steps: (1) Initial denaturation at 95 °C for 5 min; (2) 5 cycles at 95 °C for 1 min, starting with an annealing temperature of 58 °C for 1 min, which decreased by 1 °C every cycle, 72 °C for 2 min; (3) 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 53 °C for 1 min, and primer extension at 72 °C for 2 min; and (4) a final extension step at 72 °C for 10 min. All PCR amplification reactions were performed in a DNA thermal cycler (Bioer, China). The fragments obtained were then separated on a 2 % SEAKEM ME agarose gel, and analyzed using a DNA molecular weight marker Fast Ruler High Range of 1 kb (Fermentas, Life Sciences) using Kodak ID 3.0 software (Kodak, New Haven, CT).

Results and discussion

Effect of herbicides on rhizobia

2,4-D

The effect of the herbicide on the growth of strains assessed in YEMA was different. A significant number of the strains studied decreased their growth (59.3 %) and others did not grow (8.6 %) in the medium supplemented with 2,4-D, mainly in genus *Bradyrhizobium*, *Ensifer* and *Rhizobium* (Table 2). Inhibition of rhizobial strains by 2,4-D in genus *Bradyrhizobium* and *Mesorhizobium* was observed by Zabaloy and Gómez (2005). 2,4-D can affect sensitive microbial populations, which in turn are used as substrate by microorganisms resistant to herbicides (Zabaloy and Gómez 2008).

In BM, a 91.4 % of the strains evaluated (all genera) developed as the control. Only 7.4 % of the strains grew more in presence of 2,4-D and showed potential to use this herbicide as a source of nutrients. Studies in surface soil, aquifer sediment and groundwater collected from a Danish site showed that 2,4-D does not affect the bacterial density; in fact, it stimulates growth on certain decomposers even if it is implemented in 10,000 mg kg^{-1} (Lipthay et al. 2007).

Table 1 Description of oligonucleotide primers, amplicon sizes and nucleotide sequence analysis of denitrification genes

Target gene	Primer sequence	Expected amplicon size (bp)
*nap*A	(5′)-CATCATGACACGTTCACCCG (3′)	3,000
	(5′)-CATCGAAACCGCCTGAGT (3′)	
*nir*K	(5′)-CGTCTAGTTGGTGTTGGC (3′)	1,500
	(5′)-CACTGCATCCCGTCATCAAA (3′)	
*nor*C	(5′)-GCGCFCAACCGGCCTTATT (3′)	500
	(5′)-CGACAGGAATCAACCGGA (3′)	
*nos*Z	(5′)-GCTCCCGATCAGACCGATT (3′)	2,000
	(5′)-CATGAGCGACAGCGACAA (3′)	

2,4-D mineralization experiments carried out using three different soils (clay, loam and sand) showed that mineralization appeared to be the main process limiting 2,4-D availability, with each soil containing its own 2,4-D decomposers (Boivin et al. 2005). Biodegradation of 2,4-D, as several herbicides, can occur either through specific decomposers that use the herbicide as a source of carbon and nitrogen, or by co-metabolism, where the relative proportions of these populations varies with soil type. The available microbiota to mineralize this compound has been found naturally in cultivated soils. *Ralstonia eutropha* JMP134 is a well studied 2,4-D degrader (Clément et al. 2001), and other organisms capable of degrading 2,4-D belong to genera like *Achromobacter*, *Alcaligenes*, *Arthrobacter* (Ka et al. 1994). Rhizobia could be considered, along with several microbial species, as members of a consortium; and thus cover different stages in the process of biodegradation of 2,4-D.

Table 2 Effect of agrochemicals on the growth of 81 rhizobial strains in yeast extract mannitol agar, and utilization of herbicides in Bergersen medium modified

Herbicide	Genus	Resistance [a]			Utilization [b]		
		+	±	-	++	+	-
2,4-D	*Rhizobium*	5	-	3	-	8	-
	Mesorhizobium	4	1	-	1	4	-
	Ensifer	1	4	-	2	3	-
	Bradyrhizobium	16	43	4	3	59	1
Glyphosate	*Rhizobium*	8	-	-	-	8	-
	Mesorhizobium	3	2	-	1	4	-
	Ensifer	1	4	-	-	5	-
	Bradyrhizobium	14	41	8	-	59	4
Atrazine	*Rhizobium*	1	3	4	-	8	-
	Mesorhizobium	-	1	4	-	5	-
	Ensifer	2	3	-	-	5	-
	Bradyrhizobium	14	37	12	14	49	-

[a] Number of strains in the evaluation of herbicide resistance [b] Number of strains in the evaluation of utilization of the herbicides

The impact was registered as - inhibition, ± reduced growth, + normal growth, ++ increased growth, compared with growth in the control (untreated)

Glyphosate

Glyphosate effects were similar to those of 2,4-D in the study of herbicide resistance in medium YEMA (Table 2). Again, a great proportion of the strains (58 %) decreased the growth respect to the control and eight of the total strains (9.9 %) were inhibited. Results were similar to those of Zabaloy and Gómez (2005), who found a decrease in the growth of different strains of Rhizobia (*Rhizobium*, *Mesorhizobium*, *Ensifer* and *Bradyrhizobium*) due to glyphosate. However, in studies made with the same genus in YEMA supplemented with 450 μg of glyphosate—an application nine times higher than that made in our work—all *Bradyrhizobium* tested were resistant and few strains of the other genus were affected negatively by the herbicide (Drouin et al. 2010).

In the evaluation of utilization of glyphosate by the strains in BM, most of the strains had development similar to the control (94 %). Only one strain grew more on treatment with glyphosate than the control. Therefore, we cannot confirm the ability of rhizobia to use glyphosate in solid medium. Zabaloy and Gómez (2005) found one strain of the genus *Bradyrhizobium* capable of growing in glyphosate as the sole source of carbon and energy. In contrast, Liu et al. (1991) showed that the ability to degrade glyphosate is widespread among rhizobia, using the herbicide as source of phosphorus, not as source of carbon or nitrogen. In agricultural soils, temporal variation of glyphosate levels depends directly on the time of application and rainfall (Peruzzo et al. 2008). Glyphosate is moderately persistent in soil and is degraded primarily by co-metabolic microbial processes. It is strongly adsorbed to soil particles, and thus has a low mobility through the soil profile (Zabaloy and Gómez 2008).

Atrazine

Atrazine had a particular behavior in relation to the strains tested. On the one hand, in the experiment of tolerance to

herbicides in YEMA, atrazine was the herbicide that had the most influence. The herbicide decreased growth in at least one strain of each genus (54.3 %) or inhibited their development (24.7 %) (except *Ensifer*). On the other hand, in the study of the utilization of herbicides as source of nutrients in BM, atrazine was the most used herbicide. Fourteen strains of *Bradyrhizobium* (approximately 17.3 % of the total) increased their development by the presence of atrazine and were considered potential transformers of this compound (Table 2). Regardless, most of the strains (82.7 %) grew as the control, like the treatments with 2,4-D and Glyphosate. A similar study of sensitivity with atrazine in higher concentration showed that it did not affect any of the *Rhizobium* and *Bradyrhizobium* strains evaluated, and inhibited 12 of the 21 *Mesorhizobium* strains tested (Drouin et al. 2010).

Studies on atrazine mineralization in the rhizosphere of maize showed no decrease in microbial biomass due to treatment with atrazine. The development of microorganisms that degrade this herbicide can be stimulated by the exudation of organic compounds in the rhizosphere (Piutti et al. 2002). Atrazine is moderately persistent in natural environments, with an average life ranging from a few days to several months, and has been one of the most heavily used agricultural herbicides over the past 30 years (Devers et al. 2004). This heavy usage, combined with atrazine's relatively long half-life in the environment, means that agricultural fields remain as significant sources of atrazine to surface and groundwater systems (Dombek et al. 2001).

It is unusual for a single microorganism to contain all the enzymes to degrade a pesticide, but there are exceptions. It has been shown that *Pseudomonas* sp. ADP is able to completely mineralize atrazine on soils of United States (Devers et al. 2004), France (Piutti et al. 2002) and Poland (Marecik et al. 2008). In agricultural soils of Argentina, atrazine-degrading *Arthrobacter* sp. strains (Fernández et al. 2012) were isolated and in agricultural soils of Australia, a single bacterium, *Arthrobacter nicotinovorans* HIM, with significant levels of atrazine mineralization was found (Aislabie et al. 2005). Microorganisms can use different sources of carbon and nitrogen to adapt to different environments (Marecik et al. 2008), but the use of a specific substance appears to be a feature of the strain rather than species or genus level (Zabaloy and Gómez 2005).

Denitrification capacity of rhizobia

Nitrate reduction in bacteria has three functions: the utilization of nitrate as a nitrogen source for growth (assimilation), the generation of metabolic energy by using nitrate as a terminal electron acceptor (respiration), and the dissipation of excess reducing power for redox balancing (dissimilation). Denitrification is a reductive process initiated by respiratory (dissimilatory) nitrate reduction, which transforms nitrate into molecular nitrogen gas in four stages (Fernández et al. 2011).

Fifty-eight percent of the strains tested, corresponding to *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium*, showed the capacity of assimilatory reduction, and seven strains from *Bradyrhizobium* were gas producing, thus being considered potential denitrifiers. On the other hand, some of the strains either made nitrate respiration, reducing nitrate to nitrite (13 %), or did not use nitrate (26 %); thus suggesting a wide diversity among rhizobial strains in the physiological process evaluated (Table 3).

The reduction of nitrates and denitrification is rather unusual among rhizobia. Rhizobial strains isolated from *Lotus* sp. have been characterized by their ability to denitrify and were identified as *Mesorhizobium loti* species and *Bradyrhizobium* sp. (Monza et al. 2006). Moreover, Fernandez et al. (2008) found that from 250 isolates of *Bradyrhizobium* spp., 16.4 % were denitrifiers, while 17.6 % were nitrate reducers, and all denitrifying strains were *Bradyrhizobium japonicum*. However, our results demonstrated denitrification capacity in six strains of *B. japonicum* and one strain of *Bradyrhizobium elkanii*.

Legumes production like soybean (*Glycine max* L.) depends highly on the amount of symbiotic microorganisms present in the soil, particularly rhizobia. In sixteen soybean-bearing Argentine soils with different cropping histories, it was found that the larger soil number of natural populations of *B. japonicum* was in spring (mean= $5.9 \times 10^7 g^{-1}$), and that soil management did not significantly change the population size of bradyrhizobia nodulating soybean in the soils (Silva et al. 2002). The annuals bacterial inoculations allow this number to be kept elevated. *Rhizobium* inoculants seem to be an attractive and cost effective source of nitrogen for legumes cultivation (Fatima et al. 2006). On the other hand, the increase in density of these bacteria that could be denitrifiers leads to nitrate loss by denitrification in the soil, therefore decreasing availability for the cultivation. It was demonstrated that even with an important density of *Bradyrhizobium* in soils, denitrification rate in soil was not influenced significantly by these microorganisms (Fernández 2007). These bacteria can be considered environmentally friendly, since they have been used for many years with legumes without causing harm to the environment or to users, and it was demonstrated that *Bradyrhizobium* and *Rhizobium* have excellent potential to be used as plant growth promoting rhizobacteria (PGPR) with non-legumes (Antoun et al. 1998).

Strains double capacity: atrazine transforming and denitrifying

From the results of item 3.1.3 and item 3.2, it was found that three of the 81 strains tested, *B. japonicum* MSDJ 5727, *B.*

Table 3 Capacity of 81 rhizobial strains for reduce nitrate and/or nitrite in medium Bergersen modified under anaerobic conditions

Genus	Absence of NO_3^- and NO_2^-		Presence of NO_2^-	Presence of NO_3^-
	Gas present [a]	Gas absent [b]	Gas absent [c]	Gas absent
Rhizobium	-	3	2	3
Mesorhizobium	-	4	1	-
Ensifer	-	-	-	5
Bradyrhizobium	7	33	10	13
Total	7	40	13	21

NO_3^- nitrate, NO_2^- nitrite

[a] Potential denitrification;
[b] Assimilative nitrate reduction;
[c] Nitrate respiration

japonicum MSDJ 5385 and *B. elkanii* MSDJ 5567 (MSDJ: Microbiologie du Sol Dijon), were able to transform atrazine and to denitrify. Therefore, they were selected to verify their ability for atrazine utilization in liquid medium and to screen the presence of denitrifying genes.

Growth of strains monitored spectrophotometrically ($A_{620\ nm}$) revealed a similar behavior during the first 2 weeks, with pronounced growth (increase of turbidity) (Fig. 1). Maximum absorbance was on day 14 for *B. japonicum* MSDJ 5727 ($A_{620\ nm}$ = 0.4) and on day 21 for *B. japonicum* MSDJ 5385 ($A_{620\ nm}$ = 0.35) and *B. elkanii* MSDJ 5567 ($A_{620\ nm}$ = 0.3). From the fourth week on, absorbance values were stable, although they were lower than those observed in previous weeks. The variation in SDC growth shows the different ability to use the herbicide; particularly, *B. japonicum* MSDJ 5727 incremented its growth in 14 days (7 days before the others). By contrast, Devers et al. (2004) monitored the growth ($A_{600\ nm}$) of *Pseudomonas* sp. ADP and *Chelatobacter heintzii*, finding stable absorbance throughout incubation, therefore indicating that bacteria did not grow during the experiment. Also Piutti et al. (2002) found no correlation between mineralization potential and microbial biomass, but detected a correlation between *atz*C gene coding an enzyme involved in atrazine mineralization and soil mineralization ability.

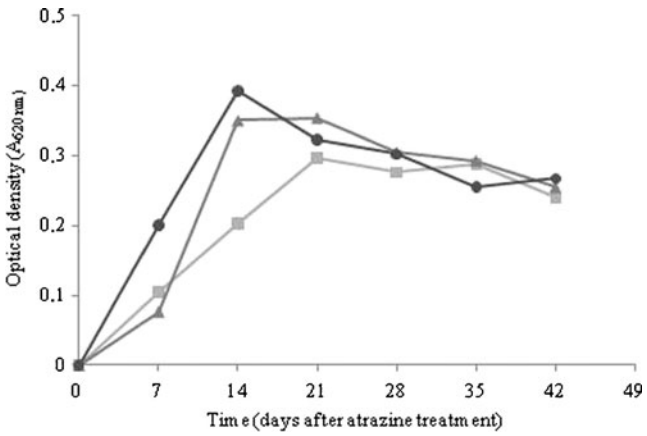

Fig. 1 Growth curves of three strains of *Bradyrhizobium* in medium Bergersen modified supplemented with atrazine (50 mg l^{-1}). (■) *B. elkanii* MSDJ 5567; (▲) *B. japonicum* MSDJ 5385; (●) *B. japonicum* MSDJ 5727

Atrazine mineralization does not always occur; for example, *Arthrobacter nicotinovorans* HIM isolated directly from an agricultural sandy dune soil 6 months after a single application of atrazine was able to grow in minimal medium with atrazine as sole nitrogen source, but was unable to mineralize ^{14}C-ring-labelled atrazine (Aislabie et al. 2005). Similarly, in our work, atrazine was the only nutrient available to bacteria; hence, we propose that their growth is due mostly to atrazine use.

It has been shown that soils repeatedly treated with atrazine show enhanced atrazine degradation, resulting in its mineralization in less than 2 weeks (Devers et al. 2004). In our study, *B. japonicum* MSDJ 5727 had the higher growth on day 14. This suggests that the strain uses atrazine to develop in a short term. Microorganisms often respond to xenobiotics input into the environment, adapting to the use of xenobiotics as nutrient and energy source (Udiković-Kolić et al. 2012). A variety of soil fungi and bacteria slowly evolved enzymes and pathways capable of degrading herbicides based on an s-triazine ring. The strain *B. japonicum* MSDJ 5727 used in our study comes from soils with corn/soybean production and repeated applications of atrazine. Therefore, we suggest that atrazine utilization by the strain could enhance, and adaptation for atrazine mineralization is possible.

The presence of atrazine in environments like groundwater, surface water, flooded and saturated soils, which are often anoxic or reductive, has generated interest in the potential for anaerobic biodegradation of this herbicide. Atrazine degradation or disappearance is rapid in soil with aerobic conditions and much slower in anaerobic or reducing conditions (DeLaune et al. 1997). Degradation of herbicides under anaerobic conditions is a characteristic of few bacteria. *Ralstonia basilensis* M91-3 is a denitrifying soil bacterium capable of using s-triazines as its sole C and N source under aerobic as well as anaerobic conditions (Stamper et al. 2002). The atrazine degradation pathway included hydroxy-atrazine formation and ring cleavage. Denitrification-coupled atrazine degradation by M91-3

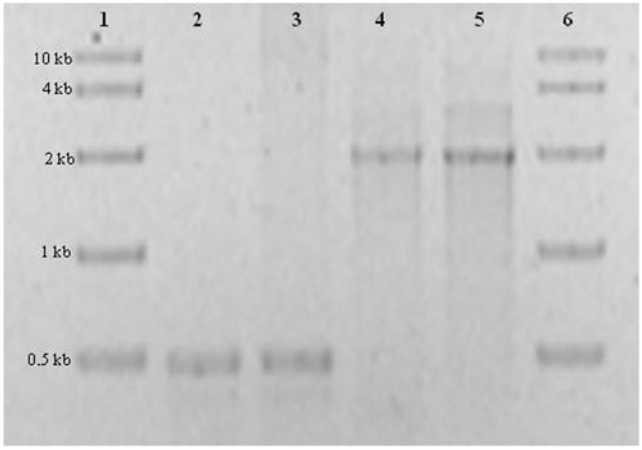

Fig. 2 PCR analysis with primers designed to amplify internal regions of *nor*C (lanes 2–3) and *nos*Z (lanes 4 and 5) genes. Lanes 1 and 6: DNA size marker; *B. japonicum* MSDJ 5385 (lane 2 and 4); *B. japonicum* USDA 110 (control) (lanes 3 and 5)

proceeds through hydroxyatrazine, ultimately to CO_2 and NH_3 (Crawford et al. 1998).

In our study, *B. japonicum* MSDJ 5385 (serogroup USDA 127) used atrazine as nutrient source and presented two genes involved in denitrification process, namely *nor*C and *nos*Z (Fig. 2). Denitrification among rhizobia is rare, and most species do not contain the whole set of denitrification genes (Monza et al. 2006). Another possible explanation to these inefficient amplification reactions could be due to differences between nucleotide sequences of the primers and genetic sequences for the enzymes among denitrifying bacteria (Fernández et al. 2008). In this case, SDC are of different groups and have different nucleotide sequences [GenBank accession: AF363141 (*B. japonicum* MSDJ 5727 serogroup USDA 123); AF363122 (*B. elkanii* MSDJ 5567, reference USDA 61)]. On the basis of the results of this study, we suggest that it could be interesting to evaluate the anaerobic biodegradation of atrazine in this microorganism of agro-environmental interest.

Conclusions

This work shows a varied response to herbicides among rhizobial strains even within the same genus. Herbicide sensitivity was detected in more than half of the strains tested and a low proportion of strains were able to use herbicides as nutrient source. Atrazine was the most used herbicide by bradyrhizobia.

Under denitrifying conditions, some *Bradyrhizobium* strains produced gas. Assimilatory reduction prevailed in most strains, while nitrate respiration was observed in low proportion.

Physiological ability to use atrazine and denitrifying was observed in three rhizobial strains. The rest of tested strains were able to do one or the other of the two physiological processes. Atrazine usage occurs principally under aerobic conditions, but the capacity to utilise this herbicide under denitrifying conditions is of interest to improve water and soil quality, and seems a reasonable compromise between crop protection and environmental concerns.

Acknowledgments The authors are grateful to Secretaría de Ciencia y Técnica of the Universidad Nacional del Sur for financial support of the research work, through PGI No. (24A/146) and to Comisión Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) for providing a fellowship to Magalí Vercellino.

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