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# Plant-Soil-Microorganism Interactions on Nitrogen Cycle: *Azospirillum* Inoculation

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## 1. Introduction

When soil is analyzed as a system, it shows great complexity. It is constituted by aggregates of mineral (sand, clay, lime) and organic (live and decomposing organisms) elements, which interact in the soil atmosphere and the water.

The most dynamic chemical fraction constituents of soil, like the compounds from organic matter degradation, the ions in the soil solution (including protons) and the gas concentration of pore atmosphere, define the main physico-chemical variables of soil.

As soil microorganisms are active transformation agents of both the mineral and the organic components of the soil, they play an essential role in plant nutrient cycles, i.e., soil fertility. The study of microbial functions, considered as the activity expression of microorganisms under the environment's conditions, constitutes an important issue in soil and plant microorganism interaction. By means of the expression of their specific functions, microbial communities may act on the environment, for example, by producing or consuming organic or inorganic compounds, or by releasing enzymes into the environment. On the other hand, the environment acts on function expression by means of biotic, abiotic and anthropic conditioners. In this context, soil inoculation with microorganisms is a biotechnological practice that may be performed within the framework of productive soil handling (Lynch, 1987). The goals to achieve include contributing to plant nutrition and soil fertility, controlling phytopathogenic microorganisms or biodegrading compounds -organic or inorganic- which contaminate the soil (Meiri & Altman, 1998).

The recognition of the benefits gained by *Leguminous* plant inoculation with *Rhizobium*, and widespread communication have led to a search for interaction *Gramineous* plants-bacteria, which may be similarly promising. The discovery of *Spirillum* (currently *Azospirillum*), a bacterium that can fix  $N_2$  in the roots of a tropical forage gramineous plant with lush growth (Döbereiner & Day, 1975), led to the hypothesis which states that growth in those plants was directly related to the association *Gramineous* plant- $N_2$  fixation bacterium (Klucas, 1991).

With full conviction about the importance of *Azospirillum* as a biotechnological tool, studies were conducted in several countries (Bashan & de-Bashan, 2010; Jain et al., 2010). As a result of these studies, information was rapidly spread about inoculation tests performed globally in several gramineous plants in soils, with different species of the genus *Azospirillum*

(Harmann & Baldani, 2006). These studies were especially carried out at an agronomic scale (Dhale et al., 2011; Okon et al., 1998). The results obtained were dissimilar, and this was attributed to different *Azospirillum* strains behavioural patterns in the various soils used (Bashan & Dubrovsky, 1996; Bashan, 1999; Hungria et al., 2010).

There was also a random effect in the inoculation with *Azospirillum* on plant production in the Argiudoll soil studied. In inoculation tests performed in field plots which are representative of the Argentine Humid Pampa, a significant increase in biomass (18-22%) was observed only in some grasses crop cuts. Wheat yield, expressed by ear weight and number, and grain weight were not affected by inoculation with these bacteria. On the other hand, when forage sorghum was inoculated under laboratory growth conditions, it was observed that the inoculated strain infected the roots and that, modifications in soil inorganic nitrogen availability were produced (Perotti, 2001).

Even if this vast experimental global study showed the relativity of the initial idea (i.e., that the effect of *Azospirillum* was directly related to its ability to fix  $N_2$ ), it also evidenced the multiple capabilities these bacteria have. As well as having the potential to fix  $N_2$ , they can produce siderophores, bacteriocins, and plant growth hormones (Bashan & de-Bashan, 2010; Jain et al., 2010). They can also increase ion absorption (e.g.,  $K^+$  and  $NO_3^-$ ), avoid plant hydric stress, and modify soil redox potential (Bagheri, 2011; Bashan, 1999; Hungria et al., 2010; Pidello et al., 1993). *Azospirillum* also showed that it can assimilate  $NO_3^-$  in aerobiosis, reduce  $NO_3^-$  to  $NO_2^-$  in anaerobiosis, and produce polyphenol oxidase (Bashan, 1999; Hartmann & Baldani, 2006). These multiple capabilities led to its classification as a bacterium that promotes plant growth (PGPR, Plant Growth Promoting Rhizobacterium). The previously described potential capabilities of *Azospirillum* spp. showed these bacteria as an optimal tool to modify the functional population structure in a complex system such as the agricultural soil.

In this work, the greatest importance was given to the study of inoculation effect on: (i) plant; (ii) soil inorganic nitrogen; (iii) soil urease activity; (iv)  $N_2$  fixation activity in soil, and (v) indigenous rhizospheric diazotrophic microorganisms. The study was based on the hypothesis that the expression of functional capabilities in microorganisms introduced into the soil depends on the accomplishment of two basic conditions: (i) the effective settlement of the bacterium in the rhizosphere (i.e., depending on its ability to survive when introduced into the soil) and (ii) that this fact affects the rizosphere environment (Perotti, 2001; Pidello, 2011). In general terms, and from the soil biotechnology perspective, it is important to know whether the bacterium introduced into the soil is able to functionally “settle” within the system (Bashan & Vazquez, 2000).

This study is intended to help understand the relationship between plant and microorganisms in soil. This contribution was meant to go beyond a descriptive approach and to advance on explanatory aspects, so as to establish some causality relationships in the bilateral relationship between the biotic and abiotic components which would best define the system and inform about the feasibility of manipulating it with a biotechnological objective. Even though these relationships focused on the non-symbiotic diazotrophic functional group, a methodological base was provided to conduct the study of other heterotrophic microbial functional groups which play a significant role in plant nutrition. The ability to survive and modify the agricultural system researched of *Azospirillum*,

bacteria chosen as a model of a functional diazotrophic population, was analyzed based on the current knowledge available.

We will first provide a brief description of the nitrogen cycle in the soil-plant-microorganism system, and later a critical analysis of the results obtained from short-term to mid-term tests with plants in molisol soil pots (Typic Argiudoll from the Argentine Humid Pampa), in a laboratory controlled setting and with soil microcosms.

## 2. The nitrogen cycle

Plant growth and development is related to soil fertility, i.e., nutrient accessibility (chemical elements which, in the form of small ions or molecules are taken by roots to contribute to plant nutrition), and water availability (the water present in the soil profile, that fill soil micro-pores, and is available for plant roots). Nitrogen is one of the most important chemical elements in the soil because it appears in large amounts in various compounds (aminoacids, nucleic acids). The largest nitrogen reservoir in our planet is found in the lithosphere, the mother rock, representing 97.8% Earth's total nitrogen, which is not available to organisms. The atmosphere's diatomic nitrogen ( $N_2$ ), only representing 2% total N, despite existing in a high proportion in air (79%), is used as a nitrogen source by several microorganisms, and is considered the main source of nitrogen in the biosphere (Frioni, 1990).

In general terms, it may be stated that the various nitrogenated compounds may undergo the following transformations:



These transformations, which are the result of very slow processes (25, 100 or 200 years may elapse before an atom produces every interconversion) (Jenkinson, 1990), guarantee the presence and diversity of nitrogenated compounds in the biosphere. As mentioned before, biological  $N_2$  fixation is the main process allowing the incorporation of atmospheric  $N_2$  into the nitrogen cycle, and is carried out by microorganisms called "diazotrophs", which have a nitrogenase enzyme complex, and whose most important habitat is the soil.

### 2.1 The nitrogen cycle in soil fertility

Nitrogen, together with carbon, hydrogen and oxygen are the fundamental components of organisms in the biosphere. The inorganic forms, ions  $NH_4^+$  and  $NO_3^-$ , or the diatomic form ( $N_2$ ) are incorporated into the biosphere by plants and microorganisms, which receive the ions from the soil or air, respectively. Moreover, the degradation of plant and animal remains, and microbial cells (soil organic matter), provides inorganic nitrogen and small nitrogenated molecules (amines and amides) which can also be taken by microorganisms and plants (Heller et al., 1998).

The transformations undergone by the various nitrogen species in nature are either biotic (mainly microorganisms and plants) or abiotic (physico-chemical) processes, which is called the "Nitrogen Cycle". This cycle was conceived in 1913 by Lohnis, who identified the various compounds and the biochemical processes involved in its operation. Abiotic factors conditioning these processes (e.g., ion adsorption and lixiviation) were included later into the nitrogen cycle concept (Paul & Clark, 1989).

From the microbial ecology perspective, understanding the significance of the various stages in this cycle, in each agro-ecosystem, and establishing the causality relationships linking them are the main objectives in the study of this cycle. The availability of this information is necessary to "manipulate" this cycle by developing management practices that enhance its efficiency, and guarantee its operation within a "sustainable" approach, without affecting other element cycles that characterize the agricultural system's operation.

2.2 The cycle processes

As shown in Table 1, nitrogen has several oxidation states (between +5 and -3) and, based on this fact, compounds in nature are extremely diverse. This diversity and the type of chemical bonds occurring among atoms (simple, double or triple covalence) produce significant differences in relation to the stability in compounds. The most stable forms found in soil are NO<sub>3</sub><sup>-</sup> and N<sub>2</sub>, the former as a free ion in the soil solution, and the latter as part of the soil atmosphere. Ion NO<sub>3</sub><sup>-</sup> stability occurs because a nitrogen atom has the highest oxidation degree, whereas N<sub>2</sub> stability is related to the high bond dissociation energy that the molecule has (941 kJ mol<sup>-1</sup>).

(+5) nitric acid (HNO <sub>3</sub> )	(+2) nitric oxide (NO)	(-1) diimide (N <sub>2</sub> H <sub>2</sub> )
(+4) nitrogen dioxide (NO <sub>2</sub> )	(+1) nitrous oxide (N <sub>2</sub> O)	(-2) hydrazine (N <sub>2</sub> H <sub>4</sub> )
(+3) nitrous acid (HNO <sub>2</sub> )	(+1) nitrous oxide (N <sub>2</sub> O)	(-3) ammonia (NH <sub>3</sub> )

Table 1. Nitrogenated compounds based on nitrogen oxidation degree

Transformation of N<sub>2</sub> into NO<sub>3</sub><sup>-</sup> is thermodynamically possible, but no microorganisms are known which, in the presence of O<sub>2</sub>, have developed a metabolic strategy to do it (McBride, 1994). For N<sub>2</sub> to become NH<sub>4</sub><sup>+</sup>, there must be a highly reductive medium, and great energy to break the triple bond in the molecule (N≡N), which is a condition that only occurs inside diazotrophic microorganisms. These microorganisms are able to create an optimal environment through the nitrogenase complex.

According to the type of transformation that occurs in relation to element oxidation degree, the nitrogen cycle processes in the soil may be classified into: (i) oxidative pathways, where energy is released; and (ii) reductive pathways, where energy is consumed.

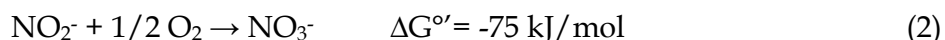
2.2.1 Oxidative pathways

In oxidative pathways, nitrogen electrons in an oxidation state of (-3) are attracted to oxygen, and nitrogen gains an oxidation state of (+3) or (+5). In this process, which is called nitrification, energy is generated and used by microorganisms to perform anabolic processes and thus sustain their biomass growth. Even though these microorganisms, called "chemolithotroph" (Heller et al., 1998), are not numerous, they are important from the agricultural soil microbial ecology viewpoint, because to gain metabolic energy they are independent of soil organic matter availability.

This process includes two steps. In the first step, soil nitrifying microorganisms take NH<sub>4</sub><sup>+</sup> and, in the presence of O<sub>2</sub>, oxidate it into NO<sub>2</sub><sup>-</sup>, performing the process called nitritation (Equation 1). The oxidation of NO<sub>2</sub><sup>-</sup> into NO<sub>3</sub><sup>-</sup> constitutes the second step called nitrataion



(Equation 2). The negative values of standard free energy variation ( $\Delta G^\circ$ ) indicate that this chemical pathway can be used by microorganisms for growth.



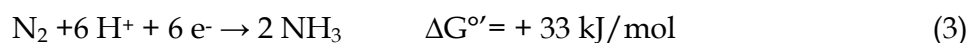
### 2.2.2 Reductive pathways

In the reductive pathways, nitrogen has a lower oxidation degree with energy consumption and reductive power. These requirements may be given by the catabolism of carbon compounds ("heterotrophic" microorganisms), the transformation of light energy ("phototroph" microorganisms), or the oxidation of elements in their elementary state ("chemolithotroph" microorganisms), as it is the case of the denitrifying bacterium *Thiobacillus denitrificans*, which obtains energy from sulfur compound oxidation, or *Micrococcus denitrificans*, which obtains energy from hydrogen oxidation (Glinski & Stepniewski, 1983). In agricultural soil, chemoheterotrophic microorganisms from the nitrogen cycle are proportionally greater in number. In the processes of heterotrophic microbial catabolism, energy is obtained from electron transfer of carbon-reduced-to- $\text{O}_2$  compounds (aerobic respiration) to an acceptor of alternative electrons such as  $\text{NO}_3^-$  (anaerobic respiration) or to organic compounds (fermentation) (Leclerc et al., 1995).

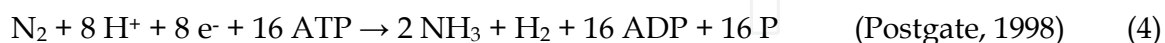
The reductive pathways of the nitrogen cycle are the most important pathways from the microbial ecology perspective due to the number of processes in which they are involved (biological  $\text{N}_2$  fixation, ammonification,  $\text{NO}_3^-$  and  $\text{NH}_4^+$  assimilation, denitrification), and to their close dependence on the transformations of soil organic matter.

#### 2.2.2.1 Biological $\text{N}_2$ fixation

In this process, the atmosphere's  $\text{N}_2$  is reduced to  $\text{NH}_4^+$  by the nitrogenase enzyme complex, as described in the following equation:



The energy required in this process is provided by the adenosine triphosphate (ATP) molecule, and each transferred electron requires a minimum of 2 ATP (a total of 12 ATP per fixed  $\text{N}_2$  molecule). As a coupled reduction ( $2 \text{H}^+ / \text{H}_2$ ) occurs, the global theoretical reaction of this process is:



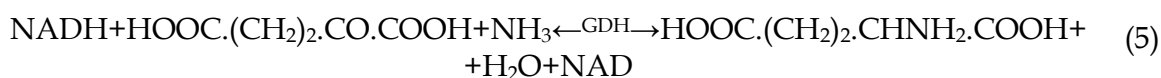
Based on the previous discussion, energy dependence of  $\text{N}_2$  fixation is high, and it is ultimately furnished by organic matter degradation or by the photosynthetic process.

#### 2.2.2.2 Inorganic nitrogen assimilation

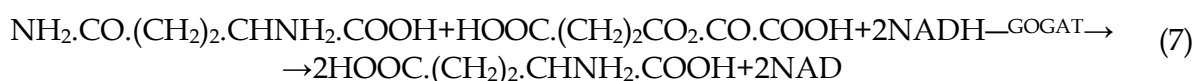
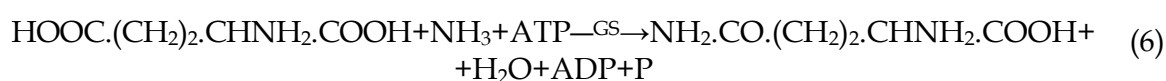
As it was previously described, microorganisms and plants add nitrogen into their cells, mainly as ions  $\text{NO}_3^-$  or  $\text{NH}_4^+$ .

Ammonium that may be obtained from the soil or by means of  $\text{N}_2$  fixation, is transformed into organic nitrogen by the following mechanisms: (i) from  $\alpha$ -ketoglutarate together with

glutamate dehydrogenase (GDH) to render glutamate, as shown by the following reaction:



(ii) from a glutamate molecule together with the glutamate synthase (GS) enzyme to render glutamine. As shown in Equation 6, the reaction requires energy as ATP. Glutamine is later transformed with a reductive power (NADH) and  $\alpha$ -ketoglutarate with the participation of glutamine- $\alpha$ -ketoglutarate aminotransferase (GOGAT) in glutamate (Equation 7). This pathway (called GS/GOGAT) is frequently used in the metabolism of soil microorganisms.



When  $\text{NH}_4^+$  comes from the soil solution (where it is naturally found in low concentration) the low affinity for the substrate having the GDH enzyme ( $K_m(\text{GDH}) = 10^{-3} \text{ M}$ ) and the high affinity of GS ( $K_m(\text{GS}) = 10^{-5} \text{ M}$ ) lead to the fact that the main  $\text{NH}_4^+$  addition mechanism in soil microorganisms is the pathway GS/GOGAT (Postgate, 1998), even though there are other nitrogen uptake pathways (e.g., small organic molecules).

### 2.2.2.3 Ammonification

$\text{NH}_4^+$  release in soil from organic nitrogen is originated in the decomposition of animal, plant or microbial cells. It is produced by glutamate deamination (Equation 5) or by the action of hydrolytic enzymes on amino acid molecules (deaminase enzymes) (Equation 8):

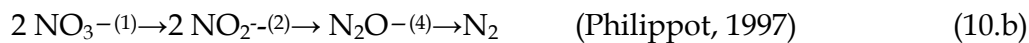
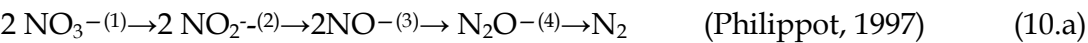


or amides (deamidases) as in the case of urea hydrolysis by the action of the urease enzyme:



### 2.2.2.4 Denitrification

This process, considered as a disassimilative pathway of the N cycle (Postgate, 1998), comprises the enzymatic reduction of  $\text{NO}_3^-$  into  $\text{NO}_2^-$ , then to  $\text{N}_2\text{O}$  and  $\text{N}_2$ . In this process,  $\text{NO}_3^-$ , instead of  $\text{O}_2$ , acts as a final electron acceptor. Electrons may come from organic matter oxidation, photosynthesis or mineral compounds (Paul & Clark, 1989). The whole process is described by the following equations (10.a) or (10.b) with or without nitrite oxidoreductase involvement. The enzymes involved in each stage are: (1) nitrate reductase; (2) nitrite reductase; (3) nitrite oxidoreductase; and (4) nitrose oxidoreductase:



3. Results

3.1 Inoculated strain survival

The bacterium used in this study was *A. brasilense* strain 7001 (streptomycin-resistant derivate of *A. brasilense* sp 7-ATCC 29145), obtained by C. Elmerich, Pasteur Institute, France). Cultures were performed in Nfb (nitrogen free broth) medium (Döbereiner, 1980). The survival and growth of the inoculated bacteria were evaluated with serial dilutions, and plate techniques on Nfb agar medium amended with streptomycin (1 mg mL<sup>-1</sup>), and congo red (Rodriguez Cáceres, 1981). Survival of bacteria inoculum was studied in pots with and without plants, and in sterilized soil by gamma radiation (25 KGy γ-irradiation from a <sup>60</sup>Co source, Atomic Energy Comission, Ezeiza, Argentina).

3.1.1 Survival in rhizospheric soil

The survival of inoculated strain in fescue plant rhizospheres was different in each experiment but, in general, during the first 2 weeks, the initial cell number decreased in one or two orders. From that period until the end of the experiment (Fig. 1), cell number increased, fluctuating between 10<sup>5</sup>-10<sup>7</sup> CFU per dry soil gram (Colony-Forming Unit, CFU). This result of inoculum survival, which may be regarded as high in degree, was not significantly affected by plant density (Perotti et al., 1987). A similar result was obtained when the strain was inoculated in soil supplemented with glucose. Even though this carbohydrate is not used by *A. brasilense* (Hartmann & Zimmer, 1994), it may be used by a large number of soil heterotrophic bacteria, suggesting that the competitive pressure with which the inoculum was confronted in this situation might be significantly increased.

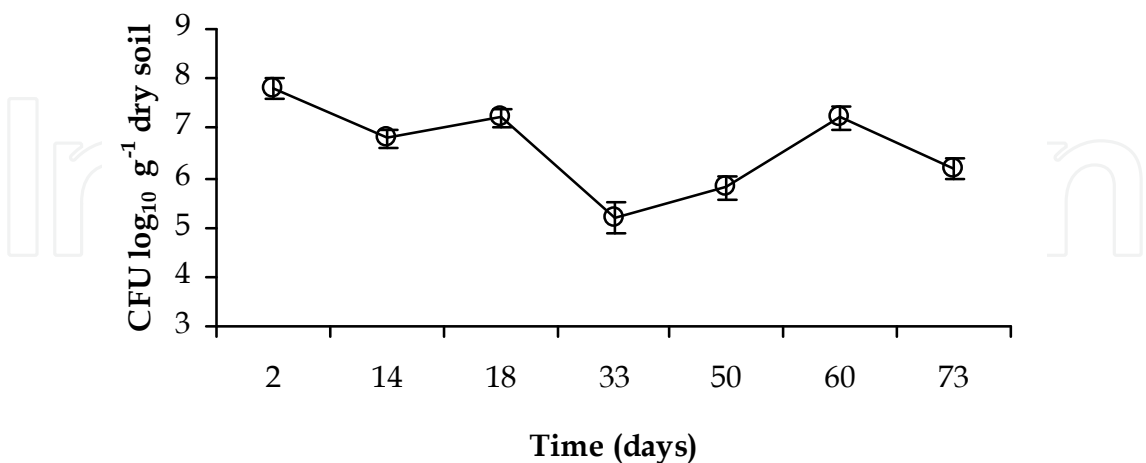


Fig. 1. Evolution of *A. brasilense* survival in fescue rizhospheric soil.

The inoculum survival was not different in the supplemented soil compared to the soil without supplement (Perotti & Pidello, 1999a).



These results suggest that rhizospheric carbon excretions would not significantly affect the survival capability of the inoculated strain.

3.1.2 Survival in non-rhizospheric soil

The survival of the studied *Azospirillum* strain in non-rhizospheric soil was tested in Typic Argiudoll soil samples with naturalized grassland vegetation taken in three different months during the same year: March and May (autumn), and September (spring); the soil characteristics are shown in Table 2. The soil samples selected from those three months differed in relation to global microbial activity, which was defined by soil dehydrogenase activity (DA) (Casida et al., 1964), and by the oxidizable carbon (CO)/NH<sub>4</sub><sup>+</sup>-N ratio. Redox potential intensity (Eh) corresponds to aerobic soils (McBride, 1994) with a lower value in September soil, and pH is within the neutrality range.

Sample month	CO (%)	NH <sub>4</sub> <sup>+</sup> -N (µg g <sup>-1</sup> dry soil)	CO/NH <sub>4</sub> <sup>+</sup> -N ratio	Dehydrogenase activity (µg triphenyl-formazan g <sup>-1</sup> h)	Eh (mV)	pH
March	2.34	4.8	0.585	120	407	7.1
May	1.83	5.3	0.366	450	437	7.6
September	1.27	26.5	0.048	60	305	7.2

Table 2. Main features of Typic Argiudoll soil obtained in three different months representing the seasons autumn (March and May) and spring (September) during a year.

Survival of the *Azospirillum* strain introduced in the soils was assessed in inoculated soil incubations, under controlled laboratory conditions (20 °C, twice the equivalent humidity, in a dark place). As time elapsed, the inoculated strain population decreased in the three samples of soil (Fig. 2). There was an approximate two order-reduction at 41 days after incubation. The second day after incubation, there was a population of approximately 10<sup>7</sup> cells per soil gram. Overall, the population evolution decreased to an approximate value of 10<sup>5</sup> on day 41. In September soil, three weeks after the inoculation, the inoculated strain evolution was more significant than the one from other soil samples. The different soil behaviour observed in September soil might be justified by means of the value shown by the CO/NH<sub>4</sub><sup>+</sup>-N ratio, which was significantly lower than the other cases. This result suggests that in heterotrophic populations the survival potencial would be related to sustrate availability. As *Azospirillum* was introduced in soil samples that had different amounts of oxidable carbon (Table 2), the inoculum probably competed with an indigenous heterotrophic population that was temporarily stimulated.

A positive relation between the inoculated strain population and dehydrogenase activity in each of the sample dates during the incubation period was also observed. This relationship suggests that the active inoculum evidently affected an enzymatic activity that shows the total system’s microbial activity, i.e., the inoculum may have affected the structure of a functional population which can oxidate organic carbon, and by this pathway have an impact on the significant decrease in soil reduction potential (Eh), as shown in Table 2.

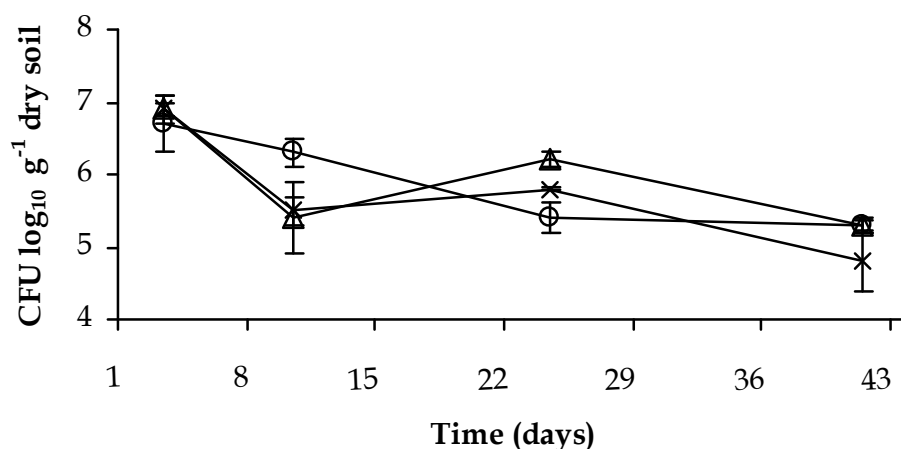


Fig. 2. Evolution of *A. brasilense* survival in soil samples taken in the months March (o), May (x) and September (Δ).

### 3.1.3 Survival in soil sterilized by gamma radiation

Inoculation experiments in soil sterilized by gamma radiation were conducted under controlled laboratory conditions (20 °C, twice the equivalent humidity, in a dark place). The *Azospirillum* population introduced in sterilized soil constantly increased during the incubation period. The population of the inoculated strain was approximately  $10^6$  CFU per gram of dry soil the second day after inoculation, and approximately  $10^8$  CFU per gram of dry soil 16 days after inoculation (Perotti & Pidello, 1999b). This result might be explained if the fact that the inoculated strain does not have indigenous microbial competitor in the irradiated soil is accepted. As reported by other authors (Cawse, 1975; Lensi et al., 1991), in the soil sterilized by gamma radiation there is a significant increase in soluble organic carbon and in ammonium ion content. In this situation, the availability of these easily assimilative substrates and the absence of microbial competitors may have produced the significant growth of the strain introduced.

### 3.2 Effects of *Azospirillum* inoculation on plant systems

As fescue is an important *Gramineous* plant in the grasslands of the Argentine Pampa region, it was used as a plant model to study the effects of *Azospirillum* inoculation. In the next experiments, shoot/root ratio, nitrate content in shoot and nitrate reductase activity in leaves were examined in presence or absence of denitrifying *Azospirillum* strains under different plant growth conditions. Seeds of *Festuca arundinacea* L. CV El Palenque (INTA, Argentina) were disinfected with hydrogen peroxide for 30 min. and washed three times with distilled water. They were later sowed in plastic pots with a mixture of soil and sand. The soil (Typic Argiudoll, 0.16-0.27 %N; 2.19% C and pH (H<sub>2</sub>O) 6.1) was sieved with a 150 mesh sieve. The pots were placed into a growth cabinet at 28-22 °C (day/night), 70% relative humidity, with a photoperiod of 16 hs. The light levels that the plantules received were 197 or 274  $\mu\text{Em}^{-2} \text{s}^{-1}$  photosynthetically active radiation, within a wavelength range of 400-700 nm (PAR), which were provided by cool-white fluorescent tubes and incandescent bulbs. In the first series of experiments ("high plant density"), twenty germinated seeds per pot were

sowed in 150 g of soil-sand mixture (1:5). Plants in Experiment 1 received  $197 \mu\text{E m}^{-2} \text{s}^{-1}$  and in Experiment 2 received  $274 \mu\text{E m}^{-2} \text{s}^{-1}$ . In the second series of experiments ("low plant density"), four germinated seeds per pot were sowed in 150 g soil-sand (1:5), in Experiments 3 and 4, and fifteen seeds per pot in 1200 g soil-sand (1:3) in Experiment 5. In Experiment 3 the illumination regime was  $197 \mu\text{E m}^{-2} \text{s}^{-1}$ , and  $274 \mu\text{E m}^{-2} \text{s}^{-1}$  in Experiments 4 and 5.

In every experiment the soil was inoculated simultaneously with  $10^8$  cell/pot using two strains: *A. brasilense* sp 7001 and *A. lipoferum* sp G (A. Pidello and J. Balandreau. Abstr. Proc. Steenboock-Kettering Int. Simp.  $\text{N}_2$  Fixation, Madison, 1978). The control pots received the same concentration of inoculums, but these were autoclaved. To guarantee the physiological state of the plants, the soil was irrigated by capillary absorption with a nutrient N-free solution. Only in Expt. 5 (the longest experiment in duration)  $50 \text{ mg L}^{-1} \text{NH}_4^+\text{-N}$  and  $33 \text{ mg L}^{-1} \text{NO}_3\text{-N}$  were added in the nutrient solution at the 5th and 6th weeks. In Expt. 2 and 4, plants were harvested after 27 days, and in Expts. 1, 3 and 5 after 22, 23 and 74 days, respectively.

Shoot and washed root dry weights were obtained by drying the plants at  $70^\circ\text{C}$  for 48 h in a forced air oven stove. "In vivo" nitrate reductase activity in leaves was determined by a method proposed by Neyra & Hageman (1974). Nitrate-N in leaves was determined by a colorimetric method (Cataldo et al., 1975). Inorganic-N in soil (Expt. 5) was determined after extraction with 2N KCl (10/100; P/V) during 1 h by a distillation method (Bremner & Keeney, 1965).

### 3.2.1 Effect on shoot/root ratio and shoot nitrate content at high and low plant density

Table 3, shows that plant density produced significant modifications ( $p < 0.05$ ) in the shoot/root ratio in both inoculated plants and control plants, regardless of the PAR values used in the experiments. According to current literature data, these values coincide with  $\text{CO}_2$  interchange values of 38% and 41% maximum value for *F. arundinacea* under experimental conditions, similar to those used in these experiments (Wilhelm & Nelson, 1978), which suggests that the plants were not in the appropriate condition to express their maximum potential to synthesize biomass. This leads to the assumption that the values observed may be conditioned by this fact. However, the ratios presented in Table 3 were the quotients of ratio of the shoot/root ratio in inoculated treatments vs controls (Bashan & Dubrovsky, 1996), which reveal that the variable showed a clearly different behaviour in both cases (in high density treatments the value was  $1.125 \pm 0.035$  and in low density it was  $0.823 \pm 0.052$ , with significant differences between both values at  $p < 0.05$ ). Consequently, the results obtained suggest that there are two opposite effects on the shoot/root ratio. When plant density was higher, the bacteria introduced increased the ratio indicating that they may, directly or indirectly, increase efficiency in root absorption (Bashan & Dubrovsky, 1996). When plant density was low, the bacterial inoculum lowered the ratio, suggesting that there would be an increase in the functional aspects of the root, increasing root mass, which might lead to an indirect increase in aerial mass.

The  $\text{NO}_3\text{-N}$  content in shoot was higher in inoculated plants, and this increase was not modified by PAR difference or plant number (Table 3). This increase effect in nitrogen uptake coincides with the results reported by other authors (Roy Mihir Lal & Srivastava Ramesh, 2010; Saubidet et al., 2002), and was explained in association with root mass stimulation (Fages, 1994).

Inoculated plant / Non-inoculated plant ratio						
PAR ( $\mu\text{E m}^{-2} \text{ sec}^{-1}$ )	Expt.	High plant density Shoot/Root Shoot $\text{NO}_3^-$		Expt.	Low plant density Shoot/Root Shoot $\text{NO}_3^-$	
197	[ 1 ]	1.09 a <sup>1</sup>	1.26 b	[ 3 ]	0.91 c	1.13 d
274	[ 2 ]	1.16 a	1.16 b	[ 4 ]	0.83 c	1.22 d
				[ 5 ]	0.73 c	1.35 e
mean (SEM)		1.12(0.03)*	1.21(0.05)ns		0.82 (0.05)	1.23 (0.06)

<sup>1</sup> The same letters in the same column indicate that the mean values are not significantly different between them (p=0.05).  
\* Significant differences between high and low plant density at p=0.05 level; ns: Non-significant differences between high and low plant density.

Table 3. Effect of *Azospirillum* inoculum on shoot/root ratio in inoculated plants vs non-inoculated plants, and  $\text{NO}_3^-$ -N shoot content in inoculated plants vs non-inoculated plants at two values of photo-synthetically active radiation, wave length range 400-700 nm (PAR).

3.2.2 Effect on nitrate reductase activity in leaves

Nitrate reductase activity in leaves, measured during a 10 week-period after inoculation (Expt. 5) is shown in Fig. 3. The results suggest that the differences observed in  $\text{NO}_3^-$ -N content in shoots were not related to nitrate reductase activity (NRA). In inoculated treatments, only after the tenth week,  $\text{NO}_3^-$ -N content in shoots significantly increased above the level observed during the experiment in both treatments (Fig. 3A). NRA constantly increased in inoculated and control plants after five weeks. This fact suggests that  $\text{NO}_3^-$ -N in leaves was not a limiting factor in NRA. The  $\text{NO}_3^-$ -N content measured possibly showed a total pool in leaves but not the nitrate flux which is related to NRA. In this experiment with nitrogen supply, inoculated rhizosphere showed there was a similar level of soil inorganic-N at different times (Fig. 3B and Fig. 3C).

3.2.3 Effect on rhizospheric diazotrophic microorganisms

An indigenous diazotrophic population was selected in order to study the impact of inoculation with *A. brasilense* on preexistent microorganisms in soil. The study was conducted in the rhizospheric soil of fescue inoculated with *Azospirillum*. A comparison was made between population evolution of microorganisms and their potential for growth in a nitrogen-free medium (Nfb). Colony morphology in Nfb agar plates, as well as microbial cell reactions to Gram stain and oxidase and catalase tests were used to individualize indigenous rhizospheric diazotrophic populations. Growth continued during the 22-day growth period in fescue plants. This fact should be considered in order to analyse the significance of the result from the population ecology perspective. Apart from the

individualized colonies mentioned above, a high number of very small colonies (oligonitrophilic microorganisms) were found, which was included in the total number of possible diazotrophic microorganisms (this data was not shown).

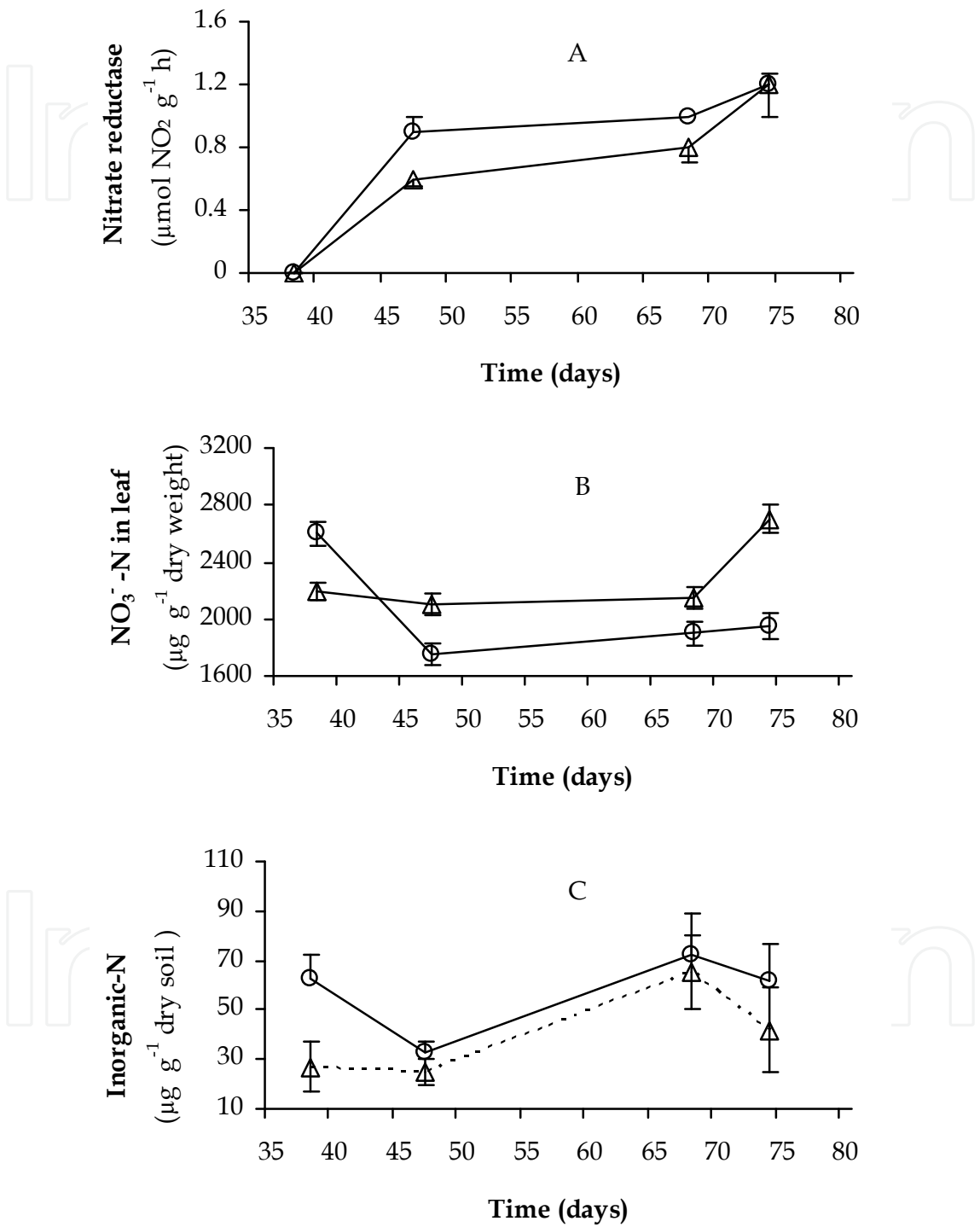


Fig. 3. Nitrate reductase activity (A),  $\text{NO}_3^-$  -N content of *F. arundinacea* leaf (B), and soil inorganic nitrogen ( $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ -N) (C) in the inoculated (Δ) and the control treatment (○).



The results indicated that immediately after inoculation the number of indigenous diazotrophs showed the maximum values observed, approximately  $10^8$  CFU g<sup>-1</sup> dry soil decreasing one order in the first week, with stability around this value until day 22. It was also observed that 10 days after inoculation, the presence of the inoculated strain produced a significant decrease in the population of two colonies identified as Gram negative. This was a reduction of approximately two orders. The difference described vanished during the course of the study showing that there were other types of interaction, e.g., an inhibiting effect of *A. brasilense* selectively affecting some microorganisms but not the whole indigenous diazotrophic population. This effect appeared to decrease towards the end of the study, when both systems (inoculated and non-inoculated) reached a similar distribution in density in the studied populations.

### 3.3 Effect on bio-edaphic parameters

The bio-edaphic parameters assessed were inorganic nitrogen, dehydrogenase, nitrogenase and urease activities, and soil reduction potential (Eh). Evolution of these parameters in root-free soil microcosms inoculated with *A. brasilense* 7001 and in non-inoculated controls was assessed. Short-term to mid-term experiments were conducted, under controlled laboratory conditions.

#### 3.3.1 Effect on inorganic nitrogen content

In the study soil, there were significant fluctuations in inorganic nitrogen during the whole year (Perotti, 2001), which shows the dynamic nature of soil microbial activity in this ecosystem. Nitrite, nitrate and ammonium ion evolution, in soil taken in March, May (autumn) and September (spring) (Table 2), was studied for 10 weeks after inoculation with *A. brasilense*.

The evolution of ammonium in inoculated soil was different with regard to the evolution in control soil (Fig. 4A). On day 3, there was a significant difference between the inoculated and the uninoculated systems. A rapid immobilization phase was observed, which might be associated to metabolic activity of indigenous microorganisms in the controls, and indigenous and introduced microorganisms in the inoculated soil. After this initial period, inoculated soils showed a constant reduction in ammonium content during a 40-day period. This situation was not observed in controls where, from day 10, there was a new mineralization phase continuing until the end of the period assessed, which showed higher ammonium concentration values than the values recorded in the inoculated soil.

These results show that *Azospirillum* modified the mineral nitrogen dynamics, leading to stabilization in the ammonium immobilization phase, independently of the existing initial differences in available oxidable carbon levels in the three soil samples (Table 2). On the other hand, inoculated bacteria produced a clearer nitrite and nitrate accumulation rate than controls (Fig. 4B). Nitrite and nitrate concentrations in inoculated soils show a linear relation with time ( $r=0.83$ ;  $p<0.01$ ). As it was previously reported, a greater ammonium accumulation suggests that in these soils, ammonium concentration was not a limiting factor for nitrification (Dommergues & Mangenot, 1974). This result should be highlighted, as it shows that *Azospirillum* has the potential to help functional autotrophic groups which are important in soil fertility (Perotti & Pidello, 1991).



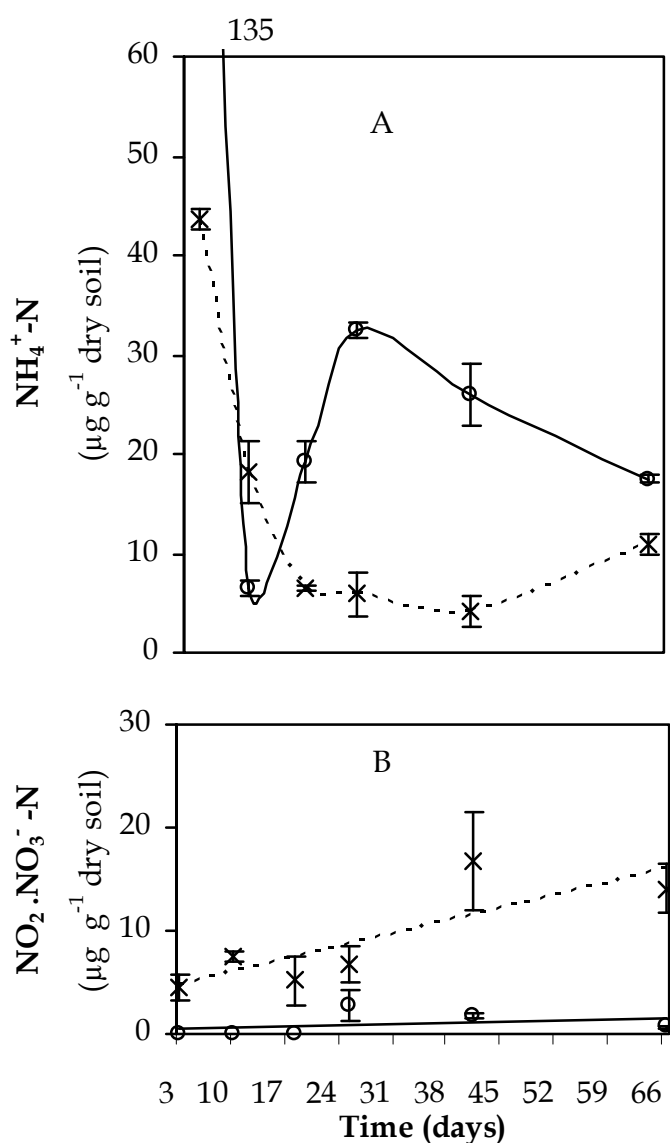


Fig. 4. Effect of *A. brasilense* inoculation on inorganic nitrogen in Argiudoll .  $\text{NH}_4^+\text{-N}$  (A) and  $\text{NO}_2^-\text{.NO}_3^-\text{-N}$  (B). Inoculated soil (x) and control soil (o). Mean from March, May (autumn) and September (spring) soil samples.

### 3.3.2 Effect on dehydrogenase activity

Inoculation produced an initial depression in this enzymatic activity in relation to controls, manifested during the first 10 days (Fig. 5). Lower activity shows there was a reduction in total microbial activity (Casida et al., 1964).

On the other hand, the relationship between dehydrogenase activity and ammonium content in the control soil was positive ( $r=0.74$ ;  $p<0.05$ ). This would indicate that the lowest concentrations observed in ammonium concentration (e.g., on day 10) appeared when there was greater microbial activity. This relationship was not observed in the inoculated soil, where ammonium appeared in lower concentrations in relation to the concentrations observed in controls. This fact would explain why in the inoculated soil there are no phases that show a clear predominance of mineralization or ammonium immobilization in soil,

with their corresponding increase or disappearance of ammonium concentration (Fig. 4A). Under these conditions, the lack of relationship between the changes in ammonium concentration and total microbial activity is logical.

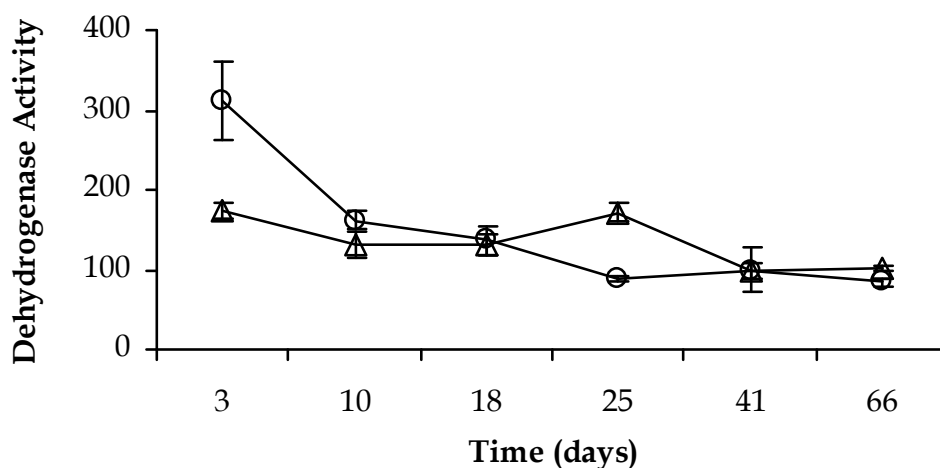


Fig. 5. Dehydrogenase activity evolution ( $\mu\text{g}$  triphenyl formazan chloride  $\text{g}^{-1}$  soil 24 h) in incubations of Typic Argiudoll inoculated with *A. brasilense*. Control soil (o) inoculated soil ( $\Delta$ ). Bars represent the standard error.

### 3.3.3 Effect on nitrogenase activity

Non-symbiotic biological  $\text{N}_2$  fixation (Equations 3 and 4) in the studied soil is naturally expressed at low levels (Perotti, 2001). This does not necessarily mean that this activity is low; it probably responds to a transitory substrate flux of readily assimilated compounds, originating in soil radicular exudates or organic matter transformation (Pidello & Perotti, 1997). This flux would produce substrate competence favouring the expression of heterotrophic microbial functions in the form of "pulses", with spatial heterogeneous distribution when located outside the strictly rhizospheric area ("hot spot") (Drewitt & Warland, 2007).

Potential nitrogenase activity (the soil contained in microcosms was supplemented with 1mg glucose per g), measured 24 h after inoculation with *Azospirillum*, was higher than the activity occurring in the control soil ( $28 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ dry soil h}^{-1}$  vs  $23 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ dry soil h}^{-1}$ , respectively,  $p < 0.05$ ). Twelve days after inoculation, the inoculated soil maintained the trend observed, with higher values than those found in control soils, although the differences were not statistically significant, which shows there was a potentially functional indigenous population.

### 3.3.4 Effect on urease activity

Urease is one of the enzymes which are responsible for organic nitrogen mineralization (Equation 9), and its activity is responsible in part for the presence of ammonium in soil. It is associated to the presence of microbial or plant cells, and it is also adhered as a free enzyme to soil colloids (Nannipieri, 1993).

We studied the effect of *Azospirillum* strain introduction in soil on urease activity as a model which would show the action of this microorganism on active enzyme in soil, whose

expression does not exclusively depend on the specific functional group number. The experimental model used was inoculation in soil sterilized by gamma radiation and in non-sterilized soil. In both treatments, 15 days after the start of the experiment, there was a significant increase in urease activity in inoculated systems (Perotti & Pidello, 1999b), and in general, the study suggests that the bacteria potential is strongly conditioned by factors such as  $\text{NH}_4^+\text{-N}$  content and the indigenous microbial population.

### 3.3.5 Effect on redox and acid-base intensity

Oxidation-reduction conditions in any microbial ecosystem are considered a “master” variable (McBride, 1994), which helps anticipate innumerable potential interrelationships between abiotic and biotic components. Within certain limits, reduction potential (Eh) - which is an intensity component defining the redox state- is a useful tool to delimit the expression range of various functional microbial groups (Pidello, 2011).

In microcosms of soil inoculated with *A. brasilense*, the values of apparent reduction potential (Eh') remained lower than in uninoculated control soils, even though these values were higher than 400 mV in both systems, which shows that in the studied systems aerobiosis conditions predominated (Pidello et al., 1993). The values of apparent reduction potential corrected for pH=7 (Eh'<sub>7</sub>) in inoculated systems were also lower, which proves that the reduction observed is due to differences in electroactive compounds associated with the presence of *Azospirillum* and not to a proton concentration effect (Glinski & Stepniewski, 1983).

## 4. Conclusions

Plant inoculation with beneficial bacteria is a biotechnological practice involving critical aspects in the relationships between plant-bacteria and soil. It involves aspects related to both, plant physiology and bacteria as well as the bilateral effects produced in the soil environment. A fundamental aspect for the end result in soil-plant microorganism inoculation depends on two conditions: the first is that the effective settlement of bacteria in the rhizosphere is produced, and the second is that the bacteria significantly affect the environment. So, from the soil biotechnology perspective it is important to know whether the bacteria introduced into the soil will be able to act over the system.

In naturally fertile soils, as in the case of the Typic Argiudoll assessed, there are mineralization-immobilization step alternations in the nitrogen cycle that may temporarily increase nitrogen concentration levels. An increase in inorganic nitrogen content in soil may compromise the expression of microbial activities related to the nitrogen cycle, as well as other physiological aspects of the bacteria introduced, which are linked with their survival and potential to act on the system.

The results obtained regarding *A. brasilense* survival in the studied soil indicate that during the longest assessment periods (approximately 40 days) there was a reduction of approximately two orders in the number of bacteria in the soil compared with the concentration in the inoculant used. This may be considered a highly positive behaviour which does not appear to be significantly affected by plant density. During the 24 hours following the inoculant introduction into an environment favouring heterotrophic population development in general, there was no change in inoculant concentration

evolution. When the *Azospirillum* strain was introduced into gamma-sterilized soil, survival was higher, and the inoculated population significantly increased towards the end of the assessment period. These results showed that the indigenous functional population affects inoculated strain survival. The results also suggest that there would be an active interaction between inoculum-native microorganisms. This would be evident in two more significant phenomena: on the one hand, the indigenous microflora affected *Azospirillum* strain survival and, on the other, the *Azospirillum* strain affected the indigenous population of diazotrophic microorganisms and their functional expression nitrogenase activity, and also the total number and functional level of total diazotrophic microflora as suggested by the differences observed in dehydrogenase activity.

In relation to the impact of inoculation on the other hierarchical variables in this study, the following conclusions may be drawn: (i) the tests conducted to assess the effect on plants showed that *Azospirillum* influenced on the partition of plant dry matter, and that this effect of *Azospirillum* on modifications in shoot/root rate was not related to the intensity of photosynthetically active radiation (PAR); and (ii) the amount of  $\text{NO}_3\text{-N}$  was higher in inoculated plants, a fact observed in plants that grew in soils with and without nitrogen supplement, which would be related to an increase observed in inoculated non-rhizospheric soil  $\text{NO}_3^-$  availability. On the other hand, an increase in root development observed in inoculated plants through an increase in the ion absorption surface might explain this effect. These facts clearly show that in the system studied there are significant changes in the structure of the functional population. This suggests that these variables, undoubtedly important for the system's eco-physiology, would operate by means of changes produced by the inoculum on the structure of the indigenous microbial population.

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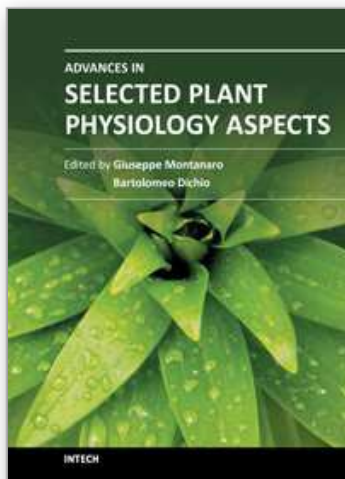


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