Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact on the production and culturable rhizosphere microflora

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**Abstract**

Scientific evidence recognizes that the operation of a terrestrial ecosystem depends on soil microbial activity. Some *Azospirillum* strains produce plant growth regulators, increase the development of roots, and fix atmospheric nitrogen (N\(_2\)). Some *Pseudomonas* strains are capable of producing cytokinins and solubilizing organic phosphorus. A sustainability analysis requires a detailed knowledge of the interrelationships between the microorganisms added to the system and those present in the soil. This study examines the effect of three commercial inoculants *Azospirillum brasilense* Az1 and Az2 as well as *Pseudomonas fluorescens* Pf on biomass production, grain yield and rhizosphere colonization of wheat, combined with two levels of N-addition. Plate counts of rhizosphere soil showed that the inoculation and N-addition did not affect the number of *P. fluorescens*, whereas it significantly affected the number of *Azospirillum*. N-addition and inoculation did not change the communities of actinomycetes and bacteria but they changed the number of fungi at the rhizosphere of wheat plants. Community-level physiological profiles of carbon-source utilization of rhizosphere soil microbial communities were changed after inoculation with Az1, Az2 and Pf depending on the phenological stage of the crop. Although no significant responses were observed, in average, PGPB inoculation increased aerial biomass by 12%, root biomass by 40% and grain yield by 16%. These increases represent important earnings for the farmer and they may help to obtain a greater sustainability of the agroecosystems.

1. Introduction

The supply with enough food of sufficiently good quality is one of the most important problems in the world today. The annual demand of wheat in developed countries is 3%, but the yield increase is less than 1% [36]. The nutrition of wheat crop is one of the limiting factors for achieving large yields without stress factors such as weeds, pests [27] and diseases [50]. The increases in potential yield, stability and quality of crops are strongly linked to the training and knowledge to decision-making and technical transformation [48]. Both in the short and long term, these increases in production would not be possible without taking into consideration the sustainability of the agroecosystem.

Microorganisms are an essential and large component of the living biomass of the soil [51]. They play a key role in the
biogeochemical cycles and have a great potential for agriculture use and environmental protection [43]. However, the current use of microorganisms in agriculture remains at a low level despite the significant investment in scientific work to understand and use natural microbial resources and to improve plant growth and health [13].

Microbial inoculants represent an emerging technology designed to improve the productivity of agricultural systems in the long run. They can be seen as a technology aligned with principles of sustainable agriculture, as opposed to the increased use of pesticides and fertilizers in recent times. Several microorganisms are used in the normal agricultural practice, and others have potential for future use [29,35]. Most of them have the ability to colonize and establish an ongoing relationship with plants producing increases in biomass, root growth and commercial yield. They are usually called plant growth-promoting rhizobacteria (PGPR) [34] or more generally plant growth-promoting bacteria (PGPB) [28].

Among the most used PGPB are bacteria of the Azospirillum genus. The potential of this bacterium-plant association to increase crop production was first reported several years ago [4]. Positive impacts on plant growth through several mechanisms include enhancement of root development, production of growth regulators and nitrogen fixation [20,41]. The content of nitrogen, phosphorus, potassium and various micronutrients is higher in plants inoculated with Azospirillum [11,20]. Significant effects have been observed in wheat [9,11,38], corn [10,19], soybean [7] and rice [3,24], among other species including more than a hundred crops and environmentally important plant species [8]. A recent publication compiled data from different countries showing the state-of-the-art in the Azospirillum inoculation responses [12].

To date, several PGPB including Pseudomonas strains have been characterized as phosphorus solubilizers with the ability to produce organic acids (such as oxalic acid, fumaric acid and citric acid) and phosphatases that facilitate the solubilization of phosphorus and other nutrients [14,44]. In addition, it has been demonstrated that some strains can produce and supply several cytokinins [22]. However, the largest amount of information about the activity of Pseudomonas strains is associated with the indirect effects, through the control of pathogenic microorganisms. This can reduce the incidence of diseases through a number of mechanisms, including increased competitive ability for available nutrients, production of antibiotics, siderophores and induction of systemic resistance [15,31].

The sustainability and profitability analysis requires a detailed knowledge of the interrelationships that exist between microorganisms commonly added as inoculants and those within the natural system. Several authors have reported the impact of Azospirillum root development with the consequent advantage on the absorption of water and nutrients [6,20,41]. However, most of this information corresponds to experiments performed under controlled conditions. Thus, it is necessary to analyze the effects of commercial inoculants in order to obtain better crops and a better use of the environmental resources. The hypothesis analyzed here is that the inoculation with PGPB such as Azospirillum and Pseudomonas can modify cultivable microbial rhizosphere communities and plant growth in field conditions.

2. Materials and methods

2.1. Bacterial inoculants

Three commercial inoculants were used in this study. Two of them contained Azospirillum brasilense and the given names at this study were “Az1” and “Az2”. The inoculant containing Pseudomonas fluorescens was named “Pf”. All of them are recommended for wheat inoculation. The market names of Az1, Az2 and Pf are RHIZOFLO Liquid, NoctinAzO and RIZOFOSlig, respectively. Az1 and Az2 are manufactured by the companies Laboratorios CKC Argentina S.A. and Síntesis Química S.A.I.C, respectively. Pf is manufactured by the company Rizobacter Argentina S.A.

2.2. Bacterial cultivation conditions

For the analysis of rhizosphere soil microbial communities, plate counts of viables on selective media [1] were used to estimate populations of cultivable bacteria, fungi and actinomycetes [33]. Tryptic soybean agar (Difco, Detroit, MI, USA) with 0.5 μg ml⁻¹ of benomyl was used to enumerate bacteria. Malt extract agar (Difco, Detroit, MI, USA) was used for both, fungi and actinomycetes. In order to suppress the development of bacteria, plates to enumerate fungi received 30 μg ml⁻¹ of streptomycin [1]. Plates to enumerate actinomycetes included the addition of 67 μg ml⁻¹ of rose bengal and pH was adjusted to 6.0–6.2, in order to suppress the development of bacteria and fungi [1]. Counts of typical colony-forming units of Azospirillum were performed for the rhizosphere of plants whose seeds were treated with control, Az1 and Az2, using a selective medium with Congo-red dye [45]. Also, counts of typical colony-forming units of P. fluorescens using a commercial medium of Pseudomonas Agar F (PAF) (Difco, Detroit, MI, USA) with 0.5 μg ml⁻¹ of benomyl were performed for the rhizosphere of plants treated with control and Pf.

2.3. Determination of the community-level physiological profiles (CLPP)

For the determination of the microbial physiological activity of the rhizosphere soil, CLPP-profiles a modified approach of the technique described by Garland and Mills [26] was used [21]. At tillering and grain-filling stages, rhizosphere samples were assessed by their microbial physiological activity through community-level physiological profiles (CLPP) of carbon-source utilization. Empty sterile microplates were used and the 10⁻³ dilution of each rhizosphere sample was exposed simultaneously to 23 carbon sources (CS). Absorbance records at 590 nm measured after 72 h at 30 °C were obtained using an automated microplate reader Multiskan EX™, (Thermo Corporation, Vantaa, Finland). Additionally, communities of filamentous fungi were evaluated using FF MicroPlates™ of Biolog Inc. (Hayward, CA, USA).
2.4. Field site

The experiment was performed at a 0.42-ha wheat field near the town of Coronel Pringles, southwest of Buenos Aires Province, Argentina (37° 54′ 04″ South and 61° 29′ 00″ West), on a Typical Argiudoll soil. The chemical soil characteristics of the experimental field determined just before sowing were: pH (1:2, soil/water) 6.2, total organic matter 4.2%, total N 145 kg ha\(^{-1}\), available P 16.5 mg kg\(^{-1}\).

The previous crops of the experiment were an eight-year pasture and a sunflower crop. The location has temperate and subhumid-dry conditions. The average annual rainfall is 870 mm concentrated mainly during autumn and spring when certain water excess sometimes occurs.

2.5. Field experiment: seeds, inoculants and treatments

Seeds of *Triticum aestivum* L. cultivar Buck Sureño\(^{\text{®}}\); supplied by Buck Semillas S. A. La Dulce, Buenos Aires, Argentina. The seeds were sown on July 5, 2006 during the indicated period for the region, based on temperatures, day length, and water and soil conditions. The sowing machine was adjusted to a plant density of 320 plants m\(^{-2}\). Before sowing, seeds were treated with the fungicides thiram and carbendazim (300 ml per 100 kg of seed) to prevent soil pathogens always present in field conditions. In order to avoid any toxic effects, fungicides were applied as indicated by the manufacturer which was the same for each inoculant. After drying, seeds were inoculated with commercial inoculants based on *A. brasilense* and *P. fluorescens*. Single inoculations were carried out at the same day of sowing using a vertical axis mixer applying 500 ml per 100 kg of seeds, as recommended by the manufacturers. The experiment had a completely randomized split-plot factorial design with four blocks as replicates with 1050 m\(^2\) each. Treatments were the Control (without inoculation) and three commercial aqueous-liquid inoculants: Az1, Az2 and Pf. Counts of culturable actinomyces and determination of contaminants in the inoculants were performed. No contaminants were detected in the inoculants. According to the yield potential of the study area and the P content in the soil at the region, based on temperatures, day length, and water and soil conditions. The sowing machine was adjusted to a plant density of 320 plants m\(^{-2}\). Before sowing, seeds were treated with the fungicides thiram and carbendazim (300 ml per 100 kg of seed) to prevent soil pathogens always present in field conditions. In order to avoid any toxic effects, fungicides were applied as indicated by the manufacturer which was the same for each inoculant. After drying, seeds were inoculated with commercial inoculants based on *A. brasilense* and *P. fluorescens*. Single inoculations were carried out at the same day of sowing using a vertical axis mixer applying 500 ml per 100 kg of seeds, as recommended by the manufacturers. The experiment had a completely randomized split-plot factorial design with four blocks as replicates with 1050 m\(^2\) each. Treatments were the Control (without inoculation) and three commercial aqueous-liquid inoculants: Az1, Az2 and Pf. Counts of culturable actinomyces and determination of contaminants in the inoculants were performed. No contaminants were detected in the inoculants. According to the yield potential of the study area and the P content in the soil at sowing, P fertilization treatments were not included. In contrast, two levels of nitrogen fertilizer applied as urea with doses of 0 and 45 kg ha\(^{-1}\) in the main plots. The different inoculation treatments were applied in the randomized design in the subplots.

2.6. Sampling schedule and determinations of plant growth and biomass attributes

Sampling dates were at three stages of the crop cycle: tillering (106 days after sowing, das), grain filling (136 das) and physiological maturity (155 das). Aerial plant-biomass was determined for the three stages by cutting the plants growing in a line of 1 m length which was representative of the canopy. Every single sample contained 56 plants of wheat. In the first two sampling dates, samples of roots, rhizospheres and soil from the 0–20 cm layer were separately obtained by using a soil-core sampler. Root samples were washed with tap water to estimate root length. Rhizosphere was considered the soil attached to roots after hand-shaking the samples and subjected to analysis of microbial communities. Root length was estimated by the intercept technique [40] and aerial and root biomasses were determined at every phenological stage by drying the samples up to constant weight after a period in the oven at 60 °C. Grain yield and number of spikes were determined when physiological maturity was reached.

2.7. Statistical analysis

Absorbance records of CLPP were analyzed using principal component analysis (PCA). Microbiological and plant data were subjected to analysis of variance and comparison of means using Tukey’s test (p ≤ 0.05). The statistical package INFOSTAT/Professional\(^{\text{TM}}\) (Version 1.1 - University of Córdoba, Argentina) was used.

3. Results

3.1. Microbial rhizosphere communities

The culturable rhizosphere microbial communities differed between treatments at grain-filling stage. The number of colonies with and without fluorescence was significantly higher in the rhizosphere of plants inoculated with Pf than in that of control plants (Table 1). Nitrogen fertilization did not show any effect on the number of fluorescing bacteria on PAF-agar, presumably in the rhizosphere of plants at tillering and grain-filling stages. This was also evident during the grain-filling stage for treatments of *Azospirillum* like bacteria, determined on Congo-red agar in the control and Az1 treatment. However for the Az2 treatment a significant reduction (p ≤ 0.05) was observed. On the other hand, the interaction between inoculation and fertilization was significant, since the rhizosphere of plants inoculated with Az2 contained less *Azospirillum* than those supplied with urea. This response was not observed in plants inoculated with Az1 because their rhizospheres were similar to those of control plants (Table 1). The presence of *Azospirillum* in control plants indicated that this bacterium is naturally present in the soil where the experiment was conducted. Counts of culturable actinomyces and total bacteria showed that neither inoculation nor Table 1 – Effect of the interaction between fertilizer-inoculation with PGPR on the numbers of typical colonies of *A. brasilense* and *P. fluorescens* in the rhizosphere of wheat plants at grain-filling stage

<table>
<thead>
<tr>
<th>Fertilizer Addition (kg ha(^{-1}))</th>
<th>Typical colonies log(_{10}) cfu g(^{-1}) root</th>
<th>Azospirillum</th>
<th>Pf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.8 b</td>
<td>8.3 ab</td>
<td>8.5 ab</td>
</tr>
<tr>
<td>45</td>
<td>7.5 ab</td>
<td>8.1 b</td>
<td>3.0 a</td>
</tr>
</tbody>
</table>

Means with the same letters for each type of PGPR are similar as compared by Tukey’s test (p ≤ 0.05).
fertilization modified these communities. The addition of urea significantly increased the number of culturable fungi in the rhizosphere at the grain-filling stage, without changing the physiological profiles of the communities of filamentous fungi in the rhizosphere of plants inoculated with Pf (data no shown).

The CLPP of carbon-source utilization of the microbial communities of samples at tillering and grain-filling stages are shown in Figs. 1 and 2, respectively. In average, PC1 and PC2 explained 47% and 9% of the total variation, respectively. At tillering, PCA showed that control plants had different microbial activities in their rhizosphere when PC1 was analyzed. PC2 was able to detect significant differences between the microbial communities of plants inoculated with Pf and Az1 (Fig. 1). At the grain-filling stage no differences were found for PC1 between the CLPP of the microbial communities of the plants of the inoculation treatments. Significant differences between the physiological activities of the communities in the rhizosphere of plants inoculated with Az2 and Pf were found only for PC2 (Fig. 2).

3.2. Root length

The inoculation treatments did not generate significant differences in the root length of wheat plants at the sampled phenological stages of wheat plants (Fig. 3). Differences were observed between tillering and grain-filling stage.

3.3. Biomass production

The analysis of plant biomass production gave indication that inoculation did not generate significant differences in the weight of the aerial parts at tillering (data not shown). At the grain-filling stage the dry weight of the aerial part of control plants (which had no fertilizer or inoculant) was lower than that of the inoculated ones, in plots both with and without urea (Fig. 4). The aerial biomass of plants inoculated with Az2 increased by 26%, whereas those inoculated with Pf increased by 23% as compared to controls without urea. However, these increases were not significant. At maturity it was noted that the dry weight of aerial parts did not increase with the addition of urea (data not shown), but did so with inoculation (Fig. 5). It was observed that Az2 produced significant (p < 0.05) increases of 20% when compared to plants without inoculation.

The dry weight of roots showed a trend different from that observed in the dry weight of the aerial parts. The most important impacts of inoculation were observed at tillering, with significant differences (p < 0.05) between the dry weight of roots in the inoculated treatments and that of controls (Fig. 6). Thus, plants inoculated with Az1, Az2 and Pf increased root weight by 105, 33 and 46% as compared to controls. At this stage, both fertilization treatments did not significantly differ in the production of root biomass (data no shown). The average increase in root biomass between both stages was 32%. However, there were no differences between treatments for this variable at grain-filling stage (data not shown).
3.4. Grain yield

Grain yield increased 17, 14, and 19%, with treatments Az1, Az2, and Pf, respectively (Fig. 7) relative to the control without inoculation and fertilization. The N-fertilization (Control CF) increased this plant attribute in 13%. The average increase for the combination of N-fertilization and inoculation (treatments Az1 CF, Az2 CF, Pf CF) with respect to the treatment Control CF was 7%. Yield increases due to N-fertilization and PGPR inoculation were 13% and 14%, respectively. However, all these effects were not significant at the $p \leq 0.05$ level. The number of spikes per square meter was also slightly affected by inoculation (Fig. 8). Only the inoculation treatment with Pf showed significant differences against the control. No differences are noted between the control and Az1 or Az2 treatments.

4. Discussion

It is known that the total number of microorganisms in any ecological niche is up to $10^8$–$10^{12}$ per gram of soil [1] and probably $10^3$ different types are present [49]. It is also recognized that the complexity and variability of microbial diversity has to be assessed at different biological levels [26]. Microbial diversity can be measured by various methodologies such as direct and plate counts as well as fatty acid profiles and molecular methods [39]. Estimations of the genetic complexity of a particular microbial community assessed by any method based on analyses of 16S rRNA or rDNA recover more than any culturing method such as plate counts, which can obtain only 0.1–14% of the total number of microorganisms, depending on the physical and chemical conditions of growth [1,49]. However, the relevance of cultural bacteria for ecosystem function has been rarely discussed. Additionally, the DNA-extraction method applied can influence the patterns obtained by molecular techniques showing the necessity of care in interpreting these molecular genetic microbial diversity data [39]. There is evidence that the culturable component of soil microbial communities could be most relevant in terms of both biomass and activity [16], showing positive correlations between culturability and both cell size and activity [2]. The culturable and non-culturable types of microorganisms present in the soil microbial community may probably interact physiologically and one could assume that some microorganisms of the non-culturable group can pass through some specific physiological changes to the culturable group [2,39]. In this work, it was assumed that the comparison of a fraction of the culturable subgroup within the community could be sufficient to indicate changes in the dynamics of the

![Fig. 4 – Inoculation and fertilization effects on aerial biomass production at the grain-filling stage of wheat. Similar letters indicate no significant differences with Tukey’s test ($p \leq 0.05$).](image)

![Fig. 5 – Effect of inoculation treatments on production of aerial biomass at physiological maturity of wheat. Different letters indicate significant differences with Tukey’s test ($p \leq 0.05$).](image)

![Fig. 6 – Effect of inoculation treatments on root biomass production at tillering of wheat. Different letters indicate significant differences with Tukey’s test ($p \leq 0.05$).](image)

![Fig. 7 – Impact of inoculation and fertilization on crop grain yield. Numbers on the bars indicate percentages of grain increases with respect to Control SF for bars SF and with respect to Control CF for bars CF. Similar letters indicate no significant differences with Tukey’s test ($p \leq 0.05$). SF: No fertilized; CF: Fertilized.](image)
whole community. This hypothesis, that the culturable bacteria have an ecological significance in soil was also raised by other authors [2,16,47] who demonstrated a very significant ecological function of culturable bacteria in any particular ecosystem.

It has been accepted that the exclusion or addition of specific bacteria does not necessarily modify the overall function of the microbial community [26]. The data presented in this work showed that inoculation with commercial products based on Azospirillum and Pseudomonas may exert different effects on the numbers of specific subgroups of culturable bacteria in the rhizosphere of wheat plants at the grain-filling stage (Table 1). Although the number of inoculated PGPB was high, the competition between native and added strains must be considerable, because the rhizosphere of inoculated and control plants had similar numbers of culturable rhizobacteria. The counts of culturable bacteria were made on a semiselective indicator media, which allow only a presumptive approximation to detect A. brasilense and P. fluorescens like bacteria because the commercial inoculants did not contain specifically marked strains which could be identified selectively. Because the inoculation procedure was applied as suggested by each manufacturer, it is clear that it is necessary to improve the quality of the inoculants in order to obtain a better colonization and ensure the inoculation response.

The profiles of carbon substrate (CS) utilization of the soil microflora were changed by the inoculation with all three PGPB at the tillering and grain-filling stages, as shown by PCA analyses (Fig. 1). At grain-filling stage it was possible to distinguish the different microbial physiological profiles between the rhizosphere of plants inoculated with Pf and Az2 (Fig. 2). The CS utilization is related to the number of microorganisms, which are able to use each CS within the well as a sole carbon supply and are concomitantly stained with the tetrazolium purple. The importance of growth indicates that the colour responses produced in this assay are a reflection of the functional potential of the original community. Therefore, it is possible to obtain a physiological fingerprint of the metabolic abilities of the complex microflora [18,25]. It was shown, that A. brasilense Sp7 inoculation did not affect substantially the bacterial rhizosphere and rhizoplane community of maize seedlings growing under controlled conditions [30]. In these studies, molecular phylogenetic probes and primers targeting the 16S rRNA and rDNA of bacteria were applied to assess the structure of the microbial communities associated to the roots. In this regard, it was observed that the effects of different levels of heavy metals on the culturable portion of the microbial community were significant, while they were minimal on the dominant bands observed in the DGGE analysis of DNA extracted directly from the soil [16]. These authors suggested that the most appropriate measurements to determine the effect of the contamination on soil health will be activity rather than the presence or absence of different cell types. Culturable bacteria are probably the largest, most active microorganisms in a given soil sample [2]. This kind of approach is often criticized for their selectivity, but this characteristic made them useful to determine the biological responses to anthropogenic activity [16]. Besides, it could be assumed that changes in the physiology of the microbial communities could be occurring before the structural ones could be detected. The ecological impact of the inoculation practices should receive more attention in order to know how the functional biodiversity is altered [26]. In this regard, studies of Azospirillum inoculation response performed during the crop sequence are necessary to know if the structural and physiological changes of the associated microbial communities are permanent or transitory.

Root growth is an irreversible increase in both mass and length [42]. In this work, root length data did not increase with PGPB inoculation, although the values were similar to those reported before [23]. The method applied here has the possibility to study root growth (length and biomass) with a standardized sampling technique using a core-sampler at a defined soil depth. In average, root length increased 49% between the tillering and grain-filling stages, as expected (Fig. 3). However, no significant differences were observed between treatments. Although root growth (length and biomass) was not measured at physiological maturity, it can be assumed that it was at least equal to the values observed at the grain-filling stage. The failure to demonstrate the expected root stimulation effect by Azospirillum inoculants [17] is possibly due to the native microflora of this bacterium was in the soil at similar numbers (Table 1). This was probably reducing the possibility to express the potential of the inoculated strains. Additionally, as it is known, the interactions between plants and endophytic microorganisms are found in grasses and crops worldwide, providing plants tolerance to biotic and abiotic stress, better ability to fix C and improved nutrient uptake [37]. Thus, endophytic microorganisms probably present in wheat plants could also reduce the inoculation effects observed in this experiment. At tillering, the effect of Az1 on root biomass was significant as it almost doubled the values of controls (Fig. 6). After harvest, these increases in root growth and aerial biomass at physiological maturity (Fig. 5) represent additions of plant residues added to the soil. As soils under intensive agriculture have to receive plant residues in order to maintain the organic matter pools [32,46], the increase in both aerial and root biomass due to Azospirillum inoculation could have a significant effect on soil quality. Although the grain yield increases were not significant, they represent important earnings for the farmer (Fig. 7). Farmers could use PGPB inoculation to obtain the same yield increase reached with 45 kg ha$^{-1}$ of urea. Sometimes, neither N-fertilization nor PGPB inoculation have the expected responses. For that reason, it is necessary to improve the level
of efficiency that these agricultural practices require. The quality of the inoculants should be addressed to ensure the establishment of PGPR-plant associations [5,41]. This point of view is in accordance with the widely accepted idea to maintain the sustainability of agroecosystems.

REFERENCES


