

Short Communication

Search for endophytic diazotrophs in barley seeds

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Abstract

Eight endophytic isolates assigned to *Pseudomonas*, *Azospirillum*, and *Bacillus* genera according to pheno-genotypic features were retrieved from barley seeds under selective pressure for nitrogen-fixers. Genetic relationships among related isolates were investigated through RAPD. Six isolates displayed nitrogen-fixing ability, while all could biosynthesize indolacetic acid *in vitro* and showed no antibiosis effects against *Azospirillum brasilense* Az39, a recognized PGPR.

Key words: *Azospirillum*, *Bacillus*, *Pseudomonas*, PGPR, RAPD.

Inoculation of crops using biofertilizers containing different plant growth promoting rhizobacteria (PGPR) has gained great acceptance over last years. This technology not only may result in increased crop yields, but it is also expected to reduce the need for chemical fertilizers and pesticides, allowing a more sustainable scheme of agricultural practice (Kumar *et al.*, 2011; Rai, 2006).

Malting barley cultivated area in Buenos Aires province, Argentina, has been growing during last decades. An increasing international demand, good yields and a shorter life cycle (which allows the anticipation of soybean sowing), as compared to other traditional winter crops, have been identified as the main factors leading to this trend (Bragachini and Peiretti, 2012). Since water and nitrogen availability are usually determinant factors for barley yield in this region, the use of *Azospirillum*-based inoculants arises as a reasonable biotechnological approach to cope, at least in part, with these constraints, as they are expected to promote root system development and, consequently, to allow better access to soil water and dissolved nutrients. On another hand, these products may contribute to N plant nutrition through biological fixation.

In previous experiments we inoculated barley seedlings with the collection strain *A. brasilense* Az39 and ob-

served that *Azospirillum* counts in the roots of inoculated plants (based on the standard MPN procedure on semisolid malate enrichment medium) were generally below those reported for other plant species studied in *Azospirillum* inoculation trials. At the same time, development of bacterial communities in the aforementioned culture medium for control units (uninoculated roots) was frequently corroborated, despite the fact that all seeds had been surface-disinfected before sowing (unpublished data). These observations led us to suspect that barley seeds could contain endophytic diazotrophic microorganisms that eventually might compete with introduced PGPR during colonization of the rhizospheric niche. In this sense, it was reported that rice seed endophytes inhibited early colonization of rice roots by *A. brasilense* (Bacilio-Jiménez *et al.*, 2001).

In this work, our goal was to detect and to identify endophytic diazotrophs in the seeds of three barley cultivars recommended for malting barley production in Buenos Aires province (Argentina) and to test under laboratory conditions their nitrogenase activity and indolacetic acid (IAA) production, to gain a basic approach of their plant growth promoting potential. Additionally, we examined possible antagonisms between these endophytic microorganisms and *Azospirillum brasilense* strain Az39, a well-

known strain from a culture collection which is included in the formulation of several biofertilizers commercialized in Argentina.

Seeds of the malting barley cultivars Scarlett, Quilmes Carisma, and Josefina INTA were surface disinfected (70% ethanol 5 min, followed by 5% sodium hypochlorite containing three drops of Tween 20 for 15 min) and thoroughly rinsed with sterilized distilled water. Then, seeds were longitudinally split and crushed in the upper part of a 5-mL vial containing NFb semisolid medium (Dobereiner, 1980), and incubated at 30 °C for 3 days. Disinfected uncrushed seeds were also placed into NFb vials and incubated in the same conditions. NFb vials showing turbidity and/or pellicle and pH rise were subcultured on Petri dishes containing RC medium (Rodríguez-Cáceres, 1982). Reddish colonies displaying different phenotypes were selected and transferred to NFb vials. After 3 days of incubation, vials showing turbidity and/or a whitish pellicle or a faint veil, and pH rise and a dominant population of motile rods observed under optic microscopy were grown on RC plates and then in NFb semisolid medium repeated times until pure cultures on RC plates were obtained.

Gram staining, cell shape, colony morphology and routine biochemical tests guided preliminary identification of the isolates obtained (Holt *et al.*, 1994; University of Idaho webpage, 2011) and 16S rRNA gene sequencing was carried out to complete identification at genus level. Total DNA was extracted from randomly chosen colonies of each selected isolate using the Illustra® Blood Genomic Prep Mini Spin Kit (GE Healthcare). A fragment of around 1500 bp of the 16S rRNA gene was amplified using the universal primers 27F and 1492R. PCR products were purified with the Wizard® PCR purification kit (Promega) and sequenced at the Genomic Unit of the Biotechnology Institute of CNIA-INTA using a capillary automatic sequencer model ABI3130XL (Applied Biosystems, USA). The Naïve Bayesian Classifier utility (Wang *et al.*, 2007) from the RDP Release 10 (<http://rdp.cme.msu.edu>) was used to assign the obtained sequences into the new bacterial taxonomy at genus level with 95% of confidence. The Seqmatch utility (type strains) from the same RDP website and the Eztaxon-e utility from the EzBioCloud platform (<http://eztaxon-e.ezbiocloud.net>) were used to find closest sequences according to their 16S rRNA gene sequence similarity, in order to suggest most probable affiliations of the isolates obtained at species level.

A RAPD analysis using three different primers was used to study the genetic variability between isolates linked to the same or closely related species. Four independent PCR reactions were carried out using random primers OPA3 (5'-AGTCAGCCAC-3'), OPA9 (5'-GGGTAA CGCC-3') and OPA18 (5'-AGGTGACCGT-3') (Operon Techn. Inc. Alameda, CA) alone and a combination of the three primers. Isolates would be categorized as unrelated strains in the presence of RAPD profiles differing by three

or more bands. Differences in band intensity (at a given position) were not taken into account for the differentiation of isolates.

IAA biosynthetic ability of the isolates obtained was estimated by Salkowski colorimetric technique using OAB medium (Okon *et al.*, 1977) amended with 100 µg mL⁻¹ of L-tryptophan (Glickmann and Dessaux, 1995). Nitrogen fixing ability of these strains was determined by the acetylene reduction assay (Hardy *et al.*, 1968).

The potential antagonistic effects of the isolates obtained against *Azospirillum brasilense* strain Az39 were investigated using an adaptation of the double agar layer plate assay described by Muñoz-Rojas *et al.* (2005). All determinations were performed from three independent experiments and analytical measurements were done with a minimum of three replicates.

Between two and six scarlet or reddish colonies with different appearance could be recognized on each RC plate, while no microbial development was observed in the NFb vials where disinfected uncrushed seeds were deposited. Further purification of these colonies through consecutive passages between semisolid NFb and solid RC media finally rendered a total of eight isolates: three of them came from cultivar Scarlett (isolates 21, 22, and 23), two from cultivar Quilmes Carisma (isolates 24 and Q), and three from cultivar Josefina INTA (isolates 2, 3, and 10).

The isolates obtained were affiliated to three bacterial genera, *Bacillus*, *Pseudomonas* and *Azospirillum* (Table 1). These genera are traditionally regarded as root-associated or soil-dwelling bacteria rather than true endophytic diazotrophs (James and Olivares, 1997; Reinhardt *et al.*, 2008). Endophytic colonization and plant growth promoting traits for different members of *Bacillus* and *Pseudomonas* were largely documented (Botelho Praça *et al.*, 2012; Compant *et al.*, 2011; Mishra *et al.*, 2009; Zhao *et al.*, 2011). Using microscopy coupled with immunogold labeling, important diazotrophs like *Herbaspirillum seropedicae* and *Azospirillum brasilense* were found within cavities in surface-sterilized rice seeds by Baldani *et al.* (1993). These authors suggested that microorganisms may be transferred from generation to generation via seeds. Sundaram *et al.* (1988) also reported *Azospirillum* spp. in the seeds of turfgrass. As far as we know, the finding of *Azospirillum* as a barley seed endophyte has not been reported before.

The partial sequences of the 16S rRNA gene of isolates 10 and 24 shared 100% identity and were closely related to the sequence of the type strain of *A. doebereineriae* (99% identity), suggesting that these isolates probably belong to this species. In a similar way, we found that isolate 21 is related with high probability to *Bacillus pumilus* and isolate 23 to *Bacillus thuringiensis*, according to the 16S rRNA gene sequence analysis. Regarding *Pseudomonas* isolates, given the high percentage of identity between the 16S rRNA gene sequences of the different type strains

Table 1 - Phenotypic features and most probable affiliation of isolates obtained from barley seeds.

| Barley cultivar | ID code | Growth on liquid, solid and semisolid media | IAA ^a | ARA ^b | Microscopy | Colony phenotype in RC | Closest related type strains (partial 16S rRNA sequence analysis) |
|-----------------|---------|--|------------------|------------------|--|---|---|
| Scarlett | 21 | | 16.7 | 1.20 | Gram positive endospore-forming rods of relatively large size. | Reddish with orange center; slightly risen. | <i>Bacillus pumilus</i> ATCC6071 (T) (ABRX0100007) 99.8% id |
| | 23 | Fast on OAB (24 h) and on RC (24-48 h). Weak turbidity and a discrete pH rise on NFb. | 7.7 | ND | | Red to scarlet, eye-shaped. | <i>Bacillus thuringiensis</i> ATCC 10792 (T) (ACNF01000156) 99.8% id |
| | 22 | | 6.8 | ND | Gram negative rods of small size. | Deep pink, convex, mucoid. Fluorescence on King B slants. | <i>Pseudomonas japonica</i> IAM15071 (T) (AB126621) 98.7% id |
| Quilmes Carisma | 24 | Relatively slow on OAB (48 h) and on RC (72 h or more). Faint veil and intense pH rise in semisolid NFb after 48-72 h. | 7.5 | 1.60 | Gram negative curved rods of medium to large size. | Scarlet, flat. | <i>Azospirillum dobereineriae</i> (T) (AJ238567) 98.7% id |
| Josefina INTA | Q | Fast on OAB (24 h) and on RC (24-48 h). Weak turbidity and a discrete pH rise on NFb. | 12.6 | 0.45 | Gram negative rods of small to medium size. | Red to orange, creamy. Fluorescence on King B slants. | <i>Pseudomonas taiwanensis</i> BCRC 17751 (T) (EU103629) 99.6% id |
| | 2 | Fast on OAB (24 h) and on RC (24-48 h). Weak turbidity and a discrete pH rise on NFb. | 13.4 | 0.60 | Gram negative rods of small to medium size. | Red to orange creamy. Fluorescence on King B slants. | <i>Pseudomonas taiwanensis</i> BCRC 17751 (T) (EU103629) 99.6% id <i>Pseudomonas plecoglossicida</i> (T) (AB009457) 99.5% id |
| | 3 | | 17.3 | 0.53 | | | <i>Pseudomonas taiwanensis</i> BCRC 17751 (T) (EU103629) 99.6% id |
| | 10 | Relatively slow on OAB (48 h) and on RC (72 h or more). Faint veil and intense pH rise in semisolid NFb after 48-72 h. | 4.4 | 1.75 | Gram negative curved rods of medium to large size. | Scarlet, flat. | <i>Azospirillum dobereineriae</i> (T) (AJ238567) 98.6% id |

ND Not determined.

^a IAA production expressed as µg mL⁻¹.^b ARA expressed as µmol ethylene h⁻¹ mg protein⁻¹.

within the genera, we can only state that isolates Q, 2, and 3 are related to the *P. putida* group according to the phylogenetic analysis reported by Mulet *et al.* (2010), while isolate 22 is related to the *P. lutea* group.

The RAPD analysis with primers OPA 3, OPA 9, and OPA 18, used independently or combined in the same PCR reaction, did not differentiate between isolates ascribed to the same *Pseudomonas* group (Figure 1, lanes 2-13), suggesting that they could correspond to the same strain, even when they were obtained from different cultivars (Quilmes Carisma and Josefina INTA). The same was observed for *Azospirillum* isolates (Figure 1, lanes 14-21). In other words, our results did not reveal genotypic variation among isolates related to the same bacterial species or phylogenetic group, but suggest an interesting relation between the taxonomic affiliation of the microorganisms isolated and the cultivar where they came from, as isolates retrieved from the seeds of Scarlett (a traditional European spring and winter barley variety) did not show overlap with the isolates obtained from the seeds of the Argentinian improved cultivars Quilmes Carisma (lodging resistant) and Josefina INTA (short cycle, cold resistant), where we corroborated the presence of the same or quite similar genotypes on comparing isolates recovered through the culture-dependent protocol here followed.

All the isolates obtained were able to produce IAA in OAB medium amended with tryptophan. Isolates that affiliated in the *P. putida* group and the isolate with maximum identity with *B. pumilus* displayed the highest *in vitro* IAA production levels, while one of the isolates with maximum identity with *A. doebereineriae* type strain (isolate 10) showed the lowest. The ability to produce IAA using tryptophan as precursor is a well-known feature of many members of the bacterial genera here identified (Spaepen *et al.*, 2007). Regarding this biosynthetic ability in our isolates, a broader spectrum of culture conditions

should be investigated before drawing definite conclusions.

Two isolates failed to grow in NFb once completely purified and were therefore considered non-nitrogen fixers. The other isolates exhibited different ARA levels (Table 1). These results suggest the existence of cometabolic interactions among seed endophytic communities, which may have allowed the survival of non-fixing organisms in the absence of a nitrogen source at the initial steps of our isolation protocol. The ability to fix nitrogen, which has long been considered to be absent from the *Pseudomonas* genus, is now well recognized in several strains of *Pseudomonas stutzeri* (Desnoues *et al.*, 2003; Yan *et al.*, 2008) and in *Pseudomonas azotifigens* sp. nov. (Hatayama *et al.*, 2005), both included in the *P. stutzeri* group, *P. aeruginosa* lineage, according to Mulet *et al.* (2010). Our *Pseudomonas* isolates, with weak or null ARA, phylogenetically diverged from the aforementioned microorganisms, as they were linked to the *P. fluorescens* lineage.

To sum up, through this research we proved that barley seeds host bacterial inhabitants that eventually may contribute to early plant growth through different plant growth promoting abilities, like indolacetic acid production and/or nitrogen fixation. On the other hand, we observed that these microorganisms had no antibiosis effects against *A. brasilense* Az39. Therefore, the low *Azospirillum* counts verified in previous experiments where barley seedlings were inoculated with this strain should not be imputed to active antibiosis mechanisms of these endophytes. The possible activity of other endophytes excluded under our isolation protocol, and eventual differences in the competitive ability of the endophytic communities under natural conditions as compared to introduced microorganisms, may rather account for our previous findings. Rhizosphere colonization is an intricate process that certainly deserves further investigation efforts.

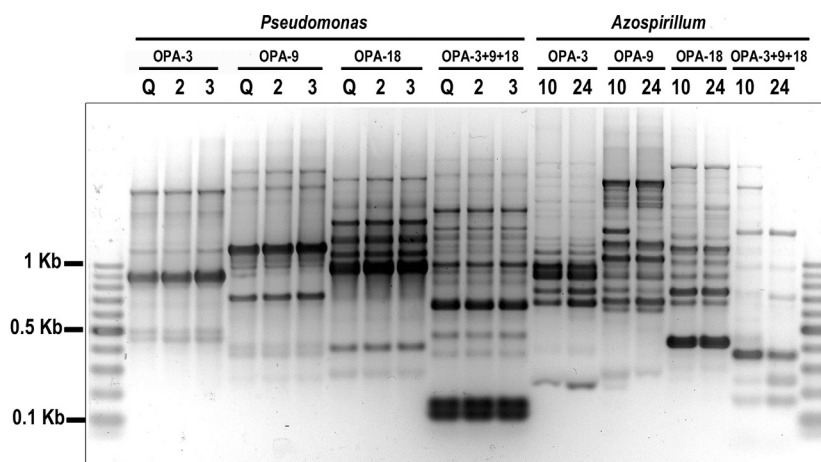


Figure 1 - RAPD analysis of *Azospirillum* and *Pseudomonas* (*P. putida* group) isolates. RAPD amplified products separated on 2.5% agarose gel and stained with GelRed®. A 100-1000 bp DNA ladder was used as molecular weight standard (Lanes 1 and 22). DNA was amplified with random primers OPA 3, OPA 9 and OPA 18, used independently or combined in the same PCR reaction, as described in the text.

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