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Quantification of the potential biocontrol and direct plant growth promotion abilities based on multiple biological traits distinguish different groups of *Pseudomonas* spp. isolates ☆

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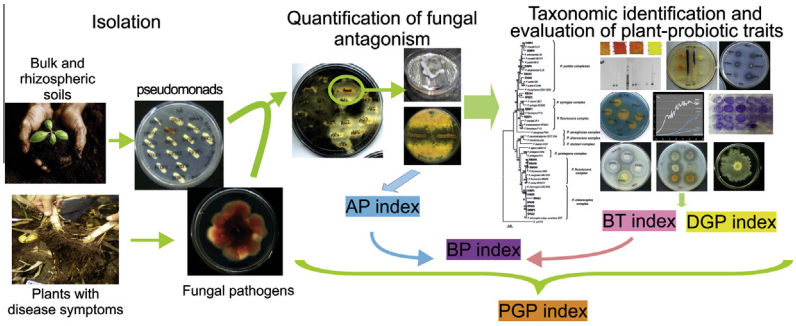
HIGHLIGHTS

- Nineteen pseudomonads with antifungal activity were isolated and characterized.
- Antagonism against 12 fungi and multiple promotion traits were quantified.
- PCA clustered isolates into two groups: biocontrol and direct promotion potentials.
- Novel indexes could help to classify native isolates by their potential as PGPR.
- Results suggest biocontrol and direct promotion traits do not converge in same strains.

GRAPHICAL ABSTRACT



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ABSTRACT

Members of the *Pseudomonas* genus colonize the rhizosphere of different plant species and display plant-probiotic traits. Therefore, they are interesting candidates for biological agricultural inputs to stimulate plant development and/or promote crop health. We have generated a collection of 19 isolates of pseudomonads obtained from either bulk soil or the rhizosphere of healthy individuals of major extensive crops from different plots under no-till management located in Argentina. Isolates were selected for their ability to antagonize several fungal pathogens recovered from infected soybean and maize plants. Partial sequencing of 16S rDNA, *oprF* and *rpoB* genes positioned isolates within all major pseudomonads groups. Most isolates colonized the rhizosphere of soybean and maize seedlings without affecting germination or development. Members of the *Pseudomonas chlororaphis* subgroup, and one isolate of the *Pseudomonas putida* subgroup, displayed the widest antifungal spectrum and strongest antagonistic potential. A principal component analysis based on the outcome of quantitative and qualitative tests related to biocontrol of fungal pathogens, direct plant growth promotion, and other root colonization-related traits, showed that isolates with the highest antagonistic potential and mostly of soil origin were grouped together, whereas rhizospheric isolates and those with strong flagellar-dependent motility, exoprotease production and biofilm development, were clustered in a separate group.

☆ Rizobacter Argentina S.A. has priority access to the bacterial isolates reported here.

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Interestingly, quantitative comparison of newly developed biocontrol and direct growth promotion indices revealed that these two plant probiotic traits are rather associated to different set of isolates, and rarely present in the same pseudomonad strains. These indices could be employed to screen and categorize isolates by their potential to act as a biocontrol or a biofertilizer agent.

1. Introduction

Development of sustainable agriculture that can feed a growing population while also preserving the natural sources demands the application of environmentally-friendly crop managements methods (Raaijmakers et al., 2009, 2002). The use of agricultural bioproducts, as biofertilizers, phytostimulators and biocontrol agents, together with appropriate crop management practices, is an attractive alternative for sustainable agricultural practices because of the possibility to reduce the agrochemical applications that have been continuously applied during the last decades leading to the environmental degradation of natural resources (Adesemoye et al., 2009; Altieri and Nicholls, 2003; Carvalho, 2006).

Bacteria of some species of the *Pseudomonas* genus are candidates for bioproducts' development as they possess a wide spectrum of plant-growth promoting properties (PGPP) (Lugtenberg and Kamilova, 2009; Pathma et al., 2011; Walsh et al., 2001). In Argentina, there are several commercially available inoculants based on *Pseudomonas* strains that are claimed to act as biofertilizers and phytostimulators of major crops like wheat, corn and soybean, whereas there are no registered commercial products yet with *Pseudomonas* spp. for biological control of plant diseases (Valverde and Ferraris, 2009).

In order to achieve satisfactory biocontrol results, pseudomonads must reach colonization levels above 10^5 CFU per gram of root (Thomashow et al., 2007) and they must survive in a very competitive environment like the rhizosphere (Fischer et al., 2010). Therefore, effective biocontrol isolates must also be excellent root colonizers of the specific target plant (Lugtenberg and Dekkers, 1999). Beyond host plant genetics, both soil abiotic properties and climatic factors also influence the establishment and development of a plant-growth promoting rhizobacteria (PGPR) population in the rhizosphere (Compant et al., 2010; Raaijmakers et al., 2002; Weller, 2007). Carbon sources found in root exudates, available minerals from soil, cell density achieved in root colonization, secondary metabolites produced by other microorganisms and other environmental factors (pH, temperature) are some of those factors that influence expression of PGPP in the rhizosphere. These may explain some inconsistencies in the performance of inoculated PGPR isolates under field conditions (Babu, 2011; Lugtenberg and Kamilova, 2009; Raaijmakers et al., 2002). For these reasons, the focus has been recently put into the isolation of PGPR from the same agricultural plots or crop rhizospheres where they would be applied later as inoculants. This strategy may help to overcome difficulties in adapting non-native bacterial species in a new environment (Cordero et al., 2012; Fischer et al., 2010; Mavrodi et al., 2012). However, one bottleneck for development of novel biocontrol formulations is the necessity to select a reduced number of appropriate candidates for conducting time-consuming field trials among a large collection of isolates. In this regard, it would be useful to count on screening tools that allow ranking of isolates based on their *in vitro* direct and indirect PGPP.

Several plant diseases affect extensive crops that are economically relevant in Argentina, like maize, wheat and soybean. Anthracnose (caused by *Colletotrichum* spp.), root, stem and/or spike rot (caused by *Fusarium* spp.), charcoal rot (caused by *Macrophomina phaseolina*), pod and stem blight (caused by *Phomopsis* spp.) and frogeye leaf spot (caused by *Cercospora sojina*), are some examples of important diseases that are difficult to control in our country (Carmona, 2014; Carmona et al., 2009; Lago, 2009; Nesci et al., 2006). In Argentina, more than 75% of the total agricultural area is under no-till management (Albertengo et al., 2014). Despite all the benefits that no-tillage provides to the soil (Derpsch et al., 2010), coupling this practice with monocropping tends to increase the severity and incidence of crop diseases, as the pathogen inoculum persists on crop residues (Leoni Velazco, 2013). For economic reasons, soybean is the major cultivated crop in Argentina (Domingo Yagüez et al., 2012). Soybean monoculture has expanded in the last years, leading to an increase of plant diseases caused by necrotrophic pathogens, most of them soil-borne fungi (Pérez-Brandán et al., 2014). Similar to what happens with bioremediation processes, finding the best plant-PGPR partners to inhibit pathogen accumulation might probably enhance the performance of the isolates, and ultimately, improve the biocontrol approach (Tapadar and Jha, 2013; Walsh et al., 2001).

In the context of the BIOSPAS consortium, a multidisciplinary project created for studying the agricultural effects on soil biology and biochemistry (from the Spanish acronym of *Soil Biology and Sustainable Agricultural Production*, Wall, 2011), we aimed to isolate and characterize pseudomonads from agricultural plots under no-till management, which display antagonistic activity against a number of pathogenic fungi obtained from soybean and maize plants cultivated in the same environments where the pseudomonads were isolated. In order to provide tools to facilitate identification of multipurpose strains appropriate for development of novel bioproducts among large collections of isolates, we here report: (i) the selection method used to screen the antagonistic potential of more than 100 pseudomonads isolates; (ii) the quantification of the antagonistic activity of the selected isolates and the calculation of a biocontrol index based on these results; (iii) the *in vitro* characterization of several traits related with the biocontrol activity, or directly with the plant growth promotion; (iv) the multivariate analysis of all quantified bacterial properties (v) the development, calculation and comparison of a biocontrol potential index (BPI), a direct growth promotion index (DGPI), and a *PGPR* index (PGPI), for comparative ranking of the isolates of interest.

2. Materials and methods

2.1. Sampling sites and sample collection

Samples were collected in agricultural fields located at four different geographical sites distributed across a 400 km West–East transect in the most productive region in the Argentinean Pampas, near the villages of Bengolea and Monte Buey (Córdoba Province), Pergamino (Buenos Aires Province), and Viale (Entre Ríos Province). At each site, top soil (0–10 cm) and plant root samples (whole plant root systems plus its surrounding soil core) were collected from plots under three different soil managements (treatments), which were mainly characterized by its intensity of crop rotation (or Good Agricultural Practices, BP from the Spanish acronym of Buenas Prácticas), very low or no crop rotation (or Bad Agricultural Practices, MP from the Spanish acronym of Malas

Prácticas), and natural grassland nearby BP and MP plots where no agriculture was practiced for at least 30 years (natural environment, AN from the Spanish acronym of Ambiente Natural). The geographical coordinates, physicochemical properties and the records of agricultural management and crop yields of the sampled fields are reported elsewhere (Agaras et al., 2012; Figuerola et al., 2012). Samples were kept at 4 °C until processing in the lab.

2.2. Isolation and conservation of pseudomonads

Soil and rhizosphere samples from samplings done in July 2009 and February 2010 were processed as described previously to determine quantitative and qualitatively their pseudomonads population (Agaras et al., 2014, 2012). After performing plate counts on *Pseudomonas*-selective medium S1 (Gould et al., 1985), those colonies with morphological differences among samplings, were re-streaked on S1 plates and on nutrient agar plates (tryptone soy agar 40 g/l; yeast extract 5 g/l; Biokar). A collection of more than 100 different isolates was generated from all agricultural treatment samples from all locations. Isolates were stored at −80 °C in multiwell plates containing per well 200 μl of an overnight culture in nutrient-yeast broth (NYB, nutrient broth 20 g/l; yeast extract 5 g/l; Biokar) supplemented with glycerol at 20%. We designated the isolates' names as acronyms of the sample type (S, bulk soil; R, rhizosphere); the sampling site (B, Bengolea; M, Monte Buey; P, Pergamino; V, Viale) and the agricultural management (AN, BP or MP, see Section 2.1 for references), followed by its serial number. For instance, isolate SVBP6 corresponds to isolate #6 from bulk soil (S) of a plot located in Viale (V) that was managed under good agricultural practices (BP).

2.3. Isolation and characterization of the fungal pathogens

Fungi associated with stems, shoots and roots were isolated during 2010 from selected diseased plants from the same aforementioned agricultural plots (Table 1). Plants were carefully removed from plots, stored in paper bags and returned to the laboratory for isolation of fungi. Symptomatic fragments of roots and shoots were washed in tap water, cut in 1 cm pieces and surface disinfested for 2–3 min in sodium hypochlorite (0.1%), washed with sterilized distilled water 2–3 min, and allowed to dry on sterilized paper. Small fragments of tissues were transferred to Petri dishes containing potato dextrose agar (Laboratorios Britania) amended with streptomycin 100 mg/L (PDAS) (Singleton et al., 1993). Plates were incubated at 25 °C for 7 days in the dark. After colony development, myceliar fragments were transferred onto PDAS plates, and pure cultures were obtained by dilution plating. Isolates were grown on PDA slants, identified using morphology (Leslie and Summerell, 2006; Scandiani and Luque, 2009), and stored in the Culture Collection of CEREMIC (Centro de Referencia de Micología, Rosario, Argentina). The fungal pathogen *C. sojina* CCC 172-2009 was originally isolated from leaves of the same area, but not the same plots, during 2009 and it was characterized in earlier studies (Carmona et al., 2009; Scandiani et al., 2012).

2.4. Antifungal-based selection of Pseudomonas isolates

To select pseudomonads isolates with biocontrol potential, bacteria and fungi were challenged in PDA plates. Fungi were grown in malt extract (1.5 g/l, Biokar, France) at 24 °C and 200 rpm (between 4 and 10 days, depending on the isolate). One hundred microlitres from each culture were spread as a lawn onto PDA plates, and then, sets of 20–30 *Pseudomonas* isolates were streaked on each PDA plate (Supplementary Fig. 1a). The incubation period was between 4 and 10 days at 24–25 °C, i.e., the time that the fungal mycelium took to reach the border of a control plate without bacteria. Those pseudomonads that showed an inhibition halo around their streaks in the screening plates were selected to perform single antagonism assays (Supplementary Fig. 1b) by streaking the isolate on opposite sides of a PDA plate, and placing an agar plug from a fresh culture of each fungal pathogen in the center. Incubation conditions were the same as for the screening assays. Isolates that confirmed their antagonistic potential against two or more fungal pathogens were finally selected for further characterization.

2.5. Taxonomical assignment of the selected Pseudomonas isolates

16S rDNA, *rpoB* and *oprF* gene fragments were amplified by PCR from thermal cell lysates and partially sequenced by Macrogen Inc. (Seoul, Korea), as previously described (Agaras et al., 2012; Tayeb et al., 2005). The obtained 16S rDNA sequences were first used to query the Seqmatch tool of the Ribosomal database project II (Cole et al., 2009), and partial *oprF* and *rpoB* sequences were analyzed with the BlastN tool in the NCBI database. In order to approach the taxonomical position within the established *Pseudomonas* complexes (Bodilis et al., 2011; Mulet et al., 2010; Ramette et al., 2011), phylogenetic analyses were carried out with concatenated partial 16S rDNA, *rpoB* and *oprF* sequences (Mulet et al., 2010; Agaras et al., 2012). For this, we selected 510 nt within the 5' region of the 16S rRNA gene (positions 110–619 in *Pseudomonas protegens* Pf-5, AJ417072) plus 480 nt within the 5' region of the *rpoB* gene (positions 1575–2085 in *P. protegens* Pf-5; NC_004129.6) and 510 nt of the *oprF* gene (positions 263–742 in *P. protegens* Pf-5, NC004129). The corresponding concatenated sequences of reference strains were included in the analysis. Neighbor-joining trees were inferred from evolutionary distances calculated with the Kimura 2-parameter formula, using the software MEGA v6 (Tamura et al., 2013). Confidence analyses were undertaken using 1000 bootstrap replicates. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). A subgroup of 16S rDNA and *oprF* sequences has been already published (Agaras et al., 2012). Novel 16S rDNA and *oprF* sequences, and the complete set of partial *rpoB* sequences have been deposited into GenBank under accession numbers KP710764 to KP71082.

2.6. In vitro quantification of the antagonistic potential and calculation of the antagonism potential index

To quantify the fungal growth inhibitory activity of selected isolates, conidia suspensions of each fungus were prepared from fresh plate cultures (4–10 days old, depending on every fungal pathogen) with 3 ml of saline solution (NaCl 0.85%), and counted in a Neubauer chamber. Suspensions were normalized to a final concentration of $2–4 \times 10^8$ conidia per ml. One hundred microlitres of these suspensions were spread onto triplicate PDA plates, and two 3 cm-streaks of each selected *Pseudomonas* isolate were made at the middle of those plates (Supplementary Fig. 1b). Upon incubation (same conditions as described above), the length of the inhibition zones was measured to the nearest mm.

Based on the results of the average inhibition zones generated by a single isolate against all tested fungi, we calculated the antagonism potential index (API) as follows: $([h_i/h^{\max}{}_i])/n) \times$ (number of inhibited fungi/n). In this formula h_i is the average inhibitory halo of the pseudomonad isolate over each of the tested fungi ($n = 12$, i.e., the total number of fungi tested; $i = 1–12$), and $h^{\max}{}_i$ is the average inhibitory halo of the pseudomonad isolate with the highest antagonistic activity in the collection against each of the tested fungi.

Table 1
Origin and characteristics of fungal pathogens used in the antagonism assays to screen *Pseudomonas* isolates as potential biological control agents.

Identification	CCC code[1]	Sample origin[2]	Target organ of susceptible crops	Reservoir[3]	Disease name	Difficulty in controlling
Colletotrichum truncatum	118-2010	Soybean stem – BP Bengolea	Stem, pods and seeds	Stubble and seed	Anthracnose	Medium
Fusarium oxysporum	119-2010	Soybean stem – BP Bengolea	Root and stem	Soil and stubble	Root rot	High
Fusarium semitectum	120-2010	Natural pasture stem – AN Bengolea	Root, stem, pods and seeds	Soil, stubble, pods and seeds	Root rot	High
Fusarium graminearum	122-2010	Natural pasture stem – AN Bengolea	Root, stem, pods and seeds	Soil, stubble, pods and seeds	Root and stem rot	High
Fusarium oxysporum	125-2010	Maize – MP Bengolea	Root and stem	Soil and stubble	Root rot	High
Fusarium oxysporum	126-2010	Maize – MP Bengolea	Root and stem	Soil and stubble	Root rot	High
Fusarium verticilloides	128-2010	Maize – MP Bengolea	Stem, root, spike and seed	Soil, seed and stubble	Root and spike rot	High
Colletotrichum graminicola	130-2010	Maize – MP Bengolea	Root and stem	Stubble and seed	Anthracnose	Medium
Macrophomina phaseolina	131-2010	Soybean – MP Bengolea	Root and stem	Soil, stubble and seed	Charcoal rot	High
Phomopsis sp.	134-2010	Soybean – MP Bengolea	Root, stem, pods and seeds	Stubble and seed	Pod and stem blight	Medium
Fusarium solani	139-2010	Soybean – MP Bengolea	Root and stem	Soil and stubble	Root rot	High
Cercospora sojina	172-2009	Soybean leaves – Monte Buey	Root, stem, leaf and seeds	Stubble and seed	Frogeye leaf spot	Medium

[1] Code given at the Culture Collection of CEREMIC, Centro de Referencia de Micología, Rosario, Argentina.
[2] Geographical location, agricultural treatment and plant from which fungi were isolated. BP, agricultural plots under Good Agricultural Practices; MP, agricultural plots under Bad Agricultural Practices; AN, natural environments surrounding agricultural plots. For details about these geographical sites and treatments, see Figuerola et al. (2012) and Agaras et al. (2012), and Material and Methods Section 2.1.
[3] Location where the fungal pathogen survives between cropping seasons.

2.7. Characterization of biocontrol-related activities and calculation of the biocontrol trait index and the global biocontrol potential index (BPI)

For plate assays, we used normalized bacterial suspensions (OD_{600} = 1.0 in saline solution) from overnight NYB cultures. Twenty microlitres of each normalized suspension was spotted on triplicate plates.

Exoprotease and phospholipase production was analyzed in skimmed milk agar or in egg yolk agar, respectively, as reported previously by Sacherer (1994). Siderophore production was estimated in CAS agar plates (Pérez-Miranda et al., 2007). The relative activity or production was expressed as (diameter of the observed halo/diameter of each bacterial spot) × 100.

HCN production was assayed qualitatively by the picrate-filter paper method (Egan et al., 1998) and quantitated by colorimetry (Voisard et al., 1989) from cultures grown in DF medium (Koo et al., 2010) supplemented with 0.1% glucose. The calibration curve was generated with KCN at concentrations ranging 0–1.2 μg/ml.

Established PCR approaches were used to evaluate the presence of four different genes related to the production of antibiotics: *phlD* for DAPG (McSpadden Gardener et al., 2001), *phzF* for phenazines (Mavrodi et al., 2010), *pltB* for pyoluteorin (Mavrodi et al., 2001) and *prnD* for pyrrolnitrin (de Souza, 2002). DNA from *P. protegens* strain CHA0 was used as positive control for *phlD*, *pltB* and *prnD* PCR detection (Ramette et al., 2011), whereas *P. fluorescens* 2–79 served as positive control for *phzF* (Mavrodi et al., 1998). PCR reactions were carried out with thermal cell lysates as templates, following the cycling protocols reported for each target gene in the aforementioned references.

Based on the results of the average biocontrol-related activities the biocontrol traits index (BTI) was calculated as follows: $([a_i/a^{\max}{}_i])/n$. In this formula a_i is the average activity of the biocontrol trait i for a given pseudomonad isolate; $a^{\max}{}_i$ is the maximum average activity detected among all tested isolates; n, the total number of activities evaluated. In order to estimate the global ability of isolates to act as a biocontrol agent, we calculated the biocontrol potential index (BPI) as (API + BTI)/2.

2.8. In vitro characterization of direct plant growth promoting activities and calculation of the direct growth promoting index (DGPI)

In agreement with the definition of Lugtenberg and Kamilova (2009), namely "Direct plant-growth-promoting rhizobacteria enhance plant growth in the absence of pathogens", we have measured different bacterial activities that can be linked to this definition.

Plate and liquid assays in NBRIP medium were performed by triplicate to evaluate the ability of the selected pseudomonad isolates to solubilize inorganic phosphate, using $Ca_3(PO4)_2$ as phosphate source (Nautiyal, 1999). In plate assays, solubilization efficiency (SE) was calculated as for exoprotease or phospholipase activity by measuring the halo and colony diameters. In liquid assays, the NBRIP medium was buffered at pH = 8.0, and the final achieved pH and the inorganic phosphorous (Pi) concentration were determined at the end of the experiment as a measure of the organic acid secretion and phosphate solubilization, respectively (Fernández et al., 2012).

The production of indole-3-acetic acid (IAA) and of similar reactive compounds was quantified in cultures grown in DF medium supplemented with 0.1% glucose and 0.5 mg/ml of tryptophan, after 72 h of incubation at 28 °C and 200 rpm (Koo et al., 2010). The standard curve was generated with 3-IAA solutions ranging 0–50 μg/ml (in ethanol 90%). *Pseudomonas putida* GR12-2 (Patten and Glick, 2002) served as positive control for IAA production.

1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase production was determined in DF medium containing 0.1% glucose and 3 mM ACC as the sole nitrogen source (Koo et al., 2010). We monitored OD_{600} every 15 min during 48 h at 28 °C in microplate wells containing 200 μl of growth medium (shaken at 200 rpm just before every measurement). *P. putida* ATCC17399/pRKAcc served as positive control for AAC deaminase production, whereas *P. putida* ATCC17399 was used as the negative control (Grichko and Glick, 2001).

A direct growth promotion index (DGPI) was calculated based on the activities explained above, as follows: DGPI = $([a_i/a^{\max}{}_i])/n$. In this formula a_i is the average activity of the direct growth promotion trait i for a given pseudomonad isolate; $a^{\max}{}_i$ is the maximum average activity detected among all tested isolates; and n, the total number of activities evaluated.

2.9. Detection of quorum sensing N-acylhomoserine lactones

Biosensor strains *Chromobacterium violaceum* (CV026 and VIR07) and *Agrobacterium tumefaciens* NTL4/pZLR4, were used to detect the production of quorum sensing (QS) signals of the N-acylhomoserine lactone (AHL) type (Farrand et al., 2002; Morohoshi et al., 2008). Two parallel streaks of *C. violaceum* strain

CV026 or strain VIR07 were done along the middle of triplicate NA plates, with a separation of 1 cm from each other. At both sides of the streaks, 3 drops (10 µl) of normalized bacterial suspensions (OD_{600} = 1.0) were spotted. Violacein production by the AHL reporter strains was recorded after 48 h of incubation at 28 °C. *P. aeruginosa* PAO1 was employed as a positive control whereas *Escherichia coli* K12 served as a negative control. For the assay with *A. tumefaciens*, 3-ml overlays of soft water agar (0.7% w/v) containing 0.5 ml of a saturated culture from the indicator strain and 40 µg/ml of 5-bromo-3-indolyl-β-D-galactopyranoside (X-Gal) was poured onto NA plates. Upon solidification of the indicator overlays, 5 µl drops of normalized bacterial suspensions (OD_{600} = 1.0) were spotted. Plates were incubated at 28 °C for 48 h. *A. tumefaciens* NT1/pTiC58ΔaccR was employed as a positive control whereas *A. tumefaciens* NTL4 served as negative control (Cha et al., 1998; Farrand et al., 2002).

2.10. Motility assays

Swimming, swarming and twitching motility were evaluated for each selected isolate by triplicate plate assays using NYB supplemented with different agar concentrations (3, 5 and 7 g/l, respectively) (Rashid and Kornberg, 2000). Bacterial progress was measured after 18 h of incubation at 28 °C.

2.11. Biofilm formation

We carried out static cultures in 96-multiwell polystyrene plates to analyze biofilm formation by the crystal violet method after 8 h of incubation at 28 °C (O'Toole and Kolter, 1998). Triplicate cultures were started with 100 µl of bacterial suspensions of OD_{600} = 0.2 in M63 medium supplemented with 0.4% of glucose and 0.4% of casaminoacids. After the incubation period, the liquid phase was removed for measurement of OD_{600}, the planktonic cells and growth medium remnants were washed out twice with saline solution and the surface attached cells were stained with 0.2% (w/v) crystal violet. After 15 min, we added 100 µl of ethanol 95% (v/v) to solubilize the dye and we measured the absorbance at 570 nm (Merritt et al., 2011). Additionally, we inspected biofilms that were developed statically during 20 h in 24-multiwell plates, under an inverted microscope (Leica DMI6000B). Images were taken with a DFC450 camera controlled by the Leica Applications Suite 3.7.0 software.

2.12. Calculation of the plant growth promoting index (PGPI)

In order to estimate an integrative index that takes into account antagonism, biocontrol, direct growth promotion and colonization traits we calculated the PGPI as follow: $PGPI = ([a_i/a^{max}_i])/n$. In this formula a_i is the average activity of the trait i for a given pseudomonad isolate; a^{max}_i is the maximum average activity detected among all tested isolates; and n, the total number of activities evaluated.

2.13. Effects of the isolates on early germination and seedling development of maize and soybean

We carried out greenhouse experiments with the 19 pseudomonads isolates using 330 cm^3 pots filled with vermiculite (n = 10). Maize seeds (*Zea mays*, experimental hybrid Sursem C3; germinative power, GP = 99%) were surfaced disinfected by soaking in 70% v/v ethanol for 1 min followed by treatment with 1% w/v sodium hypochlorite for 10 min. After six washes with 100 ml of sterilized distilled water, surface-disinfected seeds were inoculated with 10 ml of a normalized bacterial suspensions of OD_{600} = 1.0 or saline solution as a negative control. Soybean seeds (*Glycine max*, Don Mario DM4970, GP = 93%) were not disinfected, as their PG decreased strongly after this process. Two seeds per pot were sown for both plants, and pots were kept watered by sub-irrigation with sterilized Jensen solution (Vincent, 1970). Germination (% emerged plants relative to sown seeds) and plant development (whole plant fresh weight) was evaluated after 14 days (maize) or 21 days (soybean) of incubation in the greenhouse at 24–26 °C. To confirm a proper seed bacterization, inoculated seeds as well as root pieces from harvested plants, were deposited onto S1 agar plates at 28 °C and incubated during 48 h, before inspecting the aspect of the bacterial outgrowth.

2.14. Statistics

Experiments were repeated at least twice and done by triplicate each time. Average and standard deviation values were calculated from recorded data and used to carry out ANOVA, t-test or linear regression analysis with the Infostat software (Di Rienzo et al., 2013). When appropriate, multiple comparison tests were done with the algorithm Di Rienzo, Guzmán and Casanoves (DGC, based on cluster) to evaluate if the differences among values were statistically significant. The tool "Difference between proportions" of the "Inference based on two samples" analysis that belong to the Infostat software (Di Rienzo et al., 2013) was used to evaluate germination percentages of inoculated versus control seeds. Unless clarified in the text, all the analyses were done at $p < 0.05$.

Every quantified activity was considered to perform a Principal Component Analysis (PCA) with the Infostat software (Di Rienzo et al., 2013). Average values for each quantitative test determined for every selected *Pseudomonas* isolate were included in the PCA. PCR and QS results for each isolate were given the following values: 1 for positive, and 0 for negative. Other qualitative assays were not included.

3. Results

3.1. Diversity of pseudomonads with antifungal activity isolated from soils and rhizospheres of agricultural plots of the Pampean Region in Argentina

A collection of 128 isolates with morphologically different colony types was generated from bulk soil and rhizosphere samples from healthy crop plants obtained from agricultural plots under no tillage management between July 2009 and February 2010 (Agaras et al., 2012). After screening for antagonism against a panel of 12 pathogenic fungus (Supplementary Fig. 1a), 19 of those isolates proved to inhibit the growth of two or more fungal pathogens listed in Table 1. Therefore, they were selected for identification and further characterization. There was no evident trend at first glance in the geographical, soil management, type of sample (bulk or rhizosphere soil), or crop origin for the selected isolates (Supplementary Table 1).

Multiple comparison of concatenated partial 16S rDNA, *rpoB* and *oprF* gene sequences for the 19 selected isolates, together with reference strains, revealed a wide distribution of isolates among the different *Pseudomonas* complexes established in previous taxonomic classifications (Bodilis et al., 2011; Ramette et al., 2011) (Fig. 1). That is, the 19 selected isolates fell within most of the pseudomonads sub-groups, except for the *P. aeruginosa*/*P. resinovorans* complex, the *P. syringae* complex and the recently separated *P. protegens* complex (Fig. 1).

Upon quantitation of the *in vitro* antifungal potential (Supplementary Fig. 1b), we observed that each selected isolate displayed a differential inhibitory level against the collection of fungal pathogens (Fig. 2). We found seven isolates that could

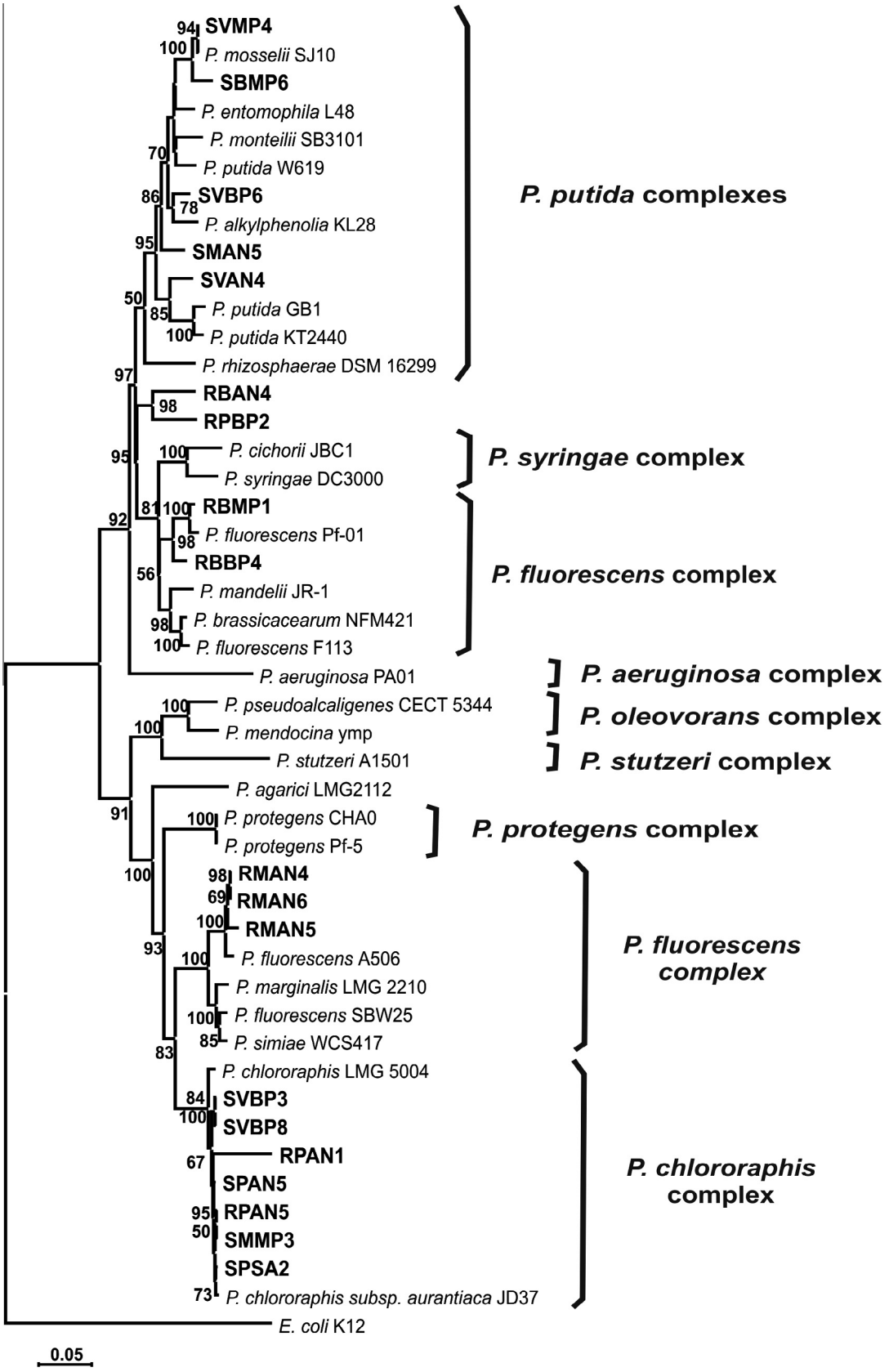

Fig. 1. Phylogenetic analysis of 16s rDNA-*rpoB*-*oprF* concatenated genes from 47 taxa, including the 19 isolates characterized in this study (Submission ID 1791069) and 28 reference strains. Phylogenetic analyses were conducted in MEGA6 (Tamura et al., 2013). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). The sequences of 16S rDNA, *rpoB* and *ompA* genes from *E.* coli K12 substr. MG1655 were employed to root the tree. Taxonomic grouping was based on the works of Mulet et al. (2010), Ramette et al. (2011) and Bodilis et al. (2011).

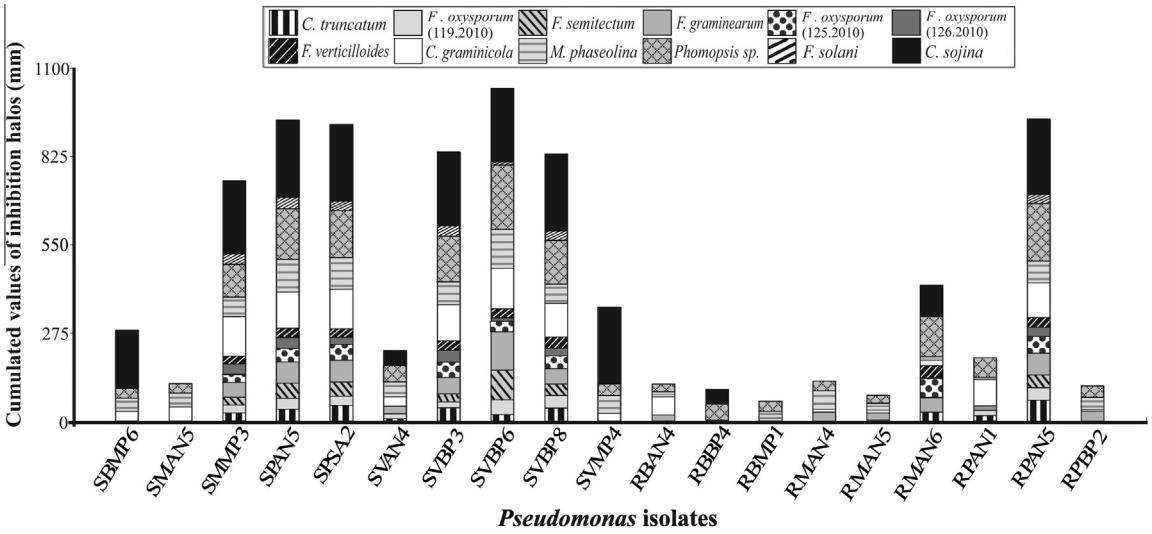

Fig. 2. Fungal antagonistic potential of selected pseudomonads isolates. Vertical axis shows the cumulative values (in mm) of the inhibition halos that were measured for every fungus challenged with each of the 19 pseudomonads isolates (*x* axis). The antagonistic potential against every fungal pathogen (i.e., the size of the inhibition halo) is represented by the high of the section bar assigned for every fungus (see legend).

Table 2
Antagonism potential against fungal pathogens, quantitative indices based on *in vitro* PGPP, and taxonomic identification of each of the 19 tested *Pseudomonas* isolates.

Bacterial isolates	Number of fungi inhibited[1]	API[2]	BTI[3]	BPI[4]	DGPI[5]	PGPI[6]	*Pseudomonas* complex[7]
RPAN5	12	0.78	0.65	0.72	0.49	0.60	*P. chlororaphis*
SPSA2	12	0.74	0.68	0.71	0.50	0.60	*P. chlororaphis*
SPAN5	12	0.77	0.64	0.70	0.48	0.58	*P. chlororaphis*
SVBP3	12	0.70	0.64	0.67	0.13	0.45	*P. chlororaphis*
SVBP8	12	0.69	0.60	0.65	0.33	0.49	*P. chlororaphis*
SMMP3	12	0.61	0.59	0.60	0.37	0.49	*P. chlororaphis*
SVBP6	12	0.75	0.32	0.53	0.46	0.47	*P. putida*
RPAN1	6	0.08	0.69	0.38	0.22	0.37	*P. chlororaphis*
SVAN4	7	0.08	0.59	0.34	0.55	0.36	*P. putida*
RBAN4	4	0.02	0.62	0.32	0.54	0.39	*P. asplenii*
SMAN5	3	0.02	0.52	0.27	0.44	0.31	*P. putida*
RBMP1	2	0.01	0.51	0.26	0.37	0.28	*P. fluorescens*
RMAN6	8	0.24	0.29	0.26	0.36	0.30	*P. fluorescens*
RBBP4	2	0.01	0.37	0.19	0.55	0.27	*P. fluorescens*
SBMP6	4	0.04	0.29	0.17	0.36	0.28	*P. putida*
SVMP4	4	0.05	0.24	0.14	0.42	0.31	*P. putida*
RMAN4	4	0.03	0.22	0.12	0.51	0.27	*P. fluorescens*
RPBP2	4	0.02	0.21	0.12	0.43	0.23	*P. asplenii*
RMAN5	3	0.01	0.17	0.09	0.35	0.17	*P. fluorescens*

Isolates are arranged by their BPI values.
[1] Number of the fungi inhibited *in vitro* from the total of 12 fungi tested.
[2] Antagonism potential index, calculated with the formula explained in Section 2.6.
[3] Biocontrol traits index, calculated with the formula explained in Section 2.7.
[4] Biocontrol potential index, calculated with the formula explained in Section 2.7.
[5] Direct growth potential index, calculated with the formula explained in Section 2.8.
[6] Plant growth promoting index, calculated with the formula explained in Section 2.12.
[7] Taxonomic positions based on major phylogenetic subgroups shown in Fig. 1.

inhibit the growth of all tested fungi (SMMP3, SPAN5, SPSA2, SVBP3, SVBP6, SVBP8 and RPAN5; highlighted in dark gray in Table 2 and Fig. 2); three isolates with an intermediate antagonistic activity (SVAN4, RMAN6 and RPAN1; highlighted in light gray in Table 2 and Fig. 2); and nine isolates that could inhibit 30% or less of the fungi tested (SBMP6, SMAN5, SVMP4, RBAN4, RBBP4, RBMP1, RMAN4, RMAN5 and RPBP2; Fig. 2). This qualitative classification is consistent with the corresponding calculated API shown in Table 2. *Pseudomonas chlororaphis*-related isolates and isolate SVBP6, which has been tentatively assigned to the *P. putida* cluster, but phylogenetically closer to the *P. alkylphenolia* species (Fig. 1), had the highest antagonistic potential in terms of the number of fungi antagonized and the individual fungal growth inhibition (Fig. 2). Interestingly, this API-based ranking also grouped *Pseudomonas* isolates by their taxonomic identity, linking the biocontrol potential with the phylogenetic classification (Table 2).

3.2. Biocontrol traits of the selected pseudomonad isolates

The antibiotic biosynthetic potential was studied by PCR detection of the corresponding genes. The *phzF* gene, involved in phenazine production, was detected in all *P. chlororaphis*-related isolates (Table 3). The *prnD* gene, required for pyrrolnitrin synthesis, was detected in some members of *P. chlororaphis* and *P. putida* complexes (Table 3). Only four isolates may be pyoluteorin producers, as they were positive for the *pltB* gene (Table 3). By contrast, none of the 19 isolates was PCR positive for the *phlD* gene, involved in the DAPG production.

Table 3
Colony morphology and results of the biocontrol-related activities (indirect PGPP) evaluated for the 19 *Pseudomonas* isolates.

Bacterial isolates	Phospholipase[1]	Exoprotease[1]		HCN	*pltB* [3]	*prnD* [3]	*phzF* [3]	Siderophore [4]
	Relative activity	Relative activity	Plate assay[2]	HCN conc. (uM)/DO600[1]	PCR	PCR	PCR	Relative production
RBAN4	97.5 ± 13.0*	108.3 ± 8.3*	+	127.7 ± 37.2*	+	+	-	116.7 ± 7.2[c]
RBBP4	106.3 ± 10.8*	111.1 ± 4.8*	+	103.4 ± 12.2*	+	-	-	0[g]
RBMP1	184.3 ± 5.6*	104.8 ± 30.6*	*n.d*	*n.d*	-	+	-	122.6 ± 17.7[c]
RMAN4	51.9 ± 3.2	118.0 ± 4.4*	-	<3	-	-	-	90.0 ± 17.3[d]
RMAN5	32.3 ± 8.0	63.6 ± 6.1	-	<5	-	-	-	0[g]
RMAN6	66.7 ± 0.0*	119.4 ± 24.4*	+	128.1 ± 76.1*	-	-	-	81.5 ± 6.4[d]
RPAN1	173.6 ± 12.0*	147.2 ± 6.6*	+	283.5 ± 42.9*	-	-	+	170.4 ± 6.4[a]
RPAN5	125.9 ± 17.0*	135.4 ± 1.8*	+	156.2 ± 60.0*	-	+	+	84.7 ± 16.8[d]
RPBP2	50.3 ± 5.1	97.2 ± 4.8*	-	<5	-	-	-	104.2 ± 7.2[c]
SBMP6	65.7 ± 9.8*	163.4 ± 16.5*	±	44.0 ± 20.1	-	-	-	92.6 ± 12.8[d]
SMAN5	120.6 ± 8.8*	126.8 ± 9.9*	+	139.9 ± 52.8*	+	+	-	116.7 ± 14.4[c]
SMMP3	130.0 ± 17.3*	120.7 ± 5.7*	+	100.2 ± 23.7*	-	+	+	58.3 ± 7.2[e]
SPAN5	153.3 ± 45.1*	121.0 ± 10.9*	+	120.9 ± 29.6*	-	+	+	84.3 ± 5.6[d]
SPSA2	164.7 ± 23.8*	139.9 ± 8.9*	+	105.4 ± 7.5*	-	+	+	104.2 ± 7.2[c]
SVAN4	93.3 ± 5.8*	125.8 ± 8.4*	±	81.3 ± 17.7*	+	+	-	100.5 ± 11.8[c]
SVBP3	157.9 ± 4.0*	148.5 ± 10.5*	+	111.4 ± 39.7*	-	+	+	57.1 ± 12.4[e]
SVBP6	70.6 ± 4.2*	142.4 ± 5.3*	±	37.5 ± 19.6	-	-	-	145.8 ± 7.2[b]
SVBP8	117.1 ± 33.0*	119.4 ± 4.8*	+	124.7 ± 65.5*	-	+	+	70.8 ± 7.2[e]
SVMP4	85.9 ± 12.2*	144.4 ± 4.8*	±	38.4 ± 22.4	-	-	-	30.4 ± 6.4[f]

Mean values ± SD are shown.
For every activity, higher values than those expressed by the positive control (*P. protegens* CHA0) are highlighted in gray.
[1] Phospholipase, exoprotease and HCN values with an asterisk show a statistically significant difference respect to the negative control (*P. protegens* CHA89) when the *t*-test was applied ($p < 0.05$).
[2] (+) symbol means a clear positive reaction (paper with a strong orange coloration); (±) means a putative positive reaction (paper with a light orange coloration); and (−) symbol means a negative reaction (paper with a yellow coloration) (Egan et al., 1998).
[3] (+) symbol means a positive reaction, and (−) symbol means the absence of a band in the electrophoresis gel. *phlD* results are not shown because no one was positive.
[4] Letters indicate statistically significant differences between isolates when the DGC test was applied ($p < 0.05$).

The production of extracellular hydrolytic enzymes was a generalized feature of the collection, although isolates could be categorized according to their relative exoprotease and phospholipase activity (Table 3). HCN production above background levels was detected for 12 isolates, being the qualitative results consistent with those from the quantitative test (Table 3). Finally, all isolates, except RBBP4 and RMAN5, secreted different amounts of iron chelating compounds in CAS plates (Table 3).

These results were used to calculate BTI (Table 2) As expected, the stronger fungal antagonists (higher API values) also shown high BTI scores (Table 2); the only exception was isolate SVBP6. However, we detected a set of isolates that had BTI scores higher than 0.50 but showed only modest or low antagonistic activity against the tested fungi (RPAN1, SVAN4, RBAN4, SMAN5, RBMP1).

3.3. Assessment of direct PGP traits

Inorganic phosphorus solubilization assays in both solid and liquid medium showed different levels of this activity among the antifungal isolates (Table 4). The highest solubilization efficiencies (SE) were detected in members of the *P. fluorescens* complex, whereas the lowest SE values were recorded for some of the *P. chlororaphis*-related isolates (Table 4). Linear correlation analysis among final pH, final Pi and SE values revealed a strong positive correlation between pH and Pi ($r^2 = 0.90$, data not shown).

With regards to auxin production, only 5 members of the *P. fluorescens* and *P. putida* complexes (namely RBAN4, RMAN4, RMAN5, SMAN5 and SVAN4) produced IAA-related compounds at statistically similar levels to that of the positive control *P. putida* GR12-22 (7.29 ± 2.71 μg/ml/OD_{600}, $p > 0.05$) (Table 4).

In terms of ACC deaminase activity, only the *P. chlororaphis*-related isolate SPSA2 was able to grow using ACC as the sole nitrogen source, reaching a similar cell density (1.28 ± 0.06) to that of the positive control *P. putida* ATCC 17399/pRKAcc (1.33 ± 0.05), after 48 h of incubation at 28 °C (Table 4). Nevertheless, other nine isolates showed a modest but significantly higher growth than the ACC deaminase negative control strain *P. putida* ATCC17399 (0.08 ± 0.06, Table 4).

These results were used to calculate DGPI (Table 2). Only five isolates (SPSA2, SVAN4, RBAN 4, RBBP4 and RMAN4) showed a DGPI index equal or higher than 0.50 (Fig. 3, Table 2), but not all these isolates had also high biocontrol potential as judged by their BPI values (Fig. 3 and Table 2).

Table 4
Results of screening assays related to direct PGPP of the 19 *Pseudomonas* isolates.

Bacterial isolates	Pi solubilization (solid)	Pi solubilization (liquid)		ACC deaminase[1]	IAA[1]	QS	Biofilm	Swimming	Swarming	Twitching
	Solubilization efficiency	μg P/ml	pH	OD_{600} after 48 h	[IAA] (μg/ml)/ DO_{600}	AHLs	8 h on PE (Abs_{570}/ OD_{600})	mm of colony development (18 h)		
RBAN4	46.7 ± 11.6^{c}	188.8 ± 5.3^{a}	4.1^{b}	<0.1	5.4 ± 1.2*	–	2.6 ± 0.8^{c}	26.6 ± 1.1^{d}	12.5 ± 2.1^{e}	4.0 ± 0.1^{b}
RBBP4	70.0 ± 10.0^{b}	87.3 ± 56.1^{c}	4.3^{b}	0.2 ± 0.0	3.0 ± 0.2*	–	6.0 ± 2.1^{c}	30.4 ± 0.4^{d}	12.3 ± 0.5^{e}	3.2 ± 0.4^{b}
RBMP1	53.3 ± 11.6^{c}	124.3 ± 13.2^{b}	3.3^{a}	0.2 ± 0.1	2.1 ± 0.5*	–	3.5 ± 1.2^{c}	28.1 ± 1.9^{d}	12.1 ± 0.4^{e}	3.8 ± 1.2^{b}
RMAN4	106.7 ± 11.6^{a}	117.0 ± 9.5^{b}	4.2^{b}	<0.1	4.1 ± 0.1*	–	2.9 ± 0.5^{c}	46.7 ± 0.5^{c}	40.0 ± 1.7^{b}	4.3 ± 0.2^{b}
RMAN5	33.3 ± 11.6^{c}	118.1 ± 4.8^{b}	3.9^{b}	<0.1	4.7 ± 0.3*	–	4.7 ± 3.0^{c}	22.6 ± 13.4^{d}	29.0 ± 0.3^{c}	4.4 ± 0.2^{b}
RMAN6	76.2 ± 8.3^{b}	121.4 ± 7.1^{b}	3.9^{b}	<0.1	1.7 ± 0.3*	–	3.0 ± 0.9^{c}	55.2 ± 4.0^{b}	40.0 ± 4.4^{b}	3.9 ± 0.0^{b}
RPAN1	0.0 ± 0.0^{d}	139.1 ± 6.2^{b}	3.3^{a}	<0.1	1.8 ± 0.1*	+	3.0 ± 0.9^{c}	34.1 ± 1.3^{d}	40.8 ± 6.0^{b}	5.5 ± 0.8^{b}
RPAN5	35.8 ± 7.4^{c}	178.0 ± 0.0^{a}	3.9^{b}	0.8 ± 0.1*	1.2 ± 0.3	+	3.1 ± 0.1^{c}	39.1 ± 1.0^{d}	42.0 ± 1.5^{b}	4.5 ± 0.1^{b}
RPBP2	93.9 ± 10.5^{a}	122.9 ± 8.8^{b}	4.1^{b}	0.2 ± 0.1	0.9 ± 0.1	–	2.4 ± 0.8^{c}	27.7 ± 1.4^{d}	13.3 ± 0.6^{e}	5.6 ± 0.1^{b}
SBMP6	33.5 ± 6.4^{c}	137.3 ± 1.6^{b}	4.0^{b}	0.3 ± 0.1*	1.9 ± 0.3*	–	1.1 ± 0.3^{c}	79.8 ± 5.2^{a}	90.0 ± 0.0^{a}	3.3 ± 0.8^{b}
SMAN5	53.3 ± 11.6^{c}	149.6 ± 15.0^{b}	4.2^{b}	0.2 ± 0.1*	3.1 ± 1.6*	–	2.6 ± 0.4^{c}	30.4 ± 3.7^{d}	12.2 ± 1.8^{e}	4.0 ± 1.0^{b}
SMMP3	11.1 ± 9.6^{d}	190.1 ± 2.5^{a}	5.0^{c}	0.4 ± 0.1*	1.4 ± 0.2*	+	5.8 ± 3.2^{c}	44.8 ± 2.5^{c}	22.2 ± 0.4^{d}	5.1 ± 0.5^{b}
SPAN5	26.7 ± 11.6^{c}	196.9 ± 1.5^{a}	4.1^{b}	0.7 ± 0.0*	1.9 ± 0.3*	+	3.1 ± 0.8^{c}	36.3 ± 2.2^{d}	18.8 ± 2.5^{d}	3.6 ± 0.5^{b}
SPSA2	0.0 ± 0.0^{d}	182.7 ± 5.4^{a}	4.3^{b}	1.3 ± 0.1*	1.0 ± 0.5	+	3.1 ± 1.5^{c}	44.1 ± 4.4^{c}	28.4 ± 2.2^{c}	7.3 ± 3.0^{b}
SVAN4	53.3 ± 11.6^{c}	206.9 ± 9.0^{a}	4.0^{b}	<0.1	4.5 ± 3.1*	–	3.8 ± 3.1^{c}	31.0 ± 0.8^{d}	12.2 ± 1.0^{e}	3.9 ± 0.0^{b}
SVBP3	0.0 ± 0.0^{d}	65.9 ± 1.6^{c}	4.9^{c}	0.3 ± 0.0*	n.d.	+	9.0 ± 5.8^{b}	29.6 ± 1.1^{d}	4.2 ± 0.1^{f}	4.2 ± 1.2^{b}
SVBP6	68.5 ± 19.9^{b}	143.9 ± 43.4^{b}	3.9^{b}	0.5 ± 0.0*	1.4 ± 0.3	–	2.2 ± 1.2^{c}	38.4 ± 1.8^{d}	16.4 ± 1.9^{d}	4.7 ± 0.2^{b}
SVBP8	35.8 ± 7.4^{c}	63.0 ± 0.4^{c}	4.9^{c}	0.6 ± 0.1*	2.4 ± 0.3*	+	13.3 ± 4.2^{a}	33.5 ± 3.4^{d}	12.6 ± 0.2^{e}	3.5 ± 0.3^{b}
SVMP4	44.4 ± 13.9^{c}	186.7 ± 2.8^{a}	4.3^{b}	0.3 ± 0.0*	1.5 ± 0.2*	–	1.1 ± 0.3^{c}	44.4 ± 6.2^{c}	90.0 ± 0.0^{a}	12.3 ± 8.9^{a}

Values ± SD are shown.
Different letters mean statistically significant differences among values, estimated with the multiple comparison test DGC ($p < 0.05$).
[1] To analyze these assays, the *t* test based on two samples was applied, comparing each sample with the negative control (*P. putida* ATCC 17399).

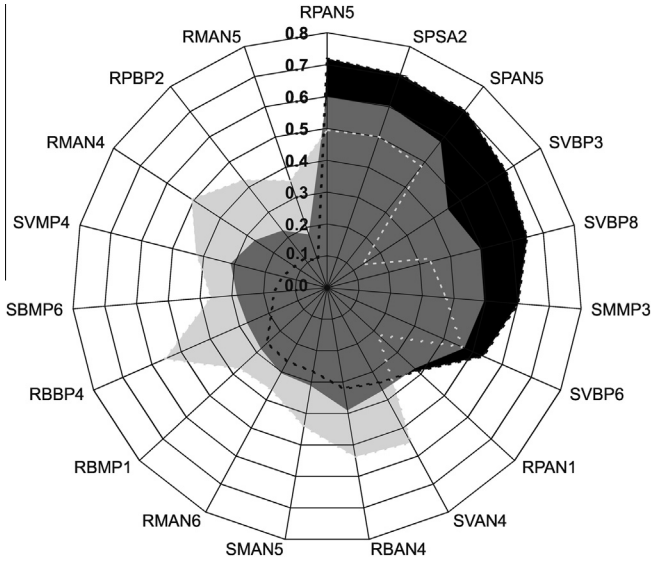

Fig. 3. Comparison of biocontrol potential index (BPI in black), direct plant growth promotion index (DGPI in light gray) and PGPR index (PGPI in dark gray), for every characterized pseudomonad isolate. See text for details on the calculation of each index.

3.4. Production of AHL-quorum sensing molecules, motility traits and biofilm formation

All isolates related with the *P. chlororaphis* cluster induced violacein production in both *C. violaceum* AHL reporter strains, CV026 (short chain AHL reporter) and VIR07 (medium chain AHL reporter), and induced *lacZ* expression in the *A. tumefaciens* long chain AHL reporter strain NT1/pZLR4 (Table 4).

Swimming motility, an individual cell movement phenomenon dependent on the polar flagella rotation, was evident in every tested isolate (Table 4). The social swarming motility was much higher in 2 of the 19 isolates, whereas the type IV pili-dependent twitching motility was evident only for the isolate SVMP4 (Table 4).

Significant attachment of cells to the microplate polystyrene surface above the negative control wells was detected after 8 h of static incubation for all isolates (Table 4). However, only isolate SVBP8 stood out over the rest of the isolates (Table 4). This isolate, along with the rest of the *P. chlororaphis*-related isolates, showed the highest Abs_{570}/OD_{600} values in the quantitative assay (Table 4). The biofilm coverage pattern was highly diverse among the isolates (Supplementary Fig. 2).

3.5. Effects of seed inoculation with antifungal isolates on soybean and maize germination and early plant development

Most of the isolates did not influence the germination rate of maize seeds, when comparing with the non-inoculated control (Fig. 4a). However, isolate SBMP6 showed a moderate negative effect on germination of both crop seeds, and other five isolates (RMAN5, RPAN5, SMMP3, SPSA2 and SVAN4) decreased the germination of soybean plants (Fig. 4a). Three isolates significantly promoted early maize development (RBBP4, RBMP1 and SMMP3, Fig. 4b). For soybean plants, two isolates significantly promoted their early development (RBAN4 and RPBP2, Fig. 4b). On the other hand, SVBP3 and RMAN4 were the only isolates that showed a statistically significant weak negative impact on maize early development (Fig. 4b). Correct seed bacterization and subsequent root colonization were confirmed by development of pseudomonads colonies after incubation of seeds or root pieces on S1 plates (data not shown).

3.6. The antifungal pattern of Pseudomonas isolates is mainly explained by their in vitro biocontrol traits and the origin of the soil sample

In order to evaluate if there is any subgrouping of the isolates according to their probiotic traits and soil sample origin, we performed a multivariate analysis. The PCA outcome, for which the two principal components explained 60% of the total variance, showed the influence of the biocontrol-related and plant growth promoting traits on the structure of the pseudomonads collection (Fig. 5). The first component (CP1, horizontal axis) explained

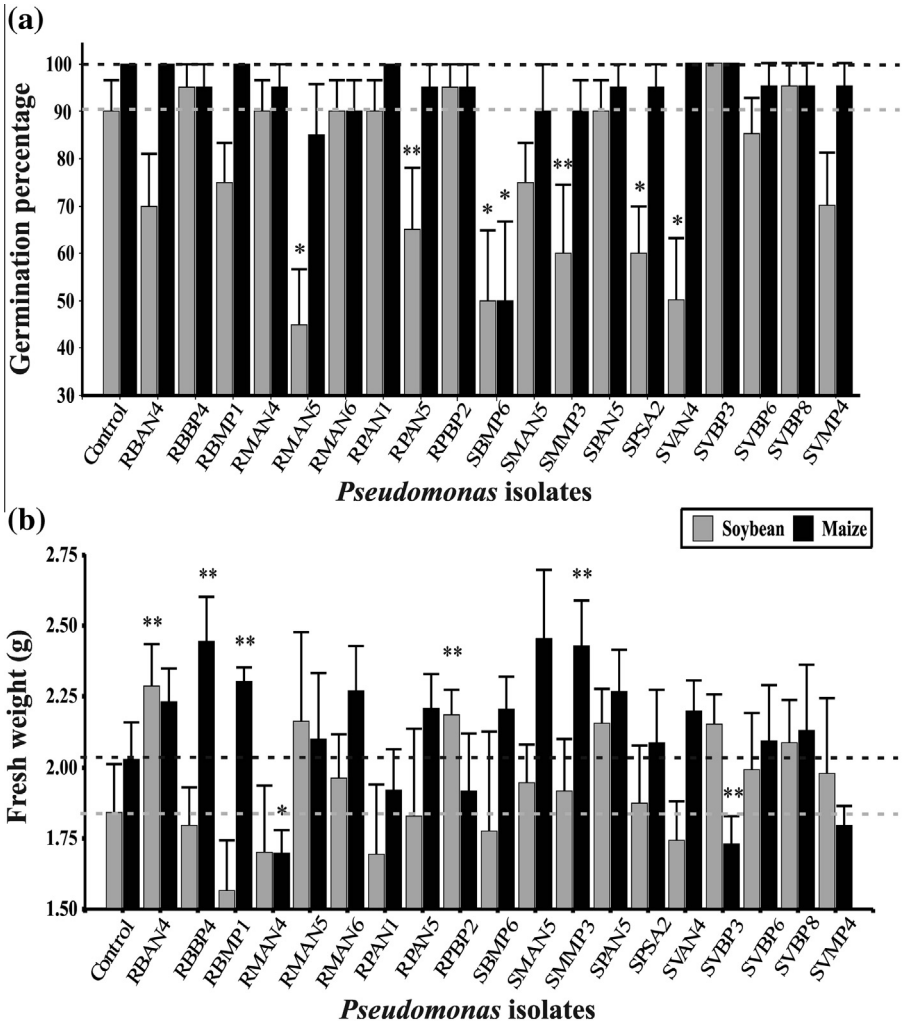

Fig. 4. Effect of seed bacterization on seed germination rate (a) and early development (b) of soybean (gray bars) and maize (black bars) seedlings. One asterisk indicates a statistically significant difference of 95% ($p < 0.05$) when comparing with the non-inoculated control in the two samples *t*-test, whereas two asterisks denotes a difference of 90% ($p < 0.10$) in the same test. Bars indicate standard errors. Dashed lines indicate the average values of control plants. (a) Germination values are expressed as the proportion of germinated seeds over the total number of sown seeds ($n = 20$). (b) Fresh weight values are averages for one pot ($n = 10$).

46.1% of the variance and separated the isolates in roughly two groups. The rightmost group along PC1 includes isolates with highest antagonistic power (Table 2 and Fig. 2) and almost all of them (6/7) were isolated from bulk soil samples. This group of isolates was also characterized by their production levels of HCN, phospholipase, AHL signals and siderophores, together with the presence of *phzF* and *prnD* genes, on top of their fungal antagonism quantification values. The leftmost group along PC1 included isolates mostly from rhizospheric soil samples and with the highest plant growth promoting activity on maize (Fig. 5). The second axis PC2, which explained 13.9% of the variance, distinguished isolates with higher motility in the upper part of the graph. Swimming, swarming and twitching influenced this separation, together with exoprotease production and biofilm formation (Fig. 5).

If we consider all the activities described above as part of an index that gives account of the general plant growth promotion, i.e., PGPI, including fungal antagonism, biocontrol traits, direct plant growth promotion traits and colonization traits, we found that those isolates that were ranked together according to their PGPI, were also grouped together by their taxonomic assignment (Table 2), as previously noticed for the BPI (Table 2). Thus, *P. chlororaphis*-related isolates and SVBP6 showed the highest PGPI values, but mainly influenced by their great biocontrol potential (Table 2, Figs. 2 and 3).

4. Discussion

Bacterial strains of the *Pseudomonas* genus are intimately related to biocontrol (Moënne-Loccoz and Défago, 2004; Walsh et al., 2001) and natural suppressiveness of soils (Mendes et al., 2011; Moënne-Loccoz and Défago, 2004; Walsh et al., 2001; Weller, 2007; Weller et al., 2002). Also, several *Pseudomonas* species are linked to direct plant growth promotion (Hol et al., 2013; Lugtenberg and Kamilova, 2009; O'Sullivan and O'Gara, 1992; Saharan and Nehra, 2011). Due to these beneficial traits, we aimed to isolate and characterize native pseudomonads with antagonistic potential against 12 fungal pathogens that affect different extensive crops in Argentina (Table 1) (Carmona and Melo Reis, 2012; Carmona, 2014; Scandiani et al., 2012). This strategy might allow us to identify novel isolates with biocontrol potential that are naturally adapted to environmental conditions of agricultural plots of our region, where they could be applied as a biological input (Cordero et al., 2012; Fischer et al., 2010; Tolba and Soliman, 2013).

The selected 19 isolates showed a diverse taxonomic distribution, based on the concatenated 16S rDNA-*rpoB*-*oprF* sequences (Fig. 1). These group of isolates did not include any pseudomonads related with the *P. aeruginosa* or the *P. syringae* complexes, generally associated with pathogenesis in humans and plants,

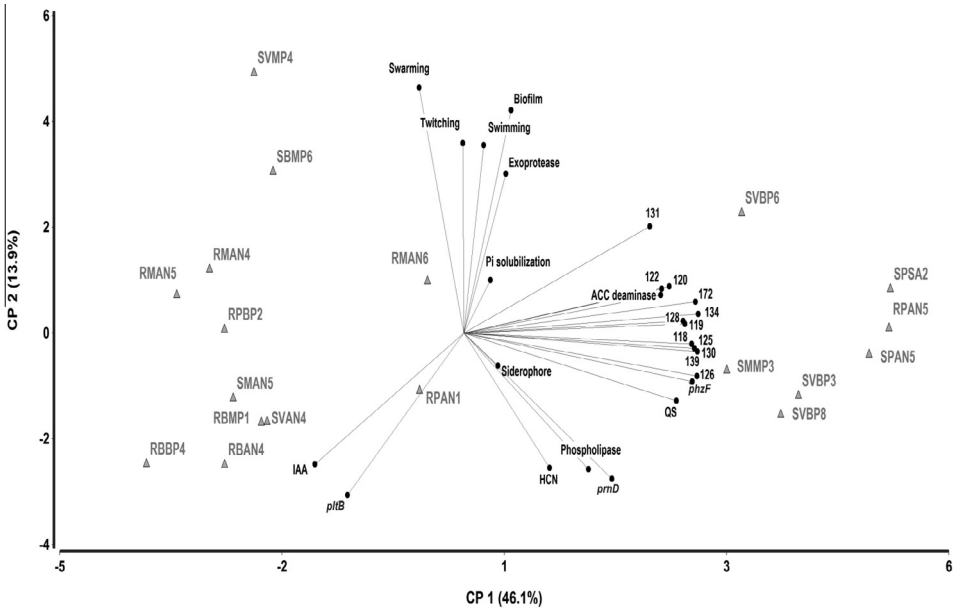

Fig. 5. Principal component analysis of the activities evaluated in the 19 pseudomonad isolates. Components 1 and 2 (CP1 and CP2, respectively) together explain 60% of the total variance. CP1 separates the pseudomonads isolates with higher fungal antagonistic potential (right side of the chart) from those that showed a lower inhibition level. CP2 separates the more mobile isolates (upper part of the chart) from the rest, influenced also by the exoprotease production and biofilm development.

respectively (Govan and Deretic, 1996; Manceau and Horvais, 1997). This degree of diversity was observed also in the antagonistic potential. Every pseudomonad isolate could neither antagonize all tested fungi, nor has the same inhibition level against every fungus (Fig. 2 and Supplementary Fig. 1). Isolates with the highest BPI values (Table 2), were clustered in the PCA (Fig. 5). Some members of this subgroup of *Pseudomonas* related with the *P. chlororaphis* complex were previously linked with growth promotion of several plants (Bolwerk et al., 2003; Cho et al., 2008; Johnsson et al., 1998; Rosas et al., 2007), and their biocontrol potential is mainly due to the production of phenazines (Chin-A-Woeng et al., 2001; Ghirardi et al., 2012; Mavrodi et al., 2006). Orange pigmentation of the *phzF*$^+$ isolates (Table 3) strongly suggests the production of 2-hydroxyphenazine-1-carboxylic acid, whereas the yellow diffusible pigmentation of isolate RPAN1 suggests the production of phenazine-1-carboxylic acid (Mavrodi et al., 2006). Isolate RPAN1 was the only phenazine-producer with a rather low antagonistic activity (API value) against fungi (Table 2), but with a relatively high BTI value (Tables 2 and 3). This particularity suggests the implication of hydroxyphenazine-1-carboxilic acid in the antagonistic potential of *P. chlororaphis*-related bacteria against the fungi tested in this work.

Phenazine-producers also showed, overall, high HCN, phospholipase, and siderophore production (Table 3), which are all activities that could contribute to their biocontrol potential (Jagadeesh et al., 2001; Kloepper et al., 1980; Sacherer, 1994; Voisard et al., 1989). The orange color of colony halos in CAS agar could indicate the production of hydroxamate-related compounds, as nocardamines; or mixed siderophores, as pyoverdines (Pérez-Miranda et al., 2007). Also, notably, all *phzF*$^+$ isolates produced QS signals of the AHL type (Table 4), which are intimately related with the regulation of phenazine synthesis (Mavrodi et al., 2006).

SVBP6 isolate, a member of the *P. putida* complex that was closely related to *P. alkylphenolia* species based on its *rpoB* sequence, resulted to be one of the best *in vitro* antagonists (Table 2, Fig. 2), and it clustered with the phenazine-producers in the PCA (Fig). Surprisingly, this isolate did not show the presence of any of the antibiotic-related genes evaluated in this work, and its HCN production was moderate (Table 3). Thus, its BTI was low compared with its antagonism ability *in vitro* (API value; Table 2). Other important metabolites involved in biocontrol are biosurfactants, as cyclic or linear lipopeptides and rhamnolipids (D'aes et al., 2010). However, provided that we did not detect surfactant activity in SVBP6 supernatants in different growth media (data not shown), we are currently investigating the genetic determinants of the high level of *in vitro* antagonism displayed by isolate SVBP6.

The observed phenotypic differences among those isolates showing the highest level of inhibition corroborate the great diversity of mechanisms that the *Pseudomonas* genus can display for antagonizing their competitors (Haas and Défago, 2005; Lugtenberg and Kamilova, 2009; Nagarajkumar et al., 2004; Pathma et al., 2011; Raaijmakers and Mazzola, 2012). In addition, *Pseudomonas* strains can activate the induced systemic response (ISR) in plants, collaborating with the plant health (Park et al., 2009; van Loon, 2007; Zamioudis and Pieterse, 2012), which is another strategy that some of our isolates (RBAN4 and SVBP6) have shown in the tomato – *P. syringae* pv. tomato DC3000 pathosystem (data not shown).

Direct plant growth promotion activities were also present in some of these isolates (Table 4), with P solubilization activity values which were well in agreement with the classification suggested by Meyer (Meyer et al., 2011): *P. chlororaphis*-related isolates presented the lowest values, whereas *P. fluorescens*-related isolates showed the highest scores (Fig. 1 and Table 4). The positive correlation between final Pi concentration and pH suggests that such solubilization levels were reached by the secretion of organic acids, as it was already demonstrated for several pseudomonads (de Werra et al., 2009). IAA production and ACC deaminase activity are both related with control of proliferation and elongation of root tissues in plants (Glick et al., 2007; Oberhänsli et al., 1991; Patten and Glick, 2002). These two properties were present in different isolates of our collection of fungal antagonistic pseudomonads (Table 4). Isolates RBAN4, SMAN5 and RBBP4, which showed high production of IAA (Table 4), increased the fresh weight of maize seedlings (Fig. 4b), and RBBP4 also increased soybean fresh weight (Fig. 4b). IAA production in the rhizosphere of maize and soybean seedlings colonized by these isolates could have favoured their root development, resulting in higher fresh weight. Interestingly,

isolates able to grow on ACC were associated with high antagonistic potential in the PCA analysis (Figs. 2 and 5). As ACC deaminase activity may enhance the survival of plant seedlings under various abiotic and biotic stresses (Grichko and Glick, 2001; Saravanakumar and Samiyappan, 2007), this property is an additional factor that could improve the biocontrol potential of pseudomonads isolates.

The screening of several *in vitro* activities is a common strategy used to evaluate the PGPP of new isolates (Laslo et al., 2012; Mavrodi et al., 2012). However, in general, those activities are mainly used to explain the results from biological control or PGP assays. The wide range of *in vitro* PGP traits that we assessed, based on simple published methods, allowed us to develop numerical indices that could express the potential of a particular isolate to act as putative biocontrol or PGP agent. Thus, it would be feasible to isolate a large number of bacteria and to select the best PGPR candidates, extending the possibilities to find new promising PGP agents. With this strategy, we linked the screening of direct and indirect PGP traits for our collection of selected fungal antagonistic pseudomonads with numerical indices. An index is a useful tool to categorize several isolates according to their properties showed *in vitro* (Laslo et al., 2012). In our work, we could categorize the *Pseudomonas* isolates based on their biocontrol, direct growth promotion and general PGPR potential (Fig. 3). Interestingly, our results revealed that taxonomically related isolates, irrespective of their geographic or biological origin, were also linked by their BPI and PGPI values (Table 2). This is the first report that connects pseudomonads taxonomy with PGPR potential. In addition, this classification suggests that, in general, the best potential biocontrol agents do not necessarily converge with the best direct plant growth promoting agents within the *Pseudomonas* genus (Fig. 3). To our knowledge, there is no published evidence in this regard. Analysis of a wide spectrum of pseudomonads could clarify this presumption. With our consistent results, we think that PGPI would be a useful tool that could be employed as a complement for classifying *Pseudomonas* isolates of agronomic interest, supplementing the genotypic and phenotypic information.

5. Conclusions

We found that in spite of the significant phylogenetic biodiversity, PCA clustered isolates in two major groups sharing, on the one hand, a bulk soil origin and superior biocontrol potential but rather weak direct PGP properties, and a second group with the opposite traits and mostly rhizospheric origin. These findings, along with the quantitative indices calculated on the basis of the individual tested traits, revealed that there are isolates with promising biocontrol potential (high BPI) but rather weak direct probiotic activities (low DGPI) (e.g., SVBP3 or SVBP8), or *vice versa*, with promising direct plant growth promoting traits and low biocontrol potential (e.g., RBBP4 or RBAN4). Thus, these indices could be employed to categorize large numbers of isolates by their potential to act as a biocontrol or a biofertilizer agent before performing time-consuming *in planta* assays with native isolates. Overall, our results support two relevant considerations related to the selection of pseudomonads of potential agronomical use: (1) biocontrol and PGPR potential of a *Pseudomonas* isolate could be linked with its taxonomy; (2) top fungal antagonistic potential together with the highest direct plant growth promotion potential rarely converge in a single *Pseudomonas* isolate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2015.07.003.

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