

Full Length Research Paper

## Screening of plant growth promoting rhizobacteria for the development of vegetable crops inoculants

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The enrichment of plant rhizosphere with beneficial bacteria is a strategy that favors the production of more vigorous seedlings, which is essential for the success of vegetable cultivation. The aim of this study was to screen fluorescent pseudomonads that are able to act as plant growth-promoting rhizobacteria. Production of indole acetic acid (IAA) and phosphate solubilization were used as traits for initial screening of 78 strains. In addition, we evaluated kale growth promoting ability under greenhouse conditions in unsterilized soil. Strains that significantly increased the dry mass of plants were characterized based on biochemical traits and by 16S rRNA sequencing. All isolates produced IAA. From these, 59 solubilized CaHPO<sub>4</sub>. Seven strains which promoted kale growth, presented low or intermediate solubilization efficiency and IAA production ability and were characterized as *Pseudomonas putida*. Seven growth promoting strains with potential for the development of inoculants were selected for future tests under field conditions. This study also shows that growth promoting capability was not consistently related *in vitro* to IAA production and phosphate solubilization.

**Key words:** Rhizospheric soil, auxin, phosphorus, kale, root colonization.

### INTRODUCTION

Vegetables are well known as very important constituents of a healthy diet, especially because of their nutrition quality, including high content of mineral salts and vitamins, which have been showing benefits in the prevention of certain types of cancers, cardiovascular diseases, diabetes, among other illnesses (Poll et al., 2012). The horticulture is a growing sector of Brazilian agribusiness, corresponding to 12.4% of the gross domestic product, which amounts to \$100 billion (Poll et al., 2012). This has led to an increasing interest in the development of new approaches to improve Brazilian's vegetable production.

A strategy that has been often proposed is the enrichment of the rhizosphere with plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009).

The inoculation of PGPR on the seeds and substrate can favor the production of more vigorous seedlings due to the protection against soilborne plant pathogens, production of growth regulators, better use of fertilizers and induction of tolerance to environmental stresses (Compant et al., 2010; Glick, 2010).

Among PGPR, fluorescent pseudomonads are good rhizosphere colonizers, although they have also been found inside tissues of flowers and fruits (Compant et al., 2010; Peix et al., 2009) and, more recently, reported to induce the formation of root nodules in black locust (Shiraishi et al., 2010). A straightforward strategy to obtain PGPR is the screening of pseudomonads isolated from agro-ecological farms, which possess higher microbial diversity than observed under conventional manage-

ment (Altieri, 1999). Besides, isolation from samples taken from the rhizosphere tends to provide bacterial strains that directly interact with plant roots. Fluorescent pseudomonads possess high rhizosphere competence and they are recognized as one of the main groups of PGPR or plant-probiotic bacteria (Höfte and Altier, 2010; Lugtenberg and Kamilova, 2009).

The ability to produce indole acetic acid (IAA) and to solubilize phosphate by pseudomonads is well correlated with the potential of these bacteria to promote growth of different plants in greenhouse and field experiments (Ahmed and Shahab, 2011; Spaepen and Vanderleyden, 2010). It has been suggested that IAA is involved in the communication between bacteria and in plant-bacteria interactions (Spaepen et al., 2007). Nevertheless, although about 80% of the rhizosphere inhabiting bacteria are IAA producer, the exact ecological role of IAA production remains unclear. Phosphate solubilization appears to be an advantageous mechanism for soil bacterial, due to the reduced availability of phosphorus in many soil environments. A great proportion of rhizosphere bacteria are able to solubilize P *in vitro*, and such bacteria could be selected to develop inoculants to reduce P deficiency in agriculture soils (Ahmed and Shahab, 2011).

Although these mechanisms have been abundantly recognized, little is known about the diversity and the potential of plant growth promoting pseudomonas of vegetable crops in tropical soils. In Brazil, where the use of bacteria as inoculants is legally restricted, there are no commercial products registered at Brazilian Ministry of Agriculture, Livestock and Food Supply containing fluorescent pseudomonads as an active ingredient (Brasil, 2012). However, strains belonging to *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* are commercialized in the USA (Kiewnick, 2007) and Europe (Alabouvette et al., 2006). These bacteria represent a great opportunity to improve vegetable crops yield and to reduce economic and environmental costs with mineral fertilizers, as already demonstrated for soybean, cowpea and more recently, for sugarcane (Rumjanek et al., 2005; Silva et al., 2009). Therefore, in the present study, rhizobacteria isolated from vegetable crops cultivated under organic production system were screened for production of IAA, phosphate solubilization and growth promotion, using kale as indicative plant.

## MATERIALS AND METHODS

### Origin of bacterial strains

Seventy eight (78) isolates of fluorescent rhizobacteria were obtained from kale (*Brassica oleracea* var. *acephala* L.), lettuce (*Lactuca sativa* L.), parsley (*Petroselinum sativum* L.) and rudbeckia (*Eruca sativa* Miller) cultivated on an experimental area (SIPA - Integrated Agroecological Production System) located in Seropédica, lat 22° 45'S, long 43° 41'N in Rio de Janeiro State, Brazil (Pinton et al., 2010). This area represents a collaborative

research effort between Embrapa Agrobiologia, Universidade Federal Rural do Rio de Janeiro and Pesagro-Rio. Three plants of each vegetable species were harvested at the last third part of the vegetative cycle and the roots shaken to remove the loosely attached soil. The adhering soil of the three plants was pooled and considered as the rhizosphere soil. Rhizosphere soil (1 g) from each vegetable species was mixed with 9 ml of distilled water and serial dilutions were prepared. One hundred microliters of each dilution were spread on plates containing King's B agar amended with the antibiotics as described by Pinton et al. (2010). Fluorescent colonies presenting growth characteristics of pseudomonad were isolated and stored in glycerol at -80°C for further analysis.

### Production of IAA and IAA-related compounds

The production of IAA and IAA related compounds was determined by spectrophotometry. Bacterial suspensions ( $10^9$  CFU ml<sup>-1</sup> 0.85% NaCl) were inoculated in 5 ml Tryptone Soy broth amended with 200 µg ml<sup>-1</sup> L-tryptophan (Sigma-Aldrich, St Louis, USA). The samples were then incubated for 72 h at 29°C under agitation (150 rpm) in the dark. Optical cell density (OD) was measured by spectrophotometer (absorbance at 600 nm). A 1.5 ml aliquot of the supernatant, obtained after centrifugation of the culture at 10,000 rpm for 5 min, was mixed with 1 ml of Salkowski's reagent that can react mainly with indole acetic acid, but also with indolepyruvic acid and indoleacetamide (Glickmann and Dessaux, 1995). After 30 min of incubation in the dark, absorbance was determined at 540 nm. The concentration of IAA and IAA-related compounds was expressed in µg ml<sup>-1</sup> per unit OD (optical density) of the bacterial cultures, using a standard curve prepared with synthetic IAA (Sigma-Aldrich, St Louis, USA).

### Phosphate solubilization ability

The isolates were tested using GY medium (Katznelson and Bose, 1959) containing CaHPO<sub>4</sub> as sole phosphate source. After 12 days of incubation at 29°C, the solubilization index (SI) was determined by dividing the halo diameter by the colony diameter (Berraquero et al., 1976). The SI was used to classify the strains in terms of solubilization efficiency (SE) as low (SI <2), intermediate (2 < SI <3) or high (SI >3) (Hara and Oliveira, 2005).

### Inoculation of kale with PGPR under controlled conditions

A total of 78 strains were evaluated in four experiments. From these, seven isolates (BR9130, BR9133, BR9128, BR9121, BR9132, BR9109 and BR9129) that increased biomass of inoculated plants higher than the control without inoculation were re-evaluated in a separate assay. The isolates were inoculated by the addition of 1 ml of bacterial suspension ( $10^9$  CFU ml<sup>-1</sup> NaCl 0.85%) on the soil surrounding kale roots in pots containing 1 kg of a non sterilized dystrophic sandy soil, with the following chemical characteristics: pH, 5.3; Al, 0.3 cmolc dm<sup>-3</sup>; Ca + Mg, 0.9 cmolc dm<sup>-3</sup>; P, 4 mg dm<sup>-3</sup>; K, 20 mg dm<sup>-3</sup>; carbon, 0.33% and organic matter, 0.57%. Ten days after sowing (DAS) controls received the same amount of sterile saline solution (0.85% NaCl). Fifteen DAS, 6 ml of nutrients solution containing (in gL<sup>-1</sup>) NH<sub>4</sub>NO<sub>3</sub> (2.8), K<sub>2</sub>HPO<sub>4</sub> (3.5), CaCl<sub>2</sub>·2H<sub>2</sub>O (10.5) and MgSO<sub>4</sub>·7H<sub>2</sub>O (0.35) were added to each pot. The assay was maintained in a greenhouse at an average temperature of 27°C and plants were irrigated with 40 ml of distilled water twice a day. The randomized blocks design was adopted, with five replicates. Dry weight of shoots and roots was determined.

### Biochemical characterization

Isolates capable of promoting plant growth were biochemically

**Table 1.** Class division of fluorescent vegetable rhizobacteria according to IAA like molecules production, phosphate solubilization and plant growth promoting.

SE <sup>a</sup>	IAA <sup>b</sup>			
	Classes ( $\mu\text{g ml}^{-1}$ per unit OD) <sup>b</sup>			
	Low	Medium	High	Total
Null	7 (0) <sup>c</sup>	8 (0)	4 (0)	19 (0)
Low	7 (1)	19 (4)	5 (0)	31 (5)
Intermediate	8 (0)	12 (2)	4 (0)	24 (2)
High	0 (0)	2 (0)	2 (0)	4 (0)
Total	22 (1)	41 (6)	15	78 (7)

<sup>a</sup> Solubilization efficiency: null = bacterium did not grow; low = solubilization index (SI) < 2, intermediate = 2 < SI < 3 and high = SI  $\geq$  3. <sup>b</sup> Production of indole acetic acid (IAA and IAA-related in culture medium supplemented with 200  $\mu\text{g ml}^{-1}$  of tryptophan), expressed in  $\mu\text{g ml}^{-1}$  per optical cell density (unit OD, absorbance at 600 nm). Isolates were classified as 'low' if < 18  $\mu\text{g ml}^{-1}$  optical cell density per unit OD was detected, 'medium' for 18 to 42  $\mu\text{g ml}^{-1}$  per unit OD and 'high' if it released > 42  $\mu\text{g ml}^{-1}$  per unit OD. <sup>c</sup> Values between parentheses indicate the number of isolates in each class that increased the biomass of kale as shown.

characterized using the commercial Kit API system 20NE (BioMérieux Laboratory, France).

### 16S rRNA gene sequencing

Total DNA from the isolates capable of promoting plant growth on kale was isolated using phenol chloroform extraction basically as described (Ausubel et al., 1992). 16S rRNA genes were amplified from 20 ng of template DNA using the universal eubacterial primer set 27f/1492r with an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. Reactions were performed in 50  $\mu\text{l}$  volume with the composition recommended by the manufacturer (Invitrogen, Brazil). Amplicons were purified using the Qiaquick PCR purification kit (Qiagen, Germany). Sequencing reactions were carried out in a 3730 ABI DNA Analyzer using the BigDye terminator 3.1 sequencing kit (Applied Biosystems, USA). Sequences were compared with the GenBank database (<http://www.ncbi.nih.gov>) using BLAST (Altschul et al., 1997) and aligned using Clustal W (EMBL-EBI, <http://www.ebi.ac.uk/clustalw/>). Neighbor-joining (NJ) analyses were performed with MEGA 4 (Tamura et al., 2007), using the Kimura-2 correction and 5000 bootstrap replicates.

### Data analysis

The data were subjected to analysis of variance (ANOVA) and the Scott-Knott test at the 0.05 significance level.

## RESULTS

### Growth promotion tests

All isolates produced IAA and IAA-related compounds *in vitro*. The amount produced varied from 0.78 to 401.62  $\mu\text{g}$

$\text{ml}^{-1}$  per unit OD. Based on the amount of IAA produced, isolates were grouped as 'low' for values below 18  $\mu\text{g ml}^{-1}$  per unit OD, 'intermediate' for values ranging from 18 to 42  $\mu\text{g ml}^{-1}$  per unit OD and 'high' for values higher than 42  $\mu\text{g ml}^{-1}$  per unit OD (Table 1). Most of the isolates (52.56%) were classified as intermediate producers and 19.23% produced high amounts of IAA. From all the isolates, 75.6% solubilized  $\text{CaHPO}_4$ . From those, 39.74% showed low, 30.76% showed intermediate and 5.13% showed high efficiency of solubilization. In the greenhouse experiments, seven out of the 78 isolates showed a positive effect on plant growth and inoculated plants presented higher shoot and root dry weight than the control (Table 2). Depending on the isolate tested, the increase in shoot growth ranged from 79.80 to 131.60%. Although the plant growth promoting ability of the strains did not differ significantly, the highest increase on shoot dry weight was observed for the strain BR9109. The effect on root growth appeared to be more pronounced. Increases ranging from 106.80 to 221.10% were observed after inoculation with the different effective isolates ( $p \leq 0.05$ ). Plants inoculated with the strain BR9129 showed the highest values of root dry mass and total dry mass.

### Biochemical characterization

As determined by the API system, all seven PGPR strains presented a biochemical profile identical to *P. putida*. All strains produced fluorescent pigments, showed positive reaction for arginine dihydrolase, assimilation of glucose, potassium gluconate, malate and trisodium citrate and negative to gelatin hydrolysis, glucose fermentation, urease activity and assimilation of mannitol and adipic acid.

### Phylogenetic analysis

The results of comparative analysis of the 16S rRNA gene sequence of the seven isolates was not conclusive, although the identification of API 20NE was confirmed to genus level. Strains are very closely related to *P. putida*, *Pseudomonas plecoglossicida*, *Pseudomonas fulva* and *Pseudomonas montellii* sequences from the database (Figure 1).

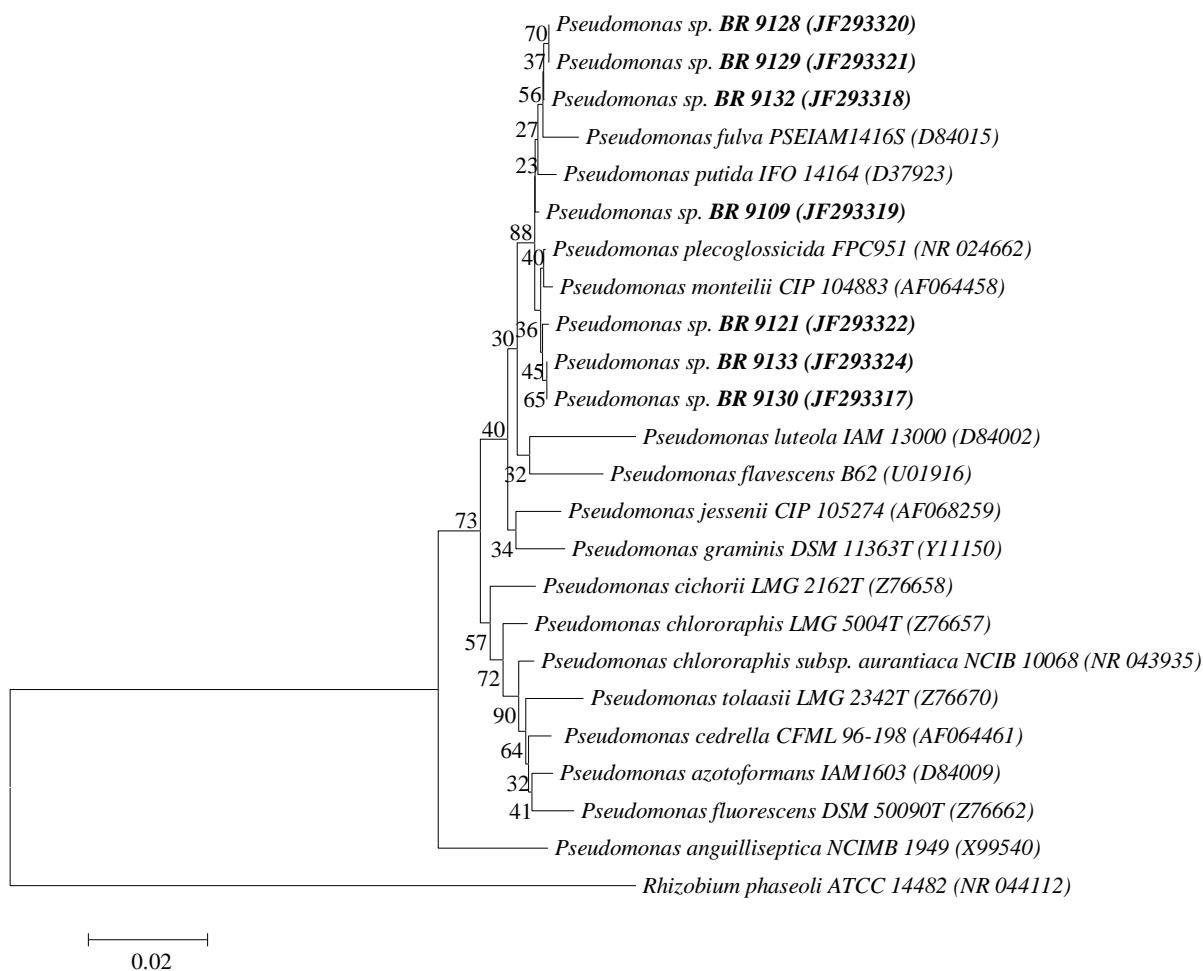
## DISCUSSION

The aim of this study was to isolate and characterize fluorescent pseudomonads that are able to promote growth of vegetable crops. Therefore, the strategy was to isolate bacteria from the rhizosphere of the target plant species: kale, lettuce, parsley and rudbeckia grown under organic management, as a starting point in the selection

**Table 2.** Plant growth promoting rhizobacteria characterized by IAA and IAA-related compounds production and solubilization of calcium monophosphate.

Treatment	Growth promotion <sup>a</sup>			IAA <sup>b</sup>	Phosphate solubilization <sup>c</sup>
	SDW (g)	RDW (g)	TDW (g)	ug ml <sup>-1</sup> unitOD <sup>-1</sup>	SI
Control	0.39 <sup>b</sup>	0.27 <sup>b</sup>	0.65 <sup>c</sup>	-	-
BR9130	0.69 <sup>a</sup>	0.41 <sup>b</sup>	1.11 <sup>b</sup>	7.22 <sup>c</sup>	1.79 <sup>b</sup>
BR9133	0.81 <sup>a</sup>	0.55 <sup>a</sup>	1.36 <sup>a</sup>	32.68 <sup>b</sup>	1.62 <sup>b</sup>
BR9128	0.80 <sup>a</sup>	0.62 <sup>a</sup>	1.42 <sup>a</sup>	41.74 <sup>a</sup>	1.87 <sup>b</sup>
BR9121	0.80 <sup>a</sup>	0.68 <sup>a</sup>	1.49 <sup>a</sup>	27.03 <sup>b</sup>	2.23 <sup>a</sup>
BR9132	0.86 <sup>a</sup>	0.60 <sup>a</sup>	1.47 <sup>a</sup>	40.01 <sup>a</sup>	1.96 <sup>b</sup>
BR9109	0.89 <sup>a</sup>	0.72 <sup>a</sup>	1.62 <sup>a</sup>	23.34 <sup>b</sup>	2.31 <sup>a</sup>
BR9129	0.82 <sup>a</sup>	0.85 <sup>a</sup>	1.67 <sup>a</sup>	24.73 <sup>b</sup>	1.76 <sup>b</sup>
VC(%) <sup>d</sup>	24.88	33.73	24.29	9.4	11.9

Values within a column followed by a same letter are not significantly different according to Scott-Knott test ( $P < 0.05$ ). <sup>a</sup>Growth promoting kale (*Brassica oleracea* cv. *acephala*) under greenhouse conditions. Evaluated parameters were shoot dry weight (SDW), root dry weight (RDW) and total dry weight (TDW). <sup>b</sup>Production of indole acetic acid (IAA and IAA-related in culture medium supplemented with 200  $\mu\text{g ml}^{-1}$  of tryptophan), expressed in  $\mu\text{g ml}^{-1}$  per optical cell density (unit OD, absorbance at 600 nm). <sup>c</sup>Index of solubilization (SI) of  $\text{CaHPO}_4$ . <sup>d</sup>Variation coefficient.

**Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences of *Pseudomonas* spp. The tree was generated by the neighbor-joining method. Numbers at nodes indicate bootstrap values (percentage of 5000 replicates). Scale bar: 0.02 substitutions per nucleotide position. Name of each type strain and/or isolate follows the sequence accession numbers.

of PGPR. We started by evaluating IAA production and phosphate solubilization as initial traits for strain selection, as these are recognized as traits of efficient PGPRs (Compant et al., 2010; Lugtenberg and Kamilova, 2009).

IAA production is more frequent among rhizosphere inhabiting bacteria than among bulk soil bacteria (Spaepen and Vanderleyden, 2011). Accordingly, in the present study, all isolates obtained from the vegetable rhizosphere produced IAA. Therefore, it seems likely that the IAA production by these isolates has a role in rhizosphere colonization, as has been suggested by Spaepen et al. (2007).

In general, the capacity to solubilize calcium phosphate by rhizobacteria is linked to type and quantity of organic acid production (Rodriguez and Fraga, 1999) and we suppose that this may explain the predominance of low and average efficiency of solubilization found among the isolates studied.

Total dry weight was higher in plants inoculated with seven out of the 78 isolates. All the plant growth promoters produced IAA and solubilized phosphate. These isolates differ significantly in the production of IAA and also in the SE, but there is no clear relationship between the amounts of IAA and P solubilization to their contribution to plant growth promotion. Besides these, it is possible that others, as yet not investigated mechanisms are responsible for the effect of these strains on plant growth. Furthermore, metabolites synthesis by bacteria in the rhizosphere depends on precursors present in root exudates that vary among plant species, growth phase and day/night periodicity of exudation.

Our results showed that a screening method based on these traits considering the activity of the bacteria *in vitro* might not to be a good approach to select PGPR, as this does not necessarily indicate its effects on the plant growth under greenhouse conditions, especially in non-sterile soils, suggesting a sensitivity to critical factors, such as establishment in the roots, competition with resident microbiota and survival under abiotic stresses. Smyth and co-workers (2011) studied rhizobacteria from cereals and found similar results. There was no significant correlation between wheat growth promotion in the glasshouse and the results of phenotypic tests. Furthermore, other mechanisms that were not investigated in this study may also be involved in the response of increased growth of plant such as nitrogen fixation, production of siderophores, exopolysaccharides and ACC deaminase enzyme (Glick et al., 2007; Compant et al., 2010).

Although, the screening of plant performance in non-sterile soil does not reveal all the mechanisms involved, from a practical point of view, this is more recommended than *in vitro* methods as discussed by Pliego et al. (2011). Possibly for that reason, only 9% of the isolates evaluated in this study promoted plant growth under non-

sterile soil conditions despite displaying favorable traits under *in vitro* conditions. Experimental conditions using non-sterile soil stimulate competition of the selected bacteria with resident microorganisms and the need to survive under abiotic stress in the soil.

In our study, we observed that the seven strains that promoted the growth of kale, showed low and medium production of IAA. These data are in accordance with results of Jayasudha et al. (2010) that showed that the growth of groundnut plants was not greatly influenced by fluorescent pseudomonads that produced high amounts of IAA, but by lower and medium producers of it.

In this study, the effect on the growth of kale by PGPR occurred mainly in the root system. The increase of the root system is advantageous for seedlings of vegetables because may dock them faster on the ground, promote the achievement of water and nutrients, increasing their chances of survival (Ahmed and Shahab, 2011). The IAA produced by bacteria can change the pool of plant auxin stimulating the emergence of lateral roots that allow plants to better explore the soil, which in turn may lead to improve access to water and minerals. This can favor the uptake by plants, phosphorus which comes into contact with the roots through radicular interception (Ali et al., 2010; Hodge et al., 2009). It may in turn lead to further increases of the shoot mass, leading to greater rates of photosynthesis, more exudation and more niches in the rhizosphere.

As expected, all PGPR strains belonged to the genus *Pseudomonas*, as shown by the API and 16S rRNA gene sequence analysis. They were grouped in the rRNA group I, which is the genus *Pseudomonas* sensu stricto (Palleroni, 1984), with the following examples, fluorescence production, assimilation of glucose and negative to glucose fermentation. All the PGPR strains had biochemical profiles identical to *P. putida*, which shows negative reaction to gelatin hydrolysis, normally used to differentiate them from *P. fluorescens*, *Pseudomonas chlororaphis*, *Pseudomonas aureophaciens* and *Pseudomonas aeruginosa* that belonged to the same homology group.

In the present study, we selected seven *Pseudomonas* strains that promoted the growth of kale plants under greenhouse conditions, showing a great biotechnological potential. Moreover, pre-selections using *in vitro* tests of some commonly used traits, such as phosphate solubilization and IAA production may mask actual PGPR strains.

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