Isolation of *Pantoea ananatis* from sugarcane and characterization of its potential for plant growth promotion

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ABSTRACT. Each year, approximately 170 million metric tons of chemical fertilizer are consumed by global agriculture. Furthermore, some chemical fertilizers contain toxic by-products and their long-term use may contaminate groundwater, lakes, and rivers. The use of plant growth-promoting bacteria may be a cost-effective strategy for partially replacing conventional chemical fertilizers, and may become an integrated plant nutrient solution for sustainable crop production. The main direct bacteria-activated mechanisms of plant growth promotion are based on improvement of nutrient acquisition, siderophore biosynthesis, nitrogen fixation, and hormonal stimulation. The aim of this study was to isolate and identify

bacteria with growth-promoting activities from sugarcane. We extracted the bacterial isolate SCB4789F-1 from sugarcane leaves and characterized it with regard to its profile of growth-promoting activities, including its ability to colonize *Arabidopsis thaliana*. Based on its biochemical characteristics and 16S rDNA sequence analysis, this isolate was identified as *Pantoea ananatis*. The bacteria were efficient at phosphate and zinc solubilization, and production of siderophores and indole-3-acetic acid *in vitro*. The isolate was characterized by Gram staining, resistance to antibiotics, and use of carbon sources. This is the first report on zinc solubilization *in vitro* by this bacterium, and on plant growth promotion following its inoculation into *A. thaliana*. The beneficial effects to plants of this bacterium justify future analysis of inoculation of economically relevant crops.

Key words: *Pantoea ananatis*; Phosphorus solubilization; Zinc solubilization; Phytohormone production; Plant growth promotion; *Arabidopsis thaliana*

INTRODUCTION

Each year, approximately 170 million metric tons of chemical fertilizer are consumed by global agriculture (IFA, 2014). Furthermore, some chemical fertilizers are toxic when used in high concentration and their long-term use may damage soil and groundwater environments (Berg, 2009) with consequences for human and animal health. The use of plant growth-promoting bacteria (PGPB) may be a cost-effective strategy for partially or completely replacing conventional chemical fertilizers, and may become an integrated plant nutrient solution for sustainable crop production (Dion et al., 2010; Babar et al., 2011).

The mechanisms by which PGPB promote plant growth include phosphate and zinc solubilization activity (Verma et al., 2001; Intorne et al., 2009), production of phytohormones (Lee et al., 2004), nitrogen fixation (Compant et al., 2005), siderophore biosynthesis (Lodewyckx et al., 2002), and assisting the host plant in overcoming environmental stresses (Ryan et al., 2008).

The discovery of this mutual relationship between plants and PGPB has driven the development of new technologies aimed at using these microorganisms as biofertilizers (Richardson, 2001). Moreover, the isolation and characterization of new species of beneficial microorganisms, as well as the selection of isolates with desirable characteristics, are important steps in studies that aim to intensify agricultural production and promote the sustainability of these systems. In this sense, this study attempted to isolate and identify further bacteria with growth-promoting activities from sugarcane. The isolate *Pantoea ananatis* SCB4789F-1 was extracted from sugarcane leaves; its profile was then characterized in terms of phosphorus and zinc solubilization, indole-3-acetic acid (IAA) and siderophores production, and its ability to colonize *Arabidopsis thaliana* with effective promotion of plant growth.

MATERIAL AND METHODS

Isolation and quantification of *P. ananatis* strain SCB4789F-1

The bacterial strain SCB4789F-1 was isolated from sugarcane plants (cv. CB4789), multiplied from stalks, and grown for 3 months in a greenhouse located in Campos dos Goytacazes, Brazil (21°36′S 41°35′W).

Sugarcane root and leaf segments were used for bacterial isolation. These segments were washed first in running water to remove any remaining waste soil, and subsequently in sterile distilled water. Soon after, 1 g of the samples (root and leaf, respectively) was superficially fumigated with 70% ethanol for 2 min, and washed twice in sterile distilled water for 3 min. For disinfestation, roots were immersed in a 1% chloramine T solution for 10 min, and leaves were again exposed to 70% ethanol for 3 min. After surface disinfection, root and leaf samples were macerated in saline and subjected to serial dilutions. The samples were plated on LGI-P medium (Döbereiner et al., 1993) supplemented with 20 mg/L yeast extract and incubated at 30°C for 7 days. Subsequently, the obtained colonies were sub-cultured to obtain a pure culture of the isolate. The isolated strain was then stored in 50% glycerol at -70°C.

16S rDNA amplification and sequencing

The genomic DNA of the isolate was extracted using a DNAzol Plant Extraction Kit (Invitrogen, Grand Island, NY, USA) according to the manufacturer instructions. The 16S rDNA sequence (1.5 kb) was amplified by polymerase chain reaction (PCR) with the following oligonucleotide primers for *Eubacteria* FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RD1 domain (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al., 1991). Amplification of 16S rDNA was performed in a 20 µL volume comprising: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM of each deoxyribose nucleoside triphosphate (dNTP); 20 pmol oligonucleotide primers; 1 U Taq DNA polymerase; and 20 ng DNA. The reaction was performed in a Mastercycler thermocycler (Gradient-Eppendorf, Applied Biosystems Inc., CA, USA) with an initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 60 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The amplified products were purified using 70% ethanol and 100% isopropanol. The nucleotide sequence was determined by using an ABI PRISM 3130 Genetic Analyzer automatic sequencer (Applied Biosystems, CA, USA) and a BigDye Terminator kit according to the manufacturer instructions and using the set of oligonucleotides described in Table 1.

Table 1. Sequences of the primers used for amplification of the 16S rDNA of the SCB4789F-1 strain isolated from sugarcane leaves.

Sequence (5'-3')	Alignment position in the sequence of Escherichia coli	References
CCT ACG GGA GGC AGC AG	341-357F	Lane, 1991
CTG CTG CCT CCC GTA GG	357-341R	Lane, 1991
GTA GSG GTG AAA TSC GTA GA	685-704F	Lane, 1991
GTA GSG GTG AAA TSC GTA GA	704-685R	Lane, 1991
GCA ACG AGC GCA ACC C	1099-1114F	Lane, 1991
GGG TTG CGC TCG TTG C	1114-1099R	Lane, 1991

Phylogenetic analysis of the 16S rDNA sequence

The sequence of 16S rDNA was compared using the GenBank database via the BLASTN algorithm (Altschul et al., 1997) to determine its similarity to other sequences. Sequences of 16S rDNA (~1500 bp) of different strains of *P. ananatis* and related genera were accessed through the GenBank database (http://www.ncbi.nlm.nih.gov/entrez) and aligned using the ClustalW 1.8 program (http://www.ebi.ac.uk/index.html). Subsequently, the adjustment of DNA sequence extremities was made with the aid of the BioEdit 5.0.6 program (Copyright©1997-2013, Carlsbad, CA). To calculate the statistical significance of similarity between the sequences, a resampling for

1000 replications was used (Swofford et al., 1996). The method of distance ("neighbor-joining") (Saitou and Nei, 1987) was used to construct the phylogenetic tree using the Geneious 4.8 program. The 16S rDNA sequence of *Escherichia coli* was used as a phylogenetic tree outgroup.

16S rDNA sequence accession numbers

The nucleotide sequence of the 16S rDNA of the bacterial strain SCB4789F-1, isolated in this study, was deposited at GenBank and assigned access number HM562980.

Isolate biochemical profile

The biochemical profile of the isolate was determined using the automatic identification system for members of the Enterobacteriaceae family, API-ID 32 GN Kit (bioMérieux SA, France). Isolate inoculation into this kit was performed according to manufacturer recommendations and, after 24-48 h incubation, strips were read and results were computed and analyzed by means of an ATB® instrument or a mini API. Oxidase and catalase tests were also performed to identify the isolate.

Antibiotic resistance profile

The resistance and/or susceptibility to antibiotics profile was determined by the diffusion method on Kirby-Bauer discs (NCCLS, 1983) in Mueller-Hinton culture medium. The resistance and/or susceptibility was tested for 20 µg/mL amoxicillin + 10 µg/mL clavulanic acid; 5 µg/mL ciprofloxacin; 10 µg/mL gentamicin; 300 µg/mL sulfazotrin; 30 µg/mL tetracycline; 30 µg/mL cephalothin; 30 µg/mL cefoxitin; 10 µg/mL ampicillin; 10 µg/mL tobramycin; and 30 µg/mL cephalexin. The plates were incubated at 30°C for 24 h.

Test of plant growth-promoting activities

To evaluate the phosphorus and zinc solubilization potential of SCB4789F-1, the tests were carried out in LGI (Cavalcante and Döbereiner, 1988). Initially, the bacterial strain was grown in liquid medium DYGS for 18 h at 30°C and 140 rpm. After bacterial growth, 10 µL aliquots of bacterial solutions (10^8 cells/mL) were placed on petri dishes containing: LGI medium (constituents in g/L: 15 agar; 10 glucose; 0.2 K_2HPO_4; 0.6 KH_2PO_4; 0.2 $MgSO_4 \cdot 7H_2O$; 0.02 $CaCl_2 \cdot 2H_2O$; 0.01 $FeCl_3$; 0.002 $Na_2MoO_4 \cdot 2H_2O$, pH 6) + 0.54% $Ca_5(PO_4)_3OH$ or 0.12% ZnO; and LGI-P medium (constituents in g/L: 15 agar; 100 crystal sugar; 0.2 K_2HPO_4; 0.6 KH_2PO_4; 0.2 $MgSO_4 \cdot 7H_2O$; 0.02 $CaCl_2 \cdot 2H_2O$; 0.01 $FeCl_3$; 0.002 $Na_2MoO_4 \cdot 2H_2O$, pH 6) + 0.54% $Ca_5(PO_4)_3OH$ or 0.12% ZnO. The petri dishes were incubated at 30°C for 7 days. Zinc and the phosphate solubilization was assessed by measuring the diameter of translucent halos formed around bacterial colonies. Production of indole compounds was tested using the Sarwar and Kremer (1995) method. Siderophore production was detected using the Schwyn and Neilands (1987) method with Chrome Azurol S dye. All experiments were performed in triplicate. *Gluconacetobacter diazotrophicus* PAL5 was used as a positive control.

Inoculation and colonization tests

The bacterial cultures were centrifuged at 12,000 *g* and, after discarding the supernatant,

the cells were washed twice in sterile ultrapure water. The final optical density (0.01), measured using a spectrophotometer, was equivalent to 10^6 cells/mL. Inoculum (20 mL) was added to the petri dishes containing 7-day-old *A. thaliana* seedlings; for the control material, an identical volume of sterile ultrapure water was added. After 3 h, the seedlings were transferred into natural substrate West (West Garden, SP, Brazil) containing processed and enriched bark, expanded vermiculite and perlite, and expanded and processed peat. For 8 weeks, the plants were irrigated every 2 days with Hoagland nutrient solution (Hoagland and Arnon, 1950) containing 25% nutrients.

Phenotypic analysis of plant growth promotion

Plant shoots and roots were collected to determine the fresh matter weight. The wet weight was determined by weighing, using analytical scales, immediately after collection. For each treatment, 12 plants were evaluated.

Statistical analysis

The results were subjected to analysis of variance at 5% significance. Tukey's test was applied to identify whether the means differed significantly from each other ($P < 0.05$). Mean values and standard errors were calculated.

RESULTS

Isolation and quantification of SCB4789F-1

In this study, a bacterial strain called SCB4789F-1, which has potential for plant growth promotion, was isolated from sugarcane leaves. The strain SCB4789F-1 was isolated in LGI-P medium (Döbereiner et al. 1993) after 7 days of cultivation at 30°C. The population of the isolate SCB4789F-1 in the sugarcane leaves was approximately 2×10^3 cells/g of plant tissue. The presence of the bacterium was not detected in the sugarcane root.

Characteristics of plant growth promotion of SCB4789F-1

The ability of the bacterium to solubilize nutrients, and produce indole compounds and siderophores was assessed. For reference, the data were compared with the endophytic bacterium *G. diazotrophicus*, which was also isolated from sugarcane and is a PGPB (Cavalcante and Döbereiner, 1988).

The ability to solubilize nutrients was assessed based on the presence or absence of a solubilization zone surrounding the colonies. According to Saravanan et al. (2007), the presence of this clear zone is related to the excretion of organic acids in the culture medium, which acidify the medium and contribute to the solubilization of the nutrients. The strain SCB4789F-1 was able to solubilize calcium phosphate ($Ca_5(PO_4)_3OH$) and zinc oxide (ZnO) in the LGI medium (Table 2). In addition, SCB4789F-1 was able to produce indole compounds in both the absence and presence of tryptophan, although production was greater when the medium was supplemented with the amino acid (Table 2). The strain was also able to produce siderophores in the culture medium (Table 2).

Table 2. Plant growth-promoting activities of SCB4789F-1. Gd (Gluconacetobacter diazotrophicus) PAL5 was used as a positive control.

Strain	Diameter of halo of P-solubilization (cm)	Diameter of halo of Zn-solubilization (cm)	Indole production (µmol/L)		Siderophore production
			Tryptophan-free medium	Presence of tryptophan	
SCB4789F-1	0.37 ± 0.05[b]	1.29 ± 0.11[b]	292 ± 12[b]	1.738 ± 68[a]	+
Gd PAL5	1.04 ± 0.1[a]	1.93 ± 0.12[a]	434 ± 65[a]	665 ± 12[b]	+

+, positive Means followed by different letters differ statistically from each other at 5% significance. The values correspond to the means of three replicates.

Biochemical characteristics of the isolate SCB4789F-1

The isolate SCB4789F-1 was originally identified by means of biochemical tests, as suggested by Bergey's Manual of Systemic Bacteriology (Holt et al., 1994). The biochemical characterization tests revealed that the isolate was gram-negative, catalase-positive, and oxidase-negative. SCB4789F-1 gave positive results for all the other biochemical reactions tested except methyl red and propionic acid (Table 3). The isolate SCB4789F-1 grew well at pH 5.0-7.0 and 30°C. Based on the biochemical tests performed it was possible to identify the isolate as *P. ananatis* SCB4789F-1.

Table 3. Biochemical characteristics of the strain SCB4789F-1.

Biochemical reactions	SCB4789F-1
Gram reaction	-
Catalase	+
Oxidase	-
Sodium pyruvate	+
Citrate	+
Methyl red	-
L-rhamnose	+
D-sucrose	+
D-ribose	+
D-mannitol	+
D-glucose	+
D-melibiose	+
D-sorbitol	+
D-maltose	+
Itaconic acid	+
Suberic acid	+
Propionic acid	-
Capric acid	+
Valeric acid	+
Inositol	+
L-arabinose	+
N-acetyl-glucosamine	+
Sodium malonate	+
Sodium acetate	+
Lactic acid	+
Potassium 5-ketogluconate	+
L-serine	+
L-alanine	+
L-histidine	+
L-proline	+

+, tested positive/utilized as substrate; -, tested negative/not utilized as substrate.

16S rDNA sequencing for molecular identification

Molecular identification of the isolate SCB4789F-1 was accomplished through the amplification and sequencing of 16S rDNA (~1.5 kb). The sequence obtained was 100% identical to sequences of *P. ananatis* available at GenBank. The phylogenetic tree (Figure 1) based on the 16S rDNA sequences of other members of the Enterobacteriaceae shows that the *P. ananatis* SCB4789F-1 isolate has a high level of sequence identity with other strains of *P. ananatis* and other species of the same genus. Henceforth, this isolate will be designated as *P. ananatis* SCB4789F-1.

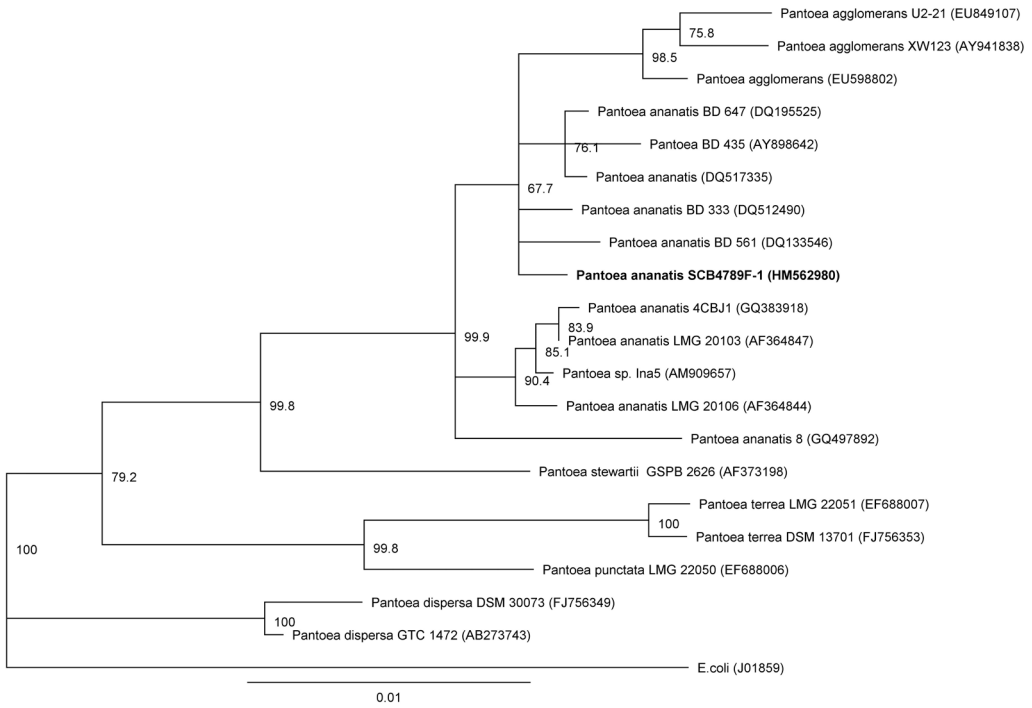

Figure 1. Phylogenetic tree showing the genetic relationship between Pantoea ananatis and other taxonomically similar strains and species of the same genus, based on 16S rDNA. GenBank access numbers are in parentheses. We used the "neighbor-joining" method and the distances were calculated using the Jukes and Cantor method. The values on each branch represent percentages of 1000 bootstrap replicates. Escherichia coli was included as the outgroup. The scale is the similarity distance of the group in question. *P. ananatis* SCB4789F-1 is indicated in bold.

Profile of antibiotic resistance in *P. ananatis* SCB4789F-1

P. ananatis SCB4789F-1 proved to be moderately resistant to 10 µg/mL ampicillin and 30 µg/mL cefoxitin, but was susceptible to the other tested antibiotics: 20 µg/mL amoxicillin + 10 µg/mL clavulanic acid; 5 µg/mL ciprofloxacin; 10 µg/mL gentamicin; 300 µg/mL sulfazotrin; 30 µg/mL tetracycline; 30 µg/mL cephalothin; 10 µg/mL tobramycin; and 30 µg/mL cephalexin.

Effect of inoculation with *P. ananatis* SCB4789F-1 on the development of *A. thaliana*

Seven-day-old *A. thaliana* seedlings were inoculated with *P. ananatis* SCB4789F-1 and after 60 days the ability of the bacterium to promote plant growth was evaluated. The inoculation with *P. ananatis* SCB4789F-1 resulted in a 37% increase in fresh weight of the plant shoots compared with non-inoculated plants (control) (Figure 2A). However, there was no significant difference in the fresh root weight (Figure 2B). These data show that inoculation of *A. thaliana* seedlings with *P. ananatis* SCB4789F-1 efficiently promoted plant shoot growth (Figure 2A and 2C).

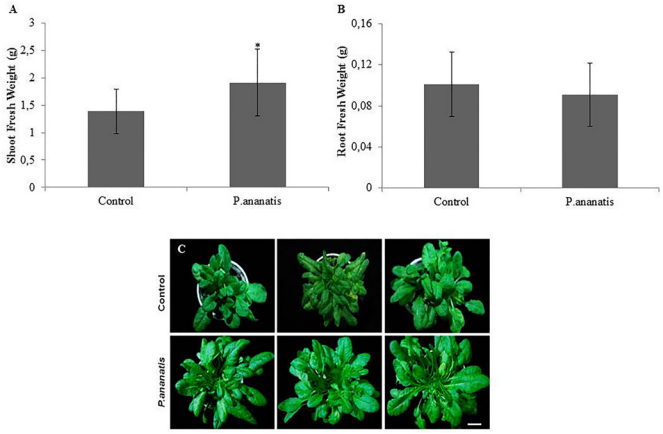

Figure 2. Plant growth promotion. Seedlings of *Arabidopsis* thaliana ecotype Col-0 were inoculated with *Pantoea ananatis* SCB4789F-1 after 7 days of germination. The control was not inoculated. After 60 days of inoculation, the shoot weight (A) and the root weight (B) were determined (N = 12). Representative photos of inoculated and control seedlings were taken (C). The asterisk (*) indicates that the treatments differed statistically from each other at 5% significance according to the Student's t-test. The experiments were repeated three times and showed similar results. The white bar equals 1 cm.

DISCUSSION

Isolation and selection of microorganisms with effective characteristics for plant growth are important steps in the development of new biofertilizers. In this study, we isolated *P. ananatis* SCB4789F-1 strain from sugarcane leaves and showed that it has multiple characteristics associated with plant growth promotion.

Although some studies have reported *P. ananatis* isolation from economically important crops, such as coffee (Nunes and de Melo, 2006), ginseng (Cho et al., 2007), papaya (Thomas et al., 2007), maize (Rijavec et al., 2007), and rice (Mano and Morisaki, 2008), they have failed to ascertain the essential growth parameters of this bacterium.

Here, the potential of *P. ananatis* SCB4789F-1 to promote plant growth has been demonstrated by its ability to solubilize phosphorus and zinc, produce siderophores, and synthesize IAA. In fact, only one other study on *P. ananatis* B1-9 strain isolated from the rhizosphere of onions showed potential for promoting plant growth in peppers, cucumbers, and melons (Kim et al., 2012).

The ability to solubilize phosphorus has been extensively reported for other species of the genus *Pantoea* (Ortega et al., 2008). However, no study has demonstrated the solubilization of zinc by *P. ananatis* or any other member of the genus. There are a few examples of bacteria

associated with plants that are demonstrably able to solubilize zinc, such as *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *G. diazotrophicus*, (Di Simine et al., 1998; Fasim et al., 2002; Saravanan et al., 2007; Intorne et al., 2009). Microorganisms that solubilize phosphorus and zinc can mineralize and solubilize these nutrients from organic and inorganic mixtures of soil samples. The ability of *P. ananatis* SCB4789F-1 to solubilize phosphorus and zinc, and to enhance phytoremediation of soils contaminated with zinc, suggests the ecological advantage of making it available to plants.

The search for siderophore-producing microorganisms is of interest because they have a significant effect on plant nutrition when a plant suffers from iron deficiency. Moreover, siderophore production provides a competitive advantage for bacteria to colonize plant tissues and, especially, antagonize pathogenic microorganisms, which compete for the same ecological niche (Sharma and Johri, 2003). In this regard, studies carried out on rice showed that *P. ananatis*, which is able to secrete siderophores, has antagonistic activity against the pathogen *Rhizoctonia solani* (Yang et al., 2008). In cotton plants, the antagonistic effect of *Pantoea* spp against pathogenic fungi arising from siderophore secretion has also been demonstrated (Li et al., 2010).

Synthesis of IAA, which is an auxin, has been described for *P. ananatis* (Halda-Alija, 2003; Enya et al., 2007). Auxins are typically used in agriculture to stimulate plant growth through root development (Benjamins and Scheres, 2008). According to Kim et al. (2012), the production of IAA by *P. ananatis* B1-9 is the most important factor for growth and fruit production in pepper plants. Another study has shown that *P. ananatis* 125NP12 isolated from tomato plants is capable of producing IAA. The authors suggested that IAA synthesis and an increased ability of the bacteria to colonize media showed a protective effect against gray mold disease on cut leaves (Enya et al., 2007).

Herein we report for the first time the successful inoculation of *A. thaliana* with *P. ananatis* SCB4789F-1. The ability to stimulate plant growth is considered to be a complex phenomenon. Therefore, current data on *A. thaliana* inoculation and its growth promotion should provide an important model system for studies on plant-bacteria interactions, with the availability of a wide range of plant mutants, knowledge of the genome, and ease of handling within a controlled environment. The demonstration that *P. ananatis* SCB4789F-1 promotes plant growth suggests future inoculation tests in other cultures of economic interest. Some studies have reported that *P. ananatis* inoculation in some crops such as pepper (Kang et al., 2007), papaya (Thomas et al., 2007), and maize (Rijavec et al., 2007) effectively improves plant yield. However, the mechanism behind the improvement has not yet been clarified. The efficient association of *P. ananatis* SCB4789F-1 with *A. thaliana* provides an important experimental tool for the elucidation of such mechanisms.

With increasing awareness of the harmful effects of agricultural practices based on chemical fertilizers, studies seeking to isolate and characterize microbial strains that can be used to promote growth in economically relevant crops have become important. In conclusion, this study reported that *P. ananatis* SCB4789F-1 has the essential characteristics for plant growth promotion, and its successful inoculation into *A. thaliana* stimulates shoot growth. Further studies evaluating the effect of this bacterium on promoting plant growth in economically relevant crops are warranted.

Conflicts of interest

The authors declare no conflict of interest.

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