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Endophytic bacteria *Bacillus safensis* and *Pseudomonas hibiscicola* and their ability to increase rice seedling growth

Roberto Lanna-Filho[1,*] https://orcid.org/0000-0003-2359-2806
Bruna Canabarro Pozzebon[2] https://orcid.org/0000-0002-2719-5472
Andréia Mara Rotta de Oliveira[3] https://orcid.org/0000-0002-2542-7540

1. Universidade Federal do Rio Grande do Sul – Departamento de Fitossanidade – Porto Alegre (RS), Brazil.
2. Biotrop Soluções Biológicas – Vinhedo (SP), Brazil.
3. Secretaria da Agricultura, Pecuária e Desenvolvimento Rural – Departamento de Diagnóstico e Pesquisa Agropecuária – Porto Alegre (RS), Brazil.

*Corresponding author: lanna.filho@ufrgs.br

ABSTRACT

Endophytic bacteria *Bacillus safensis* RS95 and *Pseudomonas hibiscicola* RS121 were evaluated for their ability to promote the growth of rice seedlings and produce indole-acetic acid (IAA) and siderophores and to solubilize phosphates. 'Guri' rice seeds were immersed in bacterial endophyte cell suspensions (separated and two-strain mixed), as well as in *Escherichia coli* DH5α, phosphate-buffered saline (PBS) and water treatments (negative controls). Seeds were sown on agar-water in Petri plates placed vertically at an angle of 65°. The ability of plant growth-promoting endophytic bacteria (PGPEB) to produce IAA and siderophores was determined by Salkowski colorimetric and chrome azurol S (CAS) assays, respectively. Mineral phosphate solubilization activity was calculated by inoculating the endophytes onto medium containing insoluble phosphate. PGPEB showed a positive effect on the growth of rice seedlings, causing a mean growth of shoots and primary-roots of 60 and 67%, respectively. Bacterial strains also showed positive traits for IAA and siderophore production, as well as phosphate-solubilization activity.

Keywords: indole-acetic acid; siderophore; phosphate-solubilization; plant growth-promoting endophytic bacteria.

Bacterial endophytes are able to promote the growth of plants by solubilizing nutrients, synthesizing plant hormones and supplying essential vitamins to plants (SANTOYO et al., 2016). Furthermore, triggering mechanisms significantly increase the plant's ability to metabolize these substances to target different physiological processes (SHISHIDO et al., 1999). The growth-promoting effect of these microbes on the host may be related to the ability to solubilize phosphates, produce indole-acetic acid (IAA) and improve iron nutrition (FADIJI; BABALOLA, 2020). Putative bacterial endophytes from rice (*Oryza sativa* L.) root- or stem-microbiome may have strong attributes for the stimulation of crop growth (WALITANG et al., 2019), as well as for the development of a biofertilizer. Thereby, plant growth-promoting endophytic bacteria (PGPEB) could be used in rice crop fertilization programs in association with chemical fertilizers.

The use of PGPEB in a rice cultivation system would improve the host plant performance for nutrient uptake (RAMOS et al., 2020), reducing the high cost of chemical nutrients and minimizing the risk of soil contamination. Furthermore, these bacteria may indirectly increase host health and vigor against plant pathogens (LODEWYCKX et al., 2002). Here we report the ability of the endophytic bacteria *Bacillus safensis* RS95 and *Pseudomonas hibiscicola* RS121 to stimulate the growth of rice seedlings, as well as the intrinsic fitness of the strains to synthesize indole-acetic acid (IAA) and siderophores, and to solubilize phosphate.

The endophytic bacteria *B. safensis* RS95 and *P. hibiscicola* RS121 were previously isolated from the stem and root of lowland rice plants (POZZEBON, 2015; TOMITA, 2019). For the study, the rice cultivar 'Guri' was chosen because it is widely cultivated in the southern Brazilian state of Rio Grande do Sul. Rice seeds were surface-sterilized with 70% (v/v) alcohol for 1 min, 1.5% (v/v) sodium hypochlorite for 2 min and rinsed in sterile distilled water. Sterilized seeds were

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bacterized in cell suspension (≈ 10^8 CFU·mL^{-1}) of *B. safensis* RS95, *P. hibiscicola* and two-strain mixed (Mix) prepared in phosphate-buffered saline (PBS) [0.1 mol·L^{-1}; pH 7.0; containing 0.05% Tween-80]. Seeds were also immersed in *Escherichia coli* DH5α (≈ 10^8 CFU·mL^{-1}), PBS and water treatments as negative controls. After about 24 h, the treated seeds were dried in flowing sterile air for 2 to 3 h and were ready for sowing. The seeds were transferred to Petri plates (five seeds per plate, in triplicate) containing 0.2% (p/v) agar-water. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the coleoptiles. Seedlings were maintained in a plant-growth chamber at 28 °C, 12 h photoperiod and 40 μmol·s^{-1}·m^{-2} light intensity. Four days later, the length (cm) of shoot and primary-root was measured with a digital caliper.

Bacterial strains were screened for IAA production by using Salkowski colorimetric assays (GLICKMANN; DESSAUX, 1995). Strains were cultured in 2 mL 96-well plates containing 1 mL of Luria-Bertani (LB) broth (without any tryptophan supplement) at 28 ± 2 °C for 48 h and 200 r·min^{-1}, followed by centrifugation at 3020 × g for 25 min. Pellets were discarded and the supernatants were filtered through 0.22 μm-pore-size filters. A 100 μL volume of the bacterial LB supernatant (BLBS) was mixed with 100 μL of Salkowski reagent (20 g·L^{-1} of $FeCl_3 \cdot 6H_2O$ in 7.9 mol^{-1} H_2SO_4). The mix was incubated for 30 min in the dark at 25 ± 2 °C, the absorbance was recorded at 530 nm with a spectrophotometer (Model UV-1100), and the concentration of IAA produced was estimated by using a standard IAA (catalogue No. 12886, Sigma) curve as a reference. Three replicates were evaluated for each BLBS sample or control. The phosphate solubilization index was calculated by inoculating the endophytes onto medium containing insoluble phosphate (10 g·L^{-1} glucose, 5 g·L^{-1} NH_4Cl, 1 g·L^{-1} NaCl, 1 g·L^{-1} $MgSO_4 \cdot 7H_2O$, 4 g·L^{-1} $CaHPO_4$, 15 g·L^{-1} agar, pH 7.2). The plates were incubated at 28 °C. The zone of clearance around the colony was observed after 48 h. The solubilization index (the ratio of the total diameter [colony + halo zone] to the colony diameter) was calculated by the formula in EDI PREMONO et al. (1996). Bacterial strains were also analyzed for their ability to solubilize calcium phosphate in liquid medium following the standard procedures (ALIKHANI et al., 2006). The assays were performed on triplicates for each strain.

Bacterial strains were checked for siderophore-producing ability by universal chrome azurol S (CAS) assay (SCHWYN; NEILANDS, 1987). Quantitative estimation of siderophores was done by taking the supernatant of bacterial cultures grown in LB broth medium (HU; XU, 2011). For this, 1 mL broth was placed in a 1.5 mL centrifuge tube (one for each bacterial culture) and, after sterilization, inoculated with 10 μL of freshly grown bacterial culture (10^8 CFU·mL^{-1}). Four replicates (tubes) were used for each strain. Apart from this, a control tube (uninoculated broth) was also maintained. After incubation at 28 °C for 48 h, bacterial cultures were centrifuged at 10,000 rpm for 10 min, cell pellets were discarded, and the supernatant was used to estimate siderophores. The supernatant (0.5 mL) of each bacterial culture was mixed with 0.5 mL CAS reagent and, after 20 min, optical density was taken at 630 nm. Siderophores produced by strains were measured in percent siderophore units (psu) which was calculated according to Eq. 1 (PAYNE, 1993)

$$\text{psu} = \frac{Ar - As}{Ar} \times 100 \quad (1)$$

where *Ar* = absorbance of reference (CAS solution and uninoculated broth), and *As* = absorbance of sample (CAS solution and cell-free supernatant of sample).

All assays were repeated twice, and the vertical agar plate assay was arranged in a completely randomized design. All data were assessed for normality (Shapiro–Wilk test) and homogeneity of variance (Brown–Forsythe test). For the vertical agar plate assay, data were subjected to Kruskal–Wallis test and means were compared by Dunn's test ($p < 0.05$). For IAA, phosphatase and siderophore synthesis assays, data were subjected to one-way ANOVA and means were compared by Tukey's honestly significant difference (HSD) test ($p < 0.05$). All data were analyzed using the software SigmaPlot 14.0 (Systat Software, San Jose, CA, United States).

Seedlings from bacterized rice seeds with *B. safensis* RS95, *P. hibiscicola* RS121 or two-strain mixed showed a higher root and shoot length compared to *E. coli* DH5α, PBS and water treatments (Fig. 1). Shoot and root length from rice seedlings did not differ between treatments with single and mixed bacterial endophytes (Fig. 2). On the other hand, treatments with bacterial strains differed significantly from control treatments. Four-day-old seedlings from bacterized rice seeds had a mean growth of shoots and primary roots of 60 and 67%, respectively, compared to control treatment (water). PGPEBs showed the ability to produce IAA, solubilize phosphate and produce siderophores (Table 1), which certainly contributed to the growth of rice seedlings (Fig. 1abc). *Bacillus safensis* RS95 showed significantly higher concentrations ($p < 0.05$) of IAA synthesis, P-solubilization and siderophore production compared to *P. hibiscicola* RS121 and *E. coli* DH5α (negative control). Although *P. hibiscicola* RS121 showed a lower concentration for the synthesis of growth factors, the results showed a significant difference ($p < 0.05$) for *E. coli* DH5α.

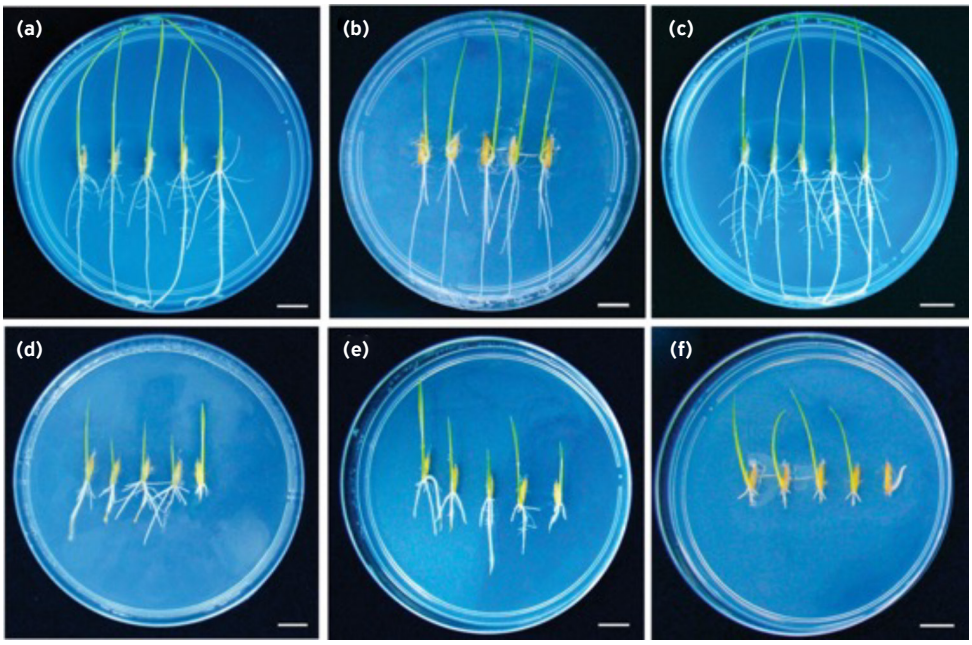

Figure 1. Effect of different treatments on seedling growth of rice (*O. sativa* 'Guri'). Four-day-old seedlings from bacterized rice seeds with *B. safensis* RS95 (a) and *P. hibiscicola* RS121 (b) and two-strain mixed (c) showed higher growth of shoot and root compared to *E. coli* DH5α (d), PBS (e) or water (f). Magnification bar = 1 cm.

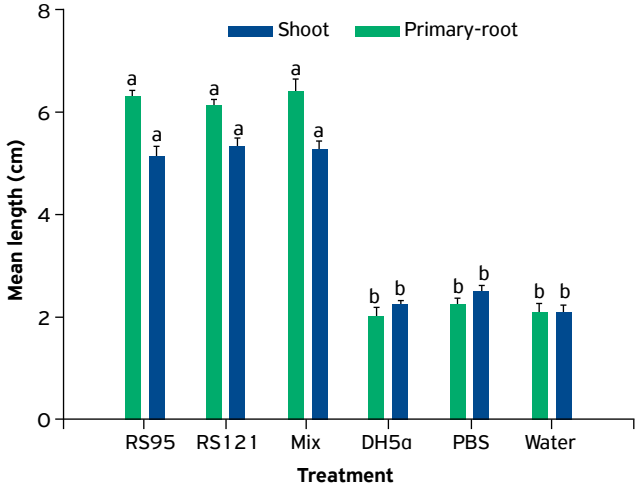

Figure 2. Growth promotion activities of endophytic strains on shoot and root length of rice seedlings (*O. sativa* 'Guri'), four days after germination. Seedlings from bacterized rice seeds with *B. safensis* RS95, *P. hibiscicola* RS121 or two-strain mixed (Mix) showed a higher root and shoot length compared to *E. coli* DH5α, PBS or water (control). Each treatment was run with five replicates per Petri-plates. Columns indicate the mean and vertical bars represent the mean ±SE from two experiments. Data of columns indexed by the same letters are not significantly different according to Dunn's test ($p < 0.05$).

Table 1. Traits of endophytic bacteria for the indole-acetic acid (IAA) synthesis, P-solubilization and siderophore production under in vitro conditions.

Bacterial strains	IAA production		P solubilization		Siderophore production (psu)
	Color intensity	Concentration (µg/mL)	Halo zone (cm)	Concentration (µg/mL)	
B. safensis RS95	+++	65.6 ± 6.69a	2.6 ± 0.36a	109 ± 7.93a	37.9 ± 3.26a
P. hibiscicola RS121	++	39.3 ± 4.09b	1.1 ± 0.08b	76 ± 6.64b	9.4 ± 0.83b
E. coli DH5α (control)	ND	00.0 ± 0.00c	0.0 ± 0.00c	00 ± 0.00c	00.00 ± 0.00c

Values are the mean; those sharing the same letters in the column are not significantly different according to Tukey's HSD at $p < 0.05$. Data are represented by the mean of four replicates ± standard deviation, (+++), high production; (++), medium production; (+), low production; and (ND), not detected.

The data clearly show the potential of PGPEB to stimulate the growth of rice seedlings. Compared to these results, WU et al. (2019) reported the positive effect of the endophytic *B. safensis* ZY16 on the growth of *Chloris virgata* Sw., as well as siderophore and IAA production ability and phosphate-solubilizing activity. In another study, GUERRIERI et al. (2020) showed that *P. hibiscicola* strains from tomato (*Solanum lycopersicum* L.) root had positive traits for the synthesis of plant growth factors: IAA, siderophores, and phosphate solubilizers. Both studies corroborate the data in this paper and reinforce the important role of these bacterial endophytes in stimulating the growth of other host plants. Although the methods employed in this may determine that endophytic agents act as PGPEBs in association with rice seedlings, the stimulus for seedling growth may have occurred only through the production of AAI or other uninvestigated phytohormones (EGAMBERDIEVA et al., 2017). This is because the seedlings were grown on medium (water-agar) without the supplementation of phosphorus or iron-based compounds that could trigger P-solubilization or iron sequestration by bacterial endophytes.

Synthesized and released by bacterial endophytes, IAA induces primary root elongation and shoot growth (EGAMBERDIEVA et al., 2017). This phenomenon was observed in this study under vertical agar plate conditions. The growth-promoting effect of PGPEBs under greenhouse or field conditions may be enhanced by their ability to solubilize complex soil organic phosphates like Al-P, Fe-P and Ca-P, as well as solubilizing and chelating iron from organic or inorganic complexes present in soil. Further studies are being carried out in order to confirm this hypothesis and to determine the ability of bacterial endophytes as putative inoculants for use in flooded rice crops. The association of endophytic bacteria *B. safensis* and *P. hibiscicola* with plants is poorly understood, especially with regard to plant-growth-promoting activities. Data presented in this study provide important contributions to a better understanding of how these endophytic agents can stimulate the growth of rice seedlings. Furthermore, it encourages us to expand our studies on other rice genotypes widely cultivated in Brazil.

AUTHORS' CONTRIBUTIONS

Conceptualization: Lanna-Filho, R. **Data curation:** Lanna-Filho, R.; Pozzebon, B.C. **Formal analysis:** Lanna-Filho, R.; Pozzebon, B.C. **Funding acquisition:** Lanna-Filho, R. **Investigation:** Pozzebon, B.C. **Methodology:** Lanna-Filho, R.; Oliveira, A.M.R. **Project administration:** Lanna-Filho, R. **Resources:** Lanna-Filho, R. **Supervision:** Lanna-Filho, R. **Validation:** Lanna-Filho, R. **Visualization:** Lanna-Filho, R. **Writing – original draft:** Lanna-Filho, R. **Writing – review & editing:** Lanna-Filho, R.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CONFLICTS OF INTEREST

All authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Not applicable.

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