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Pseudomonas putida, an endophyte of Piper tuberculatum, increases the biomass of cowpea (Vigna unguiculata L.)

Pseudomonas putida, un endófito de Piper tuberculatum, aumenta la biomasa del frijol caupí (Vigna unguiculata L.)

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ABSTRACT

Plant growth-promoting bacterial endophytes have been increasingly used in sustainable agriculture. Previous studies reported the isolation of *Pseudomonas putida* Pt12 with plant growth-promoting properties associated with the roots of *Piper tuberculatum*, a Piperaceae from the Amazon region. Thus, this study aimed to evaluate the effect of *P. putida* Pt12 on promoting the growth of cowpea (*Vigna unguiculata* L.), a legume crop with economic importance in developing countries, where its production can be affected by biotic and abiotic factors. Experiments were arranged in a completely randomized design using two methods of inoculation (seed bacterization and seedling irrigation). In seed bacterization, cowpea seeds were surface-sterilized, followed by inoculation with the Pt12 suspension (OD_{600}= 0.3, 3×10^8 cells/mL). For irrigation, 8-day-old seedlings were inoculated with the P12 suspension (OD_{600}= 0.1, $1x10^8$ cells/mL and OD_{600}= 0.3, 3×10^8 cells/mL) through soil irrigation.

Plant growth was monitored over a period of 25 days, which the parameters of biomass were assessed. The data were subjected to analysis of variance using the Scott-Knott test at 5% significance. Plants inoculated with Pt12 exhibited significantly higher growth compared to non-inoculated plants. Enhanced growth of both roots and the aerial part of the plant was observed, resulting in 206% and 59% increases in total dry biomass of plants inoculated by seed bacterization and soil irrigation, respectively. No significant differences were observed between plants irrigated with the two inoculum concentrations.

Keywords: endophytic bacteria; productivity; promotion of plant growth; seed bacterization; soil irrigation; sustainable agriculture.

RESUMEN

Los endófitos bacterianos promotores del crecimiento vegetal se han utilizado cada vez más en la agricultura sostenible. Estudios previos reportaron el aislamiento de *Pseudomonas putida* Pt12 con propiedades promotoras del crecimiento vegetal asociadas a las raíces de *Piper tuberculatum*, una Piperaceae de la región amazónica. Por lo tanto, este estudio tuvo como objetivo evaluar el efecto de *P. putida* Pt12 en la promoción del crecimiento del frijol caupí (*Vigna unguiculata* L.), un cultivo leguminoso con importancia económica en los países en desarrollo, donde su producción puede verse afectada por factores bióticos y abióticos. Se realizaron experimentos

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organizados en un diseño completamente aleatorizado utilizando dos métodos de inoculación (microbiolización de semillas y riego de plántulas). En la microbiolización, las semillas fueron esterilizadas superficialmente, seguido de la inoculación con la suspensión de Pt12 (DO_{600} = 0.3, 3×10^8 Cels/mL). Para irrigación, plántulas de 8 días fueron inoculadas con la suspensión Pt12 (DO_{600} = 0.1, 1x10^8 Cels/mL y DO_{600} = 0.3, 3×10^8 Cels/mL) mediante riego de suelo. Se monitoreó el crecimiento de las plantas hasta los 25 días, momento en el que se evaluaron los parámetros de biomasa. Los datos se sometieron a un análisis de varianza mediante la prueba de Scott-Knott al 5% de significancia. Las plantas inoculadas con Pt12 mostraron un crecimiento significativamente mayor en comparación con las plantas no inoculadas. Se observó una mejora en el crecimiento de las raíces y la parte aérea de la planta, resultando en aumentos del 206% y 59% en la biomasa seca total de las plantas inoculadas mediante microbiolización de semillas e irrigación de suelo, respectivamente. No se observaron diferencias significativas entre las plantas regadas con las dos concentraciones de inóculo.

Palabras clave: Agricultura sostenible; bacterias endófitas; bacterización de semillas; productividad; promoción del crecimiento vegetal; riego del suelo.

INTRODUCTION

Plant growth-promoting bacterial endophytes have been increasingly used in sustainable agriculture, replacing pesticides and chemical fertilizers, which can damage the environment (Maksimov *et al.*, 2018; Medison *et al.*, 2022). These bacteria can act directly in promoting plant growth through the production of phytohormones, such as indole-3-acetic acid (IAA), as well as modulating the production of substances that aid in plant nutrition, through solubilization of phosphate and potassium, and biological nitrogen fixation. In addition, they can promote plant growth indirectly by controlling pathogens and suppressing diseases (Eid *et al.*, 2021; Santoyo *et al.*, 2016; Maksimov *et al.*, 2018; Medison *et al.*, 2022).

Beneficial bacteria belonging to the genus *Pseudomonas* are among the most promising for agronomic purposes (Zboralski & Filion, 2023; Mehmood *et al.*, 2023; Ruiz-Hernandez *et al.*, 2024; Serrão *et al.*, 2025). In this context, Nascimento *et al.* (2015) isolated endophytic bacteria associated with the roots of *Piper tuberculatum*, a Piperaceae from the Amazon region with known resistance to *Fusarium solani* f. sp. *piperis*, the pathogen of rot root disease in black pepper (*Piper nigrum*) (Albuquerque *et al.*, 2001). Among them, bacterial isolate Pt12, identified as *Pseudomonas putida*, was able to inhibit the *in vitro* growth of the pathogen (Nascimento *et al.*, 2015). In addition, *P. putida* Pt12 produced compounds that are potentially bio-stimulating for plant growth, such as IAA, siderophores, and soluble phosphate (Oliveira *et al.*, 2021). Thus, this study aims to evaluate the effect of *P. putida* Pt12 in promoting the growth of cowpea (*Vigna unguiculata* L.).

Cowpea is a legume crop with social and economic importance in developing countries in Africa, Asia, and the Americas, including Brazil and Colombia, where its production can be affected by different environmental factors (Rebello *et al.*, 2011; Benchimol *et al.*, 2021; Campo-Arana & Burgos-Ayala, 2023). In the agricultural context, plants need to be able to survive and grow in adverse environmental conditions, as well as have high productivity under normal and stressful conditions. Therefore, the use of beneficial bacteria can be a sustainable alternative strategy for solving limitations on the production of cowpea.

MATERIALS AND METHODS

Preparation of bacterial suspension

The preparation of the bacterial suspension was based on the procedure described by Sá (2019), with some adaptations. *P. putida* Pt12, previously isolated by Nascimento *et al.* (2015), was cultured on tryptone soy agar at 28°C for 24 hours, followed by cell recovery in autoclaved water and adjustment of the concentrations for OD_{600}= 0.1 and OD_{600}= 0.3, resulting in 1×10^8 cells/mL and 3×10^8 cells/mL, respectively.

Bacterial inoculation of cowpea seeds and seedlings

Experiments were carried out in a completely randomized design using cowpea BRS Guariba. Two methods of bacterial inoculation (seed bacterization and seedling irrigation) were conducted separately at the Federal University of Pará (Belém, PA, Brazil, 1°28'23.9"S 48°27'27.5"W) under growth chamber conditions, with 12 hours of light per day and average temperature and relative humidity of around 28°C and 80%, respectively.

In the seed bacterization assays, cowpea seeds were first surface-sterilized by immersion in 70% alcohol for 1 minute, 1% sodium hypochlorite for 1 minute, and five washes in sterile water as described by Sá (2019), and then incubated in the Pt12 suspension (OD_{600}= 0.3) for 20 minutes. As a negative control, the seeds were incubated in autoclaved water for 20 minutes. The germinated seeds from each treatment (inoculated and non-inoculated) were planted in pots containing 240g of autoclaved soil and maintained at room temperature (26-28°C) with 12 hours of light per day and watered with autoclaved water (10mL/pot/day). Five replicates per treatment were used. The growth of the cowpea seedlings was monitored for 25 days, after which the roots and aerial parts of the plant were harvested and dried in an oven at 50°C until they reached constant mass, followed by the evaluation of the biomass parameters (dry mass of the roots, dry mass of the aerial part, and total dry mass).

For the irrigation assays, 5-day-old cowpea seedlings were grown from seeds subjected to surface sterilization and planted under the same conditions described above. First, the seedlings were treated with a nutrient solution (NPK: 4% N, 14% P_2O_5, 8% K_2O) and, three days later, they were inoculated separately with the Pt12 suspension (OD_{600} = 0.1 and OD_{600} = 0.3) through soil irrigation (10 mL/pot). As a negative control, the seedlings were irrigated with autoclaved water. Five replicates per treatment were used (inoculated OD_{600}= 0.1, inoculated OD_{600} = 0.3, and non-inoculated). Plant growth was monitored for 17 days, after which the roots and aerial parts were harvested and processed, followed by the evaluation of the biomass parameters, as described above. All data were subjected to the Scott-Knott test at 5% significance using the SISVAR software (Ferreira, 2010).

RESULTS

Results revealed that cowpea plants inoculated with *P. putida* Pt12 showed significantly increased growth compared to non-inoculated plants (Figure 1 and Table 1). Increased growth was observed in both the roots and aerial parts of the plant, resulting in a 206% increase in the total dry biomass of plants inoculated via seed bacterization, while in seedlings inoculated via soil irrigation, the increase was at least 53%. No significant differences were observed between plants irrigated with suspension OD_{600} = 0.1 and OD_{600} = 0.3 (Table 1).

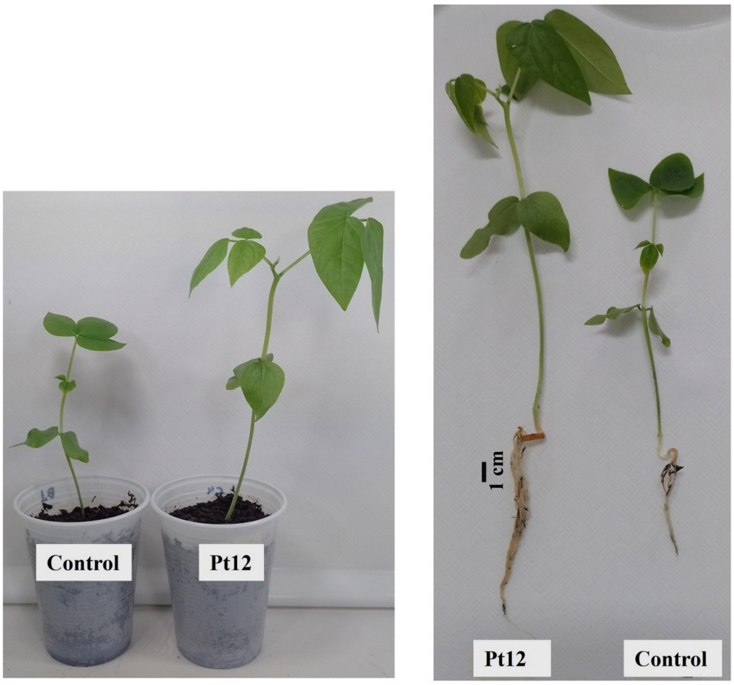

Figure 1.
Twenty-five-day-old cowpea plants (BRS Guariba) grown from seeds bacterized with P. putida Pt12 and from non-inoculated seeds (Control)

Table 1. *Evaluation of the growth of 25-day-old cowpea plants (BRS Guariba) inoculated with P. putida Pt12.*

Inoculation via seed bacterization						
	DMR		**DMAP**		**TDM**	
	(g)[1]	(%)[2]	(g)[1]	(%)[2]	(g)[1]	(%)[2]
Control	0.010 b	100	0.037 b	100	0.047 b	100
Inoculated OD= 0.3	0.027 a	270	0.117 a	316	0.144 a	306
Standard error	0.004		0.008		0.009	
Inoculation of seedlings via soil irrigation						
	DMR		**DMAP**		**TDM**	
	(g)[1]	(%)[2]	(g)[1]	(%)[2]	(g)[1]	(%)[2]
Control	0.018 b	100	0.077 b	100	0.095 b	100
Inoculated OD= 0.1	0.030 a	166	0.115 a	149	0.145 a	153
Inoculated OD= 0.3	0.033 a	183	0.118 a	153	0.151 a	159
Standard error	0.004		0.007		0.010	

** For seed bacterization, a bacterial suspension with OD600= 0.3 was used, while for seedling inoculation via soil irrigation, suspensions with OD600= 0.1 and OD600= 0.3 were used. The values shown in the table represent the means of the following parameters: dry mass of the roots (DMR), dry mass of the aerial part (DMAP), and total dry mass (TDM).*
[1]The means followed by the same letter in the same column do not differ statistically, using the Scott-Knott test at 5% probability.
[2]Percentage of parameters used.

DISCUSSION

In this study, the effect of *P. putida* Pt12 on cowpea growth was evaluated by testing two inoculation methods as well as different inoculum concentrations, since such parameters are essential for optimizing bacterial colonization in the plant host, improving the plant benefits (Lopes, Dias-Filho *et al.*, 2021; Lopes, Santiago, *et al.*, 2021).

The results confirming the ability of Pt12 to promote cowpea growth through increases in root and aerial part biomass are consistent with Oliveira *et al.* (2021), who reported the production of IAA, siderophores, and soluble phosphate by the endophyte. IAA is an auxin that stimulates root growth, including the development of root hairs and lateral roots, increasing the surface area for nutrient and water absorption, consequently promoting plant growth (Etesami & Glick, 2024). Siderophores are low-weight molecules that help plants access iron by chelating it, making it soluble and accessible to them (Ghosh *et al.*, 2020). Similarly, phosphate-solubilizing bacteria produce significant increases in plant growth and productivity, since phosphorus is involved in several metabolic processes, such as photosynthesis and energy transfer (Bargaz *et al.*, 2021). In this regard, it is known that biomass production results from the effect of photosynthesis efficiency on plant growth and yield (Sembada *et al.*, 2024). Oteino *et al.* (2015) reported increased biomass (dry weight) in pea plants inoculated with strains of *Pseudomonas* endophytes with the ability to solubilize inorganic phosphate. Thus, the increase in biomass and growth of cowpea may be related to the production of these compounds by Pt12, which produced 125 µg mL^{-1} of IAA and 56.56 µg mL^{-1} of soluble phosphate according to Oliveira *et al.* (2021).

The results are also consistent with other studies, where the effect of beneficial bacteria has been demonstrated through their inoculation in different parts of the plant, including seeds and roots, or directly in the soil or substrate. As reported by Verma *et al.* (2023), bacteria *Pseudomonas* sp., isolated from the rhizosphere of cowpea, significantly increased the growth of cowpea plants generated from bacterized seeds. *Pseudomonas libanensis* TR1, originally isolated from the rhizosphere of *Trifolium repens*, increased the total biomass of cowpea plants by 101% when inoculated through seed coating (Ma *et al.*, 2019). Ferreira *et al.* (2021) reported that inoculation of the cassava endophyte *Bacillus aryabhattai* 4W through seed bacterization increased cowpea fresh biomass by 128%. A similar increase was observed in cassava dry biomass when this endophyte was inoculated through soil irrigation (Ferreira *et al.*, 2021). *Bacillus subtilis* (LCB30) promoted increases in seed germination and dry biomass of cowpea plants (Sá, 2019). In the case of *P. putida* Pt12, despite its demonstrated effect on cowpea plant growth (increases in total biomass by 53% and 206%), no effect on seed germination was observed (data not shown).

Regarding the inoculation methods tested, Pt12 inoculation through seed bacterization produced a better effect in promoting cowpea growth when compared to seedling inoculation through soil irrigation. Similar results were observed by Cely *et al.* (2016), where seed inoculation with beneficial microorganisms was more effective in promoting growth and wood production in *Schizolobium parahyba*, compared to seedling inoculation. This difference may be attributed to the fact that, in inoculation through seed bacterization, the seeds are placed in direct and uniform contact with the bacterial suspension, which favors the penetration of bacteria into the tissues and the colonization process. Furthermore, the benefits can occur from the initial stages of plant development when seed bacterization is used. In contrast, when bacteria are inoculated into the soil, root colonization depends on several factors, such as their establishment in the rhizosphere, where interactions with plant root exudates and microbiome occur, as well as penetration into the internal tissues of the roots (Bacilio-Jiménez *et al.*, 2003; Knights *et al.*, 2021). In this regard, this scenario indicates the need for additional efforts for optimizing Pt12 inoculation conditions in cowpea through soil irrigation.

Additionally, the concentration of the bacterial inoculum was tested, which must also be considered for efficient inoculation. While high levels may be necessary to provide effective colonization, an excess could also be harmful in some way. In this regard, studies have shown that the OD values and cell concentration can be adjusted regarding the bacterial species, the host plant and the objective of the experiment. For example, suspensions of *Pseudomonas putida* R4 and *Pseudomonas chlororaphis* R5 with OD_{620} = 0.1 and cell density of 1×10^8 cells/mL were used in cotton seed inoculation (Egamberdieva *et al.*, 2015). Wheat plants were inoculated with suspensions of *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391 with OD_{600} = 0.15 (Imperiali *et al.*, 2017). Durairaj *et al.* (2017) reported on the use of suspensions of *Pseudomonas aeruginosa* and *Bacillus stratospehericus* with OD_{600} = 0.1 and OD_{600} = 0.2 in tomato inoculation, with the aim of controlling pathogenic bacteria. On the other hand, aiming at growth promotion, tomato plant substrate was inoculated with four *Pseudomonas* strains using suspensions with OD_{600} = 2.0 (Issifu *et al.*, 2023). Thus, in accordance with the literature, inoculations of *P. putida* Pt12 through seed bacterization (OD_{600} = 0.3) and seedling inoculation via soil irrigation (OD_{600}= 0.1 and OD_{600}=0.3) were effective in promoting the growth of cowpea plants. Similar results obtained with different Pt12 inoculum concentrations indicated that lower concentrations may already be sufficient to benefit the plant; however, additional experiments on seedling inoculation by soil irrigation are needed to further improve cowpea growth promotion.

CONCLUSIONS

The two inoculation methods tested were effective in promoting cowpea growth by *P. putida* Pt12, confirming its potential as a bioinoculant. Under the experimental conditions used, the inoculation of Pt12 through seed bacterization produced a greater effect on promoting the growth of cowpea. Furthermore, the use of Pt12 bacterial suspensions at different concentrations in irrigation experiments resulted in similar increases in cowpea growth, indicating that lower bacterial concentrations may be sufficient to benefit the plant.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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