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Plant growth promoting potential of endophytic bacteria isolated from cashew leaves

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Endophytic microorganisms are able to promote plant growth through various mechanisms, such as production of plant hormones and antimicrobial substances, as well as to provide the soil with nutrients, for instance, inorganic phosphate. This study aimed to evaluate the potential of endophytic bacteria isolated from cashew leaves to produce substances involved in the promotion of plant growth, such as indole-3-acetic acid, the phosphate solubilization capacity, and the antimicrobial activity. For this, 31 isolate samples were used, out of which 17 (54.8%) produce indole-3-acetic acid in concentrations ranging from 11.79 to 145.85 µg.mL^{-1}. In turn, four (12.9%) were able to solubilize phosphate and the solubilization halos range from 5 to 19 mm. Soluble phosphorus concentrations range from 62.5 to 1,605.2 mg.L^{-1}. It was observed that *Fusarium oxysporum* and *Colletotrichum* sp. were inhibited by 70 and 40% of the strains, respectively. It was found out that five bacteria (25%) were Gram-positive, predominantly the species *Staphylococcus saprophyticus* (100%), while 15 bacteria (75%) were Gram-negative. Out of these, 4 (26.6%) and 3 (20%) belong to the species *Escherichia coli* and *Shigella flexneri*, respectively. Studying the endophytic population is something important due its biotechnological applications, because it has a great potential for promoting plant growth.

Key words: *Anacardium occidentale*, auxin, endophytic microorganisms, secondary metabolites, phosphate solubilization.

INTRODUCTION

In recent decades, the search for new compounds with applications to health and agriculture has increased and

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in this context, endophytic microorganisms have shown a great potential, since they represent an important genetic diversity source and bioactive compounds (Zhao et al., 2010; Joseph and Priya, 2011).

Endophytes microorganisms spend at least one period of their life cycle beneficially associated to plants' tissues and/or organs, such as roots, branches, and leaves, and they can be isolated after disinfecting the surface of these plants. Bacteria and fungi, which are commonly found as endophytes, do not cause harm to their hosts; instead, they play important roles with regard to the plant's health (Azevedo et al., 2000; Azevedo et al., 2006; Nair and Padmavathy, 2014).

During a long co-evolution period, a friendly relationship was formed between endophytes and the host plant. Some endophytes can produce the same or similar bioactive compounds from the host plant, presenting a mutualistic association, as they receive nutrients, protection by the plant, and other benefits, such as pathogenic microorganism control, insect control, and protection against herbivores (Zhao et al., 2011).

These microorganisms facilitate the plant's growth and development through two mechanisms: Direct: when it involves in the supply of compounds which are synthesized and make easier the absorption of nutrients from the environment; and Indirect: when the microorganism reduces or prevents the harmful effects of pathogens by producing inhibitory substances or increasing the host's natural resistance.

The direct mechanisms are nitrogen fixation (N_2), phosphate solubilization, insoluble iron chelation through the production of siderophores and phytohormones, such as auxins, cytokinins, and gibberellins (Oliveira et al., 2003; Tsavkelova et al., 2007; Jha et al., 2012).

Auxins are hormones produced by endophytes which regulate plant growth and act on the cell division, elongation, and differentiation (Shokri and Emtiazi, 2010). However, the interaction of bacteria with the host plant for promoting growth depends on the concentration of hormones available to the plant cells (Oliveira et al., 2003).

Indole-3-acetic acid (IAA) is the most important auxin for the growth of roots and stems by stretching the cells (Tsavkelova et al., 2007). This hormone is usually produced by several bacteria genera which promote plant growth, such as *Actinomyces*, *Agrobacterium*, *Arthrobacter, Azospirillum, Azotobacter*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Enterobacter, Gluconacetobacter, Klebsiella, Methylobacterium, Pantoea*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Sthaphylococcus* and among others (Hayat et al., 2010; Bhattacharyya et al., 2012; Oliveira et al., 2013; Zheng et al., 2013; Rangjaroen et al., 2014).

Another important function of endophytic bacteria is related to the phosphorus cycle, being responsible for the hydrolysis of phosphorus to its soluble form. These microorganisms use different mechanisms to convert forms insoluble in soluble phosphate through the activity of enzymes such as phosphatases or phosphohydrolases with processes of acidification, chelation, exchange reactions, but usually, the main mechanism of solubilization is the release of metabolites such as organic acids (Hameeda et al., 2008; Young et al., 2013).

The ability of endophytic bacteria with regard to the solubilization of inorganic phosphate raised interest in agricultural practices and because of this, the endophytic microorganisms have been studied. They play an important role in the conversion of phosphorus to soluble phosphate.

Studying the endophytic population is important not only for acquiring knowledge on its ecological role, but also with regards to its biotechnological applications to the production of bioactive substances which can be used in the field in order to increase the agricultural production. This study aimed to evaluate endophytic bacteria producing indole acetic acid and solubilized phosphorus as well as identify them by biochemical characteristics.

MATERIALS AND METHODS

Microorganisms

We used 31 endophytic bacteria from cashew leaves (*Anacardium occidentale* L.) that belonged to the Microorganism Collection - UFPEDA, Brazil. The strains were inoculated in BHI broth (Dadook et al., 2013) for reactivation and incubated for 24 h/30°C. Afterwards, the biochemical characteristics were analyzed as described above.

Classical taxonomy (phenotypic features and microscopic analysis)

Bacteria were cultivated on Agar Nutrient (Verma et al., 2001) and after 24 h, Gram staining and biochemical tests were performed using the systems Bactray I and II (Gram-negative negative oxidase) and III (Gram-negative positive oxidase), in accordance with the manufacturer's instructions (LaborClin). Gram positive bacteria were identified by specific tests including catalase test, DNase agar, mannitol salt agar and sensitivity to novobiocin according to Winn et al. (2006).

Production of indole-3-acetic acid

The production of IAA was analyzed according to the method developed by Sarwar and Kremer (1995); inoculating 31 endophytic bacteria padronized 1.0 $\times 10^8$ UFC/ml (λ=625nm) in tubes with 10% TSA (Kuklinsky-Sobral et al., 2004) supplemented with 5 mmol.L^{-1} of L-tryptophan and cultivated for 24h/30°C in the dark under a 110 × g shake. After growth, the samples were centrifuged at 8 000 × *g* for 15 min and to the supernatant, the reagent Salkowski (0.5 mol.L^{-1} $FeCl_3$ + 7.9 mol.L^{-1} H_2SO_4) was added in a 1:1 ratio (v/v) and left in the dark for 30 min at room temperature. The emergence of pink-red color indicated the production of auxins. For quantifying IAA, spectrophotometer at 530 nm was used. A standard curve was obtained with commercial IAA solutions (Vetec) at different concentrations. Tests were performed in triplicate and *Pantoea agglomerans* UFPEDA 774 was used as the positive control and

Table 1. Concentration of indole-3-acetic acid produced by endophytic bacteria of cashew leaves.

Bacterial strain	Concentration of IAA (µg.mL^{-1})	Bacterial Strain	Concentration of IAA (µg.mL^{-1})
Pantoea agglomerans	76.31 ± 4.15	BC-26	37.29 ± 9.00
BC-1	23.12 ± 3.24	BC-46	132.79 ± 3.53
BC-2	36.95 ± 4.64	BC-47	131.73 ± 17.58
BC-8	144.43 ± 0.50	BC-48	130.7 ± 6.49
BC-9	38.81 ± 0.23	BC-49	106.63 ± 0.22
BC-10	145.85 ± 0.44	BC-51	11.79 ± 2.81
BC-21	135.29 ± 0.00	BC-55	108.91 ± 14.67
BC-22	132.79 ± 3.53	BC-63	104.29 ± 13.17
BC-24	16.66 ± 4.68	BC-147	62.07 ± 0.31

± Standard deviation.

the negative control consisted of the same medium supplemented with L-tryptophan, with no bacterial growth.

Evaluation of inorganic phosphate solubilization

For the semiquantitative assessment of phosphate solubilization, the bacteria (1.0 × 10^8 UFC/ml, λ 625 nm) were inoculated in spots on plates containing three different media: 'National Botanical Research Institute's Phosphate Solubilization' - NBRIP (Kumar et al., 2012); the medium according to Verma et al. (2001) and Phosphate medium (Kuklinsky-Sobral et al., 2004). After 120 h of incubation, clear zones around the colonies were indicative of solubilization of inorganic phosphate and the phosphate solubilization index (PSI) was calculated according to Sarkar et al. (2012). *P. agglomerans* UFPEDA 774 was used as the positive control.

Evaluation of phosphate solubilization in liquid medium

Each strain containing 1.0 × 10^8 UFC/ml (λ 625 nm), were inoculated on NBRIP and VERMA medium (previously described), according to Kumar et al. (2012) and Verma et al. (2001). After 144 h, the supernatants were analysed according molybdate-vanadate method (Inui-Kishi et al., 2012). Afterwards, the absorbance was estimated the absorbance at 420 nm. A standard curve was obtained from potassium phosphate solutions (1.0; 2.0; 4.0; 5.0; 6.0; 8.0; 10.0 mg.L^{-1}) in accordance with the Standard Methods (Clesceri et al., 1999). *P. agglomerans* UFPEDA 774 was used as the positive control.

Statistical analyses

Each experiment was performed in triplicate and analysis of variance was performed (ANOVA) at $p \leq 0.05$ and the Tukey test ($p \leq 0.05$) on the program Excel 2010 and Minitab version 15 to analyze the results of IAA's production, semiquantitative and quantitative phosphate solubilizing on VERMA and NBRIP media by bacteria of Cashew tree was done (Bluman, 2001).

Antimicrobial activity

The solubilizing phosphate and/or producing IAA strains were tested against the activity of *A. niger* UFPEDA 2003, *Colletotrichum* sp. UFPEDA 2561 and *F. oxysporum* UFPEDA 2456 (1.0 x10^6 spores/mL). The fungi were inoculated on potato dextrose agar (Ribeiro and Cardoso, 2012) and the antagonistic activity was evaluated after seven days at 28°C.

RESULTS AND DISCUSSION

Production of IAA and quantitative assessment

Out of the 31 strains tested with regards to the production of auxin, 17 (54.83%) showed reddish pink coloration after addition of the reagent Salkowski, indicating that they produce IAA through tryptophan, with concentrations ranging from 11.79 to 145.85 µg.mL^{-1} (Table 1).

The plant hormone production is part of the metabolism of several microorganisms associated to plants and they may be regarded as important agents in the regulation of the plant's growth and development (Oliveira et al., 2003). The auxin synthesized by bacteria affects the root system by increasing the number and size of adventitious roots (Gutierrez et al., 2012). One should take into account the fact that the IAA effect may vary according to their concentrations. Depending on the plant's variety, higher concentrations of IAA can have an inhibiting effect on plant growth inducing callus tissues (Ribeiro and Cardoso, 2012).

The colorimetric method for observing the production of auxins by microorganisms uses the reagent Salkowski and it is based on the oxidation of indole compounds by ferric salts (Mayer, 1958). Variation in the concentration of auxin produced by the endophytic bacteria may have been caused by differences in the behavior of each bacterium, and one needs to better understand the IAA synthesis at different times of bacterial growth, thus determining the time when the maximum synthesis of the product took place.

ANOVA indicated that the averages obtained in relation to production of IAA are not statistically equal ($p > 0.05$). Then, the Tukey shows the strain BC-10 has good potential to produce this important metabolite for plants growth.

Table 2. Phosphate solubilization index (PSI) of solubilized phosphorus in plate assay of endophytic bacteria.

Bacterial strain	VERMA	NBRIP	Phosphate medium
BC-51	-	-	2.85 ± 0.51
BC-52	-	-	1.9 ± 0.09
BC-53	2.21 ± 0.21	2.29 ± 0.66	2.31 ± 0.09
BC-56	1.54 ± 0.04	1.83 ± 0.14	3.92 ± 0.09
P. agglomerans	3.02 ± 0.42	2.00 ± 0.00	2.19 ± 0.26

± Standard deviation.

Table 3. Concentration ($mg.L^{-1}$) of phosphorus solubilized by endophytic bacteria of cashew leaves.

Bacterial strain (media)	Concentration of solubilized phosphate ($mg.L^{-1}$)
BC-51 (NBRIP)	36.73 ± 1.00
BC-51 (VERMA)	126.89 ± 21.00
BC-52 (NBRIP)	113.53 ± 15.99
BC-52 (VERMA)	400.70 ± 25.05
BC-53 (NBRIP)	1569.41 ± 11.00
BC-53 (VERMA)	1349.02 ± 62.31
BC-56 (NBRIP)	1389.09 ± 62.31
BC-56 (VERMA)	1395.77 ± 32.17
Pantoea agglomerans (NBRIP)	1095.25 ± 84.00
Pantoea agglomerans (VERMA)	808.08 ± 34.28

± Standard deviation.

Kuklinsky-Sobral et al. (2004) observed that 34% of the endophytic bacteria associated to soybean are auxin producers, standing out in the production of important substances for promoting plant growth. In this study, the BC-10 isolate showed auxin production with a 145.85 $\mu g.mL^{-1}$ concentration, higher than the result obtained by Kochar et al. (2011) through the *P. fluorescens* isolate (25.82 $\mu g.mL^{-1}$) in a culture medium containing the same tryptophan concentration (5 $mmol.L^{-1}$).

Therefore, the results found in the literature corroborate those obtained through the endophytic bacteria of cashew leaves analyzed in this study.

Phosphate solubilization assessment

The tested bacteria showed low phosphate solubilization, only 4 strains (12.9%) were able to solubilize phosphorus in the Phosphate medium (BC-52, BC-53, BC-56, and BC-51), highlighting the strain BC-51 which also produces IAA. Only two out of the four strains were able to solubilize phosphate in the VERMA and NBRIP media.

The PSI was calculated through the ratio between the phosphate solubilization halo (mm) and the colony diameter (mm), and it was observed that it ranged from 1.90 to 3.92 in the Phosphate medium and, among the four strains, three (BC-51, BC-53, and BC-56) showed a phosphate solubilization higher than the positive control, *P. agglomerans*. In the VERMA medium, PSI ranged from 1.53 to 2.21 and in NBRIP it ranged from 1.83 to 2.29 (Table 2).

After analyses of variance, it was cleared that the averages of PSI are not statistically equal ($p > 0.05$) in relation to phosphate solubilization in solid media with phosphate insoluble.

Regarding the phosphate solubilization, according to Chagas-Junior et al. (2010), the PSI is measured through the ratio solubilization halo (mm)/colony diameter (mm). Thus, the solubilization may be classified as low solubility ($PSI < 2$), middle solubilization ($2 \leq PSI < 4$), and high solubility ($PSI > 4$). Then, all of the endophytic bacteria of cashew tree have low or medium capability to solubilize phosphate.

The phosphorus concentrations solubilized by the isolates ranged from 36.73 to 1,569.41 $mg.L^{-1}$. The strains BC-53 and BC-56 solubilized phosphate at a higher concentration than the positive control in the NBRIP and VERMA media (Table 3). In liquid media, the *p*-value of ANOVA was less than 0.05 showing that there was difference between the averages of phosphate solubilization in liquid media. The Turkey test indicated that BC-53, BC-56 and the control *P. agglomerans* have

Table 4. Identification of endophytic bacterial strains which produced IAA and solubilized phosphate.

Bacterial strain	Species	Bacterial strain	Species
BC-1	*Staphylococcus saprophyticus*	BC-47	*E. coli*
BC-2	*S. saprophyticus*	BC-48	*E. coli*
BC-8	*Shigella flexneri*	BC-49	*E. coli*
BC-9	*S. flexneri*	BC-51	*Enterobacter cloacae*
BC-10	*S. flexneri*	BC-52	*Pseudomonas maltophilia*
BC-21	*Yersinia psedotuberculosis*	BC-53	*Escherichia fergusonii*
BC-22	*Escherichia coli*	BC-55	*Pseudomonas stutzeri*
BC-24	*S. saprophyticus*	BC-56	*Enterobacter sakazakii*
BC-26	*S. saprophyticus*	BC-63	*S. saprophyticus*
BC-46	*Klebsiella* sp.	BC-147	*Hafnia alvei*

the same capability to solubilize phosphate and they had higher concentrations of soluble phosphorus than BC-51 and BC-52.

The changes observed in the soluble phosphorus concentration are due to the features and requirements of each bacterial growth, for instance, different nutrient compositions in the culture medium influence the microbial development. Sarkar et al. (2012) isolated bacteria from roots of rice seedlings and tested the phosphate solubilization ability in a Pikovskaya liquid media and the concentrations ranged from 87.8 to 140.1 $mg.L^{-1}$. For this reason, the endophytic bacteria of cashew leaves have higher concentrations of soluble phosphate and because of this, they have characteristics that might promote plant growth.

Antimicrobial activity assessment

Out of the 20 endophytic bacteria which produced auxin and/or solubilized phosphate, did not observe an antimicrobial activity against *A. niger* UFPEDA 2003 was not observed. However, 14 (70%) showed activity against *F. oxysporum* UFPEDA 2456, while eight (40%) showed activity against *Colletotrichum* sp. UFPEDA 2561.

Regarding the antimicrobial activity, the term antagonist is used for the biological agents with potential to interfere in the life processes of pathogenic microorganisms, inhibiting their growth. Further studies with a deeper investigation involving biological control are needed, since the endophytic bacteria can show an activity against other pathogen types.

Characterization of classical taxonomy

Biochemical characterization showed that five (25%) are Gram-positive bacteria and the results suggest that all of them belong to species *S. saprophyticus*. It was found that 15 (75%) are Gram-negative bacteria and, among the Gram-negative bacteria with negative oxidase, it was suggested that 26.6% belong to the species *E. coli* and 20% *S. flexneri*. Other species, such as *E. fergusoni*, *H. alvei*, *Y. pseudotuberculosis*, and those from the genus *Klebsiella* sp were observed. Among the Gram-negative bacteria with positive oxidase, species *E. cloacae*, *P. maltophilia*, and *P. stutzeri* (Table 4) were observed. However, the confirmation with molecular tools needs to be done.

Stamford et al. (1998) identified the genus *Staphylococcus* as endophytic of yam bean. A similar phenomenon was observed by Velásquez et al. (2008), who were also able to identify the genus *Staphylococcus* as being endophytic of sugar cane through the analysis of 16S rRNA. Vendan et al. (2010) also identified endophytic bacteria from ginseng and observed that the genus *Staphylococcus*, confirm the results obtained in this study.

Several studies reported the occurrence of enterobacteria as plant endophytes. The presence of these microorganisms, such as *Klebsiella* spp., is usually observed in the endophytic community playing important roles in the host plants, since they are able to produce phytohormones and provide nitrogen (Dong et al., 2003; Durán et al., 2014; Guo et al., 2014).

The genus *Pseudomonas* and *Burkholderia* was identified as endophytic through biochemical and molecular tools; also they had the ability to produce some phytohormones and solubilized phosphate to growth plant (Mendes et al., 2007; Chauhan et al., 2013; Allu et al., 2014).

The assessed strains have higher potential for promoting plant growth and/or being used as biofertilizers through the production of phytohormones, such as auxins, as well as the conversion of insoluble phosphorus into soluble one, making it accessible to plant absorption. Therefore, research involving endophytes which have more than one feature for promoting plant growth is important for a better understanding of their interaction with the plant, something useful for analyzing the use of microorganisms in agriculture.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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