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Full Length Research Paper

Solubilization of calcium and iron phosphate and *in vitro* production of Indoleacetic acid by Endophytic isolates of *Hyptis marrubioides* Epling (Lamiaceae)

Luciana Cristina Vitorino*[1], Fabiano Guimarães Silva[1], Marcos Antônio Soares[2], Edson Luiz Souchie[1], Alan Carlos Costa[1] and William Cardoso Lima[2]

[1]Instituto Federal Goiano - Campus Rio Verde
[2]Instituto de Biociências, Universidade Federal do Mato Grosso (UFMT)

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Plant growth and development are associated with access to minerals and phytohormones. Some microorganisms solubilize phosphate, producing phytohormones with functional features of plant growth promoters. The capacity of endophytic isolates from the root of *Hyptis marrubioides* Epling, a Lamiaceae, to solubilize calcium phosphate in GELP medium and iron phosphate in modified Reyes basal medium was characterized. Theability of these isolates to synthesize indoleacetic acid (IAA) in DYGS medium supplemented with tryptophan was also determined. Of the 42 bacterial and six fungal strains analyzed by solubilization assays, 20% formed $CaHPO_4$-solubilization zones, although the level of solubilization was low. None of the fungi tested solubilized $CaHPO_4$. In contrast, 59% of the microorganisms solubilized high levels of $FePO_4$, and all of the fungi demonstrated this capacity. Among the bacterial isolates, 52% synthesized IAA, and strainsRF18, RG9, RF13 and RG24 were notable for their significant production of this phytohormone under test conditions (95.13, 39.28, 16.21 and 11.96 $\mu g \cdot mL^{-1}$, respectively). From a biotechnological perspective, the RG9, RG24 and RF18 strains are significant for their solubilization of high levels of $FePO_4$ and abundant IAA production.

Keywords: Solubilization halos, Phosphate-solubilizing microorganisms, Indoleacetic acid, Plant growth promoters.

INTRODUCTION

Plant growth and development are directly associated with a plant's access to essential minerals such as phosphorus (Santos et al., 2010) and the biosynthesis of phytohormones such as auxins (Multani et al., 2003; Renhardt et al., 2003; Kieffer et al., 2010). The functional role of endophytic organisms associated with the plant root system accounts for increased productivity (Rosenblueth and Martinez-Romero, 2006; Silva et al., 2009) by promoting plant growth (Payne et al., 2006; Mehnaz, 2011).

After nitrogen, phosphorus (P) is the second element that most often limits plant productivity because it is an essential constituent of both the structural components of cells, such as nucleic acids and membrane phospholipids, and of mobile storage units of metabolic energy, such as ATP (Almeida et al., 2009). Phosphorus deficiency limits agricultural production, especially in acidic soils such as the Brazilian savannah Nakayama et al., 1998). In this biome, the water-soluble P is transformed in to iron phosphate ($FePO_4$) and aluminum phosphate ($AlPO_4$) (Silva et al., 2011), which are

List of non-standard Abbreviations

IAA - Indoleacetic Acid; BCG - Bromocresol Green; SI - Solubilization Index; OD - Optical Density; PSMs - Phosphate-solubilizing Microorganisms.

*Corresponding author E-mail: lu.vitorino@hotmail.com; Tel: (64) 3620 5600, Fax: 55(64) 36205616

Moderately soluble complexes (Yadav et al., 2010). Some soil microorganisms solubilize these unavailable forms of inorganic P (Son et al., 2006; Chai et al., 2011). Recent studies have confirmed that endophytic microorganisms possess this capacity. In arbuscular mycorrhizal associations, plants acquire phosphate through a wide network of extra-radical fungal hyphae, which extend beyond the root depletion zone to the region of the soil where this mineral is found (Harrison et al., 1995).

Studies on phosphate solubilization typically evaluate the ability of both edaphic and endophytic microbiota to solubilize the Ca-P complex, and the results reflect only the environment of alkaline soils (Gadagi and Sa, 2002). Some microorganisms have the capacity to solubilize Fe-P and/or Al-Pas well, representing a more relevant analysis in the context of tropical soils (Chagas Jr et al., 2010).

There are several reports on the capacity of endophytic organisms to synthesize phytohormones (Gray and Smith, 2005). Indoleacetic acid (IAA) is known for triggering both rapid responses (cell elongation) and slow responses (cell division and differentiation) (Dobbelaere et al., 2003). This plant hormone is commonly produced by growth-promoting bacteria, such as *Aeromonas veronas, Agrobacterium* sp., *Azospirillum brasilense, Bradyrhizobium* sp., *Rhizobium* sp. And *Enterobacter* sp. (Vessey, 2003). El-Khawas and Adachi (1999) showed that *A. brasilense* (ATCC 2970) and *Klebsiella pneumonia* (ATCC 13883) have the potential to produce IAA under laboratory conditions. Moreover, they verified that the amount of plant hormone produced could be modulated *in vitro*, which is a characteristic that is strategic for promoting plant growth.

Several species of the genus *Hyptis* (Lamiaceae) are present in the Brazilian savannah and have distinct features, including medicinal properties and pharmacological relevance (Arrigoni-Blank et al., 2008; Coutinho et al., 2009). The species *Hyptis marrubioides* Epling is a medicinal plant that is native to this region (Rodrigues and Carvalho, 2001). Due to the absence of data on the biotechnological potential of the endophytic microbiota of *H. marrubioides* Epling, this study aims to identify the functional features (Friezer et al., 2011) of endophytic isolates of this species. Specifically, we seek to characterize the capacity of these strains to solubilize Ca and Fe phosphates in solid culture medium and to synthesize IAA *in vitro*.

MATERIALS AND METHODS

Evaluation of the capacity of endophytic organisms to solubilize $CaHPO_4$

Bacterial isolates obtained from the roots of *H. marrubioides* Epling and stored at the culture collection of the Laboratory of Biotechnological Microbiology and Phytopathology at the Goiano Federal Institute (IF Goiano) – Rio Verde Campus were clipped from storage flasks (penicillin flasks) and grown in nutrient broth (3 g of beef extract, 5 g of peptone and H_2O to obtain 1000mL of broth) over night at 30ºC with agitation. Petri dishes containing modified GELP medium (without addition of soil extract) were inoculated with 5µL of each culture as described by Sylvester-Bradley et al. (1982).$CaHPO_4$ was obtained by adding 1 mL of a 5% K_2HPO_4 solution and 1 mL of a 10% $CaCl_2$ solution to10 mL of medium. For fungal isolates, mycelial discs approximately 0.5 cm in diameter were activated in potato dextrose agar (PDA) for 7days at room temperature. Activated discs were distributed among petri dishes containing GELP medium (one disc per dish) and incubated in a bacteriological incubator at 30ºC for 15 days.

Evaluation of the capacity of endophytic organisms to solubilize $FePO_4$

The bacterial culture inocula and fungal culture discs grown as described above were transferred to dishes containing the basal medium described by Reyes et al. (1999) and modified by Gadagi and Sa (2002). Bromocresol green (BCG) was used as a pH indicator as follows: 5 mL of a BCG stock solution (0.5% BCG in 70% ethanol, adjusted to pH 6.5 with 1 N KOH) was added to 1 L of Reyes medium. Dishes were incubated in a bacteriological incubator at 30ºC for 15 days.

Evaluation of $CaHPO_4$ and $FePO_4$ solubilization

The evaluation of Ca and Fe phosphates in solid medium occurred through the 15th day of incubation. The diameters of the solubilization zones (halos) around the colonies were measured every 5days, and at the end of 15 days, the Solubilization Index (SI) was calculated using the following formula (Berraqueiro et al., 1976):

$$SI = \frac{\text{Halo diameter (ØH)}}{\text{Colony diameter (ØC)}}$$

For the fungal strains tested, the SI was calculated on the 5th day of incubation, before the colonies filled the entire surface of the dish. The solubilization potential of the strains was classified according to Silva Filho and Vidor (2000): low (SI < 2), medium (2 < SI < 3) or high solubilization capacity (SI > 3).

Solubilization capacity was analyzed in quadruplicate for each of 48 endophytic strains previously isolated from the roots of *H. marrubioides* Epling and 42 bacterial and six fungal strains for each phosphate tested. Data on the development of solubilization halos were subjected to

Table1. Solubilization of $CaHPO_4$ and $FePO_4$ and the production of IAA by root endophytic microorganisms of *H. marrubioides* Epling.

	Isolate code	SI_{CaHPO4} = ØH/ØC	SI_{FePO4}=ØH/ØC	(IAA) (µg·mL^{-1} of culture)
Bacterial endophytic strain	RG1	1.35^{a1}	NS	0.77^{u2}
	RG2	NS	5.75^{b}	0.60^{v}
	RG3	NS	NS	0.00^{z}
	RG4	NS	0.32^{c}	0.00^{z}
	RG5	NS	5.10^{b}	2.03^{o}
	RG6	NS	NS	0.00^{z}
	RG7	NS	6.80^{a}	4.81^{h}
	RG8	0.35^{bC}	8.48^{aA}	7.35^{f}
	RG9	0.27^{bC}	6.67^{aA}	39.28^{b}
	RG10	NS	0.30^{d}	1.17^{t}
	RG11	NS	NS	3.67^{l}
	RG12	NS	0.30^{d}	4.42^{i}
	RG13	NS	NS	1.88^{p}
	RG14	0.40^{bc}	NS	2.13^{n}
	RG15	0.32^{bC}	5.00^{bB}	0.00^{z}
	RG17	NS	NS	0.00^{z}
	RG18	0.52^{bC}	3.20^{cB}	0.00^{z}
	RG19	1.82^{a}	NS	5.53^{g}
	RG20	NS	NS	4.13^{j}
	RG21	NS	0.40^{d}	0.00^{z}
	RG22	NS	NS	0.00^{z}
	RG23	1.49^{aC}	4.30^{bB}	0.00^{z}
	RG24	NS	4.10^{b}	11.96^{d}
	RG25	NS	0.50^{d}	1.35^{s}
	RG26	0.47^{b}	NS	0.00^{z}
	RG28	NS	NS	0.00^{z}
	RG33	NS	NS	0.00^{z}
	RF1	NS	NS	1.38^{r}
	RF2	NS	1.90^{c}	0.00^{z}
	RF3	NS	6.90^{a}	10.21^{e}
	RF4	NS	NS	3.35^{m}
	RF5	NS	5.8^{a}	0.00^{z}
	RF6	NS	NS	0.00^{z}
	RF7	NS	NS	0.00^{z}
	RF8	NS	2.4^{c}	0.51^{y}
	RF9	0.54^{bC}	4.60^{bB}	0.00^{z}
	RF10	NS	NS	0.00^{z}
	RF11	NS	NS	0.00^{z}
	RF13	NS	NS	16.21^{c}
	RF15	NS	NS	0.00^{z}
	RF18	0.35^{bC}	4.90^{bB}	95.13^{a}
	RF19	NS	0.40^{c}	1.53^{q}
Fungal endophytic	RG16	NS	3.00^{c}	NT
	Trichoderma sp.	NS	3.50^{b}	NT
	RG30	NS	3.30^{c}	NT
	Papulaspora sp.	NS	4.00^{b}	NT
	Fusarium sp.	NS	1.20^{d}	NT
	RF20	NS	3.50^{b}	NT

[1]Lowercase letters compare rows, and upper case letters compare columns. Means followed by the same letter do not differ from each other by the Scott-Knott test (5%). [2]Means followed by the same letter do not differ from each other by the Tukey test (5%). NS = did not form solubilization halos; NT = strains not tested.

analysis of variance, and means were compared by the Scott-Knott test (5%) using the statistical software SISVAR (Ferreira, 2000).

Quantification of IAA biosynthesis

IAA production was assessed exclusively for the 42

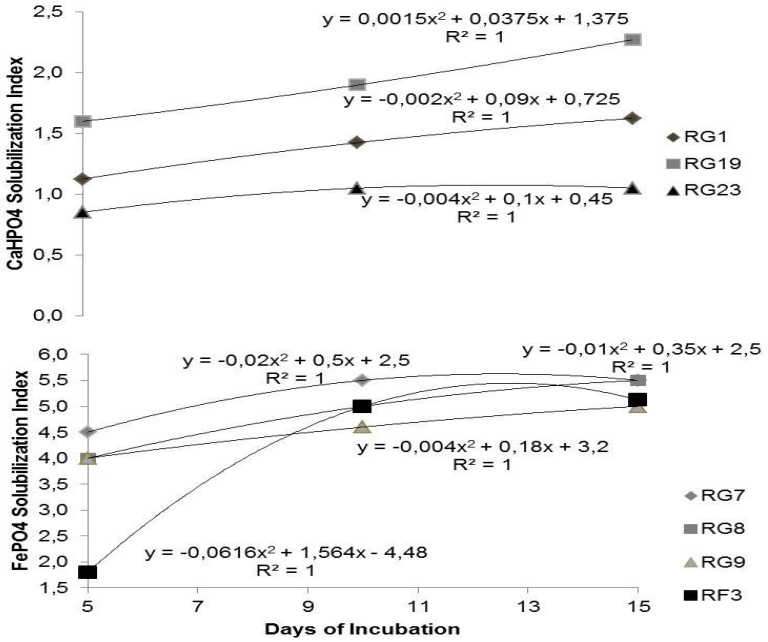

Figure 1. (A) Progression of the $CaHPO_4$ Solubilization Index in GELP medium by endophytic bacterial strains isolated from *H. marrubioides* Epling, which demonstrated an SI > 1 on the 15th day of evaluation. (B) Progression of the $FePO_4$ Solubilization Index in Reyes medium by endophytic bacterial strains isolated from *H. marrubioides* Epling, which reached an SI > 6 on the 15th day of evaluation.

Bacterial isolates from *H.marrubioides* Epling by the colorimetric method described by Gordon and Weber (1951). Initially, the bacteria were grown in 10 mL of DYGS culture medium (Rodriguez Neto et al., 1986) for 24h at 30ºC with constant agitation (160 rpm). We aseptically collected 3 mL of these cultures to determine the optical density (O. D.) at 600 nm. All samples were diluted with a 0.85% saline solution to normalize the O. D. Subsequently, DYGS medium supplemented with 100 $\mu g \cdot mL^{-1}$ of tryptophan was inoculated with 50 µL of each of the suspensions at an O. D. of 0.1. The cultures were incubated at 30°C for 72 h with constant agitation (160 rpm) prior to quantification of IAA production.

Quantification of IAA production by bacterial isolates

After 72h, the samples grown in DYGS medium supplemented with tryptophan were centrifuged for 10 min at 10,000 x g and 10ºC. Next, 1 mL of the supernatant of each sample was transferred to a test tube, and 2 mL of Salkowski reagent (1 mL of 135 $mg \cdot mL^{-1}$ $FeCl_3$ and 50 mL of 35% $HclO_4$) was added. The tubes were maintained in the dark for 30 min (Hartmann *et al.*, 1983). The presence of hormone was visualized as a pinkish color and quantified on a spectrophotometer at 530 nm. All measurements were performed in triplicate. DYGS culture medium with tryptophan was used as a negative control. The IAA concentration was determined using a standard curve equation, and the means were compared using the Tukey test with a 5% probability.

RESULTS AND DISCUSSION

The phosphate-solubilizing microorganisms (PSMs) formed a zone of clearance that was easily visible in the opaque medium, as was described by Kang et al. (2002). Among the microorganisms tested for $Ca_3(PO_4)_2$ solubilization, slightly greater than 20% of the strains exhibited a solubilization capacity in GELP medium (Table 1), and for all halo-forming strains, the solubilization capacity was characterized as low (SI < 2) (Figure 2). These results are lower than those obtained by Pedrinho et al. (2010), who observed that after 15 days of culture, all 27 isolates formed zones of clearance with diameters ranging from 12 to 55 mm. In a study by Assumpção et al. (2009), the solubilization of $Ca_3(PO_4)_2$ by 62 endophytic bacterial isolates from soy seeds was evaluated; of these, 39% showed solubilizing activity, with halos ranging from 0.2 to 8.3 cm.

The formation of halos in GELP medium by bacterial strains was assessed every 5days and analyzed for strains with an SI > 1, which exhibited quadratic behavior (Figure 1A). An SI > 1
was used as a reference because all Sis obtained in this

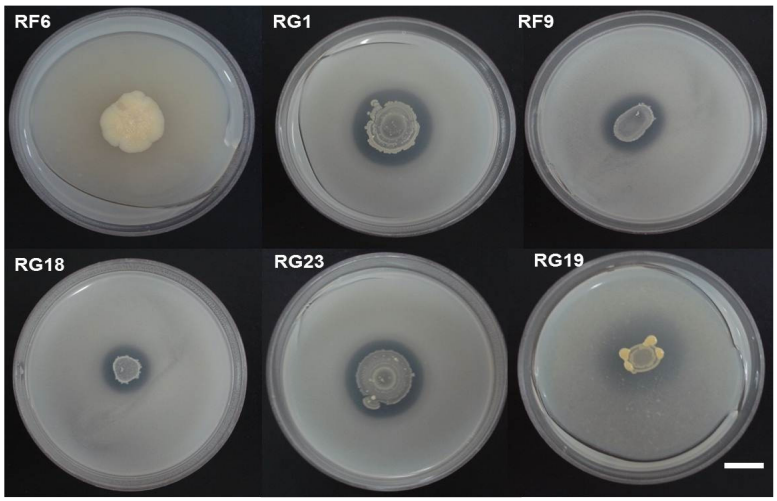

Figure 2. Dishes showing the variation in $CaHPO_4$ solubilization halos formed by different root endophytic bacterial strains of *H. marrubioides* Epling. Bar = 1 cm.

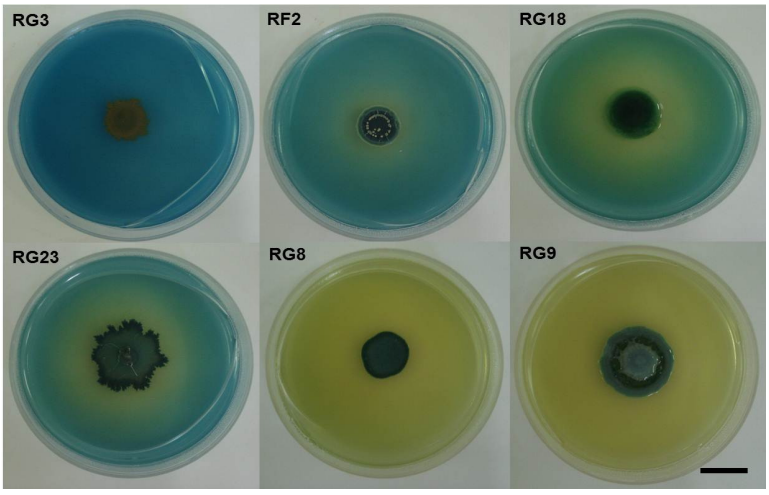

Figure 3. Dishes showing the variation in $FePO_4$ solubilization halos formed by different root endophytic bacterial strains of *H. marrubioides* Epling. Bar = 1 cm.

assay were low, and those between 1 and 2 were more significant.

None of the fungi tested solubilized $CaHPO_4$, which may be related to the fact that these fungi were isolated from the root system of a savannah-native plant, i.e., a plant adapted to acidic soil, in which P is bound to Fe^{3+} and to Al^{3+} (Khan et al., 2009). Therefore, it is possible that the functional feature of these fungi is related to the solubilization of $FePO_4$ or $AlPO_4$ but not of $CaHPO_4$. According to Illmer et al. (1995), the MSF strain utilizes biochemical strategies to solubilize inorganic P forms to soluble forms, which can occur via the production of organic acids or by a mechanism involving microbial growth and the promotion of proton (H^+) secretion. Moreover, Yadav et al. (2010) isolated a high-affinity phosphate transporter with the root endophyte *Piriformospora indica*, demonstrating that the presence of this fungus is associated with P supply to the root.

In contrast to the results from the investigation of $CaHPO_4$ solubilization, greater than 57% of the microorganisms solubilized $FePO_4$, forming visible halos and altering the pH of the medium, which appeared as a color change by the indicator dye from blue to yellow (Figure 3). In a study performed with *Aspergillus niger*, Yuan et al. (2005) revealed that the significant reduction in pH results from the production of organic acids, as a consequence of sugar consumption by the microorganism. Bizukojc and Ledakowicz (2004) and

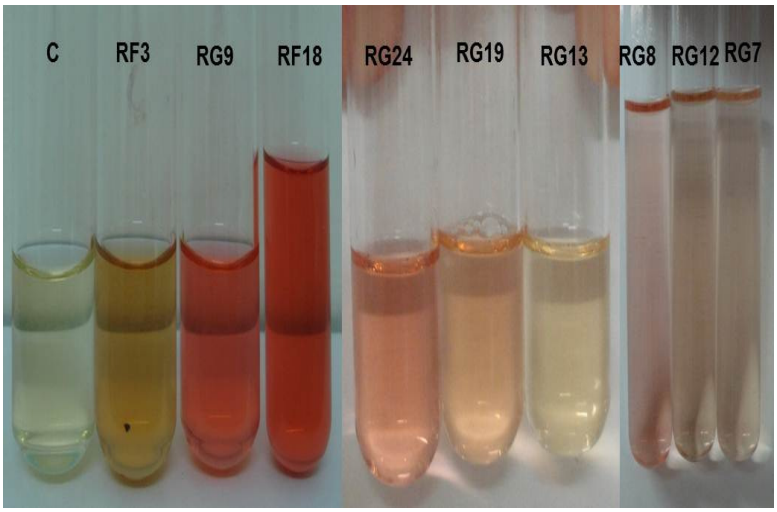

Figure 4. Colorimetric quantification of indoleacetic acid production by endophytic bacterial isolates of *H. marrubioides* Epling in DYGS medium supplemented with tryptophan. (C) Control – DYGS medium in the absence of bacteria.

Barroso and Nahas (2008) also associated the acid-producing activity of rhizosphere microorganisms with the decrease in the pH of the culture medium. These organic acids act by chelating the cations complexed to inorganic phosphate to release soluble phosphate (Whitelaw et al., 1999), which favors the availability of P to plants.

Among the $FePO_4$-solubilizing microorganisms, 59% demonstrated a high SI (SI > 3), 11% exhibited a medium SI (2 < SI < 3), and 30% showed an SI < 2. The development of halos formed by the solubilization of $FePO_4$ by bacterial strains was evaluated every 5days, and the strains with an SI > 6 and a quadratic behavior were analyzed (Figure 1B). All of the fungi tested, including *Papulaspora* sp., *Trichoderma* sp. and *Fusarium* sp., solubilized $FePO_4$.This finding disagrees with data reported by Chai et al. (2011), who isolated phosphate-solubilizing microorganisms from Chinese soil samples and found a *Penicillium* sp. with a high capacity for solubilizing rock phosphate and $AlPO_4$; however, this strain did not solubilize $FePO_4$. Rawat and Tewari (2011) explored the ability of *Trichoderma* sp. To solubilize phosphate and also found a $FePO_4$-solubilizing strain. For bacteria that solubilized both $CaHPO_4$ and $FePO_4$, the zones of clearance formed in Reyes medium were always larger than those formed in GELP medium (Table 1).

The high percentage of $FePO_4$-solubilizing endophytic microorganisms found in the roots of *H. marrubioides* Epling contrasts with several recent studies that demonstrate un satisfactory $FePO_4$ solubilization compared with other phosphates. For example, *Pantoea agglomerans*, a soy rhizosphere bacterium, solubilized hydroxyapatite, $CaHPO_4$ and $Ca_3(PO_4)_2$ more efficiently than $FePO_4$ and $AlPO_4$ (Son et al., 2006). According to Gibson and Mitchell (2004), ericoid mycorrhizal fungi solubilized $CaHPO_4$, but not $FePO_4$ or $AlPO_4$. However, recently, isolates of *A. niger* obtained from soil samples exhibited a significant capacity to solubilize $FePO_4$ in the culture medium (Barroso and Nahas, 2005; Barroso and Nahas, 2008).

Several studies have verified the existence of coevolution among endophytic species and their hosts (Barrett, 1983; Thompson, 1989; Schardl, 1997). The balance between environmental niches requires co-adapted species, and symbiotic relationships also require individuals that have coevolved that exhibit intrinsic features (Schardl, 1997), whether morphological, ecophysiological, biochemical or demographic. The reduced availability of P in potentially productive acidic soils such as the Brazilian savannah requires plants to associate symbiotically with bacteria and fungi that are adapted to those soil conditions (Zapata and Axmann, 1995) and that alter complexes that adsorb phosphate under acidic conditions, including $FePO_4$ and $AlPO_4$ (Andrade et al., 2003). Symbiotic coevolution between the endophytic microbiota and *H. marrubioides* Epling may explain the large number of iron phosphate-solubilizing organisms isolated from its roots. The fact that this plant is native to the Brazilian savannah provides justification for the high capacity for $FePO_4$solubilization evidenced by their endophytes. According to Khan et al. (2009), root-colonizing *Pseudomonas* has a Fe-collecting system based on the release of siderophores with a high affinity for this cation. Moreover, carboxylic anions can substitute for PO_4^{3-}, chelating both Fe^{3+} and Al^{3+} and thus releasing PO_4^{3-} to make it available to plants. Among the 42 bacterial strains examined for IAA biosynthesis, 52% demonstrated the capacity to produce this auxin.

However, these strains differed with respect to the percentage of phytohormone production in DYGS medium supplemented with tryptophan (Table 1). Zaharova et al. (1999) reported that 80% of the bacteria isolated from the rhizosphere are capable of producing IAA. Tryptophan functions as a physiological precursor for auxin biosynthesis in plants and microorganisms (Contreras et al., 2010), and the enzyme IpdC (indole-3-pyruvate decarboxylase – EC 4.1.1.74) is essential for the biosynthesis of this phytohormone (Kochar and Srivastava, 2011).

In particular, the strains RF18, RG9, RF13 and RG24 synthesized significant quantities of IAA under the conditions tested (95.13, 39.28, 16.21 and 11.96 $\mu g \cdot mL^{-1}$, respectively; Table 1). Kuss et al. (2007) observed values between 2.79 and 13.47 $\mu g \cdot mL^{-1}$ from endophytic diazotrophic bacteria in DYGS medium. Crozier et al. (1988) evaluated the production of IAA by isolates of *A. brasilense* originating from *Zea mays* and obtained values between 1.4 and 26.1 $\mu g \cdot mL^{-1}$ in cultures incubated for 24h at 32°C. Mascarua-Esparza et al. (1988) detected IAA production levels ranging from 6.5 to 77 $\mu g \cdot mL^{-1}$ by various cactus root endophyte isolates.

From a biotechnological perspective, the strains RG9, RG24 and RF18 demonstrated a significant potential for $FePO_4$ solubilization and IAA production. Further investigation is required to identify these strains, to quantify the solubilization we presented in this study in liquid culture medium and to determine the capacity of these isolates to promote plant growth, with the goal of using them as inoculants for acidic soils.

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