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PRODUCTION OF INDOLE-3-ACETIC ACID BY BACILLUS ISOLATED FROM DIFFERENT SOILS

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Abstract

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Bacillus sp. can be found in different environments, produce endospores and being known as Plant Growth Promoting. The aim of this study was to evaluate the ability of *Bacillus thuringiensis* and *Bacillus cereus* isolated from soil samples from various regions in the State of Tocantins, in the production of auxin. Both in the absence and in the presence of L-tryptophan all bacteria tested produced auxin. The production of auxin increased with the increasing of the concentrations of L-tryptophan, especially the isolates UFT Bt 07, UFT Bc 19 and UFT Bc 20, which presented the highest levels of production. Twenty isolates, 12 (60%) had concentrations of auxin more than 100 μg mL^{-1}. For most of the isolates the increased production of auxin occurred with 48 hours of bacterial growth. Bacilli tested have great ability to biosynthesize auxin from tryptophan as a precursor; can be considered as probable growth promoters of plant.

Key words: Plant growth promoting rhizobacteria (PGPR), phytohormones, Soil biology, indole-3-acetic acid, L-tryptophan

Introduction

Several bacteria of the genus *Azospirillum* spp, *Alcaligenes faecalis*, *Klebsiella* sp, *Enterobacter* sp, *Xanthomonas* sp, *Herbaspirillum seropedicae*, *Rhizobium* spp *Bacillus* spp and *Bradyrhizobium* spp has been listed as Plant Growth Promoting Rhizobacteria (PGPR) (Patten and Glick, 1996). The modes of action of PGPR are diverse; therefore, the growth may be favored by them both directly and indirectly. The promotion of growth is direct when the micro-organisms facilitate the uptake of certain nutrients in the soil, solubilizing minerals such as phosphorus and making it available to the plant (Rosas et al., 2006) or when they produce phytohormones, such as, indole-3-acetic acid (IAA) (Chagas-Junior et al., 2009; Patten and Glick, 2002).

The genus *Bacillus* sp. stand out as one of the main genus of PGPR used to promote of plant growth. The Bacillus has its importance in plant growth and also in disease control. *Bacillus thuringiensis* is potential to control plant parasitic nematodes in crop plants, for the control of insect vectors of diseases, production of bioinsecticide (Silva et al., 2011; Soccol et al., 2009) for potential for use in agriculture.

The auxin, IAA, is the most abundant phytohormone and also can be synthesized by various microorganisms. The IAA can synthesize both from tryptophan (Trp-dependent pathways) and from a Trp precursor but by passing Trp (Trp-inde-

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pendent pathways). Despite progress in identifying enzymes in Trp-dependent IAA biosynthesis, no single IAA biosynthetic pathway is yet defined to the level that all of the relevant genes, enzymes, and intermediates are identified (Batel et al., 2001). The L-tryptophan can be converted by IAA for two biosynthetic routes. The first route involves the Tryptophan deamination by the enzyme tryptophan transaminase, forming indol-pyruvic acid, followed by decarboxylation by the indolpiruvato decarboxylase indole-3-acetaldehyde (AIAld), which is converted by AIA indolacetaldehyde dehydrogenase. The second biosynthetic route involves decarboxylation of tryptophan by forming tryptophan decarboxylase tryptamine, which is subsequently converted to the amine oxidase AIAld and finally by IAA AIAld dehydrogenase (Salisbury and Ross, 1992; Batel et al., 2001).The main effect of IAA is to promote growth of roots and stems, through stretching of the newly formed cells in the meristem. This effect depends, however, the concentration of the hormone, in some tissues the IAA control cell division (Taiz and Zeiger, 2004).

The aim of this study was to evaluate the production capacity of auxin in bacterial of Bacillus species isolated and identified from soil samples from different regions in the State of Tocantins, and check the effect of supplementation with different concentrations of L-tryptophan.

Materials and Methods

Isolation of bacteria from samples of soil

The 20 bacterial isolates evaluated in this study were obtained from soil samples from the Laboratory of Soil Chemistry Analysis, Campus of Gurupi of the Federal University of Tocantins (UFT), from different localities in the State of Tocantins. In this procedure, the methods used to obtain the isolated were the same developed by (Monnerat et al., 2000), where each sample of 1 g of soil was homogenized in 10 mL of saline solution (0.006 mM $FeSO_4.7H_2O$, 0.01 $CaCO_3.7H_2O$ mM, 0.08 mM $MgSO_4.H_2O$, 0.07 mM $MnSO_4.7H_2O$, 0.006 mM $ZnSO_4.7H_2O$, pH 7.0) and placed in a growth period of 24 h. An aliquot of 1 mL was transferred to microcentrifuge tubes and, after heat shock (80°C for 12 minutes) to kill vegetative cells, it was diluted 10 times in saline solution. An aliquot of 100 µL from the last dilution was distributed in a Petri dish containing nutrient agar (0.5% yeast extract, 0.1% tryptone, 0.17% and 0.15 M NaCl bacteriologic agar). The material was kept in a growth chamber type BOD, 30 ± 0.5°C for 48 h. After growth, bacteria were classified according to the format of the colonies, which were classified as Bacillus isolates by presenting a circular shape and wavy edges, unpigmented, opaque and dense structure (Sosa-Gomes et al., 1998).

Identification of isolates – phase contrast microscopy

The Bacillus isolates strains were cultivated in an incubator NYSM rotating at 200 rpm, 30°C for 48–72 hours (until complete sporulation). The strains were plated in medium supplemented with 100 mg L^{-1} of penicillin. After 48 hours they were seen in phase contrast microscope to observe the presence of vegetative cells, spores and crystals. These procedures were performed to confirm that the strains that were selected were *Bacillus thuringiensis* or *Bacillus cereus* (Crickmore et al., 1998).

Assessment of IAA production

The purified isolated (colonies of 2–3 mm diameter) were transferred, about 10^8 cells mL L^{-1} Erlenmeyer flasks (150 mL) containing 25 mL of culture medium NYSM in the absence and presence of different concentrations of tryptophan (10, 25, 50, 100 and 150 mg L^{-1}).

After four days of growth on a rotatory shaking (100 rpm) at 26 ± 2°C, the bacterial material was separated by centrifugation at 12000 rpm for 15 minutes.

For IAA colorimetric analysis (Gordon and Weber, 1951) it was used part of the reagent Salkowski [($FeCl_3$ 0.5 mol L^{-1} + $HClO_4$ (35%)] and two parts of the obtained supernatant of each isolate. After verification of the qualitative presence of IAA (pink color after 25 minutes of reaction at a temperature of 26 ± 2°C), the phytohormone was quantified by spectrophotometry at 530 nm. The concentrations, in µg mL^{-1} were calculated from a standard curve with known concentrations of synthetic form of the hormone (0–100 µg mL^{-1}), whose readings were the basis for calculating the concentration of IAA in the samples.

Statistical analysis

The data were subjected to analysis of variance and the averages of the treatments were compared by Tukey's test at 5%.

Results and Discussion

We obtained twenty Bacillus isolates from various localities in the State of Tocantins, thirteen strains were characterized as being of *Bacillus cereus* and seven of *Bacillus thuringiensis* according to the methodology used in this study dividing the strains according to the presence and absence of protein crystals by microscopy phase contrast (Crickmore et al., 1998). This method of classification of Bacillus based on the presence or absence of protein crystals is used by several authors (Crickmore et al., 1998; Valicente and Barreto, 2003).

Bacillus cereus and *Bacillus thuringiensis* show phenotypic and biochemical characteristics common, but by definition, *B. thuringiensis* may be differentiated by the presence

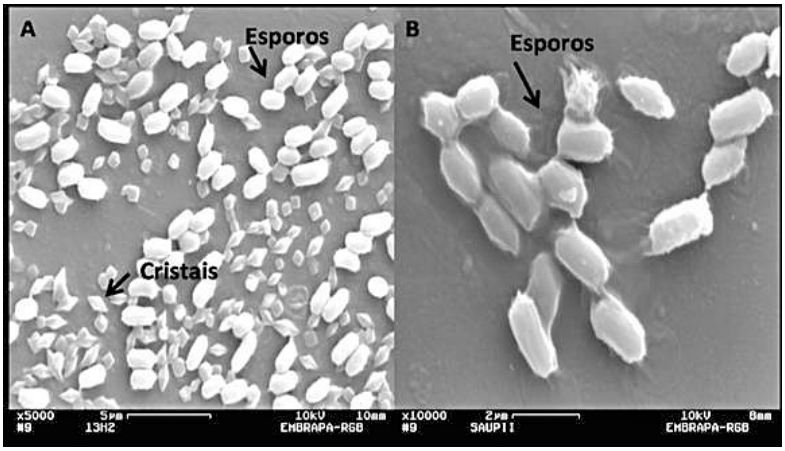

Fig. 1. Phase contrast microscopy of Bacillus:
***A*: Isolated UFT Bt 04 presence of protein crystals and spores (*Bacillus thuringiensis*).**
***B*: The presence only of spores (*Bacillus cereus*)**

Table 1

Production of Bacillus isolates by IAA in culture media in the absence and presence of L-tryptophan after 96 hours of growth period

Isolated	Concentration of L-tryptophan, mg L^{-1}						
	0	10	25	50	100	150	CV**
UFT Bt 01	25[1] a C*	17 f A	53 g A	70 h A	52 g A	71 e A	14.3
UFT Bt 02	34 bc A	26 f A	43 g A	51 h A	44 g A	58 ef A	12.9
UFT Bt 03	69 ab C	72 e C	124 f A	105 g B	127 f A	70 e C	13.6
UFT Bt 04	80 a C	69 e C	103 f B	115 g A	123 f A	93 de B	15.5
UFT Bt 05	39 bc C	159 d B	143 f B	213 f A	153 f B	179 d B	14.3
UFT Bt 06	59 b C	579 b A	556 c A	533 d A	456 d B	409 c B	11.4
UFT Bt 07	91 a C	810 a B	893 ab B	990 b B	1253 a A	1137 a A	11.5
UFT Bc 08	43 bc B	39 e B	51gB	49 h B	75 g A	88 eA	11.4
UFT Bc 09	39 bc A	50 e A	53 g A	30 h A	46 g A	46 efA	15.2
UFT Bc 10	80 a C	69 e C	104 f B	92 g B	115 f A	103 de B	11.1
UFT Bc 11	30 c C	115 d A	88 f B	104 g B	124 f A	91 de B	11.2
UFT Bc 12	10 c C	513 bc A	483 cd A	338 e B	333 de B	475 bc A	13.3
UFT Bc 13	53 b C	278 cd B	273 e B	345 e A	268 e B	368 c A	13.2
UFT Bc 14	30 c C	335 c B	323 e B	398 e B	355 de B	505 b A	12.6
UFT Bc 15	40 bc D	388 c C	470 d B	550 d B	500 cd B	1008 a A	12.1
UFT Bc 16	83 a D	599 b B	573 c B	743 c A	566 c B	533 b B	9.8
UFT Bc 17	59 b C	783 a A	853 ab A	846 bc A	646 c B	609 b B	12.4
UFT Bc 18	97 a B	413 c A	423 d A	437 de A	460 d A	473 bc A	9.9
UFT Bc 19	97 a D	610 b C	993 a A	890 bc B	1043 ab A	1037 a A	10.2
UFT Bc 20	30 c C	757 a B	730 b B	1150 a A	990 b A	1160 a A	11.2
CV**	12,2	14.2	14.5	14.2	12.2	13.2	

* Averages followed by same lower case letter in the column and capital letter on the line do not differ by 5% Tukey's test.
[1] Results in μg mL^{-1}
** C.V. Coefficient of Variation

of crystals (Luthy and Wolfersberger, 2000), visible on phase contrast microscopy (Figure 1). These are the crystals that give the toxicity of this bacterium in the biological control of various insect pests.

Bacteria grown on liquid medium without NYSM and the presence of L-tryptophan, were able to synthesize all IAA. The production of IAA was significantly different isolates and concentrations of L-tryptophan (Table 1). This transfor-

mation can be carried out by microorganisms which produce an oxidative conversion when L-tryptophan is found in the presence of peroxidase and free radicals (Zakharova et al., 1999). This result is in agreement with current work demonstrates that over 80% of the rhizosphere isolates are capable of producing this growth regulator (Khalid et al., 2004).

The production of IAA increased with increasing concentrations of L-tryptophan, especially the isolates UFT Bt 07, UFT Bc 19 and UFT Bc 20, which had the highest averages of production (Table 1). Even in the absence of inducing the synthesis of the phytohormone could be observed, ranging from 10 to 97 µL mL^{-1}, where the isolated UFT Bc 12 produced the lowest concentration and the isolations UFT Bc 18 and UFT Bc 19 had the highest concentration of IAA (Table 1). For the isolates UFT Bt 06, UFT Bc 12 to greater production of IAA occurred at a dose of 10 µL.mL^{-1} L-tryptophan. The same was observed for the concentration of 25 µL.mL^{-1} for isolates UFT Bc 09, UFT Bc 17. On the other hand, the concentration of 50 µL.mL^{-1} isolates UFT Bc 05 and UFT Bt 16 showed the highest values. The isolate UFT Bt 07 showed maximum production of IAA in a concentration of 100 µL.mL^{-1} L-tryptophan, with a decrease in output from this dose. The same result was observed with isolated: UFT Bt 03, UFT Bt 04, UFT Bc 10, UFT Bc 11 and UFT Bc 19. According (Mirza et al., 2001), IAA by the production of microorganisms may vary between different species and strains of the same species.

The culture conditions and substrate stage growth conditions may also cause variations in production. The average of the treatments also showed that there were differences among the bacteria tested. Of the twenty isolates, twelve (60%) showed higher concentration of IAA 100 µg mL^{-1} (Figure 2). These results are superior to those reported by several authors. Assumption (2008), in studies with soybean, found that Bacillus isolates were able to produce IAA production values ranging from 2.6 to 6.5 µg mL^{-1}. Araujo and Guerreiro (2010), evaluating the Bacillus growth promoters in maize (Zeamays) observed the production of IAA ranging from 0.75 to 21.3 µg mL^{-1}. Mena-Violante and Olalde-Portugal (2007), studying the culture of tomato (*Solanum lycopersicum*) found that isolates *Bacillus subtilis* promoted improvements in size, mass and texture of fruit and also the yield per plant, the authors attributed these results to a possible production of hormones by Bacillus. Araújo (2008) inoculated cells *Bacillus* sp. in corn seeds (*Zea mays*), cotton (*Gossypium hirsutum*) and soybeans (*Glycine max*) and found that bacterial inoculation led to an increase seedling emergence of cotton and soybeans, as well as significantly increased the mass of matter dried aerial parts of corn. In works carried out by Vieira and Castro (2001), studying the action of hormones on the germination of soybean seeds (*Glycine max*) found that intermediate concentrations of the hormone increased significantly the germination. It has been found that the strain *Bacillus subtilis* produce phytohormones during its development, which also provided encouragement in developing soybean root (Araujo et al., 2005).

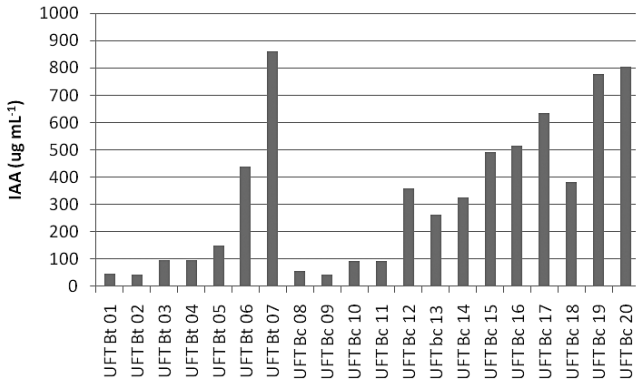

Fig. 2. Average of IAA of each isolate of *Bacillus*

Considering the nutrition of plants, two strains of Bacillus stimulated the growth of plants of *Rubus idaeus* and increased the production due to the P solubilization capacity and IAA production by the bacterium (Orhan et al., 2006). It is a heterogeneous group of bacteria chemotrophic, Gram positive, aerobic or facultative anaerobic. The ability to produce endospores heat resistant is one of the main features of Bacillus, tailoring it to design and marketing, since these microorganisms can be stored for a longer period and may stay longer in the soil (Kokalis-Burelle et al., 2006). The isolation efficient of bacteria depends on some factors of the interaction of plant-soil-climatic conditions (Chagas-Junior et al., 2012).

There are few studies related to quantification of IAA by bacteria of the species *Bacillus thuringiensis* and *Bacillus cereus*. Gomes et al. (2003) demonstrated that both bacteria, *Bacillus thuringiensis* and *Bacillus pumilus*, isolated from the cabbage plant (*Brassica oleracea*), increased the growth of lettuce (*Lactuca sativa*) in the greenhouse. The *Bacillus cereus* is known for its ability to produce gibberellic acid, IAA, zeatin and gibberellins (Karadeniz et al., 2006; John et al., 2005). Furthermore, Tilak et al. (2006) confirmed the involvement of *B. cereus* in promoting the growth of Cajanus cajan (L.) Mill sp., while Bullied et al. (2002) found that this promotes the growth of bacteria soybean (*Glycine max*).

Bacillus sp producers of auxin isolated from roots of *Cattleya walkeriana*, a Brazilian species of orchid, an endangered species, favored increases in all traits and extended the percentage of seedling survival (Galdiano Junior et al.,

2011). Pedrinho et al. (2010), assessing their ability to promote growth Bacillus isolated from the roots of maize (*Zea mays*) found that three isolates had much higher concentrations of IAA to the positive control and the other genera in 48-72 hours of growth. Use of PGPR enriched with P for increasing growth, yield and nodulation of chickpea (*Cicer arientum* L.) both under pot and field conditions (Shahzan et. al., 2008). Five of the seven *Bacillus thuringiensis* bacteria selected exhibited maximum production at two days of growth (Figure 3).

The production of *Bacillus cereus*, eight of the thirteen selected bacteria were able to obtain a maximum yield of the IAA also two days of growth (Figure 4). These results confirm previous studies (Chagas Júnior et al., 2009), under which the maximum production of IAA occurs in the initial growth of microorganisms. Halverson and Handelsman (1991), in studies performed with *Bacillus cereus* UW85, observed that the bacteria increased the nodulation of soybeans, both in a field experiment, as in growth chambers. In field experiments, differences in nodulation were detected in 25 to 35 days after planting, however over the course of the experiment 49 days; these differences were no more observed. These results demonstrate an initial growth promoting effect, but do not effect the long term. However the initial effects of growth can be beneficial in increasing the growth of young plants, helping them to better cope with environmental stresses they will face later during development (Gray and Smith, 2005).

Thus the results indicate that the bacteria tested in this study may be promising in promoting plant growth.

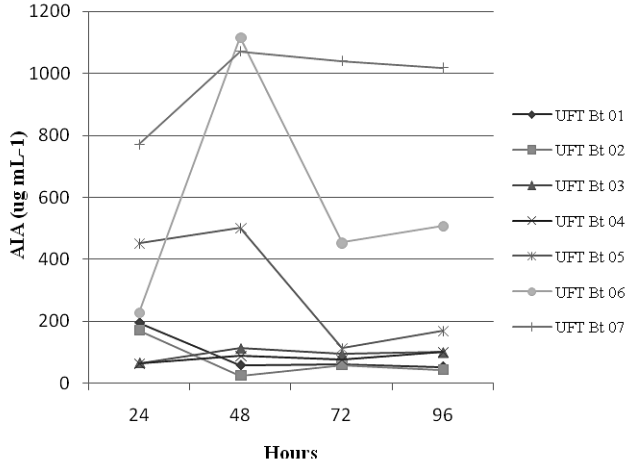

Fig. 3. Production of IAA by *Bacillus thuringiensis*

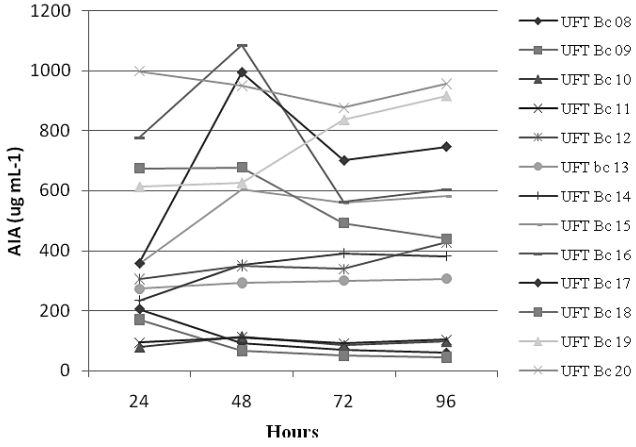

Fig. 4. Production of IAA by *Bacillus cereus*

Conclusion

All Bacillus produced IAA, independent of the concentration of the inductor L-tryptophan;

There was great variation in the production of IAA in different concentrations of L-tryptophan;

In a concentration of 150 mL^{-1} L-tryptophan there was greater production of IAA by bacteria;

For most of the isolates the increased production of IAA was 48 hours with bacterial decrease.

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