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# Phosphate solubilization and phytohormone production by endophytic and rhizosphere *Trichoderma* isolates of guanandi (*Calophyllum brasiliense* Cambess)

**Maíra Paixão Resende[1], Isabel Cristina Mendonça Cardoso Jakoby[1], Luiz Carlos Ramos dos Santos[1], Marcos Antônio Soares[2], Flávia Dionísio Pereira[1], Edson Luiz Souchie[1]* and Fabiano Guimarães Silva[1]**

[1]Instituto Federal Goiano - Câmpus Rio Verde, Cx. P. 66, 75901-970, Rio Verde, Goiás, Brasil.
[2]Universidade Federal de Mato Grosso - Instituto de Biociências. Av. Fernando Corrêa da Costa, 2367, Boa Esperança, 78060-900, Cuiabá, Mato Grosso, Brasil.

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**This work aimed to isolate and evaluate the phosphate solubilization and phytohormone production abilities of endophytic and rhizosphere fungi belonging to the genus *Trichoderma* isolated from guanandi (*Calophyllum brasiliense* Cambess). From the guanandi collected from the field, 12 isolates obtained were grown in potato dextrose broth and supplemented with the phosphate sources, to test their capacity to solubilize phosphates. A strain of *Trichoderma asperellum* from a commercial inoculant was also used. One isolate was able to solubilize calcium phosphate, 12 solubilized iron phosphate and two solubilized aluminum phosphate. Only the rhizosphere isolates were able to synthesize indole acetic acid (IAA) and none of the rhizosphere or endophytic isolates produced cytokinin or gibberellin. There are *Trichoderma* isolates that can benefit plant development, both for their known antagonistic ability against phytopathogenic fungi and for their ability to provide phosphates and or to produce phytohormones.**

**Key words:** *Trichoderma*, inoculants, calcium, iron, aluminum, solubilization, plant growth promotion.

## INTRODUCTION

The guanandi (*Calophyllum brasiliense* Camb. (Clusilaceae) is a tree species native to Brazil and is primarily found in the midwest, north, and southeast regions of the country. The guanandi is notable because it presents some important properties that are useful for reforestation programs, primarily the ability to grow in flooded environments (Oliveira and Joly, 2010). The microbial community, in general, acts cooperatively to enhance plant growth. Thus, the diversity and dynamics of these species is highlighted because plant-microorganism interactions may favor the propagation of plant species of interest (Singh et al., 2011).

*Corresponding author. E-mail: edson.souchie@ifgoiano.edu.br. Tel: (55) 64-3620-5641. Fax: (55) 64-3620-5616.

Fungi belonging to the genus *Trichoderma* are common in soils, consist of a wide diversity of species, and are characterized by different abilities emphasizing the promotion of growth and the development of plants that justify efforts aimed at the isolation and selection of species belonging to this genus, which can be used to increase the production of seedlings (Sofo et al., 2012).

Rhizosphere *Trichoderma* species can be selected for use as inoculants that act in the biological control of phytopathogens and stimulate plant growth and development (Avis et al., 2008). Some strains of this genus can be found in endophytic environment and cause no harm to the host, but enhances their growth and the control of pathogens in plants (Santos and Varavallo, 2011).

Although there are no significant registers of pathogens that seriously attack guanandi plants, the pathogenic fungi *Sclerotium rolfsii* (Ohto et al., 2007) and *Colletotrichum gloeosporioides* (Rosa et al., 2008) were reported in seedlings and older plants. In some *in vitro* tests, *Trichoderma* isolates were efficiently used as biocontrol agents against these pathogens (Sobowale et al., 2010; Bhuiyan et al., 2012).

Root colonization by *Trichoderma* spp. can substantially alter the plant metabolic profile, which reflects the energy supply required for the activation of the defense system and the promotion of plant growth (Brotman et al., 2012). An increase in the fresh and dry weights of the aerial part of the passion fruit tree with the use of *Trichoderma* spp. was observed by Santos et al. (2010).

Phosphate solubilizing microorganisms (PSM) can maximize plant growth by increasing the availability of nutrients, especially phosphorous (P). Plants associated with P-solubilizing microbial species have increased phosphate source use efficiencies because this combination can increase the sustainability of agricultural production in a system through the reuse of P residues that are by products of the microbial metabolism (Shrivastava et al., 2011).

The efficiency of phosphate solubilization depends on the phosphate source and the presence of different sources of carbon (C) and nitrogen for the PSM (Barroso et al., 2006). According to Altomare et al. (1999), *Trichoderma harzianum* was able to solubilize phosphates and promote the development of plants. The contribution of *Trichoderma asperellum* T34 in providing iron to cucumber plants in calcareous soil was demonstrated by Santiago et al. (2013). However, Oliveira et al. (2012) confirmed that all tested fungal isolates from this genus had the ability to solubilize calcium phosphate.

The production of auxins by microorganisms, in some circumstances, can be considered one of the major means through which they promote plant growth and depends on the growth conditions (Dastager et al., 2010). The interaction of a plant species with *T. harzianum* involves systemic modifications of the levels of different phytohormones and may have physiological implications on growth and plant resistance (Medina et al., 2011). Most of the *Trichoderma* genus isolates tested by Oliveira et al. (2012) produced the auxin indole acetic acid (IAA), with or without the L-tryptophan precursor.

This work aimed to isolate and evaluate the phosphate solubilization and phytohormone production abilities of endophytic and rhizosphere fungi belonging to the genus *Trichoderma* isolated from guanandi.

## MATERIALS AND METHODS

Guanandi seedlings were grown in the nursery for 120 days, in tubes containing soil from B horizon of an Oxisol, and collected in the permanent preservation area of the Instituto Federal Goiano - Câmpus Rio Verde, GO. The seedlings were irrigated daily using an automated system. To isolate fungi belonging to the genus *Trichoderma* from the plant in the field, an individual that was located at the following coordinates obtained by using global positioning system (GPS) was chosen: latitude 17° 48' 1.692" S; and longitude 50° 53' 57.0696" W. The root systems, with soil attached, were collected from a plant in the nursery and from a plant in the field, placed in an isothermal box, and taken to the Laboratory of Agricultural Microbiology for processing.

### Isolation and identification of rhizosphere and endophytic fungi

To isolate rhizosphere fungi, ten grams of root fragments with adhered soil were transferred to an Erlenmeyer flask containing 90 ml of peptone water (0.1%). This flask was left shaking (90 rpm) for 1 h to remove the soil from the rhizosphere of the guanandi, and serial dilutions were prepared, followed by the surface plating of the $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions in *Trichoderma* Selective Medium with Captan (TSMC) according to Askew and Laing (1993).

The endophytic isolation roots were washed in running water to remove the adhered soil, and subsequently left shaking with Tween-80 for 3 min, and were rinsed until the complete removal of the detergents was achieved. To eliminate the microbiota on the outer surface of the roots, the roots were treated with alcohol (70%) for 1 min, sodium hypochlorite (2%) for 3 min, 70% alcohol, and sterile distilled water to remove the previous solutions. Intact fragments were selected for further inoculation in selective medium TSMC.

For both rhizosphere and endophytic isolation the plates were incubated at 25°C. After the emergence of colony forming units (CFU) of the fungi, growing colonies were restreaked on Potato Dextrose Agar (PDA) plates. On the 13$^{th}$ day of incubation, the frequency of root fragment colonization was evaluated using the following formula: Frequency of colonization = No. of colonized fragments x 100/No. of total fragments.

The isolates obtained were identified by observation of their morphologic characteristics on PDA, and a micro-culture of each fungus was performed. The ANATIQUANTI program was used to measure the sizes of the spores observed under a microscope (Aguiar et al., 2007).

### Determination of *in vitro* phosphate solubilization ability

Fungi belonging to the genus *Trichoderma* which was isolated from the rhizosphere and root tissues of the guanandi plants were cultured on glucose + yeast extract + peptone (GELP) medium with

$CaHPO_4$ precipitate (10%), according to Sylvester-Bradley et al. (1982), to verify the occurrence of a transparent halo around the mycelium of solubilizing isolates.

The isolates were cultured separately on potato dextrose broth (PDB) supplemented with $CaHPO_4$ (5 g $l^{-1}$), $FePO_4$ (3 g $l^{-1}$), or $AlPO_4$ (1 g $l^{-1}$) and incubated with shaking for 15 days. For the solubilization of $CaHPO_4$ and $AlPO_4$, the strain *T. asperellum* T22, isolated from the commercial inoculant Trichodermax EC®, was also tested.

To quantify the solubilization ability, a calibration curve was created with increasing concentrations of phosphoric acid, using a stock solution (20 mg $ml^{-1}$). From this stock solution 0, 100, 200, 300, 500, 700 or 900 µL was added to distilled water for a final volume of 1.0 ml. Subsequently, 1.0 ml of the working reagent [0.4 g of ascorbic acid; 100 ml of solution 725 (1.0 g of bismuth subcarbonate dissolved in 68 ml of sulfuric acid and added to 300 ml of distilled water, combined with 20 g of ammonium molybdate dissolved in 68 mL of sulfuric acid and added to 300 ml of distilled water) and 900 ml of distilled water] was added to all tubes of the standard curve.

A 1.5 ml aliquot was removed from the cultures for centrifugation at 8000 rpm for 10 min at 4°C. Then, 1.0 ml of the supernatant was transferred to test tubes, and 1.0 ml of the working reagent was added. After stirring and left to stand for 20 min, the phosphate solubilization was determined using a spectrophotometer (725 nm) according to Braga and DeFelipo (1974) modified by Reis et al. (2008).

#### Production of indole acetic acid (IAA)

All isolates evaluated in the phosphate solubilization tests had spore concentrations that were standardized to $10^5$ CFU $ml^{-1}$ by dilution with saline solution (0.9%). The isolates were grown on PDB medium supplemented with tryptophan (1%). Incubation was performed with shaking (90 rpm) for 13 days, in the absence of light.

For the quantitative evaluation, a calibration curve was initially created using a stock solution of IAA (300 mg $ml^{-1}$) that was diluted with distilled water to obtain increasing concentrations (0, 10, 20, 50, 100, 210, 430 and 500 µL $ml^{-1}$).

After centrifugation of the cultures (8000 rpm for 10 min at 4°C), 1.0 ml was removed from the supernatant and transferred to test tubes, and 1.0 ml of Salkowski reagent (0.62 g $FeCl_3.6H_2O$; 33 ml $H_2O$; and 50 ml $H_2SO_4$) was added. The test tubes were allowed to stand for 15 min in the dark, and then, the production of IAA was determined by colorimetric spectrophotometry (530 nm) (Gordon and Weber, 1950; Pereira et al., 2012).

#### Production of cytokinin and gibberellin

To evaluate the production of cytokinin and gibberellin by the *Trichoderma* isolates, the method suggested by Cattelan (1999) was used. Fungi were cultured in PDB medium for 8 days and centrifuged (6000 rpm for 10 minutes at 4°C). Radish seeds were passed through a sieve with 2 mm opening (ABNT 10) and germinated on wet filter paper in Petri dishes. These dishes were incubated at 25°C for 35 h in the dark.

After germination, the hypocotyls and cotyledons were separated and transferred to Petri dishes containing filter paper moistened with the supernatant from each isolate. Specifically, 10 smaller cotyledons and 10 hypocotyls fragments standardized to 3 mm were used. They were then incubated at 24°C for 72 h under continuous weak fluorescent light to compare the biomasses of the cotyledons and the lengths of the hypocotyls with the control, which consisted of filter paper moistened only with sterile medium.

Data were subjected to an analysis of variance, and the results of the phosphate solubilization and IAA, cytokinin, and gibberellin synthesis assays were compared by the Scott-Knott test (5%) using the SISVAR statistical software (Ferreira, 2011).

## RESULTS AND DISCUSSION

### Phosphate solubilization

Thirty (30) rhizosphere and 12 endophytic fungi were isolated from the seedlings of the guanandi plant grown in the nursery, whereas 83 rhizosphere and 18 endophytic fungi were isolated from the seedlings grown in the field. Among the isolates from the plant cultivated in the nursery, none were found to belong to the genus *Trichoderma*, whereas, among the fungal isolates from the guanandi plant collected in the field, 8 isolates belonging to the genus *Trichoderma* were found in the rhizosphere and 4 in the endophytic environment of the root (Figures 1 and 2).

The guanandi grown in the field allow the isolation of a higher density of microorganisms and fungi belonging to the genus *Trichoderma*, likely due to the increased concentration of organic matter and the presence of soil with a greater diversity of plant species than the soil used for growing seedlings in the nursery. Given that the nursery seedlings were grown under high stress conditions, including a low concentration of organic matter, a root system confined to tubes, and the soil of B horizon, a lower density of microbial species was expected.

The frequency of colonization for the root tissue fragments was 47.5% after 13 days of incubation. There was a higher incidence of *Trichoderma* species among the endophytic fungi, with 9.63% of the rhizosphere fungi and 22.22% of the endophytic fungi belonging to the genus *Trichoderma* (Table 1).

In the GELP solid medium, the ability to solubilize $CaHPO_4$ was not observed, due to the rapid growth of the isolates tested. Among the isolates grown in PDB that was supplemented with the phosphate sources mentioned above, 1 isolate was capable of solubilizing $CaHPO_4$, 12 isolates solubilized $FePO_4$, and 2 isolates solubilized $AlPO_4$. Thus, there are *Trichoderma* isolates that can benefit plant development, both for their known antagonist capacity against phytopathogenic fungi and for the solubilizing capacity and/or availability of phosphates.

All *Trichoderma* isolates demonstrated an ability to solubilize $FePO_4$ (Table 2). In the case of $AlPO_4$ solubilization, only isolates EC10M and EC12M demonstrated this capacity. Similarly, the same isolates and isolate RC28M were able to solubilize $CaHPO_4$. *T. asperellum* T211 demonstrated the highest ability to solubilize $AlPO_4$ but was unable to solubilize $CaHPO_4$ (Table 2).

Kapri and Tewari (2010) isolated 14 strains of *Trichoderma* spp. from the rhizosphere of different plant species and found the ability to solubilize phosphates in all of them, in different proportions. The P uptake mechanisms depend on the ability of the microorganism to

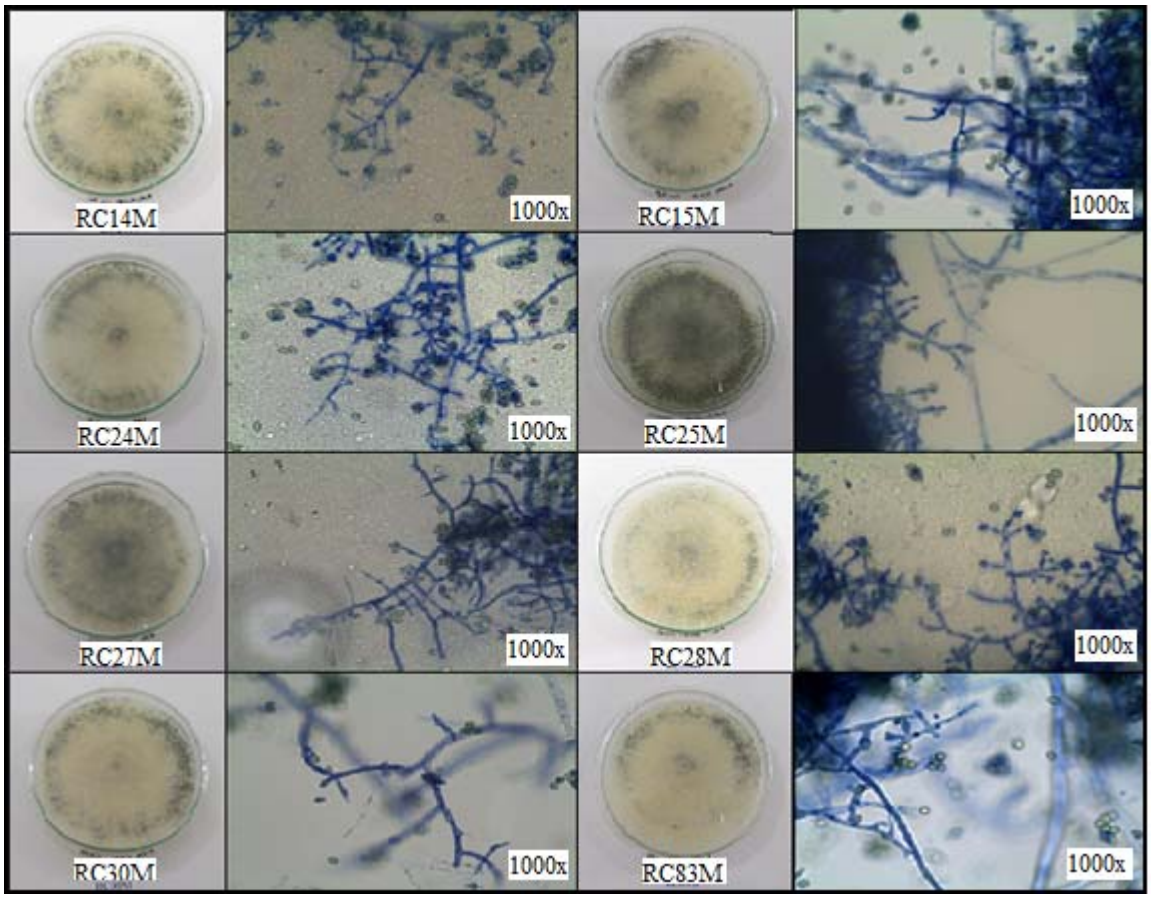


**Figure 1.** Isolates of *Trichoderma* isolated from the rhizosphere of a guanandi plant collected in the permanent preservation area, in Rio Verde, GO, Brazil. Spores: RC14M: L - 25.19 µ, W - 16.41 µ; RC15M: L - 25.07 µ, W - 18.05µ; RC24M: L - 24.47 µ, W - 15.89 µ; RC25M: L - 25.54µ, W - 16.89 µ; RC27M: L - 27.11 µ, W - 15.78 µ; RC28M: L - 26.91 µ, W - 16.94 µ; RC30M: L - 28.58 µ, W - 19.20 µ; RC83M: L - 28.92 µ, W - 17.57 µ (L = length, W = width).

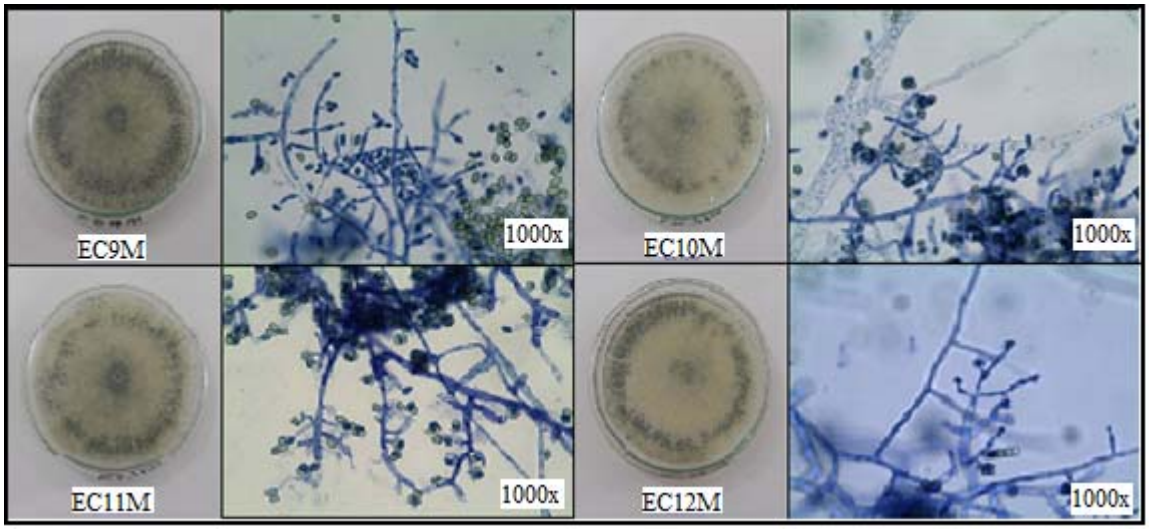


**Figure 2.** Isolates of *Trichoderma* isolated from the root interior of a guanandi plant collected in the permanent preservation area, in Rio Verde, GO, Brazil. Spores: EC9M: L - 28.39 µ, W - 18.81 µ; EC10M: L - 29.33 µ, W - 17.54 µ; EC11M: L - 25.77 µ, W - 16.16 µ; EC12M: L - 25.31 µ, W - 17.53 µ (L = length, W = width).

**Table 1.** Total of rhizospheric and endophytic fungi and *Trichoderma* isolates obtained from the root fragments of guanandi.

| Fungi isolate | Number of fungi isolates | Number of *Trichoderma* isolates | Percentage (%) |
|---|---|---|---|
| Endophytic | 18 | 4 | 22.22 |
| Rhizospheric | 83 | 8 | 9.63 |

**Table 2.** Contents of P solubilized by *Trichoderma* isolates of guanandi on PDB supplemented with calcium phosphate, iron phosphate and aluminum phosphate.

| *Trichoderma* isolates | $CaHPO_4$ (µg $ml^{-1}$) | $FePO_4$ (µg $ml^{-1}$) | $AlPO_4$ (µg $ml^{-1}$) |
|---|---|---|---|
| *RC14M | $4.12^{d}$ | $5.24^{b}$ | $0.73^{e}$ |
| RC15M | $4.39^{d}$ | $5.13^{b}$ | $0.56^{h}$ |
| RC24M | $4.53^{c}$ | $5.52^{b}$ | $0.59^{g}$ |
| RC25M | $4.33^{d}$ | $5.38^{b}$ | $0.41^{i}$ |
| RC27M | $4.21^{d}$ | $5.54^{b}$ | $0.56^{h}$ |
| RC28M | $5.54^{a}$ | $6.09^{a}$ | $0.61^{g}$ |
| RC30M | $4.41^{d}$ | $6.45^{a}$ | $0.58^{h}$ |
| RC83M | $5.43^{b}$ | $5.99^{a}$ | $0.75^{e}$ |
| **EC09M | $5.35^{b}$ | $5.74^{a}$ | $0.67^{f}$ |
| EC10M | $5.67^{a}$ | $5.80^{a}$ | $1.00^{c}$ |
| EC11M | $5.20^{b}$ | $5.67^{b}$ | $0.67^{f}$ |
| EC12M | $5.96^{a}$ | $5.93^{a}$ | $1.61^{b}$ |
| *T. asperellum* | $4.19^{d}$ | ND | $3.17^{a}$ |
| Control | $5.32^{b}$ | $2.92^{c}$ | $0.91^{d}$ |

Means followed by the same letter, in each column, do not differ by the Scott-Knott test (5%). *RC = rhizosphere isolate; **EC = endophytic isolate; ND = not detected.

colonize the plant roots, which demonstrates the importance of the distribution and metabolic activity of the microbial isolates for the preparation of inoculants (Behbahani, 2010).

According to John et al. (2010), soybean plants showed greater shoot and root growth when inoculated with *T. viride*. Phosphate solubilization by microorganisms plays an important role in providing P to the plants. Therefore, other fungal genera, such as *Aspergillus* and *Penicillium*, are equally efficient (Coutinho et al., 2012).

The use of phosphate solubilizing species as biofertilizers is an effective approach to replace or reduce the dependence on chemical fertilizers (Mamta et al., 2010).

The application of *Trichoderma* spp. combined with other microbial species may be a good strategy to increase growth, nutrient uptake and plant yield (Rudresh et al., 2005).

Carvajal et al. (2009) reported the potential to solubilize phosphates in 20% of 101 isolates of *Trichoderma* spp. Badawi et al. (2011) showed that *T. harzianum* had a greater ability to solubilize phosphate when compared with *Bradyrhizobium* spp. and *Serratia marscescens*.

## Production of phytohormones

The capacity for IAA synthesis in PDB medium containing tryptophan was observed in 2 *Trichoderma* isolates obtained from the rhizosphere of guanandi. The production of this phytohormone by the endophytic isolates was not detected (Table 3).

The production of cytokinin and gibberellin was not detected in any of the isolates tested because only differences among the hypocotyls but not among the radish cotyledons were observed for all treatments (Table 4).

Auxins promote the growth of stems and coleoptiles and inhibit root growth (Taiz and Zeiger, 2009). The *Trichoderma* isolates tested by Oliveira et al. (2012) were able to produce IAA, and the use of the L-tryptophan precursor provided a positive effect as an inducer for the synthesis of this phytohormone. Gravel et al. (2007) observed the production of IAA by *T. atroviride*, using the precursors L-tryptophan, tryptamine and tryptophol. The species *T. harzianum* also demonstrated the ability to produce this phytohormone, according to Badawi et al. (2011).

**Table 3.** Indole acetic acid (IAA) contents produced by endophytic and rhizosphere *Trichoderma* isolates of guanandi on PDB supplemented with tryptophan (1%).

| *Trichoderma* isolate | IAA(μgml$^{-1}$) |
|---|---|
| *RC14M | 1.82b |
| RC15M | 1.30c |
| RC24M | 1.46c |
| RC25M | 1.38c |
| RC27M | 1.56c |
| RC28M | 2.18a |
| RC30M | 1.49c |
| RC83M | 1.32c |
| **EC09M | 1.21c |
| EC10M | 1.25c |
| EC11M | 1.24c |
| EC12M | 1.47c |
| *T. asperellum* | 1.36c |
| Control | 1.27c |

Means followed by the same letter do not differ by the Scott-Knott test (5%). *RC = rhizosphere isolate; **EC = endophytic isolate.

**Table 4**. Cytokinin and gibberellin contents produced by endophytic and rhizosphere *Trichoderma* isolates of guanandi.

| *Trichoderma* isolate | Cytokinin cotyledons (g) | Gibberellin hypocotyls (mm) | Gibberellin + cotyledons (g) |
|---|---|---|---|
| *RC14M | 0.050a | 3.625c | 0.050a |
| RC15M | 0.015a | 3.375c | 0.015a |
| RC24M | 0.020a | 3.875b | 0.020a |
| RC25M | 0.050a | 4.750a | 0.050a |
| RC27M | 0.035a | 3.125c | 0.035a |
| RC28M | 0.040a | 5.250a | 0.040a |
| RC30M | 0.035a | 3.500c | 0.035a |
| RC83M | 0.035a | 4.250b | 0.035a |
| **EC09M | 0.050a | 4.000b | 0.050a |
| EC10M | 0.040a | 4.125b | 0.040a |
| EC11M | 0.040a | 4.500b | 0.040a |
| EC12M | 0.035a | 3.000c | 0.035a |
| *T. asperellum* | 0.040a | 4.125b | 0.040a |
| Control | 0.040a | 3.125c | 0.040a |

Means followed by the same letter in each column do not differ by the Scott-Knott test (5%). *RC = rhizosphere isolate; **EC = endophytic isolate.

Following the inoculation of cytokinin-producing microorganisms, increases in the weights of the cotyledons were observed, and for the microorganisms producing gibberellins, increases in the sizes of the hypocotyls and in the weights of the cotyledons were observed (Cattelan, 1999). In addition to playing a role in cell division in the shoots and roots, cytokinins affect other processes, such as vascular development, apical dominance and leaf senescence. The gibberellins stimulate both elongation and cell division (Taiz and Zeiger,

2009).

The *Trichoderma* spp. should be selected for the production of phytohormones, with subsequent use as inoculants to increase the growth of guanandi, given that this is not an ability found throughout the genus.

## Conclusions

The *Trichoderma* isolates evaluated in the present study were able to solubilize phosphates in different proportions and have potential for use as inoculants to increase the formation of guanandi seedlings. The rhizosphere *Trichoderma* isolates RC14M and RC28M synthesized IAA and have potential for use as inoculants to promote the growth of guanandi seedlings.

The rhizosphere *Trichoderma* isolates were more efficient for IAA production than the commercial strain, *T. asperellum* T211.

The endophytic *Trichoderma* isolates did not demonstrate an ability to produce IAA, and none of the rhizosphere or endophytic isolates belonging to this genus produced neither cytokinin nor gibberellin.

## Conflict of Interests

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

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