Chapter 90

Use of Trichoderma species as a growth promoter in mint and basil plants

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ABSTRACT
The use of microorganisms in medicinal plants may provide an increase in biomass. The objective of this work was to evaluate *Trichoderma* as a plant growth promoter, for the capacity of phosphate solubilization and synthesize of indole acetic acid (IAA) *in vitro*, and biomass production of two medicinal plants, basil and mint. Cultures were inoculated with two species of *Trichoderma*. The crop biomass as well as the relative efficiency were determined. *Trichoderma* species that solubilized phosphate and produced IAA, provided significant results in the accumulation of biomass of the crops, with relative efficiency of 276% for mint and 141% for basil, in relation to the control. The *Trichoderma* isolates showed phosphate solubilization capacity and IAA synthesis. Therefore, the studied crops presented biomass increase. These strains verified their capacity as plant growth promoters.

Keywords: Medicinal plants, Biomass, *Trichoderma harzianum*, *Trichoderma asperelloides*

1 INTRODUCTION

The influence of microorganisms on plant development includes the beneficial effects on seed germination, seedling emergence, grain growth, and yield. The use of microorganisms that promote the growth of plants and consequently the increase of agricultural production is one of the alternatives in the reduction of chemical fertilizers as well as environmental risks (MACHADO et al., 2012).

The use of these microorganisms in medicinal plants to increase biomass production is a promising alternative, considering that medicinal plants must be cultivated without using chemical fertilizers and pesticides to not interfere with their active principle.

Fungi of the genus *Trichoderma* are microorganisms that can promote increase in plant growth, positively influence seed germination, and assist in the development and yield of plants. This ability is related to the production of growth-promoting substances, phosphorus solubilization (OLIVEIRA et al., 2012; SILVA et al., 2012) and the synthesis of indole-acetic acid (GRAVEL et al. 2007; OLIVEIRA et al., 2012), as important agents for the production of plants. They also act in the control of diseases and they are inducers of resistance of several plants (CONTRERAS-CORNEJO et al., 2009; SANTOS et al., 2012; SILVA et al., 2012; ASUMING-BREMPONG, 2013; MACHADO et al., 2015).

Therefore, the objective of this work was to evaluate the efficiency of *Trichoderma* fungi, their capacity for solubilization of phosphate, synthesis of indole acetic acid (IAA) *in vitro* and its influence on the increase of the biomass of mint (*Mentha spicata* L.) and basil (*Ocimum basilicum* L.) in a greenhouse.

2 MATERIALS AND METHODS

The experiments were carried out in the Microbiology Laboratory and in a greenhouse at the experimental station of the Federal University of Tocantins (UFT), Gurupi campus, located in the southern region of Tocantins State (Brazil), latitude 11°43'45" S and longitude 49°04'07" W, at 280 m altitude.

Two species of *Trichoderma* were used. *T. asperelloides* (UFT 201) and *T. harzianum* were identified through ITS (Internal Transcribed Spacer) region sequencing, at the Instituto Biológico, São Paulo. The GenBank access number and references are shown in Table 1.

Table 1. *Trichoderma* species identified through the ITS region sequencing, used in the experiments (São Paulo, SP, 2015).

Trichoderma species	GenBank access	Similarity index (%)	Reference
T. harzianum CIB T23	EU279989	100	Hoyos-Carvajal et al. (2009)
T. asperelloides GJS 04-217	DQ381958	100	Samuels et al. (2010)

The *Trichoderma* species were initially inoculated in PDA medium (potato dextrose agar) at 25 ± 2 ° C for seven days to evaluate the phosphate solubilization capacity. Discs of approximately 8.0 mm in diameter were removed from these colonies, containing mycelium and spores, and transferred to Erlenmeyer (250 mL) in modified NBRIP media (NAUTIYAL, 1999) containing the following ingredients (g L^{-1}): 10.0 glucose, 5.0 $MgCl_2.6\ H_2O$, 0.25 $MgSO_4.7H_2O$, 0.2 KCl, 0.1 $(NH_4)_2SO_4$. 50 mL K_2HPO_4 (10%), and 100 mL $CaCl_2$ (10%) were added to medium for the formation of insoluble precipitate of calcium phosphate ($CaHPO_4$). The pH was adjusted to 7.0.

The quantitative estimate of phosphate solubilization was performed in three replicates in a completely randomized design. Incubation was performed at 25 ± 2 °C on an orbital shaker at 150 rpm for eight days. The concentration of soluble phosphorus (P) was determined by the colorimetric method of Murphy & Riley (1962), subtracting the soluble P contained in the treatments by the contained in the control sample (culture medium with phosphate and without inoculum). One part of the reagent was used for the assays, 0.5 ml of the filtered sample plus 5 mL of distilled water for each sample. The reaction was carried out for 20 minutes. Then, the soluble P was quantified in a spectrophotometer at 725 nm wavelength. The standard curve for phosphorus (P) quantification was made from monobasic potassium phosphate (KH_2PO_4) and concentrations calculated in µg mL^{-1}.

The species were cultivated in PDA medium as in the evaluation of phosphate for the production of indole acetic acid *in vitro* for seven days. 8.0 mm diameter discs containing the mycelium and fungus spores were transferred to 250 mL Erlenmeyer flasks containing 50 mL of PD (potato and dextrose) medium in the absence (control) and presence of L-tryptophan. The concentration of L-tryptophan used was 100 mg L^{-1}. Three replicates per treatment (isolates) were used in a completely randomized design.

The strains were grown under an orbital shaker (150 rpm) at 26 ± 2 °C for eight days. Then, the fungus mass was separated by centrifugation at 12,000 rpm for 15 minutes. A part of Salkowski's reagent

[$FeCl_3$ 0.5 mol L^{-1} + $HClO_4$ (35%)] and two parts of the supernatant obtained from each isolate were used for the colorimetric analysis of indole-3-acetic acid (IAA) (GORDON & WEBER, 1951). After the qualitative verification of the presence of IAA (pink color after 25 minutes of reaction at 28 °C in the dark), the phytormonium was quantified in a spectrophotometer at 530 nm. Concentrations in µg mL^{-1} were calculated from a standard curve with known concentrations of the synthetic form of the hormone (0 to 100 µg mL^{-1}), which readings were the basis for calculating the concentration of IAA in the samples.

Twenty-four pots were used for the installation of greenhouse experiments. Twelve pots for each culture of mint (*Mentha spicata* L.) and basil (*Ocimum basilicum* L.). Commercial seeds treated with 0.15% Captan (Captan 750) were used. The experiments were performed following the completely randomized design with three treatments and four replicates separately for each specie. The treatments were done with inoculation of *T. asperelloides*, *T. harzianum* (standard) and one control without inoculation.

The pots were filled with commercial substratum (Plantmax) and soil (sieved) classified as a dystrophic Yellow Red Latosol with a medium texture, in a ratio of 1:1. The soil was removed from a depth of 0-20 cm, obtained at the UFT Experimental Station, where the following characteristics were found: 4.0 $cmol_c$ dm^{-3} Ca, 0.9 $cmol_c$ dm^{-3} Mg, 0.1 $cmol_c$ dm^{-3} K, 2.8 mg dm^{-3} P, 0.06 $cmol_c$ dm^{-3} Al, 8.3 $cmol_c$ dm^{-3} CTC, 5.0 $cmol_c$ dm^{-3} sum of bases (S), 61% base saturation (V), pH 5.8 in water, 1.7% organic matter, 79, 5.0, and 16% sand, silt and clay, respectively. P and K - Mehlich extractor 1, Al^{3+}, Ca^{2+} and Mg^{2+} - KCl extractor (1 mol L^{-1}) (EMBRAPA, 1997).

The *Trichoderma* species were grown separately in PDA petri plates and incubated at 25 ± 2 °C with photoperiod of 12 hours for seven days for inoculants preparation (DIANESE et al. 2012). Six 5 mm diameter disks of each strain containing mycelia, spores and PDA medium were removed after growth. The strains were inoculated in polypropylene bags containing 300 g of commercial rice previously autoclaved at 121 °C for 1 hour with 300 mL of distilled water. The bags were grown in a BOD incubator (Bio-Oxygen Demand) at 25 ± 2 °C and photoperiod of 12 hours for seven days. Non-inoculated (control) bags by *Trichoderma* were incubated only with autoclaved rice. Then, every two days the rice cooked and inoculated by *Trichoderma* was revolved to facilitate gas exchange, breakage of mycelial aggregates and increase of sporulation. After the seven days of incubation, 30 g of the colonized rice (inoculant) was removed for each 1.7 L pot filled with substrate and soil and colonized for 7 days for subsequent planting of the seeds of the different cultures. 30 g of the uncolonized rice was used for the control pots.

The concentration of *Trichoderma* used in the experiments was determined by quantification of the number of conidia. 1 g of colonized rice was washed in 10 ml of sterilized water, followed by stirring for 1 min, and subsequent counting of the conidia in Neubauer chamber with the aid of an optical microscope. Mean concentrations of 1 x 10^9 conidia per gram of colonized rice were used.

Five seeds of mint and five of basil were sown in each pot. The thinning was carried out ten days after germination, leaving two plants per pot. After 35 days of planting (DAP), the plants were harvested.

The following biomass evaluations were performed: root length (RL), number of leaves (NL), root dry mass (RDM), dry mass of the aerial part (DMAP), and total dry mass (TDM). The relative efficiency of each treatment was determined using the DMAP data and calculated according to the formula: Relative Efficiency (RE) = (DMAP inoculated by *Trichoderma* / DMAP without inoculant) x 100.

The data were submitted to analysis of variance by the F test and the means of the treatments grouped by the Duncan test at 5% of significance using the statistical program Assistat.

3 RESULTS AND DISCUSSION

The results of phosphate solubilization capacity, pH and indole acetic acid synthesis by *Trichoderma* species are shown in Table 2.

Table 2. Mean values of calcium phosphate solubilization and indole acetic acid synthesis (IAA) in absence (AT) and presence (PT) of L-tryptophan by *Trichoderma* species (Gurupi, TO, 2015)[1].

Species	Calcium phosphate solubilization			IAA synthesis (µg mL^{-1})		
	µg mL^{-1}	% Solub.[2]	pH	AT	PT	% IAA[3]
T. asperelloides	24.76 a	121.7	5.4	2.6 aB	3.9 aA	156
T. harzianum	20.34 b	100	4.5	1.9 bB	2.5 bA	100
Control	0.44 c	-	6.1	0.2 cA	0.2 cA	-
CV (%)	6.7	-	-	7.8	8.1	-

Means followed by the same lowercase letter in the column and the same capital letter in the row do not differ by Duncan's test at 5% probability. [2] Percentage of phosphate solubilization in relation to the standard *T. harzianum*. [3] Percentage of IAA production of the isolates grown in the presence of L-Tryptophan in relation to the standard *T. harzianum*. CV- Coefficient of variation.

T. asperelloides was significantly superior ($p < 0.05$) to *T. harzianum* (standard) for phosphate solubilization, with a mean percentage of solubilization higher than 20% (Table 2).

There was a reduction of pH in the culture media with *Trichoderma asperelloides* and *T. harzianum* in the liquid medium with calcium phosphate (Table 2). The ability of phosphate solubilization by microorganisms may be related to the acidification of the culture medium, due to the decrease of the pH by the release of organic acids in the medium. Reduction of pH in culture medium cultivated by several fungus species was also observed by Vassilev et al. (2006) with *Aspergillus niger*. However, Kapri & Tewari (2010), in an experiment with *Trichoderma* spp., concluded that, although pH reduction for individual cultures occurs up to 48 hours and after acquisition of constancy, soluble phosphate concentrations continue to increase after 48 h. This clearly suggests that pH drop is not the single factor for phosphate solubilization.

Although phosphate solubilization capacity is related to acid production or pH decrease by microorganisms, these factors do not always correlate with the amount of soluble phosphate produced (STAMFORD & NAHAS, 2010). In addition to these factors, fungus growth should be considered as another important factor for phosphate solubilization (BARROSO et al., 2006).

On the other hand, the *Trichoderma* isolates used in this study were able to produce IAA in PD culture medium supplemented or not with L-tryptophan, being higher in the presence of the inducer (Table 2). *T.*

asperelloides presented a significantly higher value ($p < 0.05$) than *T. harzianum* in the presence of the inducer.

The results of root length (RL), number of leaves (NL), root dry mass (RDM), dry mass of aerial part (DMAP), and total dry mass (TDM) of the mint and basil are presented in Table 3.

Tabela 3. Mean values of root length (RL), number of leaves (NL), root dry mass (RDM), dry mass of aerial part (DMAP), and total dry mass (TDM) of the mint and basil inoculated by *Trichoderma*[1] and without inoculation (Gurupi, TO, 2015) [1].

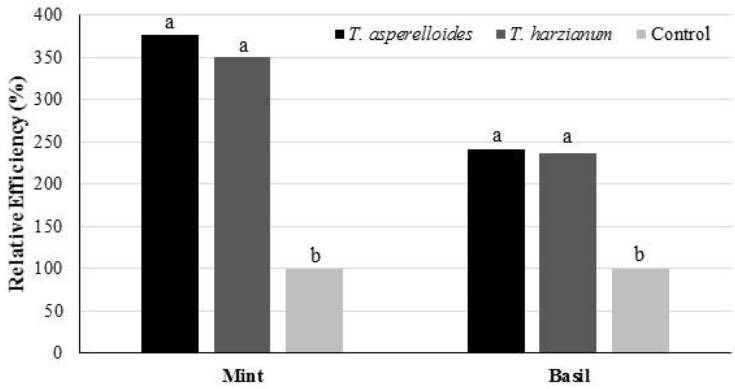
Treatments	RL ( )	NL	RDM ( )	DMAP ( )	TDM
		Mint			
T. asperelloides	26.0 a	7.3 a	2.00 a	2.86 a	4.82 a
T. harzianum	24.5 a	6.9 a	1.95 a	2.66 a	4.61 a
Control	17.7 b	4.0 b	0.74 b	0.76 b	1.41 b
CV (%)	9.4	21.5	32.9	19.6	26.3
		Basil			
T. asperelloides	30.0 a	26.8 a	2.87 a	5.32 a	8.19 a
T. harzianum	29.0 a	24.5 a	2.80 a	5.22 a	8.02 a
Control	29.5 a	21.3 a	1.24 b	2.21 b	3.45 b
CV (%)	9.0	14.5	23.7	15.1	15.7

[1]Means followed by the same lowercase letter in the columns do not differ by Duncan test at 5% significance. CV (%): Coefficient of Variation

Significantly higher values ($p < 0.05$) in treatments with *Trichoderma* inoculation than in control were observed in all traits analyzed for the efficiency of inoculation of the strains in the substrate for growth of mint (Table 3). Higher values ($p < 0.05$) for treatments with inoculation of *Trichoderma* species only for RDM, DMAP and TDM, compared to control were observed for basil. These results verified the positive effects of *Trichoderma* inoculation on growth and induction of resistance to diseases in vegetables, such as Silva et al. (2011) in cucumber plants, Benítez et al. (2004) in tomato, tobacco and cotton and Chacón et al. (2007) in tobacco and tomato.

Figure 1 presents the results of the relative efficiency of the two medicinal plants, which relates the DMAP of the treatments inoculated by *Trichoderma* in relation to the control without inoculation. A significant difference ($p < 0.01$) between treatments was observed. The relative efficiency was higher for treatments with inoculation of *Trichoderma* species for both cultures. The cultures showed an increase in mean above 250% for mint and 140% for basil compared to the control (Figure 1).

Figure 1. Relative efficiency in *Trichoderma*-inoculated mint and basil cultures. Means followed by the same lowercase letter do not differ by Duncan test at 5% significance (Gurupi, TO, 2015).



The production of auxins is one of the mechanisms most frequently presented to explain the effects of *Trichoderma* on plant growth (CONTRERAS-CORNEJO et al., 2009, 2016). Recent studies demonstrate that in the early stages of the *Trichoderma*-plant interaction, metabolites such as auxins and protein compounds released by *Trichoderma* are perceived by the roots, altering multiple hormonal mechanisms that control the growth and development of the plant under normal or stress conditions (BAE et al., 2011; GARNICA-VERGARA et al., 2015; CONTRERAS-CORNEJO et al. 2016). As a result of the colonization of the root system by *Trichoderma*, it could be the protection in the rhizosphere against phytopathogens, as well as a more developed root system, favoring the absorption of nutrients and water (CONTRERAS-CORNEJO et al. 2013, 2015, 2016).

Several authors also suggest the ability to increase plant growth and productivity by *Trichoderma* species by the ability to solubilize mineral nutrients (HARMAN et al., 2004, SILVA et al., 2012; CHAGAS et al., 2015; CONTRERAS-CORNEJO et al., 2016).

The increase in biomass and the promotion of plant growth by the fungus efficiency of the genus *Trichoderma*, as can be observed in this work, have already been reported by several other researchers in the corn (RESENDE, 2004), tomato (GRAVEL et al. 2007), cucumber (SILVA et al., 2011), cowpea (OLIVEIRA et al., 2012), and rice (ASUMING-BREMPONG, 2013, CHAGAS et al., 2015).

This work demonstrates the potential of *Trichoderma* as a plant growth promoter, which may be related to phosphate solubilization capacity and indole acetic acid (IAA) synthesis.

4 CONCLUSIONS

The species of *Trichoderma asperelloides* (UFT 201) and *T. harzianum* presented phosphate solubilization capacity and synthesized IAA.

The inoculation of these *Trichoderma* species provided an increase in the biomass of the mint and basil cultures, cultivated under greenhouse conditions.

The results obtained with the use of *Trichoderma* species in the cultivation of medicinal plants can subsidize the implementation of the agroecological production system in medicinal plants, aiming at optimizing the use of available natural resources with economic and ecological sustainability.

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