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Antagonistic effect of *Trichoderma* isolates and its metabolites against *Fusarium solani* and *F. oxysporum* in chickpea

Efeito antagonista de isolados de *Trichoderma* e seus metabólitos contra *Fusarium solani* e *F. oxysporum* em grão-de-bico

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Brazilian Journal of Development

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ABSTRACT
The aim of this work was to study and select *Trichoderma* sp. strains with biocontrol potential against *Fusarium solani* and *F. oxysporum*, the causal agents of root rot and wilt in chickpea. Antagonism against pathogenic isolates of *F. solani* and *F. oxysporum* of twenty-one isolates of *Trichoderma* sp. obtained from the rhizosphere of chickpea plants were evaluated *in vitro* through competition in dual culture tests as well as the production of volatile and non-volatile organic compounds exhibiting fungicidal and/or fungistatic activity. In the *in vivo* experiment, four isolates of *Trichoderma* sp. were selected and their antagonism was evaluated separately and combined with a commercial product based on *T. asperellum* under greenhouse conditions. *Trichoderma* sp. isolates were efficient competitors and produced metabolites capable of inhibiting mycelial growth of both species of *Fusarium*. Our results show the great versatility of the mechanisms of action from *Trichoderma* isolates, mainly associated with the production of volatile organic compounds. Despite the antagonistic effect of *Trichoderma* isolates observed *in vitro*, these isolates did not control *Fusarium* neither promote chickpea growth in *in vivo* conditions.

Keywords: *Cicer arietinum*, biological control, fusariosis, secondary metabolites.

RESUMO
O objetivo deste trabalho foi estudar e selecionar *Trichoderma* sp. com potencial de biocontrole de *Fusarium solani* e *F. oxysporum*, que causam sintomas de murcha e podridão radicular em grão-de-bico. Vinte e um isolados de *Trichoderma* sp., obtidos da rizosfera de plantas de grão de bico, foram avaliados *in vitro* quanto ao antagonismo a isolados patogênicos de *F. solani* e *F. oxysporum* por meio da competição em testes de cultura pareada e produção de compostos orgânicos voláteis e não voláteis com atividade fungicida e/ou fungistática. No experimento *in vivo*, quatro isolados de *Trichoderma* sp. foram selecionados para avaliar o antagonismo separadamente e combinados em comparação com um produto comercial à base de *T. asperellum* em casa de vegetação. Os isolados de *Trichoderma* sp. foram eficientes competidores e produziram metabólitos capazes de inibir o crescimento micelial das duas espécies de *Fusarium*. Nossos resultados demonstram uma grande versatilidade dos mecanismos de ação dos isolados de *Trichoderma*, principalmente relacionados à produção de compostos orgânicos voláteis. Apesar do efeito antagonista dos isolados de *Trichoderma* observado *in vitro*, os mesmos não controlaram a fusariose e nem promoveram o crescimento do grão-de-bico nas condições *in vivo* estudadas.

Palavras-chave: *Cicer arietinum*, controle biológico, fusariose, metabolitos secundários.

Brazilian Journal of Development

1 INTRODUCTION

Two important diseases caused by *Fusarium* spp. are known in chickpea (*Cicer arietinum*): *Fusarium* wilt, caused by *F. oxysporum* f. sp. *ciceris* and black rot of root, caused by *F. solani*. Both species are present in various countries in the world where chickpea is grown (Nene et al., 2012). In Brazil, Azevedo et al. (2017) have identified strains of *F. solani* and *F. oxysporum* causing root rot in chickpea, with consequent wilting and death of plants.

Due to the survival of the chlamydospores in the soil, these species are difficult to manage by conventional methods, such as chemical control and crop rotation (Singh et al., 2007). The resistance conferred by some cultivars is not durable due to the great variability of *Fusarium* (Jiménez-Gasco et al., 2005). *Trichoderma* spp. have been widely studied as antagonists to plant pathogens, being isolated from the soil and rhizosphere of plants and having great success to control root diseases (Aguiar et al., 2012). Isolates of *Trichoderma* have great versatility of mechanisms of action, acting against other fungi through antibiosis and production of volatile metabolites, mycoparasitism, production of cell wall degrading enzymes and competition for nutrients and substrate (Harman et al., 2004; Vinale et al., 2008; Zhang et al., 2014), they may also act promoting growth and inducing resistance against various pathogens (Harman et al., 2004; Contreras-Cornejo et al., 2009).

Bioproducts based on *Trichoderma* spp. are marketed in several countries around the world (Bettiol et al., 2012). In Brazil, their application by farmers is limited by the reduced availability of bioformulation duly registered with the Ministry of Agriculture, Livestock and Food Supply for crops of economic importance, combined with the lack of identification of new isolates with biocontrol potential and lack of information regarding benefits from using these agents (Machado et al., 2012). Therefore, the prospection of isolates and the assessment of their antagonistic potential against pathogens are the primary steps in the development process of biological products to control plant diseases. Based on this principle, this study aimed to evaluate and select isolates of *Trichoderma* sp., by means of *in vitro* and *in vivo* tests, concerning their antagonistic potential against *F. solani* and *F. oxysporum*, causal agents of root rot and wilt in chickpea.

2 MATERIAL AND METHODS

2.1 ISOLATION OF *TRICHODERMA* SPP.

Soil samples were collected from a commercial chickpea field, in the municipality of Cristalina, state of Goiás, Brazil. Samples were obtained from the rhizosphere of plants of different chickpea genotypes showing fusariosis symptoms (yellowing, wilting and discoloration of the vascular tissue). The isolation of *Trichoderma* was performed using the serial dilutions technique using 25 g of each soil sample which was homogenized in 225 mL of sterile distilled water (SDW)

Brazilian Journal of Development

(dilution 10^{-1}). Serial dilutions were performed until the dilution 10^{-4}, from which aliquots of 100 µL were spread in Petri dishes containing Potato Dextrose Agar (PDA, Sigma Aldrich), and incubated at 25°C. The typical colonies of *Trichoderma* were transferred to Petri dishes containing the same culture medium, obtaining pure colonies of the isolates from the chickpea genotypes: CNPH 0233 (T1, T2, T6, T15), CNPH 0334 (T3, T4, T12, T13, T14, T20, T21), Cicero (T5, T10, T11, T16, T19) and Jammu 96 (T7, T8, T9, T17, T18). The morphologic characteristics of each isolate were examined to confirm the genus. From the pure cultures achieved, monosporic cultures were obtained and preserved in SDW (Castellani, 1939) and in test tubes containing PDA which were stored at 4°C until use.

2.2 ISOLATES OF *FUSARIUM*

The isolates CML 2878 and CML 2870 of *F. oxysporum* and *F. solani*, respectively, belonging to the mycological collection of the Department of Plant Pathology, Federal University of Lavras, Lavras-MG, Brazil, were used in the experiments. Both isolates were obtained from root tissues of chickpea plants showing symptoms of fusariosis in Cristalina-GO, Brazil. The isolates are known as being pathogenic and aggressive in chickpea cv. Cicero (Azevedo et al., 2017).

2.3 *IN VITRO* EXPERIMENTS

To test *Trichoderma* sp. antagonism *in vitro* against *Fusarium* spp., the isolates of *Trichoderma* sp. were evaluated regarding to their potential antagonism by means of the competition in dual culture method. Thirteen isolates were selected and evaluated for production of secondary metabolites with fungicidal/fungistatic activity (non-volatile) and the production of volatile organic compounds (VOC).

2.4 COMPETITION FOR NUTRIENTS

Trichoderma sp. isolates were evaluated against *Fusarium* spp. isolates using the method of dual cultures (Dennis and Webster, 1971c). A 4 mm mycelium plug of each isolate, from seven days old colonies grown on PDA, were placed in Petri dishes containing the same culture medium on opposite sides, equidistant at 1cm from the margin of the Petri dishes. In the control, only the *Fusarium* mycelium plug was placed in the periphery of the dishes. Each pairing was done in triplicate and the dishes were incubated at 25°C under 12 h photoperiod for 7 days. After this period, the radial growth of *Fusarium* colonies was measured to calculate the inhibition of mycelial growth, according to the equation: $IMG(\%) = \frac{C-T}{C}\ x\ 100$; where IMG= inhibition percentage of radial growth, C= radial growth of *Fusarium* in control, and T= radial growth of *Fusarium* in the presence

of *Trichoderma* sp. The isolates of *Trichoderma* sp. were classified on the basis of 1-5 scale (Bell et al., 1982).

2.5 VOLATILE ORGANIC COMPOUNDS (VOC) PRODUCED BY *TRICHODERMA* AGAINST *FUSARIUM*

The isolates of *Trichoderma* sp. were grown in 90 mm diameter Petri dishes containing PDA medium and incubated at 25°C under 12 h photoperiod. Three days later, in separate dishes with the same diameter and containing the same culture medium, isolates of *Fusarium* spp. were grown by a plug of mycelium of 4 mm. To evaluate the production of volatile organic compounds, the method of Dennis and Webster (1971b) was used, where the covers of dishes containing *Fusarium* spp. were replaced by the bottom of those containing isolates of *Trichoderma* sp. The sides were sealed with plastic film. The control consisted of dishes inoculated with *Fusarium* spp. with lids replaced by bottoms containing only PDA. Each treatment was done in triplicate, and the dishes were incubated at 25°C under 12 h photoperiod. The diameter of the *Fusarium* colonies were measured the 4th, 7 th and 10 th day after incubation, to calculate the IMG, using the equation previously mentioned.

2.6 INHIBITION OF *FUSARIUM* SPP. BY NON-VOLATILE ORGANIC COMPOUNDS (NVOC) PRODUCED BY *TRICHODERMA*

The method proposed by Dennis and Webster (1971a) was used with modifications. From each *Trichoderma* isolate, grown on PDA for seven days at 25°C under 12 h photoperiod, eight mycelial plugs (4mm) were removed and transferred to flasks containing 100 mL of Potato Dextrose Broth (200 g potatoes-20 g dextrose-1 L of water). After 20 days at 25°C and under agitation, the liquid medium of the flasks was centrifuged at 3,000 rpm for 10 minutes. The supernatant was filtered in Millipore membrane of 0.45 μm pore diameter using a vacuum filter system. The fungal filtrates obtained were autoclaved at 121°C for 20 minutes and then incorporated into melted PDA at 20% (v/v) proportion. The control treatment consisted of PDA incorporated with sterile water in the same proportion of filtered. The culture medium was poured into Petri dishes and after solidification a mycelium plug of *Fusarium* was inoculated in the center of the Petri dishes. The experimental design was completely randomized with three replicates for each treatment. The dishes were incubated at 25°C under 12 h photoperiod. The diameter of the *Fusarium* colonies was measured the 4 th, 7 th and 10 th day after incubation, to calculate the IMG as described earlier.

Brazilian Journal of Development

2.7 *IN VIVO* EXPERIMENT

Individual and in mix *Trichoderma* sp. isolates T1, T3, T7 and T13 were used for the *in vivo* antagonism test. These isolates were selected because they showed good results in the *in vitro* experiments. The commercial product Quality®, based on *T. asperellum*, was used as a control. Isolates of *Fusarium* were grown in BDA medium for 15 days. Then, the culture medium with the fungal colonies was crushed in a blender at a rate of 10 plates per liter. An aliquot was removed from the suspension for analysis of colony-forming units. The isolates of *Trichoderma* were grown in the same medium and incubated for 7 days. After this period, a spore suspension was prepared in water, quantified in a Newbauer chamber and set to 5 x 10^7 conidia.mL^{-1}. The commercial product was diluted with water in order to obtain the same concentration. The mix was obtained by mixing equal parts of the calibrated suspensions of isolates T1, T3, T7 and T13. Then, 50 mL of the *Trichoderma* and *Fusarium* suspensions were homogenized in 700g of substrate composed of soil and sand, previously autoclaved, in the proportion of 1:1. Roots of chickpea seedling cv. Cicero with 7 days old, produced in propylene trays, were injured with a scalpel and transplanted in pots containing substrate previously infested with the different treatments. The control consisted in injured plants transplanted to non-infested soil. The plants were kept in a greenhouse under daily irrigation for 5 weeks.

The experiment was carried out in a completely randomized design, in a factorial scheme (7x2) +1, with six treatments with *Trichoderma* sp. (T1, T3, T7, T13, mix and commercial product) and one without *Trichoderma* x two isolates of *Fusarium* spp. (*F. solani* and *F. oxysporum*) + additional treatment without inoculation (control), totaling 15 treatments with 8 repetitions. For evaluation purposes, plants were removed from the pots, and the symptoms were evaluated visually. Then, the lengths of the root system and the aerial portion were measured and their respective fresh and dry weights were obtained. To determine the dry weight of the plants, the material was packed in paper bags and dried in a forced ventilation oven at 65 ° C for 48 hours.

2.8 DATA ANALYSIS

The data obtained was submitted to analysis of variance and the means were compared using the Scott-Knott test at 5% probability. Data related to inhibition by non-volatile thermostable metabolites was transformed to “square sine arc of y/100”. Before data transformation, the “zero” values were transformed to 1/4n, where n = maximum number of observations (mean value of the control). The analyses were performed with the aid of the statistical software SAEG.

Brazilian Journal of Development

3 RESULTS AND DISCUSSION

3.1 TESTING OF COMPETITION FOR NUTRIENTS

All isolates of *Trichoderma* sp. inhibited the growth of *F. solani* and *F. oxysporum* (Table 1), however, there were variations between the isolates in relation to their ability to suppress *Fusarium* species growth on the seventh day of evaluation. Although there was no significant difference for some isolates of *Trichoderma* sp. inhibiting the mycelial growth of *F. solani* and *F. oxysporum*, numerically we observed greater inhibition, between 37.43 to 50.29% of mycelial growth in *F. solani*, with *Trichoderma* isolates T1, T2, T3, T4, T5, T7, T10, T11 and T12. For *F. oxysporum*, inhibition ranged from 21.58 to 45.32% with isolates T1, T3, T6, T6, T12, T16, T18 and T19. In the control, the mean diameter of *Fusarium* colonies was of 5.7 cm for *F. solani* and 4.6 cm for *F. oxysporum*, respectively.

The major mechanisms involved in the antagonism to fungi by *Trichoderma* sp. are the competition for nutrients and space, the mycoparasitism and the production of antibiotic substances and hydrolytic enzymes (Harman et al., 2004). Most of the isolates of *Trichoderma* sp. assessed in pairing with *F. solani* or *F. oxysporum* showed rapid growth in culture medium. According to Benítez et al. (2004), some pathogenic fungi are sensitive to the lack of some nutrients, which makes *Trichoderma* sp. a fungus quite competitive due to its rapid growth. Zhang et al. (2014) found that in the antagonism of *T. harzianum* T-E5 to *F. oxysporum* f. sp. *cucumerinum* occurred mycoparasitism, production of extracellular substances such as excretion of enzymes and volatile compounds. In this experiment, the presence of inhibition halos was observed, which are typical of antibiosis expression. A large part of the isolates evaluated in this study (T1, T2, T4, T5, T7, T9, T11, T12, T15, T20, T21) was statistically more effective inhibiting the growth of *F. solani* than *F. oxysporum*. Therefore, the variation observed in the inhibition in this study can be explained by the fact that species of *Trichoderma* being differentially selective against various fungi and due to the control exercised varying among species or among isolates of the same species (Louzada et al., 2009).

Brazilian Journal of Development

Table 1. Inhibition of mycelial growth (IMG) ± standard error of *Fusarium solani* and *F. oxysporum* by isolates of *Trichoderma* sp. in dual cultivation and classification notes of *Trichoderma* isolates corresponding to the antagonism, according to the scale of notes proposed by Bell et al. (1982).

	IMG (%)		NOTE-SCALE	
Trichoderma Isolates	*F. solani*	*F. oxysporum*	*F. solani*	*F. oxysporum*
T1	49.71 aA	40.96 aB	2.0 bA	1.7 aA
T2	45.61 aA	35.21 bB	2.0 bA	1.0 bB
T3	45.28 aA	45.03 aA	1.3 bA	1.7 aA
T4	43.86 aA	31.61 cB	1.7 bA	1.3 bA
T5	46.20 aA	32.33 cB	1.7 bA	2.0 aA
T6	37.43 bA	38.80 aA	2.3 aA	2.0 aB
T7	45.03 aA	34.49 bB	2.0 bA	1.7 aA
T8	39.77 bA	34.49 bA	2.7 aA	1.7 aB
T9	39.18 bA	31.61 cB	2.0 bA	1.3 bA
T10	42.69 bA	34.49 bB	2.0 bA	2.0 aA
T11	44.44 aA	27.29 cB	1.7 bA	2.0 aA
T12	50.29 aA	40.24 aB	2.0 bA	1.0 bB
T13	40.94 bA	42.59 aA	1.7 bA	1.3 bA
T14	38.60 bA	35.93 bA	1.7 bA	2.0 aA
T15	40.35 bA	28.73 cB	2.0 bA	2.0 aA
T16	41.52 bA	40.96 aA	2.0 bA	1.7 aA
T17	39.77bA	36.65 bA	2.0 bA	1.7 aA
T18	40.35 bA	43.12 aA	2.0 bA	1.0 bB
T19	40.45 bA	40.24 aA	2.0 bA	1.3 bA
T20	37.43 bA	24.41 dB	2.0 bA	1.0 bB
T21	38.60 bA	21.53 dB	3.0 aA	2.0 aB
CV (%)	11.34		23.82	

*Means followed by the same uppercase letter in the row and lowercase in the column do not differ by the Scott-Knott test at 5% probability.

According to Bell et al. (1982) an isolate is considered having good antagonist potential when attaining notes (average value) ≤ 2, i.e., the isolate that grows on at least 2/3 of the surface of the medium and colonizes partially the pathogen. In this way, all isolates of *Trichoderma* sp. were considered good antagonists to *F. oxysporum*, emphasizing isolates T2, T4, T9, T12, T13, T18, T19 and T20. Only isolates T6, T8 and T21 were not good antagonists to *F. solani* with notes ≥ 2.7 but statistically superior to the other isolates.

Isolates T2, T6, T8, T12, T18, T20 and T21 were more efficient inhibiting *F. oxysporum* growth, than *F. solani*. The ability of *Trichoderma* sp. to parasitize pathogenic fungi has been reported (Louzada et al. 2009; Ram and Kendurkar, 2014). *Trichoderma* sp. are capable of recognizing the hyphae of other fungi and grow towards its direction presumably in response to chemical stimuli produced by the hypha stewardess, and, after infection, use the content of host

hyphae as a source of nutrients by means of the production of cell wall degrading enzymes, as glucanases, chitinases and proteases (Harman et al., 2004). Despite growth and curling of the hyphae being important in the parasitism process this fact does not guarantee success in biological control (Almeida et al., 2007).

Based on the performance in the experiment of dual cultures, the isolates T1, T2, T3, T4, T5, T6, T7, T11, T12, T13, T16, T18 and T19 were selected for the evaluation of production of volatile organic and not volatile compounds.

3.2 INHIBITION OF *FUSARIUM* SPP. BY VOC

All isolates of *Trichoderma* produced VOC which significantly inhibited the mycelial growth of *F. solani* and *F. oxysporum* from the third day of incubation onwards (Table 2). The pathogens affected by volatile metabolites showed a less dense mycelium. In *F. solani*, the inhibition of mycelial growth was high, above 57 % on the 3rd day of assessment, with no significant difference between the isolates of *Trichoderma* sp. On the 7 th and 10 th day of evaluation, the isolates T1, T2, T3, T6, T7 and T16 differed numerically in terms of the production of metabolites, causing further inhibition of *F. solani* growth.

In regard to *F. oxysporum*, the biggest inhibition numerically were caused by VOC produced by isolates T2, T7 and T16, both on the seventh day as on the tenth day of evaluation. Only the isolated T7 and T16 were effective in inhibiting the growth of both species of *Fusarium* above 60%, from the third to the tenth day of evaluation. The inhibitory effect of volatile metabolites produced by *Trichoderma* sp. has been observed in plant pathogens, such as *Ceratocystis paradoxa* (Eziashi et al., 2006), *F. oxysporum* f. sp. *ciceris* (Dubey et al., 2007), *Gaeumannomyces graminis* var *tritici* (Zafari et al. 2008), *Rosellinia necatrix* (Batson-Girona et al., 2014), *Sclerotium rolfsii* and *Verticillium dahliae* (Isaias et al., 2014). Some isolates produced VOC effectively inhibiting both *F. solani* and *F. oxysporum* (T2, T7 and T16), while others were effective in inhibiting a single species of *Fusarium*. These results show that isolates of *Trichoderma* sp. production of volatile compounds varied, with greater or lesser inhibitory activity to the pathogen. Harman et al. (2004) observed that the ability of *Trichoderma* in producing substances and the fungicide effect may vary among species and isolates of the same species.

Keszler et al. (2000), using gas chromatography coupled with spectrophotometry mass, identified 21 volatile compounds produced by *T. atroviride* derived principally from Pyrones and dioxolane. Siddiquee et al. (2012) identified more than 278 volatile compounds produced by *T. harzianaum*, which include saturated hydrocarbons (C7-C30), cyclohexane, cyclopentane, fatty acids, alcohols, esters, compounds containing sulfur, Piran simple and derived from benzene. Many

of these compounds may have antibiotic action alone (Batson-Girona et al., 2014; Vinale et al., 2006), or may act in synergy, which explains the great capacity of these metabolite compounds to paralyze mycelial growth of plant pathogens.

Isolates of *Trichoderma* sp. studied in the present work produced volatile compounds that showed great inhibition potential against *F. solani* and *F. oxysporum* and these compounds should be identified to understand better their composition and their roles in biological interactions.

3.3 INHIBITION OF *FUSARIUM* SPP. BY NVOC

Non-volatile organic compounds produced by isolates of *Trichoderma* sp. had little effect ihibiting *F. solani* and *F. oxysporum* and ranged as to its action (Table 3). The non-volatile compounds obtained from isolated T4, T6, T13, T16 and T19 were the most efficient reducing the growth of colonies on the third day of incubation of *F. solani*. On the seventh day of incubation, all isolates, except T5, T12 and T19, have caused a significant reduction in the growth of the pathogen. However, this effect has not continued until the 10 th day of incubation, in which *F. solani* began its growth, and there was no statistical difference between the treatments. The results showed that NVOC produced by isolates of *Trichoderma* sp. showed low activity against *F. solani*.

Table 2. Inhibition of mycelial growth (IMG) of *Fusarium solani* and *F. oxysporum* by volatile organic compounds produced by *Trichoderma* sp. isolates measured on the 3rd , 7th and 10th day after incubation.

	IMG (%)					
Isolates	*F. solani*			*F. oxysporum*		
	3rd Day^{ns}	7th Day	10 th Day	3rd Day^{ns}	7th Day	10 th Day
T1	71.87	82.34 a	80.68 a	52.17 b	58.15 b	53.21 b
T2	67.18	75.92 a	69.74 a	50.24 b	70.66 a	76.02 a
T3	74.48	78.86 a	77.65 a	46.37 b	50.93 b	53.41 b
T4	69.79	71.11 a	70.67 a	51.69 b	50.46 b	51.27 b
T5	58.33	49.97 b	45.76 b	44.06 b	35.79 c	35.48 b
T6	65.63	75.65 a	72.99 a	54.11 b	53.10 b	58.28 a
T7	73.95	80.47 a	79.98 a	66.18 a	78.35 a	81.48 a
T11	66.67	54.78 b	49.95 b	18.84 d	31.94 c	30.40 b
T12	59.89	67.63 a	59.03 b	35.27 c	42.28 c	45.42 b
T13	59.74	53.71 b	44.59 b	47.83 b	57.43 b	60.03 a
T16	64.06	78.33 a	77.88 a	63.76 a	66.81 a	70.17 a
T18	57.29	47.03 b	41.11 b	22.70 d	15.34 c	27.68 b
T19	65.10	69.77 a	67.18 a	56.04 b	49.49 b	49.12 b
CV(%)	12.84	11.24	17.58	16.73	24.41	28.68

*Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability. ^{ns}Not significant.

Brazilian Journal of Development

Table 3. Inhibition of mycelial growth (IMG) of *Fusarium solani* and *F. oxysporum* by non-volatile organic compounds produced by *Trichoderma* sp. isolates on the 3rd , 7th and 10th days of incubation.

	IMG (%)[1]					
Isolates	*F. solani*			*F. oxysporum*		
	3rd Day[ns]	7th Day	10 th Day	3rd Day[ns]	7th Day	10 th Day
T1	0.082 (1.26) c	0.514 (24.55) a	0.228 (12.22)	0.166 (3.47) b	0,260 (6,74) a	0,357(12,42) a
T2	0.187 (4.77) b	0.350 (12.77) a	0.157 (5.93)	0.323 (10.67) a	0,329 (10,87) a	0,336 (11,10) a
T3	0.026 (0.00) c	0.298 (11.56) a	0.164 (3.70)	0.168 (3.75) b	0,230 (5,20) a	0,254 (6,38)b
T4	0.397 (14.34) a	0.331(10.76) a	0.017 (0.00)	0.155 (2.88) b	0,156 (3,31) b	0,119 (1,93) c
T5	0.026 (0.00) c	0.032 (0.12) b	0.017 (0.00)	0.093 (1.62) b	0,048 (0,39) b	0,081(1,45)c
T6	0.334 (11.08) a	0.336 (11.16) a	0.017 (0.00)	0.301 (8.87) a	0,340 (11,90) a	0,283 (7,89) b
T7	0.026 (0.00) c	0.476 (21.17) a	0.380 (13.9)	0.267 (7.07) a	0,318 (10,36) a	0,270 (7,32) b
T11	0.125 (2.44) b	0.383 (14.37) a	0.204 (4.63)	0.077 (1.01) b	0,241 (5,72) a	0,227 (5,05) b
T12	0.026 (0.00) c	0.118 (2.32) b	0.151 (5.56)	0.030 (0.00) b	0,104 (2,45) b	0,188 (3,93) b
T13	0.389 (13.87) a	0.393 (14.77) a	0.047 (0.37)	0.346 (11.87) a	0,445 (18,60) a	0,416 (16,38)a
T16	0.374 (13.40) a	0.465 (20.77) a	0.296 (11.85)	0.280 (7.67) a	0,284 (8,81) a	0,248 (6,95) b
T18	0.048 (0.32) c	0.294 (9.16) a	0.145 (2.96)	0.269 (7.67) a	0,129 (2,28) b	0,202 (4,11) b
T19	0.351 (12.01) a	0.211 (4.76) b	0.017 (0.0)	0.215 (4.68) a	0,252 (6,75) a	0,226 (7,03) b
CV(%)	37.87	35.85	115.89	38.46	38.34	33.93

*Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability. [1]Data transformed to "square sine arc of y/100". In parentheses original averages in percent. [ns]Not significant.

Although there was no statistical difference, there was numerically greater inhibition effect against *F. oxysporum* from NVOCs produced by isolates T2, T6, T7, T13, T16, T18 on the third day of incubation, and by isolates T2, T6, T7, T13 and T16 on the seventh day. On the tenth day, the greatest inhibition occurred by NVOCs produced by isolates T1, T2 and T13, and the compounds produced by isolates T4 and T5 had the lowest inhibitory effect against *F. oxysporum*. Zafari et al. (2008) observed the percentage of inhibition of *G. graminis* var. *tritici* by a filtrated obtained from isolates of *Trichoderma* spp. decreased with the increase of the incubation period, suggesting that part of the inhibitory effect was due to volatile metabolites present in the filtrate.

Isaias et al. (2014) found that the response of different fungi may vary according to the metabolites produced by the antagonist. However, the inhibitory effect of NVOC compounds of *Trichoderma* sp. is due to the presence of substances with antibiotic action (Dennis and Webster, 1971a). Although the effect of NVOCs of the majority of the isolates of *Trichoderma* has been significant, there was low inhibition in relation to the action of volatile organic compounds produced by the same isolates. In this experiment, the highest inhibition observed was of 21.2% in *F. solani* incubated for seven days in culture medium containing the filtrate of isolated T7. Mulatu et al. (2013) worked with filtrates (non-volatile) of *Trichoderma* sp. added to the culture medium in the same concentration used in this study (20% v/v), and observed inhibition of *F. xylarioides* growth from 18.9 to 60%. In another study, Eziashi et al. (2006), using nontoxic metabolites compounds from

Trichoderma spp. by the cellophane method, observed inhibition of *C. paradoxa* growth ranging between 0 to 74%. Isaias et al. (2014) evaluated the effect of non-volatile metabolites and non-volatile thermostable compounds of different *Trichoderma* spp. isolates of and observed inhibition of *S. rolfsii* growth between 0 to 100% for non-volatile metabolites compounds and from 0 to 72.05% for thermostable non-volatile metabolite compounds. For these authors, although there has been a decrease in the percentages of inhibition, the metabolites of *Trichoderma* spp. remained active, even after being autoclaved. Therefore, in the present study, the autoclaving of filtrate at 121°C for 20 minutes may have degraded compounds with antifungal activity and/or occurred reaction between the compounds, forming other substances with low fungitoxic action and even null. On the other hand, the dilution of filtrates in culture medium may also have interfered in the expression of their inhibitory capacity. Daniel et al. (2014) observed a positive correlation between the concentration of the extract of the isolate *T. asperellum* T2-31 in culture medium and the inhibition of mycelial growth of *F. oxysporum*. Therefore, due to the methodology adopted in this experiment, we can conclude that the autoclaving process provided a decrease in the inhibitory activity of secondary metabolites produced by the isolates of *Trichoderma* evaluated.

3.4 *IN VIVO* EXPERIMENT

In the greenhouse evaluation, it was not possible to verify the antagonistic potential of *Trichoderma* sp. against *F. solani* and *F. oxysporum*, because the plants inoculated with the pathogens (positive control) did not develop the expected symptoms and did not differ from the negative control in relation to the evaluated variables (Table 4).

Brazilian Journal of Development

Table 4. Effect of soil treatment with *Trichoderma* sp. on the growth and fresh and dry weight of chickpeas cultivated in soil infested with *Fusarium oxysporum*.

	Length (cm)		Fresh weight (g)		Dry weight (g)	
Treatments	Aerial portion	Root ^{ns}	Aerial portion	Root	Aerial portion ^{ns}	Root
T1	32.8 a	30.1	4.77 a	3.81 b	0.91	0.44 a
T3	22.0 c	25.5	2.28 b	1.27 c	0.46	0.14 b
T7	24.7 c	29.1	2.94 b	3.51 b	0.57	0.33 b
T13	28.8 b	33.2	2.69 b	2.93 b	0.74	0.35 b
[1]Mix	28.5 b	27.1	3.93a	4.49 b	0.72	0.31 b
Quality®	28.8 b	26.5	4.55 a	6.63 a	0.94	0.54 a
[2] Control (+)	36.5 a	30.6	4.52 a	5.54 a	0.91	0.41 a
[3]Control (-)	34.2 a	27.2	4.31 a	6.91 a	0.87	0.60 a
CV (%)	17.13	27.75	37.73	40.09	45.56	49.94

[1]T1+T3+T7+T13 *Trichoderma* isolates. [2]Plants inoculated with *F. oxysporum* only. [3]Non-inoculated plants. ^{ns} not significant. Data followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability.

Some studies show the potential of *Trichoderma* sp. as plant growth promoters in crops (Souza Pedro et al., 2012; Aguiar et al., 2012), including chickpea (Jyotsna et al., 2008). *Trichoderma* can produce hormones and solubilize nutrients in the soil that are used by plants and result in increased growth (Contreras-Cornejo et al., 2009), but this potential was not observed in this study. Treatments with *Trichoderma* sp. had no significant effect on the development of chickpea plants grown in soils infested by *F. solani*. In plants grown in soils infested with *F. oxysporum*, treatments with *Trichoderma* sp. did not show a significant effect for any of the variables analyzed (Table 4). Ethur et al. (2005) evaluated isolates of *T. virens* in the control of *Sclerotinia sclerotiorum* in cucumbers, and observed that some isolates did not interfere with plant growth, while others interfered negatively.

The analysis of variance showed a significant interaction between treatments and *Fusarium* species for variables of shoot length and fresh root weight (Table 5). In plants grown in soil infested with *F. oxysporum*, less effect from isolates T3 and T7 was observed to promote the development of the aerial portion of plants. A similar result for fresh root weight was also observed in treatments T3, T7 and mix. The results obtained in this experiment confirm observations reported by Bell et al. (1982), where satisfactory results in antagonism *in vitro* may not be confirmed *in vivo*, since there is a complex interaction between several biotic and abiotic factors. This can affect the mechanisms of action of *Trichoderma* sp. and the reactions of the host and the pathogen.

4 CONCLUSIONS

Table 5. Length of aerial portion and fresh root weight of chickpea as according to the interaction between treatment with different isolates of *Trichoderma* versus *Fusarium* species.

	Shoot length		Fresh root weight	
Treatments	*F. solani*	*F. oxysporum*	*F. solani*	*F. oxysporum*
T1	29.2 aA	32.8 aA	6.02 aA	3.81 bA
T3	30.7 aA	22.0 cB	4.96 aA	1.27 bB
T7	32.8 aA	24.7 cB	5.98aA	3.51 bB
T13	29.8 aA	28.8 bA	4.11aA	2.93 bA
[1]Mix	30.8 aA	28.5 bA	7.01aA	4.49 aB
T. asperellum	31 aA	28.8 bA	4.55 aA	6.63 aA
[2]Control (+)	32.2aA	36.5 aA	6.02aA	5.54aA
CV (%)	15.96		41.43	

[1]T1+T3+T7+T13 *Trichoderma* isolates. [2] Plants inoculated only with *F. oxysporum* or *F. solani*. Data followed by the same lowercase letter in the column and uppercase in the lines do not differ by the Scott-Knott test at 5% probability.

Only isolates T1, T3 and T12 were efficient in inhibiting both *Fusarium* species studied in paired culture. All evaluated isolates of *Trichoderma* sp. produced volatile metabolites capable of inhibiting the mycelial growth of *F. solani* and *F. oxysporum*. Thermostable non-volatile metabolites produced by *Trichoderma* sp. have little inhibitory activity against *F. solani* and *F. oxysporum*.

In the *in vivo* evaluation of antagonism, there was not effect of treatments with isolates of *Trichoderma* sp. on the control of *F. oxysporum* and *F. solani*. Treatments with *Trichoderma* sp. T1, T3, T7 and T13 did not promote the growth of chickpea under the evaluated conditions, even when mixed.

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