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Artigo/Article

Production of antifungal compounds by *Trichoderma* spp. to control *Phytophthora nicotianae*, causal agent of gummosis on citrus

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SUMMARY

Orange crops in Brazil are affected by various diseases such as gummosis caused by *Phytophthora nicotianae*, which is controlled by chemical fungicides, by preventive nature through cultural practices and utilization of tolerant rootstock. However, the high costs and environmental problems caused by the intensive use these fungicides have led to the search for alternative methods of control. The objective this study was to evaluate the *in vitro* antagonistic activity of *Trichoderma* spp. isolates against *P. nicotianae* by the production of antimicrobial compounds and an alfalfa seedling bioassay, thus, 12 out of the 50 isolates of *Trichoderma* spp. evaluated were selected based on *in vitro* screening to compose bioassay treatments. The paired culture showed that all isolates tested inhibited the mycelial growth of the pathogen. In the production of antimicrobial compounds, 41 isolates produced volatile compounds using dextrose as carbon source; however, further assays showed that the use of sucrose or maltose as carbon source increases the production of these compounds. Seven isolates of *Trichoderma* spp. produced thermostable compounds and 14 isolates produced cell-free antimicrobial compounds of the antagonist. In the alfalfa seedling bioassay, four isolates of *Trichoderma* spp. inhibited the formation of sporangia and mycelia growth. The *in vitro* evaluation concerning to the antimicrobial compound production by the *Trichoderma* spp. isolates and the alfalfa seedling bioassay were able to select biocontrol agents to control *P. nicotianae.* The four most promising isolates were TB12, TB14, TB28, and TB30 and the mechanisms of action that may be involved in the biocontrol are production of volatile compounds and cell-free filtrates by the antagonists.

Index terms: alfalfa seedling bioassay, *Citrus* spp., antimicrobial compounds.

Produção de compostos antifúngicos por *Trichoderma* spp. para controle de *Phytophthora nicotianae,* agente causal de gomose em citros

RESUMO

A cultura da laranja no Brasil é afetada por várias doenças, como a gomose, causada por *Phytophtora nicotianae*, cujo controle é comumente realizado por aplicações de produtos químicos. Porém, devido aos custos financeiros e problemas ambientais ocasionados pelo uso intensivo destas aplicações, faz-se necessário a busca por métodos alternativos de controle. Este trabalho teve por objetivo avaliar a ação antagônica de isolados de *Trichoderma spp*. para controle de *P. nicotianae*, através da produção de compostos antimicrobianos, por meio destes microganimsos, além da

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realização de um bioensaio com plântulas de alfafa. Para compor os tratamentos do bioensaio, foram selecionados 12 dos 50 isolados de *Trichoderma* spp. avaliados *in vitro*. No cultivo pareado observou-se que todos os isolados inibiram o crescimento micelial do fitopatógeno. Na produção de compostos antimicrobianos, 41 isolados produziram compostos voláteis utilizando como fonte de carbono a dextrose, no entanto, em estudos posteriores, foi verificado que a utilização de sacarose ou maltose, como fontes de carbono, aumentou a produção destes compostos. Sete isolados de *Trichoderma* produziram compostos termoestáveis e 14 produziram compostos antimicrobianos livres de células do antagônico. No bioensaio com plântulas de alfafa foi observado que quatro isolados de *Trichoderma* inibiram a formação de esporângios e de micélios do fitopatógeno. Diante dos resultados obtidos, concluiu-se que as avaliações *in vitro* com relação à produção de compostos antimicrobianos pelos isolados de *Trichoderma* e o bioensaio com plântulas de alfafa mostraram capacidade para selecionar isolados com potencial para o controle de *P. nicotianae*. Os quatro isolados mais promissores foram: TB12, TB14, TB28 e TB30 e, os mecanismos de ação que podem estar envolvidos no biocontrole são a produção de compostos voláteis e livres de células pelos antagonistas.

Termos de indexação: bioensaio com plântulas de alfafa, *Citrus* spp., compostos antimicrobianos.

INTRODUCTION

Orange is one of the leading exports in Brazil. In the state of São Paulo, the area occupied to citrus growing for the 2013/14 crops was estimated at 501.8 hectares, and the producing area was estimated at 464.4 hectares. The orange production of the 2013/14 crops was around 270 40.8-kg boxes (CONAB, 2014). In addition, Brazil is the world's largest exporter of frozen concentrated orange juice, which, along with other by-products, has generated nearly 1.5 billion dollars in exports per year (FAO, 2014).

Among the various citrus phytosanitary problems, *Phytophthora* gummosis causes a reduction in productivity which leads to profit reduction. This disease has affected citrus trees world wide and is caused by *Phytophthora parasitica*, also referred to as *P. nicotianae* (Breda de Haan) (Tucker) var. parasitic (Dastur) Watherhouse. (Graham & Timmer, 1992).

P. nicotianae is responsible for the greatest damages in nurseries in Brazil although *P. citrophthora* (Sm. & Sm) Leonian and other *Phytophthora* species have already been described as causing this disease. The major damage is stem and root rot, which can hinder the proper development of citrus seedlings in the seed bed or even result in death of the tree if the damage affects the entire trunk circumference (Feichtenberger, 2001).

P. nicotianae is an oomycete that consists of hyaline and non-septate hyphae and hyaline sporangia, which can reproduce both sexually and asexually. It produces structures of resistance, such as oospores, zoospores, and chlamydospores, and, to a lesser degree, sporangia, under favorable environmental conditions. Sporangia can germinate either directly, by the formation of germ tubes, or indirectly, by the formation of zoospores in free water, stimulated by a drop in temperature (Graham & Menge, 1999).

The disease is controlled by preventive measures that include the use of healthy seedlings and rootstocks or the applications of fungicides such as fosetyl-Al and metalaxyl in the seedlings (Matheron & Porchas, 2000). However, the high costs and environmental impacts of these chemicals have led to the search for alternative methods of control. As a result, new alternatives for control of this disease have been investigated, especially those that are more compatible to agroecosystems, more economical, and incorporate the fundamental principles of sustainability (Corrêa et al., 2011).

Among the biological control agents, fungal species belonging to the genus *Trichoderma* spp. have been widely studied as biological control agents against soil-borne pathogens such as *Phytophthora* spp. In addition to being biocontrol agents, these microorganisms are also plant growth promoters and can induce plant resistance to the pathogens (Saba et al., 2012; Saksirirat et al., 2009).

Currently, more than 250 products based on *Trichoderma* spp. are manufactured and marketed for use on several crops in the international market and according to Woo et al. (2014), a large distribuition of *Trichoderma* formulations has been found in all geographical regions worldwide. Some products based on this antagonistic fungus are effective in reducing incidence of root rot and in the severity of diseases caused by soil-borne pathogens, such as *Fusarium, Phytophthora, Pythium, Rhizoctonia, Sclerotium, and Sclerotinia*. These biological products can be used either for the treatment of substrates as for the treatment of

seeds, as can be used in spraying onto the aerial parts of the plant (Harman et al., 2004).

Therefore, this study aimed to evaluate the production of antimicrobial compounds by different isolates of *Trichoderma* spp. and the use of the alfalfa seedling bioassay methodology for the selection of promising isolates of *Trichoderma* spp. in the biocontrol.

MATERIAL AND METHODS

Microorganisms

The following isolates were evaluated: 25 *Trichoderma* spp. isolates obtained from banana producing regions in the states of São Paulo and Rio de Janeiro, Brazil (TB01, TB02, TB03, TB04, TB06, TB07, TB08, TB09, TB1O, TB11, TB12, TB13, TB14, TB16, TB17, TB18, TB21, TB22, TB25, TB28, TB29, TB30, TB31, TB32, TB34); 25 *Trichoderma* spp. isolates obtained from soybean producing regions in the state of Goiás, Brazil (CE200, CE300, F1A10O11, F1A2T1001, F1A2T1014, F1A2T2, F1A9T2002, F1A9T2006, F2A3T1024, F2A3T1O24, F2A3T18030, F3A3T1010, F3A5T1015, F3A5T1025, F4A5T1, F4A5T1004, F4A5T1005, F4A1T1008, F4A1T1009, F4A4T1022, T1A1029, T1A2F1017, T2T1A2, T2A2F1018, T2A2F1021); and 01 *P. nicotianae* isolate (IAC-01/95). All of these microorganisms belong to the culture collection of the Plant Pathology and Biological Control Laboratory at the "Sylvio Moreira" Citrus Center/ IAC, Cordeirópolis/SP.

Influence of *Trichoderma* spp. isolates on the mycelial growth of *Phytophthora nicotianae*

The antagonistic effect of *Trichoderma* spp. on the mycelial growth of the phytopathogen was evaluated using paired culture in Petri dishes containing Potato Dextrose Agar (Dennis & Webster 1971).

Mycelium discs (5mm) cut from the actively growing colony margins of 7-day old *P. nicotianae* colonies grown on carrot agar medium (CA) were transferred to Petri dishes containing potato dextrose agar (PDA) and placed 3 cm apart from same size discs of each 7-day old culture of *Trichoderma* spp isolate. Plates with the pathogen without the presence of the potential antagonists were used as control. The cultures were incubated in a BOD incubator at 27°C and 12-h photoperiod. The *P. nicotianae* colonies were evaluated for mycelial growth by measuring colony diameter in two perpendicular directions on each culture plate after 7 days of incubation. A completely randomized design with five replications was used. Data were subjected to analysis of variance (ANOVA), and means were compared using the Tukey test at 5% probability level.

Evaluation of antimicrobial compound produced by the isolates of *Trichoderma* spp.

Production of volatile compounds

In order to assess the production of volatile compounds by *Trichoderma spp.* isolates, the assays were carried out on split plates containing PDA medium to prevent any physical contact between the non-volatile exudates produced by the fungus and *P. nicotianae* in the culture medium. One 5-mm disc of the medium containing *Trichoderma* spp. isolate was placed one side of the split plate, and one 5-mm disc of the medium containing the pathogen was placed on the other side of plate (the opposite side). The plates were sealed with parafilm and incubated in a BOD chamber at 27°C and 12h photoperiod. The diameter of the *P. nicotianae* colonies was evaluated in two perpendicular directions on each culture plate after 7 days of incubation.

Production of thermostable antimicrobial compounds by *Trichoderma spp*. isolates

The thermal stability of the antifungal compounds produced by the isolates of *Trichoderma* spp. was verified according to the method described by Frighetto & Melo (1995). Three discs of mycelium *Trichoderma* spp. (7-day old) were transferred to 250 mL Erlenmeyer flasks containing 50 mL of PDA. The cultures were then incubated at environmental conditions under agitation at 150 rpm for 120 hours. The broth obtained was filtered through Whatman No.4 filter paper, and an aliquot of 10 mL was transferred to Erlenmeyer flasks (250 mL) containing 90 mL of PDA. The culture media were autoclaved at 120°C and 1 ATM pressure for 20 minutes and poured into Petri dishes. After solidification, one disc (5 mm) of the medium containing actively growing *P. nicotianae* colonies was placed at the center of each Petri dish containing the medium and the metabolite produced by the antagonist. Plates with the phytopathogen

without the presence of the fungal metabolites were used as control. The cultures were incubated in a BOD chamber at 27°C and 12h photoperiod. The *P. nicotianae* colonies were evaluated for mycelial growth by measuring colony diameter in two perpendicular directions on each culture plate after 7 days of incubation.

Production of cell-free antifungal compounds by *Trichoderma* spp. isolates

Three discs of mycelium *Trichoderma* spp. (7-day old) were transferred to 250 mL Erlenmeyer flasks containing 50 ml of PDA, and the cultures were incubated at environmental conditions under agitation at 150 rpm for 120 hours. An aliquot of 15 mL of the fermentation broth correspond to each *Trichoderma* spp. isolate was centrifuged, filtered through Whatman No. 4 filter paper, and then filtered again through a Millipore membrane (0.45 uM) in order to obtain a cell-free filtrate of *Trichoderma* spp. (Frighetto & Melo, 1995).

An aliquot of 10 mL of each filtrate was transferred to 250 mL Erlenmeyer flasks containing 90 mL of melted PDA (approximately 70 °C). The medium of each treatment was poured into Petri dishes. After solidification, one disc (5 mm) of the medium containing 7-day old phytopathogen was placed at the center of the dishes. Plates with PDA without the presence of the fungal metabolites were used as control. The cultures were incubated at 27 °C for seven days, and subsequently the *P. nicotianae* colonies were evaluated for mycelial growth by measuring colony diameter in two perpendicular directions.

Statistical analysis

A completely randomized design with five replications was used in all assays of antimicrobial compound production by *Trichoderma* spp.. Data were subjected to analysis of variance (ANOVA), and means were compared using the Tukey test at 5% probability level.

Effect of carbon sources on the production of volatile compounds

Based on the results obtained in the previous assays, 12 *Trichoderma* spp. isolates (TB10, TB12, TB13, TB14, TB21, TB22, TB28, TB30, F1A2T1001, F2T1A1029, F1A9T2006, and T2A2F1021) were inoculated again to produce volatile compounds using PDA culture medium supplemented with different carbon sources: 20g/L glucose, sucrose, or maltose. The production of volatile compounds followed the same method previously described, using split plates. Plates with the phytopathogen without the presence of *Trichoderma* spp. were used as control. The plates were sealed with parafilm and incubated at 25°C ± 3°C and 12h photoperiod. The *P. nicotianae* colonies were evaluated for mycelial growth by measuring colony diameter in two perpendicular directions on each culture plate after 7 days of incubation. A completely randomized design with five replications was used, and the data were subjected to analysis of variance (ANOVA) and means were compared using the Tukey test at 5% probability level.

Alfalfa seedling bioassay

The same 12 *Trichoderma* spp. isolates (TB10, TB12, TB13, TB14, TB21, TB22, TB28, TB30, F1A2T1001, F2T1A1029, F1A9T2006, and T2A2F1021) were evaluated following the method and rating scale introduced by Leoni & Ghini (2002) in order to identify the most promising isolates for the biocontrol of *P. nicotianae* using alfalfa seedlings. Alfalfa's seeds (*Medicago sativa*), previously sanitized with sodium hypochlorite (2% v/v), were germinated. After seven days, the seedlings were transferred to 20-well polyethylene plates (5 mL well volume). In each well, it was added: 2 mL of sterile distilled water (SDW), one 5 mm disc of the CA medium containing 7-day old *P. nicotianae,* and a 5 mm disc of the BDA medium containing the potential antagonist. Plates with the alfalfa seedlings in SDW only were used as control. The plates were maintained at room temperature and 12h photoperiod, and after four days, a 20 mm piece was cut from the lower end of the radicle, stained with methylene blue, and evaluated under optical (light) microscope.

In this bioassay, in order to determine the antagonist potential of one or more *Trichoderma* spp. isolates, two rating scales that classify the level of *P. nicotianae* infection in the alfalfa seedlings were used. With regard to the presence of sporangia (Z), the rating scaleranged from 0 to 4 where: 0 = no sporangia detected; 1 = 1-5 sporangia; 2 = 6-10 sporangia; 3 = 11-50 sporangia; and 4 = more than 51 sporangia; with regard to the presence of mycelium (M) the rating scale ranged from 0 to 3 where: 0 = no mycelium; 1 = too little mycelium; 2 = medium amount of mycelium, and 3 = high amount of mycelium. The bioassay was carried out using a completely randomized design with four replications. Data were subjected to analysis of

variance (ANOVA), previously and means were compared using the Tukey test at 5% probability level.

RESULTS

Influence of *Trichoderma* spp. isolates on the mycelial growth of *Phytophthora nicotianae*

The results show that all 50 *Trichoderma* spp. isolates tested were able to significantly inhibit *P. nicotianae* colony growth, with inhibition values that ranged from 29% (F1A9T2002) to 83% (CE200), when the microorganisms were evaluated using paired culture in Petri dishes containing PDA (Tables 1-5).

Evaluation of antimicrobial compound production by *Trichoderma* spp. isolates

Production of volatile compounds

The results show that 41 out of the 50 isolates tested were able to produce volatile compounds that significantly inhibited *P. nicotianae* colony growth. In the first assay, TB10 was the only isolate able to affect the size of the pathogen colony (Table 1). In the other assays, all isolates produced volatile compounds that affected the development of the pathogen, with inhibition values ranging from 14% (F2A3T1024) to 65% (TB28) (Tables 2-5).

Production of antimicrobial thermostable compounds by *Trichoderma* spp. isolates

As for the antimicrobial compounds produced by *Trichoderma* spp. that were resistant to high temperature, it was observed that TB06 (Assay 1); TB21, TB22, TB28, and F1A2T1001 (Assay 2); TB13 and T2A2F1021 (Assay 4) were the only isolates able to inhibit *Phytophthora* colony growth, with inhibition values that ranged from 10% (F1A2T1001) to 48% (TB06). All other isolates tested did not produce thermostable compounds in sufficient quantities to affect the development of the pathogen (Tables 1-5).

Production of cell-free antifungal compounds by *Trichoderma* spp. *isolates*

The analysis of the production of cell-free culture filtrates of *Trichoderma* spp. showed that the following isolates produced antimicrobial compounds in sufficient quantities to inhibit pathogen colony growth: TB02, TB04, TB06, TB08, TB09, and TB010 (Assay 1, Table 1); TB21,

Table 1. Mean colony diameters of *Phytophthora nicotianae* after being paired with different *Trichoderma spp.* isolates or under the influence of antimicrobial compounds produced by the fungi. Assay 1

	Antimicrobial Compounds							
	Paired		Volatile		Thermostable		Cell-free culture	
Isolates	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)
Controls	5.65 a[(1)]	-	4.93 a	-	3.95 a	-	3.90 a	-
TB01	3.58 bc	36	4.90 a	0.6	3.69 ab	6	3.67 a	6
TB02	3.15 bc	44	4.55 ab	8	2.72 ab	31	2.40 c	38
TB03	3.30 bc	41	4.94 a	-0.2	3.92 ab	0.8	3.93 a	-0.8
TB04	3.85 bc	32	4.58 ab	7	2.86 ab	27	3.06 b	21
TB06	3.64 bc	36	4.55 ab	8	2.05 b	48	2.65 bc	32
TB07	3.94 b	36	4.62 ab	6	3.77 ab	4	3.66 a	6
TB08	3.85 bc	32	4.76 ab	3	2.64 ab	33	2.14 c	45
TB09	3.07 c	46	4.33 ab	12	2.30 ab	42	2.60 bc	33
TB10	3.38 bc	40	4.20 b	15	3.15 ab	20	2.40 c	38
TB11	3.49 bc	38	4.73 ab	4	3.30 ab	16	3,98 a	-2
CV %	10.08	-	6.41	-	28.10	-	8.88	-

[(1)]Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

Table 2. Mean colony diameters of *Phytophthora nicotianae* after being paired with different *Trichoderma spp.* isolates under the influence of antimicrobial compounds produced by the fungi. Assay 2

Isolates	Antimicrobial Compounds							
	Paired		Volatile		Thermostable		Cell-free culture	
	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)
Controls	6.96 a[(1)]	-	7.15 a	-	3.92 ab	-	3.79 a	-
TB18	3.69 b	47	3.12 b	56	3.75 abc	4	3.72 a	2
TB21	2.88 bc	59	2.98 b	58	2.08 e	47	1.47 c	61
TB22	1.73 c	75	3.19 b	55	3.47 cd	11	2.79 b	26
TB25	2.54 bc	63	2.96 b	59	3.94 ab	-0.5	3.62 a	4
TB28	2.38 bc	66	2.52 b	65	3.26 d	17	1.91 c	50
TB32	3.03 bc	56	3.29 b	54	4.12 a	-5	4.03 a	-6.3
F1A2T1001	2.05 bc	70	3.86 b	46	3.52 cd	10	3.64 a	4
F1A2T1014	2.00 bc	71	2.84 b	60	3.60 bcd	8	3.92 a	-3.4
CE200	1.20 c	83	3.00 b	58	3.98 ab	-1.5	3.74 a	1
CE300	1.40 c	80	3.06 b	57	4.00 a	-2	3.71 a	2
CV %	32.32	-	21.86	-	5.15	-	8.08	-

[(1)]Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

Table 3. Mean colony diameters of *Phytophthora nicotianae* after being paired with different *Trichoderma spp.* isolates under the influence of antimicrobial compounds produced by the fungi. Assay 3

Isolates	Antimicrobial Compounds							
	Paired		Volatile		Thermostable		Cell-free culture	
	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)
Controls	6.85 a[(1)]	-	7.25 a	-	4.29 abc	-	4.20 bc	-
TB29	2.25 bc	67	2.88 c	60	3.57 abc	17	4.06 bc	3
TB30	2.41 bc	65	2.97 c	59	3.94 abc	8	1.90 e	55
F1A2T2013	3.64 b	47	4.62 b	36	3.80 abc	11	5.06 ab	-20.5
F1A9T2006	2.05 c	70	3.11 c	57	3.15 bc	27	2.03 e	52
F2A3T1830	3.67 b	46	3.20 c	56	4.77 a	-11	4.15 bc	1
F2T1A1029	3.20 bc	53	2.50 c	65	4.70 a	-10	2.90 de	31
F3A5T1015	2.83 bc	59	3.30 bc	54	4.15 abc	3	3.70 cd	12
F3A5T1025	2.34 bc	66	2.90 c	60	3.78 abc	12	4.70 abc	-12
F4A5T1003	3.30 bc	52	3.49 bc	52	2.82 c	34	5.50 a	-31
F4A4T1022	3.25 bc	52	2.80 c	61	4.40 ab	-2.6	4.20 bc	0
CV %	22.75	-	17.80	-	18.06	-	13.13	-

[(1)]Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

Table 4. Mean colony diameters of *Phytophthora nicotianae* after being paired with different *Trichoderma spp.* isolates under the influence of antimicrobial compounds produced by the fungi. Assay 4

Isolates	Antimicrobial Compounds							
	Paired		Volatile		Thermostable		Cell-free culture	
	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)
Controls	6.00a[(1)]	-	7.08 a	-	3.95 bc	-	3.69 abc	-
TB12	4.10 b	32	3.15 bc	55	3.92 cd	0.8	2.13 d	42
TB13	2.45 d	59	3.33 bc	53	3.45 de	13	2.45 cd	34
TB14	2.70 d	55	3.75 b	47	4.05 bc	-2.5	1.80 d	51
TB16	3.00 cd	50	2.72 c	62	3.97 bc	-0.5	4.12 a	-12
F3A3T1010	3.90 bc	35	3.27 bc	54	4.43 ab	-12	4.05 ab	-10
F4A1T1009	2.95 cd	51	3.67 bc	48	4.79 a	-21	4.80 a	-30
F4A5T1005	2.75 d	54	3.18 bc	55	4.06 bc	-3	4.86 a	-32
T1A1029	3.85 bc	36	3.28 bc	54	4.05 bc	-2.5	4.69 a	-27
T2A2F1021	3.25bcd	46	3.13 bc	56	3.22 e	18	4.58 a	-24
TK2002	3.30bcd	45	3.99 b	44	3.84 cd	3	2.69 bcd	27
CV %	12.95	-	12.33	-	5.66	-	18.28	-

[(1)]Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

Table 5. Mean colony diameters of *Phytophthora nicotianae* after being paired with different *Trichoderma spp.* isolates under the influence of antimicrobial compounds produced by the fungi. Assay 5

Isolates	Antimicrobial Compounds							
	Paired		Volatile		Thermostable		Cell-free culture	
	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)	diameter (cm)	Inhibition (%)
Control	4.48 a[(1)]	-	6.00 a	-	3.78 abc	-	4.09 a	-
TB17	2.53 def	44	4.75 bcd	21	3.71 abc	2	3.87 a	5
TB31	2.73 cde	39	5.00 bc	17	3.65 bc	3	2.92 a	29
TB34	2.97 cd	34	4.85 bcd	19	4.01 abc	-6	4.01 a	2
F1A1011	2.18 efg	51	4.30 cde	28	3.48 c	8	3.42 a	16
F1A9T2002	3.16 bc	29	4.90 bc	18	4.20 a	-11	3.30 a	19
F2A3T1024	3.16 bc	29	5.15 b	14	4.25 a	-12	3.85 a	6
F4A1T1008	2.75 cd	39	4.80 bcd	20	4.23 a	-12	4.14 a	-1.2
T1A2F1017	1.95 g	56	4.15 de	31	4.15 ab	-10	4.10 a	-0.2
T2A2F1018	2.68 cde	40	3.65 ef	39	3.91 abc	-3	3.42 a	16
T2T1A2016	2.05 fg	54	3.20 f	47	3.91 abc	-3	3.16 a	23
CV %	9.25	-	7.23	-	6.49	-	21.17	-

[(1)]Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

TB22, and TB28 (Assay 2, Table 2); TB30, F1A9T2006, and F2T1A1029 (Assay 3, Table 3); TB12 and TB14 (Assay 4, Table 4). None of the isolates tested in assay 5 (Table 5) produced cell-free antifungal compounds in sufficient quantities to affect the *Phytophthora* mycelial development.

Effect of carbon sources on the production of volatile compounds

The analysis of the effect of different carbon sources that were used to supplement the PDA culture medium to test the production of volatiles by *Trichoderma* spp. isolates showed that compound production depended not only on the isolate of the antagonist, but also on the carbon source used. Sucrose and maltose favored the production of these volatiles (Table 6), and although all isolates tested produced volatile compounds in sufficient quantities to inhibit the pathogen colony growth, the inhibition values of the *Phytophthora* colony ranged from 30% (F1A2T1001) to 54% (TB010) (Table 7).

Table 6. Effect of different carbon sources on the production of volatile compounds by *Trichoderma* spp. and on *Phytophthora nicotianae* colony growth

Carbon Sources	Mean colony diameter (cm)
Sucrose	2.78 b[(1)]
Maltose	2.86 b
Glucose	3.32 a

[(1)] Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

Alfalfa seedling bioassay

The data of alfalfa seedling bioassay showed that the isolates TB10, TB12, TB14, TB21, TB28, TB30, and F1A9T2006 inhibited the production of *P. nicotianae* sporangia. With respect to the amount of mycelia, TB12, TB14, TB28, and TB30 were the only isolates that significantly inhibited mycelial growth (Table 8). Moreover, it was found that these last ones were able to affect both sporangia and mycelium production, and therefore they are considered as promising biological control agents against *P. nicotianae* by the infestation of alfalfa seedlings' method.

DISCUSSION

The high incidence of gummosis, disease caused by *P. nicotianae,* results in significant economic losses to the Brazilian citrus industry. Therefore, new methods of

Table 7. Mean colony diameter of *Phytophthora nicotianae* under the influence of volatile compounds produced by *Trichoderma* spp. grown on PDA medium supplemented with different carbon sources

Isolates	Sucrose	Maltose	Glucose
	Mean colony diameter of *Phytophthora nicotianae* (cm)		
Control	3.85 a[(1)]	5.25 a	5.18 a
TB10	2.25 cd	2.38 b	2.31 c
TB12	2.62 bcd	2.60 b	3.20 bc
TB13	2.67 bcd	3.07 b	3.25 bc
TB14	2.99 bc	3.07 b	2.90 c
TB21	2.68 bcd	2.75 b	2.52 c
TB22	3.30 ab	2.62 b	3.16 bc
TB28	2.60 bcd	2.38 b	3.08 bc
TB30	2.15 d	2.84 b	3.24 bc
F1A2T1001	3.00 bc	2.56 b	4.38 ab
F2T1A1029	2.82 bcd	3.25 b	4.38 ab
F1A9T2006	2.70 bcd	2.80 b	3.56 bc
T2A2F1021	2.56 bcd	2.45 b	2.50 c
CV%	13.14	16.98	20.25

[(1)] Means followed by the same lower case letters within a column are not significantly different at 5% probability level by the Tukey test.

Table 8. Selection of *Trichoderma* spp. isolates in terms of antagonistic activity against *Phytophthora nicotianae* using the infestation of alfalfa seedlings' method

Treatments	Level of infestation with *P. nicotianae* in alfalfa seedlings	
	presence of sporangia	presence of mycelium
Non-inoculated Control	0.71 b[(Z)]	0.71 b[(M)]
Inoculated Control	1.56 a	1.48 a
TB10	0.71 b	0.84 ab
TB12	0.71 b	0.71 b
TB13	0.88 ab	0.97 ab
TB14	0.71 b	0.71 b
TB21	0.71 b	0.84 ab
TB22	1.39 ab	1.22 ab
TB28	0.71 b	0.71 b
TB30	0.71 b	0.71 b
F1A2T1001	1.05 ab	0.97 ab
F2T1A1029	0.88 ab	0.84 ab
F1A9T2006	0.71 b	0.84 ab
T2A2F1021	1.22 ab	0.97 ab

[(Z)]Mean rating of the number of sporangia formed per alfalfa plant. Rating scale for number of sporangia: 0 = no sporangia detected; 1 = 1-5 sporangia; 2 = 6-10 sporangia; 3 = 11-50 sporangia; and 4 = more than 51 sporangia. [(M)] ean rating of the amount of mycelium per alfalfa plant. Rating scale for amount of mycelium: 0 = no mycelium; 1 = too little mycelium; 2 = medium amount of mycelium, and 3 = too much mycelium. Means followed by the same lower case letters within a column are not significantly different at 5% probability by the Tukey test.

controlling this disease are necessary; biological control has become an important alternative to the use of fungicides.

This study aimed to evaluate the *in vitro* antagonistic activity of different *Trichoderma* spp. isolates against *P. nicotianae* by the production of antimicrobial compounds and by an alfalfa seedling bioassay for the selection of the most promising biocontrol isolates. The results show that all 50 *Trichoderma* spp. isolates tested were able to reduce the development of *P. nicotianae* using paired culture. Corrêa et al. (2011) used the same antagonistic technique and found that the reduction of *P. parasitica* colony growth, in the presence of *Trichoderma* spp., could be attributed to the release of toxic compounds and nutrient depletion in the culture medium. According to Howell (2003), several *Trichoderma* species have the ability to produce toxic substances that are able to inhibit the growth of pathogens.

The evaluation of the production of antimicrobial compounds by *Trichoderma* that could affect the development of *P. nicotianae* in citrus plants showed that 41 *Trichoderma* spp. isolates produced volatile compounds capable of inhibiting the pathogen colony growth.

Isaias et al. (2014) found that only volatile compounds produced by *T. harzianum, T. koningiopsis, and T. asperellum* inhibited the growth of *Sclerotium rolfsii* and *Verticillium dahliae* colonies, with values significantly higher than 60% and between 40% and 60%, respectively. Martins-Corder & Melo (1998) found that 4 out of 7 *Trichoderma* spp. isolates produced volatile compounds with higher inhibitory effect on the growth of *V. dahliae* colony although there were no statistical differences between them. Fialho et al. (2010) studied the biological control of the *Phyllosticta citricarpa* using the yeast *Saccharomyces cerevisiae* and related plant pathogen control up to 87.2% by production of volatile compounds, according to these authors this was attributed to the production of eight substances, mainly alcohols.

However, based on the results obtained in the present study, it can be said that not only the isolates, but also the carbon source used in the *Trichoderma* spp. culture medium can favor the production of these volatile compounds, which, in this study, was favored by the addition of sucrose or maltose in the culture medium. According to Ezra & Strobel (2003) the composition of the medium used to

support the growth of *Muscodor albus,* which inhibits and kills fungi and bacteria by emitting volatile organic compounds, greatly influences the quality and effectiveness of the volatiles emitted. To the authors, a sucrose enriched medium mostly yielded methyl isobutylketone and acetic acid, butyl ester as the primary volatiles and neither of these substances appeared in any other medium tested.

On the other hand, Rossi-Rodrigues et al. (2009), investigating the growth of four species of *Trichoderma* in media supplemented with sucrose and glucose, found that the growth rate in the media with glucose was five times higher than that in the media with sucrose and that the growth of *T. hamatum* was 40% higher with glucose than with sucrose.

The thermostable compound production analysis showed that only 7 out of the 50 isolates tested (TB06, TB21, TB22, TB28, F1A2T1001, TB13, and T2A2F1021) inhibited the mycelial growth of *P. nicotianae,* showing that even at high temperatures these isolates were capable to release metabolites that inhibited the development of the pathogen. The same was found by Isaias et al. (2014) when studying the production of thermostable non-volatile metabolites by the 20 *Trichoderma* spp. isolates against *S. rolfisii* and *V. dahlia* growth; they observed that 4 isolates inhibited the growth of these pathogens, with inhibition values higher than 50% and from 54% to 60%, respectively.

The effect of cell-free antifungal compounds produced by *Trichoderma* spp. showed that 14 *Trichoderma* spp. isolates (TB02, TB04, TB06, TB08, TB09, TB10, TB12, TB14, TB21, TB22, TB28, TB30, F1A9T2006, and F2T1A1029) showed significant inhibition of *P. nicotianae* colony growth. It is assumed that the metabolites produced by most isolates were protein *in nature*, since, with the exception of TB06, TB21, TB22 and TB28 isolate, no other antifungal substances produced were able to withstand the high temperature. Corrigir: According to Monte (2001), the most of the enzymes tested as purified proteins have presented strong antifungal activity against fungi and that *Trichoderma* strains have shown great potential in agriculture as active components in fungicidal formulations. Studying the effect of cell-free extracts of *Trichoderama* spp. on the mycelial growth of *Rhizopus stolonifer,* Bomfim et al. (2010) found that 4 isolates inhibited the pathogen mycelial growth, exhibiting a remarkable antifungal activity.

It is important to mention that TB06, TB21, TB22 and TB28 produce metabolites that affect the *P. nicotianae* colonies development and, these substances maintained their antagonistic activities even after exposure to high temperature. On the other hand, the *Trichoderma* isolates F1A2T1001, TB13, and T2A2F1021 only produced antifungal compounds when subjected to autoclaving. A hypothesis is that components present in the microrganisms with activity against to *P. nicotianae* were released to the medium after exposure to high temperatures, according to Kupper & Fernandes (2002).

In the alfalfa seedling bioassay, which aimed to select the *Trichoderma* spp. isolates with the best biocontrol potential, it was observed that the isolates TB12, TB14, TB28, and TB30 significantly inhibited the formation of sporangia and mycelium and were thus considered as promising biological control agents against *P. nicotianae*. Moereover, Leoni & Ghini (2002), who evaluated the antagonistic potential of different bacteria, actinomycetes, and fungi isolates against *P. nicotianae*, reported that only 2 isolates, F9.1 (*Aspergillus sp*.) and A12.1 (actinomycete, not identified), were considered promising biocontrol agents and that one *Trichoderma* spp. isolate (F12.3) inhibited the formation of sporangia only at the root of the seedlings.

The present study also indicates that the *in vitro* antagonistic interactions among the microrganisms do not necessarily lead to the reduction in the number of phytopathogen propagules and consequently to the suppression of the disease. However, the data presented here suggest that the *Trichoderma* isolates TB12, TB14, TB28, and TB30 are promising biocontrol agents against *P. nicotianae* and that among the mechanisms of action that may be involved in the biocontrol are the production of volatile compounds and cell-free filtrates. These results are partly in agreement with the *in vitro* results reported by Corrêa et al. (2011). According to these authors, *T. pseudokoningii and T. virens* were the main growth inhibitors of *P. parasitica,* and the mechanisms responsible for controlling the disease in Rangpur lime plants were, probably, the competition for nutrients and antimicrobial compound production. Malajczuk (1983) also reported that the main mechanisms involved in the biocontrol against *Phytophthora* spp. can be nutrient competition and antibiosis.

As a conclusion of this work we can say that four out of the 50 *Trichoderma* spp. isolates evaluated, TB12, TB14, TB28, and TB30 exhibited potential for use as biological control agents, both *in vitro* (by production antimicrobial compounds) and in the alfalfa seedling bioassay against *P. nicotianae* since these isolates were able to affect the production of sporangia and mycelia. However, further studies on citrus plants are necessary to confirm the potential of these isolates as biocontrol

agents against the disease. The mechanisms of action that may be involved in the biocontrol are production of volatile compounds and cell-free filtrates by the antagonist. Interactions among plant, pathogen, and fungal antagonist in different environmental conditions and the knowledge about the survival of these *Trichoderma* isolates in the soil are important factors to be addressed in future studies.

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