



RESEARCH ARTICLE

ANTAGONISM OF *TRICHODERMA* ISOLATES AGAINST PLANT PATHOGENS OF AGRICULTURAL INTEREST

^{*}¹Gerusa Pauli Kist Steffen, ¹Joseila Maldaner, ²Ricardo Bemfica Steffen, ³Ricardo Mezzomo, ³Breno Bevilaqua Heinz, ³Zaida Inês Antonioli, ³Marlove Fátima Brião Muniz and ⁴Sabrina de Fátima Barbosa Dahmer

¹State Foundation of Agricultural Research, Rio Grande do Sul, Brazil

²Renovagro - Renewable Agriculture, Brazil

³Department of Soils, Federal University of Santa Maria, Brazil

⁴Rural Federal University of Rio de Janeiro, Brazil

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ABSTRACT

Trichoderma fungi are the main pathogens of biocontrol agents used in agriculture. The isolation and *in vitro* selection of these antagonistic fungi are essential steps in establishing biological control programs and the composition of commercial products. This study aimed to identify, evaluate and select *Trichoderma* spp. for biological control of plant pathogens of agronomic importance. The antagonist potential of eighteen *Trichoderma* isolates was tested with *Sclerotinia sclerotiorum*, *Alternaria alternata*, *Ceratocystis fimbriata*, *Fusarium oxysporum* and *Phoma* sp. through direct *in vitro* confrontation tests. The degree of isolate antagonism was assessed according to the criteria of Bell et al. (1982). All isolate antagonists were effective in competing for space and nutrients, with high or intermediate antagonistic action against different species of plant pathogens confronted. There were differences in the biocontrol potential of the same *Trichoderma* sp. in relation to different plant pathogens, as well as among isolates belonging to the same species. The *Trichoderma* sp. isolates tested in this work were effective in the *in vitro* control of *Sclerotinia sclerotiorum*, *Alternaria alternata*, *Ceratocystis fimbriata*, *Fusarium oxysporum* and *Phoma* sp.

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INTRODUCTION

Phytopathogens lead to significant economic losses and require efficient control practices. Chemical control generates the accumulation of residues in the environment, hindering the benefits of microbiota in the soil (Ethur et al., 2007) and causing increased resistance of the phytopathogens to the synthetic compounds (Amaral & Bara, 2005). In addition, synthetic fungicides only present a temporary effect and require successive applications during the crop cycle (Ávila et al., 2005), consequently promoting residue accumulation and environmental contamination. Thus, biological control is a more sustainable alternative (Ethur et al., 2007), and biocontrol agents are capable of establishing, colonizing and dispersing in the ecosystem (Ávila et al., 2005). Fungi of the genus *Trichoderma* Person ex Fries are the most important biological control agents in agriculture. Species of *Trichoderma* spp., which are popularly known as *Trichoderma*,

have been used successfully in the biological control of plant pathogens in soil and are the basis for numerous commercial organic products. This is because they are effective in the competition for space and nutrients (Harman, 2006; Karthikeyan, 2016), produce antifungal compounds (Edward et al., 2007; Almeida, 2009), have low production cost, ease of mass production and dispersal of propagules (Mendonza et al., 2016). Research has shown that early seed treatment with *Trichoderma* spp. reduces the incidence of plant pathogens in field and semi-field conditions and provides a higher percentage of normal plants providing there is the presence of phytopathogenic fungi in the soil or substrate (Edward et al., 2008; Gava & Pinto, 2016). Some species of *Trichoderma* sp. act as inducers of plant resistance against diseases and have the ability to promote plant growth through the synthesis of hormones, increased availability of nutrients present in the soil and stimulus for absorbing nutrients, among other factors (Hoyos-Carvajal et al., 2009). The potential use of *Trichoderma* spp. in biological control is assigned to different mechanisms of action, which vary according to the specificities of plant pathogens and isolated antagonists. The

*Corresponding author: Gerusa Pauli Kist Steffen,
State Foundation of Agricultural Research, Rio Grande do Sul, Brazil.

main mechanisms used by these fungi are the competition for space, nutrients and oxygen (Harman, 2006), antibiosis or production of volatile and non-volatile metabolites (Almeida, 2009; Edward *et al.*, 2007) and hyperparasitism (Bashir *et al.*, 2010). Among the species of *Trichoderma* sp., *T. harzianum* is the most studied due to its use as a biocontrol agent, although isolates from the species *T. hamatum*, *T. viride*, *T. koningii* and *T. pseudokoningii* also present this action potential (Milanesi *et al.*, 2013). Considering that the isolation and subsequent *in vitro* selection of antagonistic agents are key steps in the biological control of phytopathogens, this study aimed to identify, evaluate and select *Trichoderma* spp. isolates in terms of their antagonist potential for *Alternaria alternata* (F.: Fr.) Keissl., *Fusarium oxysporum* f. sp. phaseoli Kendrick and Snyder, *Sclerotinia sclerotiorum* (Lib.) of Bary, *Ceratocystis fimbriata* Ellis & Halsted and *Phoma* sp.

MATERIALS AND METHODS

Obtaining *Trichoderma* sp. isolates and plant pathogens

The *Trichoderma* sp. isolates were obtained from soil samples of native forest fragments, rhizosphere of sugar cane and tomato plants as well as yerba mate seeds, all collected in the central region of the state of Rio Grande do Sul (RS). The *Sclerotinia sclerotiorum* isolate was isolated from fungal propagules of typical lesions of this pathogen (white cottony mycelium) present in the stem and leaves of tomato plants grown in the greenhouse. Identification of the pathogen was based on morphological parameters. *Fusarium oxysporum* and *Phoma* sp. isolates were obtained through *in vitro* insulation of nodal segments of *Axonopus affinis* and *Stipasetigera*, respectively. The identification of these pathogens was confirmed through molecular techniques, with the extraction and amplification of DNA carried out in the laboratory of Molecular Biology of the Rural Sciences Center of the Federal University of Santa Maria (UFSM). *Ceratocystis fimbriata* and *Alternaria alternata* isolates were obtained from the Phytopathology Laboratory of the Plant Protection Department of the Rural Science Center (UFSM).

In vitro antagonism tests

In order to evaluate the antagonism of the *Trichoderma* sp. isolates against the different species of plant pathogens, the method of paired culture was used (Dennis & Webster, 1971), with the phytopathogens transferred 48 h before the antagonists and in opposite positions on the Petri dishes (90 mm). The dishes were kept in a climatic chamber BOD for seven days at 25° C (± 2° C) with photoperiod of 12 h. The evaluations were carried out at the end of the incubation period by measuring the diameter of the colonies of each pathogen and its respective antagonist with a millimeter ruler and grouping the isolates into classes, according to the scale described by Bell *et al.* (1982). A completely randomized design was used with four replicates for each *Trichoderma* sp. isolate. Based on the values of average diameter of the colonies (ADC) of the plant pathogens in the presence and absence of the antagonists, the control index (CI) of plant pathogens was determined for each *Trichoderma* sp. isolate through the equation: $IC (\%) = [(ADC \text{ in the absence of antagonists} - ADC \text{ in presence of antagonists}) / (ADC \text{ in absence of antagonists})] \times 100$. Data were subjected to statistical analysis using the Scott-Knott test ($P \leq 0.05$) with the aid of the computer program Sisvar (Ferreira, 2011).

Molecular identification of some fungal isolates

Molecular identification of five *Trichoderma* sp. isolates and two plant pathogens (*Fusarium oxysporum* and *Phoma* sp.) was conducted by sequencing the ITS region of the genomic DNA. To extract the DNA, the Zr Fungal/Bacterial DNA Miniprep® (Zymo Research) kit was used according to the manufacturer's instructions. The DNA was extracted from the fungal culture in potato-dextrose-agar medium. The isolates were subjected to the Polymerase Chain Reaction Technique (PCR) through the ITS region rDNA ITS1 (TCCGTAGGTGA ACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The purification of the PCR product was performed via GenElute kit™ PCR Clean-Up Kit® (Sigma Aldrich) following the manufacturer's instructions. The company Ludwig Biotec using an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems) conducted the sequencing.

RESULTS AND DISCUSSION

Five *Trichoderma* sp. isolates were identified by molecular techniques and belonged to the species *T. asperellum* (isolates T02 and T04), *T. hamatum* (isolates T03 and T05) and *T. harzianum* (isolate T08). Identification data, origin and date of isolation of the different strains obtained are shown in Table 1. Significant differences were observed in the antagonistic potential of *Trichoderma* sp. isolates in relation to the control of the various plant pathogens (Table 2). In regards to the criteria of Bell *et al.* (1982), all isolated antagonists were efficient in the competition for space and nutrients, showing high (Classes 1 and 2) or intermediate (Class 3) antagonistic action according to the phytopathogenic fungal species. By analyzing just the isolates identified at species level, it was possible to observe that *T. asperellum* was more efficient than *T. hamatum* in the control of *S. sclerotiorum* (Table 2) and *Phoma* sp. (Table 3), although both presented similar efficiency in controlling *C. fimbriata* and *F. oxysporum* (Table 3). The difficulty in controlling *S. sclerotiorum* is due to its ability to form structures of resistance in the soil (sclerotia), together with the broad spectrum of the hosts, which limits the use of practices such as crop rotation. In most situations, chemical control is ineffective and dependent on several factors, such as mode of action and dose of the product, volume of the syrup, favorable environmental conditions for the development of the phytopathogenic fungus at the time of application and culture stage of development (Zhan *et al.*, 2015). In this study, nine *Trichoderma* sp. isolates showed a high degree of *in vitro* antagonism (Classes 1 and 2) for *S. sclerotiorum* and six presented intermediate antagonistic action (Class 3). Only one isolate (T08) was inefficient in controlling *S. sclerotiorum* (Class 4) (Table 2).

All evaluated antagonists measured showed a high degree of antagonism for *A. alternata* and *C. fimbriata* (Tables 2 and 3). After contact between the antagonist and pathogenic colonies, the *Trichoderma* sp. isolates continued growing over the colony of the pathogen, thus producing conidia. These results are likely related to the lower growth rate of *C. fimbriata* and *A. alternata*, which is a characteristic that facilitates the antagonist to control the pathogen, in addition to the high competitiveness of the antagonistic isolates for space and nutrients. Considering the difficulty of control and the great impact of *A. alternata* and *C. fimbriata* in agricultural activity due to the damage caused in the production of fruits and vegetables (Zambolim *et al.*, 2002; Tumura *et al.*, 2012), these

results demonstrate the possibility of biological control of these plant pathogens through the use of *Trichoderma* spp. Furthermore, there was an inhibition of mycelial growth and differences between the average radius of the colonies of the five species of plant pathogens confronted in relation to the average radius of the control group (pure culture of the phytopathogens) after seven days of confrontation (Tables 2 and 3).

The reduction in the growth of plant pathogens in the presence of the antagonists can be attributed to the competition for space and nutrients and/or hyperparasitism (Vinale *et al.*, 2008). The results obtained in this study show the antagonistic effect of *Trichoderma* sp. isolates against the phytopathogenic fungi confronted and corroborate with Hu *et al.* (2016), who proved the hyperparasitic effect of *Trichoderma* spp. over *S. sclerotiorum*.

Table 1. Identification, origin, place and date of collection of the 18 *Trichoderma* sp. isolates evaluated

Identification	Numberoftheisolate	Origin	Municipality	Date ofisolation
NI	T01	Nativeforestsoil	Santa Maria (Fepagro)	07-04-2013
<i>T. asperellum</i>	T02	Nativeforestsoil	Santa Maria (Fepagro)	07-04-2013
<i>T. hamatum</i>	T03	Nativeforestsoil	Santa Maria (Fepagro)	07-04-2013
<i>T. asperellum</i>	T04	Nativeforestsoil	Santa Maria (Fepagro)	07-04-2013
<i>T. hamatum</i>	T05	Nativeforestsoil	Santa Maria (Fepagro)	07-04-2013
<i>T. harzianum</i>	T08	Rhizosphereof sugar cane	Santa Maria	07/15/2013
NI	T12	Rhizosphereof oats	Santa Maria	10-03-2013
NI	T13	Rhizosphereof oats	Santa Maria	10-03-2013
NI	T18	AraucariaRhizosphere	Santa Maria	09/27/2013
NI	T21	Yerba mate seeds	Ilópolis	11/22/2013
NI	T23	Yerba mate seeds	Ilópolis	11/29/2013
NI	T24	Yerba mate seeds	Ilópolis	11/29/2013
NI	T25	Rhizosphereoftomatoplants	Santa Maria	02-05-2014
NI	T26	AraucariaRhizosphere	Santa Maria (Fepagro)	02-05-2014
NI	T27	AraucariaRhizosphere	Santa Maria (Fepagro)	02-05-2014
NI	T28	AraucariaRhizosphere	Santa Maria (Fepagro)	02-05-2014
NI	T29	AraucariaRhizosphere	Santa Maria (Fepagro)	02-05-2014
NI	T30	Rhizosphereoftomatoplants	Caçapava do Sul	02-12-2014

¹NI = isolate unidentified at species level.

Table 2. Diameter of the colonies (ADC) of the antagonist isolates and the phytopathogens *Sclerotinia sclerotiorum* and *Alternaria alternata* at 7 days of paired cultures, *in vitro* control of phytopathogens (IC) and classification of *Trichoderma* sp. isolates for antagonism, according to the scale by Bell *et al.* (1982)

Species of Phytopathogen	Antagonist isolate	ADC (mm)		ADC (mm) of the phytopathogens in absence of antagonist	IC (%) ^(a)	Scale by Bell <i>et al.</i> (1982) ^(b)
		Antagonist	Pathogen			
<i>Sclerotinia sclerotiorum</i>	T01	50.59 d [*]	39.40 c	51.61 c	23.65 d	3.25 b
	T02	63.30 c	58.39 a	85.00 a	31.31 c	2 c
	T03	46.23 d	45.18 b	85.00 a	46.84 b	3 b
	T04	90.00 a	47.99 b	85.00 a	43.54 b	1 e
	T05	43.18 e	46.82 b	85.00 a	44.92 b	3 b
	T08	34.37 e	55.63 a	85.00 a	34.56 c	4 a
	T12	44.25 e	45.75 b	63.37 b	27.80 d	3 b
	T13	41.70 e	48.29 b	51.61 c	6.43 e	3.25 c
	T18	57.70 c	32.30 e	51.61 c	37.41 b	2.25 c
	T23	79.14 b	14.98 f	42.85 e	56.31 a	1.75 c
	T24	62.18 c	35.69 d	45.36 d	21.31 d	1 e
	T25	58.61 c	33.74 d	51.61 c	34.62 c	2 c
	T26	55.68 c	34.32 d	51.61 c	33.51 c	3 b
	T27	75.38 b	33.28 d	45.36 d	26.63 d	1 e
	T28	63.42 c	22.89 e	42.85 e	47.56 b	2 c
T30	72.39 b	25.32 e	42.85 e	42.57 b	1.5 c	
CV (%)		11.28	9.74	4.75	20.78	13.64
<i>Alternaria alternata</i>	T01	80.65 a	16.17 c	32.12 c	49.67 a	1.5 b
	T02	66.40 c	29.14 a	40.50 a	28.05 c	1.25 b
	T04	90.00 a	28.18 a	40.50 a	30.42 c	1 c
	T08	61.53 c	28.68 a	40.50 a	29.19 c	2 a
	T12	68.92 c	19.44 b	32.12 c	39.47 b	2 a
	T13	75.47 b	15.62 b	32.12 c	54.35 a	1.75 a
	T18	74.45 b	14.37 b	32.12 c	55.26 a	1.75 a
	T21	65.22 c	24.78 a	36.03 b	31.22 c	2 a
	T23	67.05 c	24.15 a	36.03 b	32.97 c	2 a
	T24	65.64 c	24.36 a	36.03 b	32.39 c	2 a
	T25	59.78 c	20.15 b	32.12 c	37.26 b	2 a
	T26	63.58 c	26.42 a	40.50 a	34.76 c	2 a
	T27	64.57 c	25.43 a	36.03 b	29.43 c	2 a
	T28	90.00 a	26.66 a	40.50 a	34.17 c	1 c
	T29	69.91 c	25.07 a	36.03 b	30.41 c	2 a
T30	90.00 a	26.89 a	36.03 b	25.37 c	1 c	
CV (%)		12.84	10.59	8.45	20.67	13.56

^(a)IC = mycelial growth inhibition (%)

^(b)Class 1: *Trichoderma* grows on the pathogen and occupies the whole surface of the medium; Class 2: *Trichoderma* grows on at least 2/3 of the surface of the medium; Class 3: *Trichoderma* occupies approximately half of the surface of the medium; Class 4: *Trichoderma* grows on 1/3 of the surface of the medium; Class 5: *Trichoderma* does not grow and the pathogen takes up the whole surface of the medium.

*Values followed by the same letter in the columns for each pathogen do not differ by the Scott-Knott test ($P \leq 0.05$).

The five *Trichoderma* sp. isolates confronted with *F. oxysporum* and *Phoma* sp. presented a high degree of antagonism against the plant pathogens (Table 3). In regards to their *in vitro* control of *Phoma* sp., the isolates T02 and T04, which belong to the species *T. asperellum*, excelled and were grouped into Class 1 according to the criteria of Bell *et al.* (1982) (Table 3).

The *T. asperellum* isolate obtained from a 2-year-old compost pile showed better performance in the control of phytopathogens tested when compared to three isolates from the commercial product belonging to the same species (*T. asperellum*). The results were attributed to the higher speed of growth and colonization of the culture medium of isolates from the compost pile, when compared to the isolate from the

Table 3. Diameter of the colonies (ADC) of the antagonist isolates and the plant pathogens *Ceratocystis fimbriata*, *Fusarium oxysporum* and *Phoma* sp. at 7 days of paired culture, *in vitro* control of phytopathogens (IC) and classification of *Trichoderma* sp. isolates for antagonism according to the scale by Bell *et al.* (1982)

Species of Phytopathogen	Antagonist isolate	ADC (mm)		ADC (mm) of the phytopathogens in absence of antagonist	IC (%) ^(a)	Scale by Bell <i>et al.</i> (1982) ^(b)
		Antagonist	Pathogen			
<i>Ceratocystis fimbriata</i>	T01	90.00 a*	25.48 c	35.60 b	28.42 c	1 c
	T02	85.00 a	22.16 d	42.23 a	47.51 a	1 c
	T03	85.00 a	23.75 d	42.23 a	43.75 a	1 c
	T04	85.00 a	23.36 d	42.23 a	44.68 a	1 c
	T05	85.00 a	24.23 c	42.23 a	42.61 a	1 c
	T08	90.00 a	28.63 a	44.77 a	36.06 b	1 c
	T12	90.00 a	25.19 c	41.06 a	38.66 b	1 c
	T13	90.00 a	23.54 d	32.97 b	28.60 c	1 c
	T18	66.28 c	23.72 d	35.60 b	33.38 b	2 a
	T21	75.18 b	26.03 b	32.97 b	21.04 d	2 a
	T23	90.00 a	28.14 a	32.97 b	14.66 d	1 c
	T24	72.19 b	22.83 d	32.97 b	30.77 c	1.5 b
	T25	90.00 a	28.19 a	35.60 b	20.83 d	1 c
	T26	88.68 a	26.66 b	32.97 b	19.14 d	1 c
T27	88.01 a	27.03 b	32.97 b	18.01 d	1 c	
T28	81.91 b	25.27 c	32.97 b	23.35 d	1 c	
T29	90.00 a	24.91 c	32.97 b	24.46 c	1 c	
T30	66.40 c	25.86 b	42.54 a	39.21 b	2 a	
CV (%)		14.82	5.50	6.83	12.12	9.87
<i>Fusarium oxysporum</i>	T02	52.56 a	32.44 b	65.30 a	50.32 a	2 a
	T03	52.94 a	32.06 b	65.30 a	50.90 a	2 a
	T04	54.60 a	30.40 b	65.30 a	53.45 a	2 a
	T05	53.90 a	31.10 b	65.30 a	52.38 a	2 a
	T12	51.37 a	38.63 a	63.99 b	39.63 b	2 a
CV (%)		6.94	6.72	3.30	8.16	12.18
<i>Phoma</i> sp.	T02	85.00 a	33.44 b	59.60 a	43.90 a	1 c
	T03	47.86 c	37.14 a	59.60 a	37.68 b	2 a
	T04	85.00 a	34.34 b	59.60 a	42.38 a	1 c
	T05	48.96 c	36.04 a	59.60 a	39.54 b	2 a
T12	60.11 b	33.09 b	59.93 a	44.79 a	1.6 b	
CV (%)		7.36	3.30	2.98	4.61	16.12

^(a)IC = mycelial growth inhibition (%)

^(b)Class 1: *Trichoderma* grows on the pathogen and occupies the whole surface of the medium; Class 2: *Trichoderma* grows on at least 2/3 of the surface of the medium; Class 3: *Trichoderma* occupies approximately half of the surface of the medium; Class 4: *Trichoderma* grows on 1/3 of the surface of the medium; Class 5: *Trichoderma* does not grow and the pathogen takes up the whole surface of the medium.

*Values followed by the same letter in the columns for each pathogen do not differ by the Scott-Knott test ($P \leq 0.05$).

The results confirmed the occurrence of parasitism of species of the genus *Trichoderma* on the plant pathogens studied, as indicated by Papavizas (1985). Direct confrontation tests showed that there are differences in the biocontrol potential of a single *Trichoderma* isolate against different plant pathogens, as well as between isolates belonging to the same species (Tables 2 and 3), as suggested by Louzada *et al.* (2009). According to them, species of *Trichoderma* sp. may be differentially selective against different fungi, reflecting differences in the antagonism capacity of each species or isolate. Probably, these differences are related to behavioral characteristics of each isolate, whose expression is closely related to soil and climate conditions of the microorganisms' place of origin, as well as to the different mechanisms of action against the phytopathogenic species. Fungi of the genus *Fusarium* cause diseases in a range of cultivated plants (Rezende *et al.*, 1997). Evaluating the potential of *in vitro* control of two *Trichoderma* sp. isolates in regards to *Fusarium solani*, *Sclerotinia sclerotiorum* and *Rhizoctonia* sp., Brito *et al.* (2010) also observed differences in the performance of the isolates, as observed in this study.

commercial product. In relation to the phytopathogens *F. solani* and *Rhizoctonia* sp., the isolates from the compost pile and the commercial product, both from the species *T. asperellum*, were grouped into the Classes 1 and 3, respectively, in accordance with Bell *et al.* (1982). The study of Louzada *et al.* (2009) reinforces the need for *in vitro* evaluations of *Trichoderma* sp. isolates to control different plant pathogens. Among the 230 isolates assessed in the control of *S. sclerotiorum* and *F. solani* f. sp. phaseoli, through the paired culture test, 48% inhibited the growth of *S. sclerotiorum* and only 21.7% inhibited the growth of *F. solani*. From the total number of *Trichoderma* sp. isolates tested, only 10% presented antagonism for the two phytopathogens confronted. Barbosa & Meza (2009) classified *T. harzianum* isolates as competent in the fight for nutrients and space since they presented an increased growth rate. Similarly, the *T. harzianum* isolate used in the present study was also found to be effective in the control of phytopathogens. Knowing that the use of biocontrol agents is a viable, efficient, practical and promising alternative to synthetic fungicides, which, in addition to raising the production costs and contaminating the environment (Vinale *et al.*, 2008), increase the resistance of

phytopathogenic microorganisms (Amaral & Bara, 2005), it is essential to invest in the evaluation and selection of microorganisms with potential for use in biological control. The results of this study indicate that the isolates assessed have biotechnological potential for biological control of important diseases of agricultural interest. Future field trials to assess the efficiency of the isolates in the *in vivo* control of these plant pathogens may indicate more efficient and interesting strains for composition of commercial products.

Conclusion

The *Trichoderma* sp. isolates evaluated in this study were efficient in the *in vitro* control of *Sclerotinia sclerotiorum*, *Alternaria alternata*, *Ceratocystis fimbriata*, *Fusarium oxysporum* and *Phoma* sp. Differences were observed in the biocontrol potential of a single *Trichoderma* sp. isolate against different plant pathogens, as well as between isolates belonging to the same species.

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