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Original Article

Silviculture

Pine Seeds Treatment with Trichoderma for Fusarium Control

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ABSTRACT

This study analyzes the in vitro antagonistic activity of *Trichoderma* sp. isolates against *Fusarium subglutinans* and evaluates the effect of the pine seeds treatment with *Trichoderma* sp. on the incidence of root rot. Twelve *Trichoderma* sp. isolates and two *F. subglutinans* isolates were included in the study. *Trichoderma* sp. inhibited *F. subglutinans* mycelial growth through direct contact with hyphae and the production of volatile antifungal compounds. Pine seeds treatment with the antagonist *Trichoderma* sp. reduced the incidence of root rot, increased the emergence and initial growth in the height of seedlings, and improved seedling health.

Keywords: biological control, forest seeds, forest pathology.

1. INTRODUCTION

The planted pine (*Pinus* spp.) area in Brazil has approximately 1.6 million hectares and is concentrated in the southern region (IBÁ, 2016). In their pioneering study on imported seeds of several *Pinus* species, Lasca et al. (1971) demonstrated for the first time the presence of fungi in their seeds, including *Pestalotia*, *Fusarium*, *Mucor*, *Aspergillus*, *Trichothecium*, *Alternaria*, *Diplodia*, *Botryodiplodia*, *Helminthosporium*, *Chaetomium*, *Rhizopus*, *Neurospora*, and *Penicillium*. Recently, the quarantine pest *Fusarium circinatum* was detected in pine seedlings from imported seeds (Pfenning et al., 2014). Nevertheless, the health quality of pine seeds has been little studied in Brazil (Santos et al., 2011, 2015; Maciel et al., 2014).

In addition to the study on the risk of using imported pine seeds (Pfenning et al., 2014), the few works performed in Brazil with national pine seeds have shown the association of several *Fusarium* species with pine seed rot and damping-off (Maciel et al., 2014; Homechin et al., 1986; Santos et al., 2011). In the 1980s, *F. moniliforme*, *F. oxysporum* and *F. semitectum* were reported in seed lots of *Pinus taeda* and *Pinus elliottii* var. *eliottii* from Paraná and Santa Catarina states (Homechin et al., 1986). In addition, Maciel et al. (2014) recently found *F. sambucinum* in seed lots of *Pinus elliottii* from the Rio Grande do Sul state, which were causing stem-base girdling and damping-off.

Although the treatment of forest seeds can be used to avoid the spread of pathogens and to guarantee plant health and silvicultural quality, this tools has been still little studied (Santos et al., 2011, 2015). Moreover, routine treatments of pine seeds are not performed due to the lack of tested and registered biological and chemical products (Santos & Parisi, 2011; Santos et al., 2011). Restrictions on the use of fungicides and environmental care reinforce the search for viable alternatives that are less damaging to nature and human health, like the bio-protectors. Biological seed treatment has advantages because it is non-polluting, contributes to a more stable disease control, and is useful in controlling pathogens in several crops (Ludwig et al., 2009; Carvalho et al., 2011a, b, 2014, 2015; Pedro et al., 2012; Junges et al., 2016). However, in-depth studies are necessary for the extension and improvement of this technique to be applied in forest seeds.

Limited information available on seed pathology has been pointed out as a determining factor in the low adoption of pine seed treatment (Santos & Parisi, 2011; Santos et al., 2011). Seed treatment with antagonistic fungi against phytopathogens can be useful for the sustainable phytosanitary management of pine nurseries. *Trichoderma* species have been widely used in seed treatments to control seed and soil pathogens, and to promote benefits in the growth of various agricultural plants (Carvalho et al., 2011a, 2015; Pedro et al., 2012; Maciel et al., 2014; Junges et al., 2016). In this context, this study aims to a) evaluate the *in vitro* antagonism of *Trichoderma* spp. against *F. subglutinans* through the confrontation of cultures (CC) and production of volatile and non-volatile metabolites (VM and NVM, respectively); and b) to evaluate *Fusarium* control through the seed treatment of pine seeds with *Trichoderma*.

2. MATERIAL AND METHODS

2.1. Fungal isolates

Trichoderma sp. (T106, TER, TRA, TRC, TRD, TRF, TRS, TRB1, TRB2, TR0506, TR2A, TR2B) and *F. subglutinans* (FS1 and FS2) isolates were obtained from the Collection of Forest Fungi of the *Embrapa Florestas*, Colombo, Paraná state, Brazil.

2.2. Antagonism in CC

Trichoderma sp. (T106, TER, TRA, TRC, TRD, TRF, TRS, TRB1, TRB2, TR0506, TR2A, TR2B) and *F. subglutinans* (FS1 and FS2) isolates were grown in potato dextrose agar (PDA) medium for seven days in the dark, at 24 °C. A PDA disc (5 mm in diameter) containing actively growing mycelium of *F. subglutinans* was placed in Petri dishes, each one containing PDA near its edge. After 48 hours, a PDA disc (5 mm in diameter) containing actively growing mycelium of *Trichoderma* was placed on the opposite side, near the edge of each Petri dish. The cultures were incubated in a 12-hour photoperiod for seven days, at 24 °C. The evaluation consisted in determining the degree of antagonism according to the score scale determined in Bell et al. (1982). Growth inhibition of the pathogen was calculated according to Edgington et al. (1971). The design was completely randomized with 13 treatments and four replicates. The experiment was conducted twice.

2.3. Effect of VM and NVM

The methodology described in Mariano (1993) was used for the VM. *Fusarium subglutinans* (FS1 and FS2) and *Trichoderma* sp. (TRB1, TRB2, TR0506) isolates were cultivated in Petri dishes with PDA medium for seven days in the dark, at 24 °C. PDA discs (5 mm in diameter) containing actively growing mycelium of *F. subglutinans* and *Trichoderma* sp. were placed separately in the center of Petri dishes containing PDA medium. After 24 hours, the plate containing *F. subglutinans* was superimposed on that of *Trichoderma* sp. and the two plates were gathered using PVC film to prevent the escape of VM. The plates were incubated in a Bio-Oxygen Demand (BOD) incubator under the same conditions mentioned in the above experiment. The control received only mycelial discs from both pathogens, both at the top and at the bottom of the Petri dish. The evaluation consisted of measuring the colony diameter of the pathogen and was performed on the seventh day after setting up the experiment.

The methodology described in Michereff et al. (1993) was used for the NVM. Herein the PDA medium inside the Petri dishes was covered with a sterile cellophane disc. Then, a PDA disc containing actively growing mycelium of *Trichoderma* sp. was placed in the center of the plate and incubated in a 12-hour photoperiod for 72 hours, at 24 °C. Subsequently, the cellophane paper with the adherent culture of *Trichoderma* sp. was removed and a PDA disc containing actively growing mycelium of *F. subglutinans* was transferred to the center of the plates. The control consisted of *F. subglutinans* culture after the cellophane removal, without previously superimposing the antagonist. The evaluation was performed on the seventh day after the replication by measuring the diameter of the *F. subglutinans* colonies. The experiment was conducted twice. The design was completely randomized with four treatments (three antagonist isolates plus control) and four replicates. Colony diameter values were submitted to analysis of variance, and the means were compared by the Tukey's test at 5% probability.

2.4. Treatment with Trichoderma sp. of pine sees carrying Fusarium sp.

Three pine (*P. taeda*) seed lots were used with different levels of *Fusarium* incidence: high (94%), medium (49%), and low (12%). *Trichoderma* sp. isolates (TRB1 and TR0506) were cultured in Petri dishes with PDA medium and incubated for seven days at 24 °C. Sterile distilled water (10 mL) was added to each Petri dish, and the sporulating mycelial mass was scraped with a glass stick. Next, the material was filtered on sterile gauze, and the conidial suspension was adjusted to 1×10^6 conidia/mL. *Pinus taeda* seeds were immersed in the conidia suspension for five minutes. For fungicide treatment, thiophanate-methyl + chlorothalonil (2.15 g of commercial product (c.p.)/kg of seeds) was used. The control seeds received no treatment. After treatment, the pine seeds were placed in a selective medium (Anderson, 1986) for detecting *Fusarium* sp. Petri dishes were incubated at 20 °C under fluorescent light in 12-hour photophase for 14 days. After this period, the seeds were evaluated for the presence of *Fusarium* sp. under stereoscopic and optical microscopes. The experimental design was completely randomized, with four treatments and four replicates of 50 seeds each, totaling 200 seeds per treatment.

2.5. Effect of the treatment with Trichoderma sp. of pine seeds carrying Fusarium sp. on seedling emergence

Three pine (*P. taeda*) seed lots were used with different levels of *Fusarium* incidence: high (94%), medium (49%), and low (12%). The seeds were kept in a cold room (4 °C-6 °C) for 28 days to overcome dormancy. Seed treatments with *Trichoderma* sp. (TRB1 and TR0506) isolates and with thiophanate-methyl + chlorothalonil were performed as described in the previous experiment. The seeds were then individually planted in polyethylene trays with vermiculite and kept in a greenhouse. During 90 days, weekly evaluations were performed by determining the number of healthy and symptomatic seedlings. The height of healthy seedlings was measured. The symptomatic seedlings and the non-germinated seeds (NGS) were placed in a humid chamber for the observation of fungal structures. The experiment was completely randomized, with four treatments and four replicates of 50 seeds each. Data from all tests were submitted to analysis of variance, and the means were compared by the Tukey test at 5% probability.

3. RESULTS AND DISCUSSION

In vitro antagonistic action of *Trichoderma* sp. against *F. subglutinans* was observed in the CC (Table 1) and VM production (Table 2); however, it was not detected in NVM production (Table 2).

Table 1. *In vitro* antagonism of *Trichoderma* sp. against *Fusarium subglutinans* (FS1 and FS2 isolates) evidenced by the confrontation of cultures.

Trichoderma isolates	*F. subglutinans* colony diameter (mm)		Inhibition of mycelial growth *F. subglutinans* (%)		*Trichoderma* sp. antagonistic class[2]	
	FS1	FS2	FS1	FS2	FS1	FS2
T106	45.5 a[1]	41.0 a	-	-	4.0	4.0
Control	44.1 a	39.4 a	-	-	-	-
TER	36.8 b	34.9 ab	16.4	11.5	2.5	2.3
TRA	36.2 bc	32.9 a	17.8	16.3	2.5	2.0
TRD	35.1 bc	31.9 ab	20.3	18.9	2.3	2.0
TRC	34.5 bc	31.7 ab	21.6	19.5	2.5	2.1
TRF	33.9 bc	31.5 ab	22.9	19,9	2.0	2.3
TRB2	32.9 bcd	31.3 ab	25.3	20.5	2.1	2.0
TRB1	32.2 bcd	29.1 b	26.9	26.0	2.0	1.9
TR0506	31.5 bcd	27.6 b	28.4	29.8	2.5	2.0
TRS	31.1 bcd	27.3 b	29.4	30.6	2.0	2.0
TR2B	29.9 cd	27.3 b	32.0	30.6	2.0	2.0
TR2A	27.1 d	26.4 b	38.4	32.9	2.0	2.0

[1]Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability; [2]Antagonism according to Bell et al. (1982): Class 1, *Trichoderma* grows on the pathogen and covers the entire medium surface; Class 2, *Trichoderma* grows on at least 2/3 of the medium surface; Class 3, *Trichoderma* occupies approximately half of the medium surface; Class 4, *Trichoderma* grows on at least 1/3 of the medium surface; Class 5, *Trichoderma* does not grow, and the pathogen occupies the entire medium surface.

Table 2. Inhibition of *Fusarium subglutinans* (FS2 and FS1) mycelial growth by volatile (VM) metabolites and non-volatile metabolites (NVM) of *Trichoderma* sp. (TRB1, TRB2 and TR506).

Isolates of *Trichoderma* sp. × *Fusarium subglutinans*	*F. subglutinans* mycelial growth (mm)	
	VM	NVM
TRB1 × FS2	43.3 b[1]	75.2 ns[2]
TRB2 × FS2	39.8 b	72.6
TR0506 × FS2	42.4 b	72.4
Control	52.2 a	72.8
Coefficient of variation (%)	5.3	1.4
TRB1 × FS1	41.8 a	60.8 ns
TRB2 × FS1	43.3 a	60.7
TR0506 × FS1	32.6 b	57.7
Control	49.4 a	61.7
Coefficient of variation (%)	9.0	4.3

[1]Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability; [2]not significant.

The CC test showed a significant reduction in the *F. subglutinans* mycelial growth due to the antagonism of *Trichoderma* sp. isolates (Table 1) from 11.5% to 38.4%, except for the TR106 isolate. Regarding the Bell et al. (1982) score scale, we also observed the antagonism degree 2 (Table 1) corresponding to the antagonist occupation of at least 2/3 of the Petri dish surface at seven days of incubation, except for the *Trichoderma* sp. T106 isolate. This hyperparasite antagonistic activity of *Trichoderma* against *F. subglutinans* verified in CC has already been reported for *T. harzianum* and *F. oxysporum* f. sp. *phaseoli* (Carvalho et al., 2014), and for *Trichoderma* spp. and *F. sambucinum (*Maciel et al., 2014).

This study verified a reduction of the *F. subglutinans* mycelial growth by the VM action of *Trichoderma* sp. (Table 2). *Trichoderma* sp. isolates differed significantly from the control regarding colony reduction of the *F. subglutinans* FS2 isolate. However, when confronted with the *F. subglutinans* FS1 isolate, only the *Trichoderma* sp. TR0506 isolate differed from the control, causing 33.9% of inhibition (Table 2).

Carvalho et al. (2011a) also reported the VM action of *T. harzianum* against *F. oxysporum* f. sp. *phaseoli*. In line with this study, the antagonistic actions of *Trichoderma* due to hyperparasitism and antibiosis are commonly reported (*e.g.*, Maciel et al., 2012; Harman, 2006; Carvalho et al., 2014).

There was no antagonistic action of *Trichoderma* sp. against *F. subglutinans* by the production of NVM diffusible in cellophane (Tabela 2). Carvalho et al. (2014) observed this result in *T. harzianum* against *F. oxysporum*.

There was a reduction in the *Fusarium* sp. incidence in the three pine seed lots treated with *Trichoderma* sp. (Table 3). The TR0506 isolate significantly reduced the *Fusarium* sp. incidence in the three seed lots with low, medium, and high pathogen incidence when compared to the control (P = 0.05). However, the TRB1 isolate had a significant effect only on lots with low and medium pathogen incidence. In spite of an overall lack of studies focused on the treatment of forest seeds (Santos et al., 2011, 2015), Junges et al. (2016) showed the control of *Fusarium* sp. with *Trichoderma* spp. in some seeds of native forest species such as the canafístula tree (*Peltophorum dubium*). Our results are promising and highlight the potential of *Trichoderma* in the treatment of pine seeds, as previously shown for crops (Pedro et al., 2012; Carvalho et al., 2015).

The treatment of pine seeds with *Trichoderma* sp. resulted in increased seedling emergence and NGS reduction (Table 4), as well as increased seedling emergence speed (Table 5) and seedling height (Table 6). Treatment with *Trichoderma* sp. significantly increased the percentage values of healthy seedlings in comparison to the control, whereas treatment with thiophanate-methyl + chlorothalonil did not differ from the control (Table 4). On the other hand, the percentage values of seedlings carrying *Fusarium* sp. did not differ between treatments. There was a significant reduction in the percentage values of NGS with *Fusarium* sp. for the *Trichoderma* sp. TRB1 isolate and the fungicide, whereas the *Trichoderma* sp. TR0506 isolate did not differ from the control (Table 4).

There was an increase in the emergence speed of pine seedlings from seeds carrying *Fusarium* sp. and treated with *Trichoderma* sp. (Table 5). The effect of *Trichoderma* sp. was evident in the lots with low and medium *Fusarium* sp. incidence at the 14th, 28th and 42nd days after sowing, especially for the *Trichoderma* sp. TRB1 isolate. However, in the lot with high *Fusarium* sp.

Table 3. *Fusarium* sp. incidence (%) in pine seed lots treated with *Trichoderma* sp., considering three initial incidence levels.

Treatment	*Fusarium* sp. incidence (%)		
	Low initial incidence[1]	Medium initial incidence[1]	High initial incidence[1]
Trichoderma sp. (TRB1)	10.0 b[2]	10.0 b	67.5 a
Trichoderma sp. (TR0506)	10.0 b	19.5 b	60.0 b
thiophanate-methyl + chlorothalonil	11.0 b	16.0 b	44.0 c
Control	37.5 a	63.5 a	98.5 a
Coefficient of variation (%)	39.2	25.9	9.8

[1]Lots with low (12%), medium (49%) and high (98%) *Fusarium* sp. incidence; [2]Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

Table 4. Seedling emergence and non-germinated seeds (NGS) originated from pine seeds carrying *Fusarium* sp. and treated with *Trichoderma* sp.

Treatment	Emergence (%)		NGS with *Fusarium* sp. (%)
	Healthy seedlings	Diseased seedlings	
Trichoderma sp. (TRB1)	64.6 a[1]	2.8 a	19.5 b
Trichoderma sp. (TR506)	65.0 a	3.4 a	24.0 ab
thiophanate-methyl + chlorothalonil	61.5 ab	4.6 a	11.7 b
Control	39.6 b	6.1 a	40.4 a
Coefficient of variation (%)	14.0	37.8	24.9

[1]Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

incidence, the effect was significant until the 20th day after sowing (Table 5).

Seedling height values were significantly higher in the biocontrol treatments with *Trichoderma* sp. in comparison to the control (Table 6). The chemical control did not differ from the treatment control only in the lot with medium *Fusarium* sp. incidence (Table 6).

The use of *Trichoderma* in the biocontrol of several pathogens leading to the growth of treated plants has been reported in some studies (Harman et al., 2004; Carvalho et al., 2011a; Pedro et al., 2012). However, little is known about the microbial treatment of seeds of forest species (Santos et al., 2015). In this study, we showed that the treatment of pine seeds with *Trichoderma* sp. reduced non-germinated seeds, and increased emergence and height of seedlings. Thus, the treatment of pine seeds with *Trichoderma* seems to be a promising strategy to control *Fusarium* sp. in forest nurseries. The action of *Trichoderma* spp. as a promoter of germination and plant growth is complex, performed by interactions with biochemical factors and production of various enzymes and beneficial compounds (Baugh & Escobar, 2007).

Table 5. Emergence speed of pine seedlings from three pine seed lots with different initial incidence levels of *Fusarium* sp. and treated with *Trichoderma* sp. at the 14th, 28th and 42nd days after sowing.

Treatment	Emergence (%)		
	14th day	28th day	42nd day
Low initial incidence[1]			
Trichoderma sp. (TRB1)	16.2 a[2]	31.2 a	39.2 a
Trichoderma sp. (TR0506)	13.0 b	26.7 ab	34.0 ab
thiophanate-methyl + chlorothalonil	4.5 c	21.2 b	29.0 b
Control	5.5 c	20.2 b	25.7 b
Coefficient of variation (%)	9.6	17.9	12.9
Medium initial incidence[1]			
Trichoderma sp. (TRB1)	4.7 b	26.5 a	37.0 a
Trichoderma sp. (TR0506)	12.0 a	30.2 a	40.5 a
thiophanate-methyl + chlorothalonil	12.0 a	30.7 a	36.2 a
Control	3.2 b	13.7 b	20.5 b
Coefficient of variation (%)	19.6	12.9	13.7
High initial incidence[1]			
Trichoderma sp. (TRB1)	8.5 a	22.2 a	26.7 a
Trichoderma sp. (TR0506)	3.0 b	18.5 a	27.7 a
thiophanate-methyl + chlorothalonil	1.5 b	10.7 b	25.0 a
Control	1.2 b	11.2 b	18.5 a
Coefficient of variation (%)	23.9	21.2	17.0

[1]Lots with low (12%), medium (49%) and high (98%) *Fusarium* sp. incidence; [2]Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

Table 6. Seedling height (mm) from pine seed lots with different initial incidence levels of *Fusarium* sp. and treated with *Trichoderma* sp. and the fungicide thiophanate-methyl + chlorothalonil.

Treatment	Seedling height (mm)		
	Low initial incidence[1]	Medium initial incidence[1]	High initial incidence[1]
Trichoderma sp. (TRB1)	85.3 a[2]	74.5 b	80.6 a
Trichoderma sp. (TR0506)	85.6 a	77.4 ab	76.7 ab
thiophanate-methyl + chlorothalonil	81.1 ab	83.3 a	77.1 ab
Control	77.2 b	70.0 b	73.7 b
Coefficient of variation (%)	2.8	4.7	2.7

[1]Lots with low (12%), medium (49%) and high (98%) *Fusarium* sp. incidence; [2]Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

Another action mechanism of *Trichoderma* is to compete with other phytopathogens for substrate through its rapid multiplication, suppressing their development (Bettiol, 1991). Besides, antagonists may act by increasing plant resistance (Agrios, 2005). These mechanisms may have influenced the findings of this study, in combination or individually, leading to better development of seedlings in the germination, emergence, and vegetative development phases.

4. CONCLUSIONS

- *Trichoderma* sp. isolates reduced the *F. subglutinans* mycelial growth in the CC and through VM production;
- Pine seeds treatment with *Trichoderma* sp. reduced the *Fusarium* sp. incidence;
- Pine seeds treatment carrying *Fusarium* sp. with *Trichoderma* sp. lead to a higher seedling emergence speed, higher percentage of healthy seedlings, lower percentage of NGS with *Fusarium* sp., and higher seedling height.

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