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Trichoderma spp. Isolated from Soils from the Southern State of Tocantins for the Control of *Sclerotinia Sclerotiorum* in Vitro

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Abstract - The objective of this work was to select isolates of *Trichoderma* spp. with potential of antagonism against *Sclerotina sclerotiorum,* in vitro. We used ten isolates of *Trichoderma* spp. and an isolate of *S.sclerotioruma* long with "inhibition by Volatile Products" and "direct confrontation" techniques. The results of in vitro procedures, lead to the selection of the isolates JCOUFT-28, JCOUFT-37, JCOUFT-45, JCOUFT-63 and JCOUFT-85, which were *Trichoderma* spp. that showed better antagonistic activity on the isolated *S.sclerotiorum*.

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I. Introduction

The state of Tocantins is partially located on the cerrado (savanna), an area with great potential for agriculture, cattle raising and cultivation of cereal grains. However, there are several factors that can lead to low productivity of agricultural areas. Primarily, plant disease caused by soil borne plant pathogens result in severe yield loss.

Sclerotinia sclerotiorum is a soil borne plant pathogen that causes white mold, which key characteristics are white, fluffy my celial growth on the host plant eventually producing specialized survival structures known as sclerotia (Cardoso, 1990). The increasing severity of the fungus is a reason for concern because the sclerotia can easily contaminate non-infected areas and can survive for years in the soil; moreover there are no resistant varieties to this disease (Niderasementes, 2009).

Isolates of *Trichoderma* spp. are considered efficient against several plant-pathogenic fungi. The mode of action of *Trichoderma* spp. is one or an association of the following: Parasistism, antibiosis, competition. *Trichoderma* is of saprophytic nature and produces extracellular enzymes and antibiotics that increase its parasitic capability, competitiveness and efficiency in biological control (Harman et al., 2004; Samuels, 2006).

Several researches have been done to evaluate the antagonist capability of *Trichoderma* spp against S. sclerotiorum, in vitro (Delgado et al., 2007; Louzada et al., 2009). However, no biological control study has been developed with isolates from the south of Tocantins, against *Sclerotinia sclerotiorum,* also isolated from the same region.

The present study´s objectives were to select isolates of *Trichoderma* spp. from the South of To cantins with potential to control *Sclerotinia sclerotiorum.*

II. Materials and Methods

In order to select isolates of *Trichoderma* spp., soil samples were cultured as a possible *Trichoderma* spp. sources. Thirty-seven soil samples were collected from the experimental areas of the Federal University of Tocantins-Gurupi campus, localized at 11°43'Se49°04'W with 300 m altitude and in valley areas of Lagoa da Confusão– TO (TABLE 1). The samples were taken at adepth of 0-10 cmin soil that had numerous crops and were cultivated by different methods. Afterwards, the samples were sent to the Plant Pathology Lab at the Federal University of Tocantins-Gurupi campus, where they were kept in a cold chamber until analysis.

Subsequently, five fragments of approximately 2 mm in diameter were placed in each petri dish, using the technique of direct isolation of fungi, with 3 repetitions for each sample, in the PDA (Potato, dextrose, agar) culture medium, and incubated at a temperature of 28 °C and a photoperiod of 12 hours. The period for colony growth of *Trichoderma* spp., was determined according to Watts et al. 1988. After 5 days, the petridishes with colony growth similar to *Trichoderma* spp. were selected, resulting in a total of 19 isolates found in the soil samples. In order to confirm the genus, the colonies were transferred to petri dishes that had PDA culture medium and after seven days of incubation at a temperature of 28 °C and a photoperiod of 12 hours they were identified based on morphology with the help of a microscope (Barnett & Hunter, 1998). All the isolates were maintained in the refrigerators, cultivated periodically on PDA and conserved on mineral oil for preservation of the isolates of *Trichoderma* spp. obtained.

The plant pathogen, S. sclerotiorum, was isolated from beans (*Phaseolus vulgaris* L.), that had high incidence of the disease and was cultivated in the experimental area of the Federal University of Tocantins-Gurupi Campus, and it was preserved in the same

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manner of the *Trichoderma* spp. The botanical material was collected and processed in 24 hours and it was previously abundantly washed in running water and neutral detergent to remove the epiphytic excess. Later, inside an aseptic chamber, the material was immersed in 70% alcohol for 1 minute and 3 % hypochlorite for 4 minutes and again in 70% alcohol for 30 seconds to remove the excess of hypochlorite. The same material was then washed in sterile distilled water of which a sample of 50 μL was taken for control. After the sterilization, small fragments measuring approximately 8 x 8 mm were place on PDA culture medium that had terramicina (100 μL) to inhibit bacterial growth during the fungus isolation. According to Monteiro et al. (2012) the agar substrate presents better carpogenic germination of sclerotia of *S. sclerotiorum*. The petri dishes containing the fragments were incubated at 28 °C.

Conidia counting of *Trichoderma* spp. of the 19 isolates previously obtained were done to compare the production of conidia to the control isolate, *T. harzianum* of the commercial product Trichoplus – JCO Fertilizantes, produced in Barreiras- Bahia.

Discs of 5 mm in diameter with mycelia growth of these isolates on PDA for 7 days were transferred to petri dishes (90 mm of diameter) also with the PDA culture medium. They were incubated in growth rooms acclimatized to 28°C ± 2°C, with a photoperiod of 12 hours. In order to evaluate the conidial production, 10 ml of distilled water was added to the petri dishes containing the colonies grown for 5 days. Using a Drigalski spatula, the medium containing the my celial and conidial growth, was lightly scraped off and agitated so that the spores loosen. The resulting suspension was filtered in double gauze and the concentration of the conidia was determined with the help of a Neubauer chamber. For both tests, complete randomized blocks were used with three repetitions per isolate.

With the intention of verifying if the isolates produced volatile metabolites a inhibition test for volatile products was done based o Bharat et al. (1980), where the lids were placed on top of each other after pouring PDA culture medium in each of them. The antagonist was positioned on the lower part of the petri dish while the pathogen was on the upper part. The antagonist was inoculated on the first, third and sixth day of growth of the pathogen. The lids were closed, laterally sealed by a flexible plastic and incubated at room temperature for seven daysunder continuous florescent light. The evaluation method consisted in measuring the radius of mycelia development to obtain the area of the colony.

In order to verify interaction between the pathogen and antagonist, the 19 isolates of *Trichoderma* spp. previously used were tested and evaluated with the methodology of direct confrontation (Bell et al., 1982; Ethur et al., 2005). Confirming the antagonisms of the *Trichoderma* spp. against the *S. sclerotiorum* was done using direct opposition method described by Dennis & Webster (1971). Initially, isolates were cultured on PDA medium. Petri dishes containing 20 ml of PDA medium each, received 2 discs of mycelia/agar of 9mm in diameter, which were placed on opposite ends and 1 cm apart from the side of the dish. One of the discs was a pathogen and the other one was the possible bio control agent.

As a control, petri dishes were cultured only with the pathogens. The colonies were placed in a BOD Incubator at a temperature of 28 °C ± 2°C and a 12 hour photoperiod. After seven days my celial growth of the colonies were measured with the help of a caliper, starting at the culture disc. The evaluation was done following the criteria proposed by Bell et al. (1982) with grading scales varying from 1 to 5. In this scale when the antagonist grows all over the dish (87, 5 to 100%) it is considered 1 and 6 when it does not present satisfactory growth (under 33, 2%). The isolate was considered efficient as an antagonist when the grade was lower or equal to 3. All the treatments were conducted with 3 repetitions and analyzed using a completely randomized factorial design.

The results obtained were examined using analysis of variance (ANOVA) and partitions performed by the Scott-Knott test at 5 % probability.

Table 1 : Origin of the isolates of *Trichoderma* spp. obtained from soil samples

Identification of Isolate	Origin	Crop
JCO-UFT 02	UFT campus - Gurupi-TO	Sorghum (*Sorghum bicolor*)
JCO-UFT 03	UFT campus - Gurupi-TO	Rice (*Oryza sativa*)
JCO-UFT 06	UFT campus - Gurupi-TO	Barbados Nut(*Jatrophacurcas*)
JCO-UFT 09	UFT campus - Gurupi-TO	Corn (*Zeamays*)
JCO-UFT 12	UFT campus - Gurupi-TO	Sugar-cane (*Saccharumofficinarum*)
JCO-UFT 14	UFT campus - Gurupi-TO	Capim Napier (*Pennisetumpurpureum*)
JCO-UFT 15	UFT campus - Gurupi-TO	Watermelon (*Citrulluslanatus*)
JCO-UFT 18	UFT campus - Gurupi-TO	Castor oil plant (*Ricinuscommunis*)
JCO-UFT 19	UFT campus - Gurupi-TO	Lemon grass (*Cymbopogoncitratus*)
JCO-UFT 22	UFT campus - Gurupi-TO	Vegetação de Mata
JCO-UFT 23	UFT campus - Gurupi-TO	Pinaple (*Ananascomosus*)
JCO-UFT 25	UFT campus - Gurupi-TO	Banana (Musa spp.)

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JCO-UFT 28	UFT campus - Gurupi-TO	corn(*Zeasmays*)
JCO-UFT 32	UFT campus - Gurupi-TO	corn(*Zeamays*)
JCO-UFT 34	UFT campus - Gurupi-TO	Pumpkin (*Cucúrbita moschata*)
JCO-UFT 35	UFT campus - Gurupi-TO	Cerrado vegetation
JCO-UFT 37	UFT campus - Gurupi-TO	Degradedpasture
JCO-UFT 41	UFT campus - Gurupi-TO	Balm (*Melissa officinalis*)
JCO-UFT 45	Lagoa da Confusão-TO	Sunnhemp(*CrotaláriaJuncea*)PD*
JCO-UFT 48	Lagoa da Confusão-TO	Sunnhemp(*CrotaláriaJuncea*) PC*
JCO-UFT 56	Lagoa da Confusão-TO	Crotalaria (*CrotaláriaSpectabilis*) PC*
JCO-UFT 57	Lagoa da Confusão-TO	Velvetbean(*Mucunaaterrima*) PD*
JCO-UFT 63	Lagoa da Confusão-TO	Jack bean (*Canavaliaensiformis*) PD*
JCO-UFT 67	Lagoa da Confusão-TO	Jack bean(*Canavaliaensiformis*) PC*
JCO-UFT 70	Lagoa da Confusão-TO	Hyacinth bean (*Lablabpurpureus*) PD*
JCO-UFT 74	Lagoa da Confusão-TO	Hyacinth bean (*Lablabpurpureus*) PC*
JCO-UFT 76	Lagoa da Confusão-TO	Pigeon Pea(*Cajanuscajan*) PD*
JCO-UFT 78	Lagoa da Confusão-TO	Pigeon Pea (*Cajanuscajan*) PC*
JCO-UFT 85	Lagoa da Confusão-TO	Calopogônio(*Calopogoniummucunoides*) PC*
JCO-UFT 87	Lagoa da Confusão-TO	Radish(*Raphanussativus*) PD*
JCO-UFT 92	Lagoa da Confusão-TO	Radish(*Raphanussativus*) PC*
JCO-UFT 95	Lagoa da Confusão-TO	No cultivationof legumes
JCO-UFT 96	Lagoa da Confusão-TO	No cultivationof legumes
JCO-UFT 99	Lagoa da Confusão-TO	Cowpea (*Vignaunguiculata*) PD*
JCO-UFT 102	Lagoa da Confusão-TO	Cowpea (*Vignaunguiculata*)PC*
JCO-UFT 110	Lagoa da Confusão-TO	Sorghum(*Sorghum bicolor*) PC*
JCO-UFT 111	Lagoa da Confusão-TO	Natural Meadowvegetation

* PD- Plantio direto; PC- Plantio convencional.

III. Results and Discussion

The concentration of conidia of *Trichoderma* spp. was determinedand compared to the control isolate. Only the isolates JCO-UFT 28 and JCO-UFT 35 presented a lower number of conidia when compared to the control (Trichoplus). The other isolates had a superior number of conidia than the control, as shown on table 2.

Table 2 : Results of conidia counting of *Trichoderma* spp. collected and isolated from the South of state of Tocantinsand the control sample of the commercial formulation Trichoplus JCO from Barreiras- BA

Identification	Numberofconidia
JCO-UFT 22	$1,7.\ 10^6$
JCO-UFT 28	$2,5.\ 10^5$
JCO-UFT 32	$1,5.\ 10^6$
JCO-UFT 35	$1,6.\ 10^5$
JCO-UFT 37	$6,3.\ 10^6$
JCO-UFT 41	$7,0.\ 10^6$
JCO-UFT 45	$8,2.\ 10^5$
JCO-UFT 57	$1,1.\ 10^6$
JCO-UFT 63	$5,5.\ 10^5$
JCO-UFT 67	$1,4.\ 10^6$
JCO-UFT 76	$5,5.\ 10^5$
JCO-UFT 78	$1,1.\ 10^6$
JCO-UFT 85	$5,3.\ 10^6$
JCO-UFT 87	$2,7.\ 10^6$
JCO-UFT 92	$6,4.\ 10^5$
JCO-UFT 95	$1,3.\ 10^6$
JCO-UFT 99	$2,7.\ 10^6$
JCO-UFT 102	$1,9.\ 10^6$
JCO-UFT 110	$1,6.\ 10^6$
CONTROL	$3,9.10^5$

The test for inhibition of volatiles products the isolates JCO-UFT 63, JCO-UFT 78, JCO-UFT 92 and JCO-UFT 102 produced some type of volatile compound, showing a percentage of growth reduction (GR) of the mycelia of *S. sclerotiorum*. When inoculated 3 days after the pathogen (figure 1), the antagonist was

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able to reduce up to 60 % of the pathogen growth when compared to the control. These results are in agreement with Mace do et al. (2007) findings that show the inhibition of S. *rolfssimycelial* growth by 12 isolates of *Trichoderma* spp. tested.

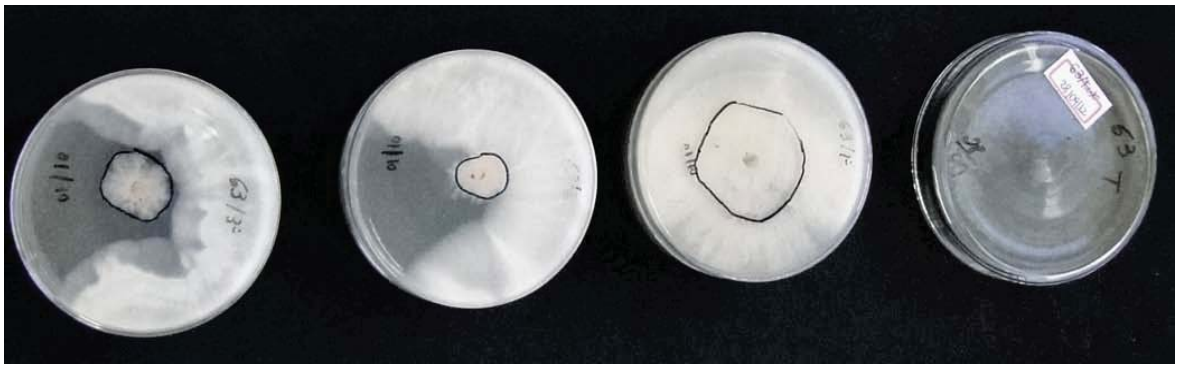

Figure 1 : Inhibition of the pathogen *(S. sclerotiorum)* by *Trichoderma* JCO-UFT 63, inoculated 3 days after the pathogen, part of the volatile production test

As for the direct opposition test, of the 19 isolates identified belonging to the *Trichoderma* genus, 16 isolates inhibited the mycelial growth of *S. sclerotiorum* with evaluations smaller than 3 as shown on table 3.The isolates JCO-UFT 28, JCO-UFT 37, JCO-UFT 45, JCO-UFT 63 and JCO-UFT 85 were significantly efficient as antagonists when compared to the other isolates. They presented grades equal to or smaller than 2, where they took over more than 2/3 of the petri dish. Only the isolates JCO-UFT 22, JCO-UFT 41, JCO-UFT 95 and JCO-UFT 99 were considered inefficient ranking 3 or higher, in other words, occupying less than half of the petri dish.

The direct opposition between the pathogen and the antagonist showed the isolates of *Trichoderma* utilized different methods of antagonism besides the production of volatile metabolites. According to Melo (1998) the mode of action of *Trichoderma* is a result of an association of mechanisms such as parasitism, antibiosis and competition. A dark colored halo was observed along the contact line between the colonies of the antagonist and the plant pathogen, as previously described by Bell et al. (1982) and Durman et al. (1999). The sclerotia formed in the petri dishes were colonized by the isolates of *Trichoderma* spp., and then they fragmented and lost their rigid consistency. Melo (1991) stated that the fungus *Trichoderma* has been found growing on sclerotia, structures that are known to be very resistant to parasitism.

Table 3 : Antagonistic activity, in vitro, of the isolates of *Trichoderma* spp., to the isolates of *S. Sclerotinia*[1]

Identification	Distance (cm)	Grade	Medium %
JCO-UFT 22	2,69	3,5	40 c
JCO-UFT 28	1,87	2	63 a
JCO-UFT 32	1,78	2,5	55 b
JCO-UFT 35	2,19	2,5	57 b
JCO-UFT 37	1,58	1,5	68 a
JCO-UFT 41	2,33	3,5	48 c
JCO-UFT 45	1,93	2	63 a
JCO-UFT 57	1,90	2,5	58 b
JCO-UFT 63	1,66	2	66 a
JCO-UFT 67	2,27	2,5	54 b
JCO-UFT 76	2,11	2,5	55 b
JCO-UFT 78	2,10	2,5	57 b
JCO-UFT 85	1,83	2	65 a
JCO-UFT 87	2,13	2,5	59 b
JCO-UFT 92	1,85	2,5	58 b
JCO-UFT 95	3,30	4	37 d
JCO-UFT 99	3,56	5	28 d
JCO-UFT 102	2,10	2,5	56 b
JCO-UFT 110	2,07	2,5	58 b
CONTROLE	2,18	2,5	55 b

[1]The colonies developed from 2 discs of the 2 fungi, place in opposite direction, at the borders of the petri dish containing PDA medium. Medium followed by the same letter are not different in the Scott-knott 5% test.

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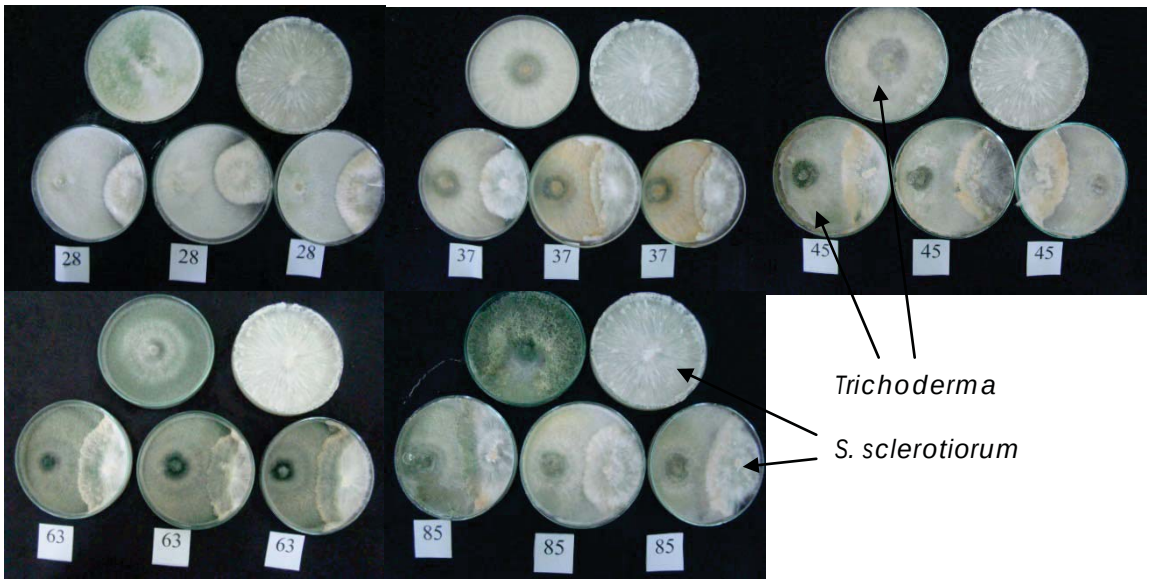

Figure 2 : Inhibition of the pathogen *(S. sclerotiorum)* by isolates of *Trichoderma spp.*, using direct opposition

IV. Conclusions

- The isolates JCO-UFT 63, JCO-UFT 78, JCO-UFT 92 and JCO-UFT 102 caused the most inhibition of the *S. sclerotiorum* growth when the antagonist was inoculated 3 days after the pathogen.
- The isolates JCO-UFT 28, JCO-UFT 37, JCO-UFT 45, JCO-UFT 63 and JCO-UFT 85 were efficient antagonist, inhibiting more than 60% of growth of the pathogen.

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