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Efficiency of different strains of *Trichoderma* on the control of *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Sclerotium cepivorum*

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ABSTRACT. The aim of this work was to verify the efficiency of different isolates of *Trichoderma* spp. on the control of *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Sclerotium cepivorum*, and the influence they pose on the conidia production of *Trichoderma* spp. For mycelial growth, discs with inoculum of phytopathogens were placed on the center of the Petri dishes followed by the addition of two *Trichoderma* sp. discs on the opposite sides of the plate after 24 hours. Every 12 hours data were collected from colonies diameters and used for the analyses of Mycelial Growth Index (MGI) and Area Under the Curve of Mycelial Growth (AUCMG). The analyses were performed by a completely randomized design with two controls, a negative one without *Trichoderma* sp. and one with a commercial strain of *Trichoderma harzianum*. Spore solution for evaluation of conidia production were made by adding 10 mL of distilled water and scratching the surface of the colonies. For *S. cepivorum*, all *Trichoderma* spp. strains reduced both indexes tested. However, while for MGI *S. sclerotiorum* also presented some reduction on the growth rate, the total area of this fungus was not affected. *Sclerotium rolfsii* strains of *Trichoderma* sp. from Lages and Curitibanos showed an effect on the reduction of AUCMG of this fungus, although none of the *Trichoderma* affected the growth rate of this phytopathogen. On the presence of *S. sclerotiorum* and *S. cepivorum*, none of the *Trichoderma* spp. showed any difference on conidia production when compared among themselves, nonetheless we did notice that on the presence of *S. cepivorum*, the strain from Rio do Sul retained its reproductive ability compared to control. Results obtained from this research can demonstrate the importance of biocontrol agents against different plant pathogens since it might have a specific antagonist-pathogen relation.

Keywords: biocontrol; soil fungi; plant pathogens; soil born disease; sclerotia.

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Introduction

In the current food production system, the use of chemical pesticides and fertilizers has had adverse effects on the natural ecosystem, resulting in the elimination of beneficial organisms, increase in agricultural pests and the presence of chemical residues in food (Bae et al., 2011).

Within this theme, the use of less toxic products of natural origin has been widely discussed due to the environmental problems caused by the irregular use of pesticides, opening opportunities for research involving the use of alternative methods of disease control, such as the use of fungi of the *Trichoderma* genus.

Trichoderma harzianum is a biological control agent used to protect against various plant pathogens and is used in foliar application, seed treatment and it is applied directly to the soil (Mikkola et al., 2012). It is a widely studied genus to produce enzymes such as cellulase (Mandels, Parrish, & Reese, 1962). Its main use is in the control of soil-borne phytopathogens, which in addition to causing diseases also compete for resources, produce inhibitory compounds, and secrete chitinolytic enzymes (Druzhinina & Kubicek, 2005). In Brazil, there are 27 registered products composed of *Trichoderma* sp. of which 19 have *T. harzianum* cells as active ingredient (Brasil, 2020).

Sclerotinia sclerotiorum, *Sclerotium rolfsii* and *Sclerotium cepivorum* are among the most relevant soil-borne phytopathogenic fungi able to cause great economic damage to the crops affected by them. *S. sclerotiorum* is known worldwide for the damage it causes in different cultures, having more than 400 species of host plants

(Boland & Hall, 1994). The use of *Trichoderma* sp. to control this pathogen in Brazil has been increasingly emphasized among producers (Bettiol, Silva, & Castro, 2019).

Sclerotium rolfsii and *S. cepivorum* are responsible for causing diseases in a wide variety of agricultural crops, especially in horticultural crops such as carrots and onions in southern Brazil. Currently, in Brazil, there are two biological control products registered against *S. rolfsii* (Brasil, 2020) but none of them are specific for these cultures. *S. cepivorum* is responsible for bulb rot in garlic and onion crops (Crowe, Hall, Greathead, & Baghott, 1979).

However, there are no products registered for the control of this pathogen (Brasil, 2020). To minimize the impact of this phytopathogen and in the absence of a registered biocontroller, the solution employed by producers has been crop rotation and the use of resistant varieties. These methods are not highly effective when the area is already contaminated by *S. cepivorum* due to its aggressiveness and persistence in the soil (Coley-Smith, 1990).

These three species of phytopathogenic fungi have in common the ability to produce a resistance structure called *sclerodium* that can survive in soil for years. Therefore, its control is difficult (Liarzi, Benichis, Benichis, & Ezra, 2020). Thus, studies indicate the use of *Trichoderma* sp. as a viable alternative due to its inhibitory potential on the development of phytopathogens and for acting with different modes of action: antibiosis, parasitism, and predation (Chagas Junior et al., 2018; Ferreira de Sá, Souza Lima, Jesus, Perez, & Gava, 2019).

The present work aimed to evaluate the efficiency of different *Trichoderma* spp. against the phytopathogens *S. sclerotiorum*, *S. rolfsii* and *S. cepivorum*.

Material and methods

The phytopathogenic fungi used were stored in the Castellani method (Gonçalves, Alfenas, & Mafia, 2007). *Sclerotium rolfsii* was isolated in 2016 from tomato plants in the region of Curitibanos-SC. *Sclerotinia sclerotiorum* was obtained from a soybean plant that showed signs of the disease in Brunópolis-SC in 2016. *Sclerotium cepivorum* was isolated from sclerotia in Petri dishes donated by Epagri-SC. All these isolates are part of the mycotheca of the Phytopathology Laboratory of the *Universidade Federal de Santa Catarina*, Curitibanos Campus.

Trichoderma spp. were obtained after performing serial dilutions of soils collected in three cities: Lages, Rio do Sul and Curitibanos, Santa Catarina state, Brazil. Serial dilution used the 10^{-2} dilution plated on Petri dishes containing culture medium Potato-Dextrose-Agar (PDA) and antibiotic (penicillin + streptomycin 500 mg L^{-1}). Petri dishes were stored in a BOD-type incubator for 48 hours at a temperature of 25ºC and 12 hours of photoperiod. The largest colony of *Trichoderma* sp. present on the plate was isolated and morphologically identified at the genus level under an optical microscope (Olympus©, model CX22).

All isolates were stored in the mycotheca of the Phytopathology Laboratory of the *Universidade Federal de Santa Catarina*, Curitibanos Campus (Table 1). As a positive control, *T. harzianum* IBLF006 (code URM 7663, Micoteca URM, Instituto Biológico – *Universidade Federal de Pernambuco*) isolated from a commercial product (Ecotrich®) sold as a biological controller was used. Commercial strain was obtained from a Petri dish with Potato-Dextrose-Agar (PDA) as a culture media, where 0.25 mL from a solution of sterile distilled water with 10% of the Ecotrich® powder (1 g in 9 mL of water) was used based on the spread plate method. As negative control, the absence of *Trichoderma* sp. was used.

Table 1. Strains of *Trichoderma* spp. with basic information about its origin location and codes of identification of the strains on the laboratory.

Strain	Species	City	Coordinates	Alt.
SC1146 - TCOM	*Trichoderma harzianum*[1]	-	-	-
SC1147 - TLAG	*Trichoderma* sp.	Lages	27°47'12"S/50°18'20"W	915m
SC1148 - TRDS	*Trichoderma* sp.	Rio do Sul	27°11'16"S/49°39'22"W	673m
SC1149 - TCUR	*Trichoderma* sp.	Curitibanos	27°16'05"S/50°32'04"W	1096m

Coordinates based on latitude and longitude; Alt. = altitude in meters; Coordinates and altitude for *T. harzianum* not shown once its location is not described in the product label. [1] *T. harzianum* IBLF006 is from the microbiological fungicide Ecotrich®, which is preserved under the code of URM 7663 in the Mycotheca URM, from the Biological Institute of the *Universidade Federal de Pernambuco*).

An adaptation of the Petri dish pairing method was used for the analysis of mycelial growth (Cassiolato, Bakes, & Melo, 1997). Mycelium discs with 5 mm in diameter from colonies of phytopathogenic fungi with seven days, were added to the center of the Petri dish containing BDA culture medium and kept in a BOD type incubator at 25ºC and 12 hours photoperiod. After 24 hours, two discs of *Trichoderma* sp. were added at the

opposite ends of the Petri dishes and aligned with the phytopathogen. Every 12 hours, the diameter of the phytopathogenic fungi colonies was measured. For this, two perpendicular straight lines were drawn on the outside of the Petri dishes with an intersection point in the center of the mycelial disk of the phytopathogen (Figure 1). Measurements were stopped when all the repetitions of the same treatment reached the limit of growth on the plates.

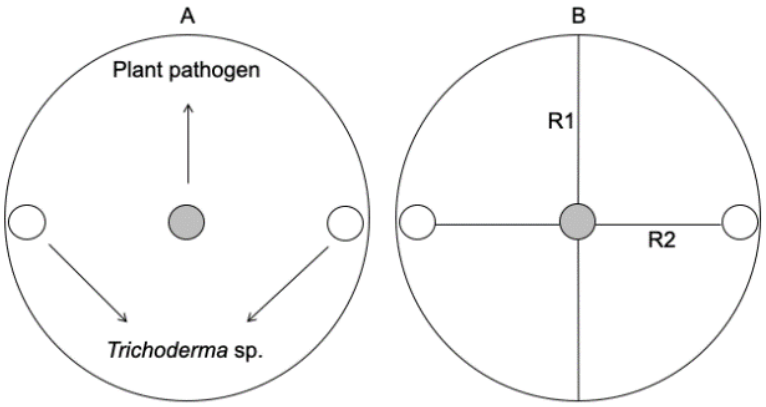

Figure 1. Illustrative scheme of the confrontation method in Petri dish used to evaluate the biological control effect of the *Trichoderma* species against the phytopathogens. (A) Superior view of the Petri dishes showing how the 5 mm mycelial discs of the *Trichoderma* species were arranged to test their antagonistic effect; (B) Inferior view of the plate indicating the measurement aspect of the mycelial growth diameter towards two lines (R1 and R2) intersected at 90° angle in the center of the plant pathogen fungus.

For each species of phytopathogenic fungus, a completely randomized experimental design with 5 treatments (three *Trichoderma* sp. isolates and two controls) and five replications was used.

Statistical analysis was performed by calculating the mycelial growth velocity index (MGVI) and area under the mycelial growth curve (AUCMG). The MGI and the AUCMG was calculated according to the formula obtained by applying the formula proposed by Campbell and Madden (1990). The formulas are shown below (Equations I and II):

$$MGI = \frac{\sum\left(\frac{y_{i+1}-y_i}{t_{i+1}-t_i}\right)}{(n-1)} \quad \text{(I)}$$

$$AUCMG = \sum\left(\frac{y_{i+1}+y_i}{2}\right) \times (t_{i+1} - t_i) \quad \text{(II)}$$

where:
y_i = diameter of the pathogen on the day;
y_{i+1} = diameter of the pathogen on the following day;
t_i = evaluation time in hours on the day;
t_{i+1} = evaluation time on the following day;
n = number of the total day's evaluations made.

Data were subjected to analysis of variance and post-hoc Tukey's test using the R software. After the end of the mycelial growth assay, Petri dishes were used to evaluate the sporulation of different *Trichoderma* spp. in the presence of the tested phytopathogens.

The control treatment in this test was represented by the pure culture of each *Trichoderma* spp., cultivated under the same conditions and within the same period in which the mycelial growth experiment took place.

For this, 10 mL of Mili-Q water were added to each Petri dish, followed by mycelial scraping with a Drigalski spatula. The resulting solution was filtered through double gauze into an autoclaved 100 mL beaker. The solutions were separated by treatment, in which all the repetitions of the same treatment were homogenized in a beaker, from which 10 ml was removed and transferred to a test tube containing 1 mL of 96% alcohol to prevent conidia germination.

The spore counts were made using a Neubauer chamber under an optical microscope (Olympus©, model CX22). Five counts were taken in the 1 mm^2 quadrant of the chamber to obtain concentration on 10^4 spores mL^{-1} (Alfenas, Zauza, & Mafia, 2007).

Results and discussion

For the mycelial growth velocity index, we found that all strains of *Trichoderma* spp. tested was effective on reducing this index compared to negative control (only the pathogen) for *S. sclerotiorum* (Figure 2A) and *S. cepivorum* (Figure 2C) with a p <0.01 for both pathogens. For *S. cepivorum* the reduction ranged from 40 to 60% approximately, and although there was no difference between the strains pointed out by Tukey test with 95% confidence level, the highest reduction rate observed was from TRDS (*Trichoderma* sp. from Rio do Sul).

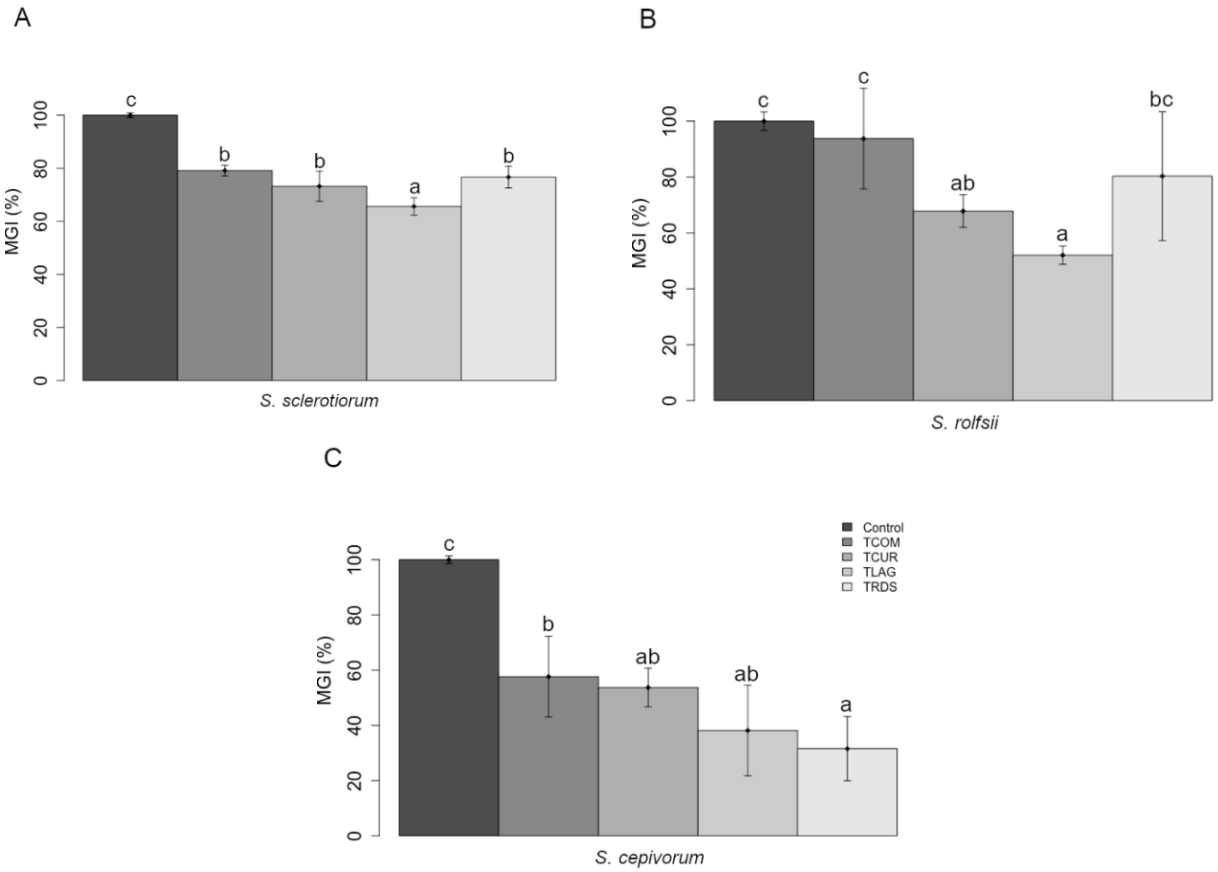

Figure 2. Mycelial growth velocity index (MGI) for three phytopathogens paired with different strains of *Trichoderma* spp. Labels on the graphs correspond to the treatments Control (only the pathogens – used as negative control), TCOM (*T. harzianum* IBLF006 – used as a positive control), TLAG (*Trichoderma* sp. from Lages), TRDS (*Trichoderma* sp. from Rio do Sul) and TCUR (*Trichoderma* sp. from Curitibanos). All strains described were used to test the impact on the MGI of *Sclerotinia sclerotiorum* (A), *Sclerotium rolfsii* (B) and *Sclerotium cepivorum* (C). For each graph, same letters showed no difference between treatments. Tukey test ran at 95% confidence level.

The area under the mycelial growth curve (AUCMG) in *S. cepivorum* showed a significant reduction for all strain tested (p <0.01), with a range of 30 to 50% compared to negative control (absence of *Trichoderma* spp.), where the higher reduction rate (50%) was achieved using strain TRDS and the lowest by *T. harzianum* (Figure 3C). For *S. rolfsii*, AUCMG there was a slight reduction when the pathogen was paired *in vitro* with TCUR and TLAG, showing approximately 20% reduction compared with control (p <0.01) (Figure 3A), although the comparison between *S. sclerotiorum* and *S. rolfsii* pointed out similar results for the *Trichoderma* spp. from Curitibanos (TCUR) and Lages (TLAG), with approximately 20% reduction.

Conidia production analysis of all *Trichoderma* spp. tested was able to demonstrate the differences within each strain when growing in the presence of the pathogen (p <0.001). For *S. sclerotiorum* and *S. rolfsii*, we observed an impact on spore's production of TCOM and TRDS, with more than 70% reduction TCOM strain spores compared with the control, determined by the growth of the strain without the pathogen (Figure 4). For *S. cepivorum*, these strains (TCOM and TRDS) were able to maintain the rate of conidia production compared with control.

For TRDS strain, the same characteristics seen on TCOM, related to conidia production, was observed. However, for this strain the reduction on the number of spores when paired with *S. sclerotiorum* and *S. rolfsii*

achieved 90% compared to control (p <0.001) (Figure 4). Our results did not find any significant difference on the production of conidia for TLAG when paired with the plant pathogens. However, the strain from Curitibanos (TCUR) indicates that in the presence of *S. sclerotiorum*, its conidia production was impaired, reducing more than 50% in comparison with control.

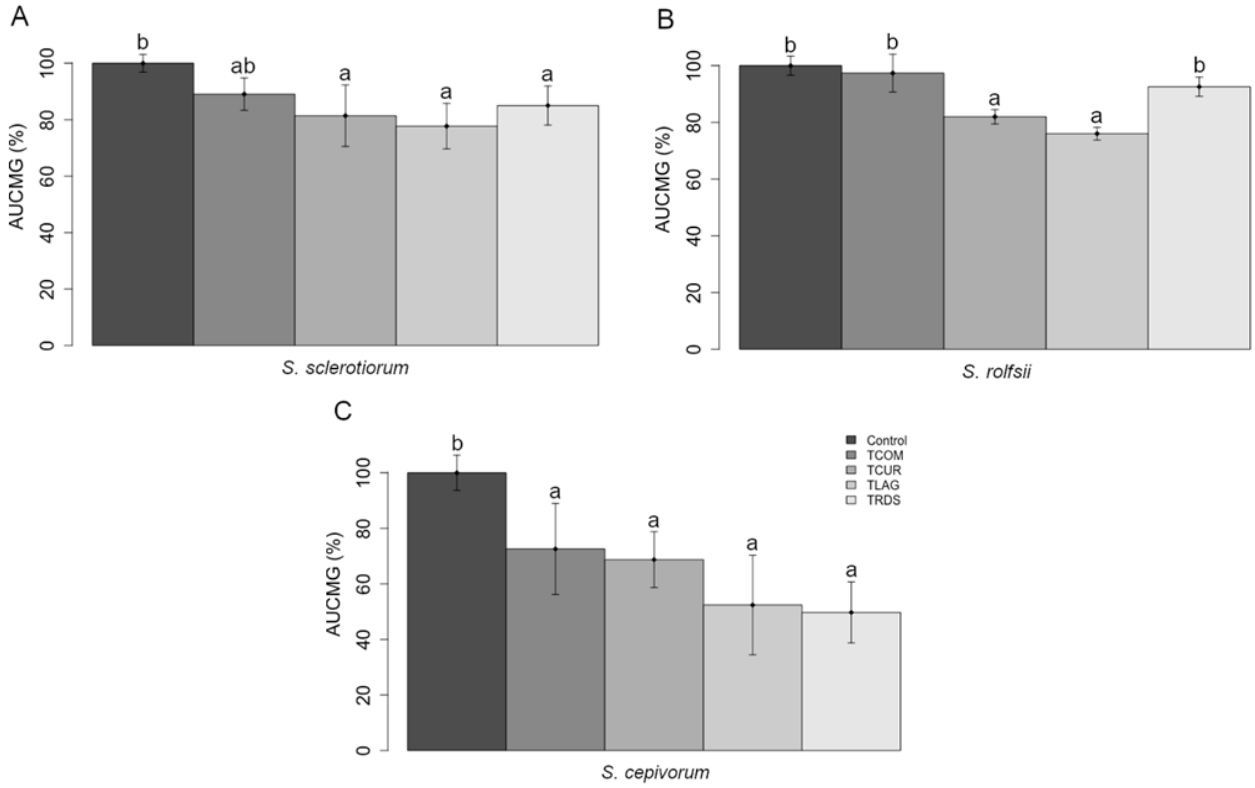

Figure 3. Area Under the Mycelial Growth Curve (AUCMG) for three plant pathogens, when paired with different strains of *Trichoderma* spp. Labels on the graphs correspond to the treatments Control (only the pathogens – used as negative control), TCOM (*T. harzianum* IBLF006 – used as a positive control), TLAG (*Trichoderma* sp. from Lages), TRDS (*Trichoderma* sp. from Rio do Sul) and TCUR (*Trichoderma* sp. from Curitibanos). All strains described were used to test the impact on the AUCMG of *Sclerotinia sclerotiorum* (A), *Sclerotium rolfsii* (B) and *Sclerotium cepivorum* (C). For each graph, same letters showed no difference between treatments. Tukey test ran at 95% confidence level.

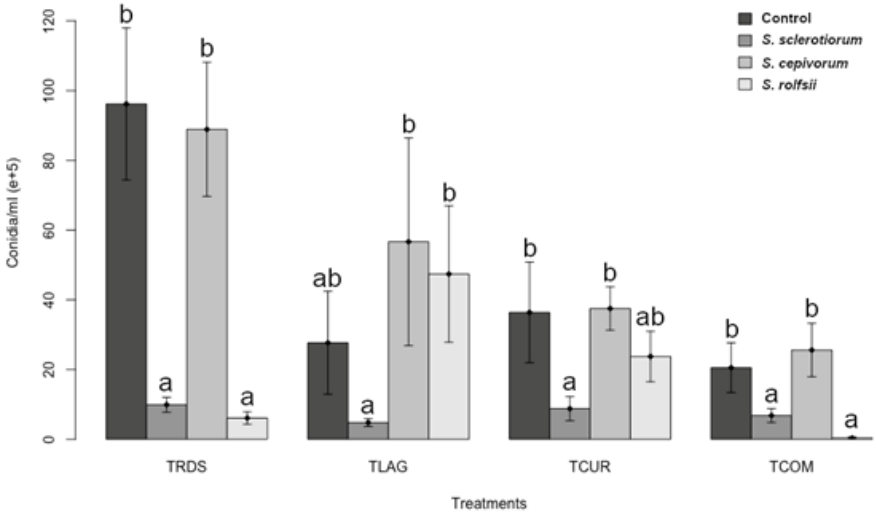

Figure 4. Conidia production comparison of each *Trichoderma* spp. strain when paired with different plant pathogen. Each treatment refers to the isolates of *Trichoderma* spp. Tukey test was run out with a 95% confidence interval. Same letters within treatments show no statistical differences between the pathogenic fungi and control (only the *Trichoderma* spp.). P values for TRDS (*Trichoderma* sp. from Rio do Sul), TLAG (*Trichoderma* sp. from Lages), TCUR (*Trichoderma* sp. from Curitibanos) and TCOM (*T. harzianum*) are less than 0.001.

Our findings related to production of spores by the different strains have also shown that the plant pathogens, based on *in vitro* analyses, might impose different levels of stress on the antagonistic strains tested. We saw that, although *S. cepivorum* and *S. sclerotiorum* did not show difference statistically significant (p >0.01), to *S. cepivorum*, the strain TRDS had the highest rate of spore production, with an average of approximately 7x106 conidia mL-1 (Figure 5).

Regarding *S. rolfsii*, from all the four strains analyzed, the one that showed the highest spore's production rate was TLAG, with an average of 4x106 conidia mL-1, followed by the strain from Curitibanos (TCUR) with an average of 2x106 conidia mL-1. These two strains were not as affected as the commercial one (TCOM – *T. harzianum* IBLF006) regarding its spore production in the presence of this plant pathogen. The other strain, TRDS did not show differences on the production of conidia in comparison with control (TCOM), which had shown the lowest value for spores' production counted throughout the experiment, with an average of $3.5x10^4$ conidia mL^{-1} (Figure 5).

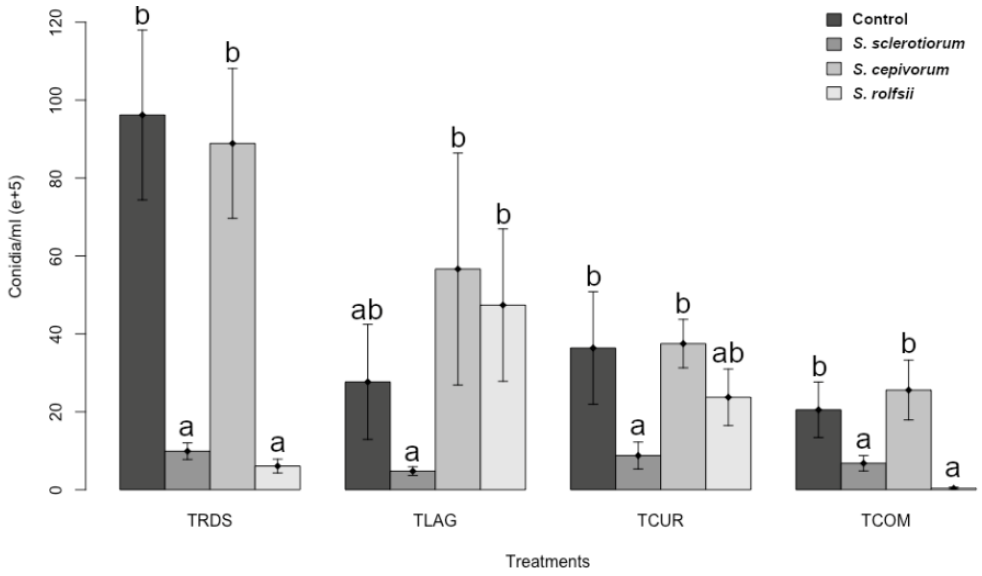

Figure 5. Comparison of conidia production of the different isolates of *Trichoderma* spp. in each phytopathogenic fungus. Each treatment refers to different phytopathogenic fungi. Tukey test was run at 95% confidence interval. Same letters within treatments show no statistical differences between the *Trichoderma* strains TCOM (*T. harzianum*), TCUR (*Trichoderma* sp. from Curitibanos), TRDS (*Trichoderma* sp. from Rio do Sul) and TLAG (*Trichoderma* sp. from Lages). Control is referred to the conidia production of *Trichoderma* strains on the absence of the plant pathogens. P values for all treatments were less than 0.01.

According to Howell (2003), the best method to obtain a potential biological control agent is through the isolation of *Trichoderma* species from areas where the disease occurs. Studies show the efficiency of this genus against different pathogens, with results that reach almost 60% reduction in the growth of phytopathogens treated with this antagonist (Amaresh, Chennappa, Avinash, Naik, & Sreenivasa, 2019).

After antagonist isolation, the paired culture assay is an important methodological process to select isolates that present greater antagonistic and mycoparasitism activity (Correa et al., 2007).

Some studies have shown that most *Trichoderma* species are capable of parasitizing phytopathogens with different levels of aggressiveness (Kubicek et al., 2011; Druzhinina et al., 2018). Despite the breadth of the genus, most studies of mycoparasitism by *Trichoderma* spp. were carried out with only a few species, highlighting *T. harzianum, T. virens, T. viride, T. atroviride* and *T. asperellum* (Meyer, Mazaro, & Silva, 2019).

It is possible that there is a selectivity in the parasitic capacity of *Trichoderma* in relation to fungi of the phylum Ascomycota, which can differentiate this genus compared to other mycoparasites in its ability to colonize sclerotia (Chaverri & Samuels, 2013).

In experiments carried out by Druzhinina et al. (2018), interactions of *Trichoderma* spp. with several phytoparasitic ascomycetes, revealing interactions between hyphae of *Trichoderma* species and other fungi belonging to this phylum, in which processes including directed growth and penetration of host hyphae, were verified.

A study by Mathews, Sivparsad and Laing (2019) corroborates the results obtained in the present study on the influence of *Trichoderma* spp. in reducing the MGI of *S. sclerotiorum*. In this study, the authors provide an

indication that mycoparasitism is probably the most likely method of action involved in suppressing the growth of this pathogen and reducing the production of sclerotia. In observations through ultrastructural analyses, it was found that there is a coiling of *T. harzianum* hyphae around the hyphae of the phytopathogen, which allows the entry of antagonist hypha fragments into the lumen of the parasitized fungus, causing assimilation of cell content, cell wall degradation and lysis (Chiuraise, Yobo, & Laing, 2015).

In experiments carried out by Isaias, Martins, Silva, Silva and Melo (2014), 20 isolates of *Trichoderma* spp. were tested and most of them presented an inhibitory effect on the mycelial growth of *S. rolfsii* in strawberry (*Fragaria* sp.). The authors also reported that the most effective control may be related to the place of origin of the isolates, since the isolate originated from the culture itself presented the best control. Correa et al. (2007) also presented results from 20 different *Trichoderma* spp. in inhibiting the growth of *S. rolfsii*. In this study, they observed that all 20 *Trichoderma* spp. reduced the growth of the phytopathogen, highlighting five isolates that occupied the entire growth area of the pathogen.

The use of *Trichoderma* spp. also shows positive results in the control of *S. cepivorum*. Fuga, Lopes, Vieira and Cunha (2016) inferred that six *Trichoderma* spp. tested showed positive results, acting mainly as hyperparasites of the fungus *S. cepivorum*. In field studies, the use of *Trichoderma* spp. against *S cepivorum* was the most used chemical treatments, presenting off as a viable and less aggressive to the environment alternative in combating diseases caused by this phytopathogen (Rivera-Mendez, Zúñiga-Vega, & Brenes-Madriz, 2016; Rivera-Méndez, Obregón, Morán-Diez, Hermosa, & Monte, 2020).

Conclusion

In conclusion, our results indicate that although *Trichoderma* species maintain their position as important antagonistic fungi presenting positive effects against two highly virulent plant pathogenic fungi such as *S. sclerotiorum* and *S. cepivorum*, we did not find, within the strains tested, a possible biocontrol agent against *S. rolfsii*.

Considering that there are few registered products for phytopathogens, the *Trichoderma* could be an important fungus to use on *in vivo* and *in situ* experiments to verify its behavior on natural conditions.

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