Agrisost | Vol. 28, January-December, 2022: 1-7

ISSN-e: 1025-0247

Antagonistic Capacity of *Trichoderma harzianum* Rifai against Fungal Pathogen *Sclerotium oryzae* (Catt.)

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Citation: Padilla Navarro, L., Buil Martin, K., Rodríguez Saldaña, M., & Hernández Torres, D. (2022). Antagonistic Capacity of Trichoderma harzianum Rifai against Fungal Pathogen Sclerotium oryzae (Catt.). *Agrisost*, *28*, 1-7. https://doi.org/10.5281/zenodo.7689003

Received: December 23rd, 2021 Accepted: March 11th, 2022 Published: April 28th, 2022

Funding source: Not declared.

Conflict of interest statement: Not declared.

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Abstract

Context: The antagonistic capacity of Trichoderma harzianum Rifai against fungal pathogen Sclerotium oryzae (Catt.) strain A-34, and the action mechanisms against the fungal pathogen of *Sclerotium oryzae* (Catt.), which caused crop losses.
Aim: To evaluate the antagonistic capacity of *Trichoderma harzianum* Rifai against the fungal pathogen *Sclerotium oryzae* (Catt.).
Methods: The concentration of spores/ml^{-1}, the viability and mycelia growth inhibition of the pathogen, and the antagonistic capacity of *Trichoderma harzianum*, through the action mechanisms. The results were processed using SPSS, version 22.0, for Windows, and Tukey's test results had a 0.05% probability. A completely randomized experimental design was used for each pathogenic fungus.
Results: No antibiosis was observed at 24 hours because the antagonistic did not inhibit pathogen growth; at 72 hours, there was hyphal interaction between *S. oryzae* and *T. harzianum*, and there was no mycoparasitism by the antagonist. *S. oryzae* grew more than the antagonistic at 96 hours, and at 120 hours, the antagonistic grew more than *S. oryzae*; therefore, no antagonistic or hyper-parasitic activity were observed in the pathogen.
Conclusions: The antagonist did not affect the mycelial growth of *S. oryzae* during the dual challenge. Likewise, *T. harzianum* (strain A-34) showed no antagonistic activity against *Sclerotium oryzae*, with a negative inhibition percent of Radial Growth, seen through antibiosis and the action mechanisms.

Keywords: *concentration, inhibition, action mechanisms, Sclerotium.*

Introduction

Rice (*Oryza sativa L*) was first observed almost 10 000 years ago in many humid tropical and subtropical Asian regions. This crop is the staple food of over half the world's population. Globally, it ranks second after wheat, according to the area harvested. However, rice provides more calories by hectare than any other grain. According to FAO, the world rice output in 2019 was 1.6 million tons higher in November (515 million tons), which entailed a 0.5% decrease compared to the absolute record of production in 2018. (LaagriculturaDigital.com, 2019).

In Cuba, in 2019, rice production was estimated at 200 000 tons for consumption, which accounts for 430 000 tons of humid grain. (Camellón, 2019, cited in Monreal, 2019).

The provincial yearbook of Camagüey, Cuba, issued by ONEI (2018) recorded 7 903.1 ha of rice, with an overall production of 31 952.4 tons (4.04 t/ha^{-1}) in the spring.

In Camagüey, following the historical record of production in 2018 of **1**00 thousand tons of humid grain, the Ruta Invasora Agroindustrial Complex **(**CAGRI) witnessed a steep fall in production in 2019 which caused an adjustment of the initially 90 407.3 t planned to a little over 52 thousand tons (ONEI, 2019).

The fungus-caused diseases found in rice plantations are numerous. The severity and incidence of the worst diseases cause yield losses that might reach 70%. Stem rot is caused by the soil pathogenic fungus *Sclerotium oryzae* (Catt.) Krause & Webster (1972), which affects the crop throughout its development, generating damage to the stem, and affecting production. The utilization of foliar fungicides to reduce the severity of the disease is a common practice in rice, with early applications starting during panicle initiation through flowering (Castañeda et al., 2017).

The applications of *Trichoderma* upon mudding performed to control the fungus, has several advantages, since it grows very quickly, but it also produces a large number of enzymes, secondary metabolites as organic compounds, and action mechanisms, such as a competition over space and nutrients of the environment, the production of secondary metabolites with antibiotic or antifungal activity, direct parasitism or the induction of resistance, with over 100 species. All of them have beneficial effects on agriculture and other branches (Castañeda et al., 2017). Accordingly, the aim of this paper is to evaluate the antagonistic capacity of *Trichoderma harzianum* Rifai strain A-34 against fungal phytopathogen of rice *Sclerotium oryzae* (Catt.).

Materials and Methods

This research study was conducted at the Bioprocess Laboratory of the Faculty of Applied Sciences from the Ignacio Agramonte Loynaz University of Camagüey, located at 21°23'19" north latitude, and 77°52'59" west longitude, and the provincial Laboratory of Plant Health (LPSV), at the north latitude and 77º 53´ 55´´ west longitude, 95 meters above sea level. The study took place between September 2019 and May 2020.

The highly pathogenic *S. oryzae* monosporic isolate was supplied by the provincial Laboratory Stock of Plant Health in Camagüey, and it was identified according to the corresponding mycological keys (Tarr, 1964). *T. harzianum* strain A - 34 was provided as the antagonistic agent by the Institute of Plant Health (INISAV).

The samples were observed on plates on 5.0 mm diameter discs containing 1.0 g of biomass per treatment, which were used to prepare the mother solution in graduated tubes, with the addition of 1.0 mL sterile distilled water. Then 0.1 mL was diluted in 0.9 mL sterile distilled water to make the seriated solutions according to the concentration needed for Neubauer chamber count, which required a drop from the last dilution.

A volume of 0.1 µL of the titer dilution was added to a drop of PDA medium on a slide (24 and 48 hours) and incubated for 12h at 28±1°C, which was observed through the optic microscope at 40x. The germinated, non-germinated, and deformed spores were counted in ten visualization fields.

The *S. oryzae* inoculum was prepared seven days before, in Potato Dextrose Agar (PDA)-Biocen medium, pH 5.5, and incubated at 28±1 °C in the dark. The *T. harzianum* antagonist used in the double challenge was obtained at the optimum time and concentration.

To evaluate the antagonistic effect, 5.0 mm diameter discs were taken from the pathogenic fungus and the antagonist, and were placed on a 90.0 mm diameter Petri dish containing PDA-Biocen, pH 5.5, and incubated at 28±1 °C in the dark. Culturing was performed using the double method (Bell et al., 1982), according to the recommendations of Rincón et al. (1992).

The action mechanisms of space competition, micro parasitism, and antibiosis of *T. harzianum* (strain A-34) on pathogenic agent *S. oryzae*. Moreover, the antibiotic effect of *T. harzianum* on the phytopathogenic agent was evaluated using the Radial Growth Inhibition Percentage (RGIP) in a dual culture, at 24 h, when there was no physical contact between the hyphae of the antagonist and those of the pathogens.

A completely randomized experimental design was used for each pathogenic fungus to determine the action mechanisms, through different treatments.

1. *T. harzianum* – *S. oryzae* 2 interactions. Control (pathogenic fungi and the antagonist), replicated three times each. The radial growth of the colonies of the phytopathogenic fungus was measured while interacting with the antagonist, as well as the controls, after 24 h, until one of the microorganisms covered the plate.

The antagonistic capacity was determined through the Bell et al. (1982) five-grade scale (Table 1), and the effect of the biocontrol agent over the mycelial growth of the plant pathogenic fungus was checked through RGIP, using the formula described by Samaniego et al. (1989), RGIP= [(R1-R2)/R1] x100. Where R1 is the radial growth of the pathogenic fungus interacting with the antagonist.

Grade	Antagonic capacity
1	The antagonist grows entirely on the pathogen, covering the surface of the culture medium.
2	The antagonist grows entirely on two-thirds of the surface of the culture medium.
3	The antagonist and pathogen colonize half the surface of the culture medium, with no dominance of one over the other.
4	The pathogen colonizes, at least, two-thirds of the surface of the culture medium.
5	The pathogen grows over the antagonist, almost covering the entire surface of the culture medium.

The study evaluated the action mechanisms by mycoparasitism, and several fragments were collected from the mycelium at the hyphal interaction area, since some contact was observed between the *T. harzianum* hyphae and those of every phytopathogen. An optical microscope (Novel, 400x) was used to observe the mechanisms (coiling, penetration, vacuolization, and lysis), which were graphically recorded using a portable digital camera.

The results were processed using SPSS, version 22.0, for Windows. The Tukey's test was performed with 0.05% probability to compare the means (95.0% confidence), through a simple analysis of variance (simple ANOVA). The data collected during titration were transformed according to the expression $Log_{10}(x+10)$ (Lerch, 1977).

Results and Discussion

Spore concentration mL^{-1} of *T. harzianum*

Upon the analysis of variance, the concentration of spores/mL^{-1} of *T. harzianum* in the three samples chosen at random from the strain before the assay (Figure 1) showed the significant reciprocal influence. The concentration of spores is the indicator required for the product's usage criterion, from which viability and mycoparasitism can be assessed.

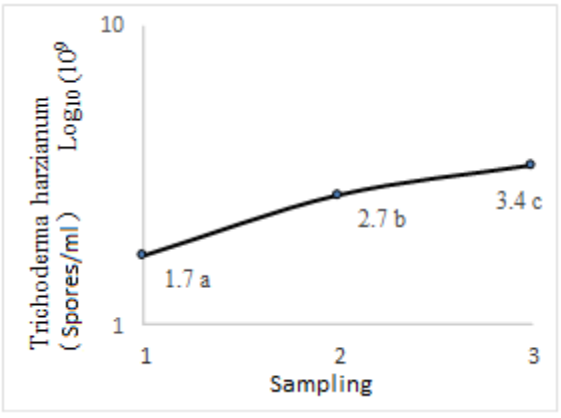


Fig. 1. Concentration spores/mL-1 of *T. harzianum* (strain A-34) Different scripts differ significantly for p≤0.05 ESẋ= 1.6

The concentration reached the highest exponential peak in the third sample. The optimal concentration for the plate challenge was 3.4 x 10^9 spores/mL^{-1}; there was a significant difference from the other samples, and they also differed from each other.

The study conducted on *T. harzianum* against several types of fungal phytopathogens of rice (Muñoz, 2019) used the same antagonist with strain A-34 from a culture in tubes containing PDA medium, pH 5.5, stored at 4±1 °C, resulting in a 10^9 concentration.

Another author with similar results to this study, in terms of concentration, though in a different culture (Pérez Torres, 2016), noted that *T. harzianum* Rifai strain A-34 in a liquid culture medium containing glucoside potato broth, Czapek, Richard and Fries, pH 5.5, with NaOH(ac) and HCl(ac), which matched the parameters of this research as to concentration, but in a different culture medium.

Concentration is a critical element for future use and the final observation of the microorganism, coinciding with the current research Quesada-Mola et al. (2019) where they describe that *T. harzianum* and the biological activity of new Cuba strains of *Trichoderma spp* are effective to control *Meloidogyne incognita* (Kofoid and White) Chitwood, particularly strain A-34 in the flasks containing the PDA medium, incubated at 30 °C until sporulation, at 10^9 concentration, showing that the highest effect was over 80%.

PDA is mostly used in the main procedures for the production of several fungus types, including *T. harzianum*. The combination of potato with glucose provides the perfect energy source for satisfactory fungal growth, whereas agar provides consistency to the medium. In all the cases, the medium was used with pH 5.5, the best to grow several fungi, such as the antagonist used in this research.

Viability took place 24 hours before the assay, with evaluations at 12 hours. The germinated, non-germinated and deformed spores at 28±1 °C, were counted, with 97.0% germination of the total of spores.

Antagonistic activity of Trichoderma harzianum against Sclerotium oryzae in dual culture.

The antagonistic activity of *T. harzianum* strain A-34 against the mycelium of plant pathogenic fungus *S. oryzae* in dual culture was observed after 72 h of challenge, demonstrating the hyper parasitic effect of the pathogen due to hyphal overgrowth those of the antagonist, as well as by the antagonist's colonizing capacity, which outgrew the plant pathogen (Figure 2).

The growth coefficient by hour during the challenge was 0.056 cm/h for the antagonist, whereas the phytopathogen reached 0.062 cm/h, evidencing that *T. harzianum* grows in a lower proportion than *S.*

oryzae. Besides, the pathogen covered the plates at 72 h completely.

These results did not coincide with the reports of Pérez Torres et al. (2017), who studied the antagonistic activity of *T. harzianum* Rifai against the rice sheath blight causing agent (*Pyricularia grisea* Sacc.), and the competence action mechanism was observed to show the antagonist at 48 h, covering three-fourths of the plate, demonstrating a quick *T. harzianum* strain A-34 growth, and the reduction of hyphal growth of *P. grisea*.

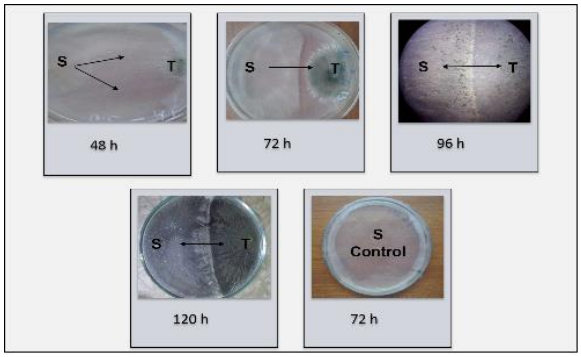


Fig. 2. Competence of dual culture between *T. harzianum* (strain A-34), (T), and *S. oryzae* (S)

The mycelial growth of the pathogen was not affected by the antagonist, causing no inhibition of radial growth during the dual challenge, which coincided with a higher growth capacity of the pathogen in the medium compared to the antagonist. It coincided with a research study done in Brasilia by Correa (2007), who identified 20 native strains, of which 15 belonged to *T. harzianum*, and the others were from *T. aureoviride* (rtwo), *T. crassum* (two), and *T. viride* (one), which upon challenge in the dual culture against fungus *Sclerotium rolfsii* (a pathogen of the same genus, but from a different species), where strains CEN224, CEN250, CEN251, CEN263, and CEN265 from *T. harzianum* did not show any antagonistic action on the pathogenic fungus, as it grew over them, colonizing all the surface of the medium. The *Trichoderma* isolates did not have strain A- 34.

Coincidentally, the rationale for this outcome (Correa, 2007) was given by several authors who demonstrated certain characteristics of this antagonist. They noted that though most agents used for biological control of pathogenic fungi have certain levels of specialization, a few *Trichoderma* species have been referred to as parasitic of a broad range of pathogens.

A significant aspect of these assays was demonstrated by Correa (2007), who said that a change of the substrate or culture medium for dual challenge is needed to assess the response of the two microorganisms involved, as the case of strains CEN219, CEN220, and CEN249, among the suitable antagonists against the pathogenic fungus, which did not show the same effect in this assay. Therefore, it can be inferred that there is no probable action caused by biologically diffusible toxic metabolites in the culture medium with the colony filtrate of any of the three strains.

A similar result was observed by Michel-Aceves et al., 2013, who picked 12 different strains of *Trichoderma spp*, based on the classification of Bell et al. (1982), by means of which, the isolates were placed between grades two and five. The Tcn-11 isolate was the only grade-two aggressive element capable of stopping growth of the phytopathogen, and inhibiting the formation of sclerotias, whereas isolate Tcn-7 remained grade five, meaning that *S. rolfsii* grew over *Trichoderma*. Of the six isolates capable of inhibiting the mycelial growth of *S. rolfsii* through secondary metabolites in the cellophane test, only three could attack them directly in the dual culture. However, the phytopathogen was more aggressive in the other isolates.

In that sense, Garrido & Vilela (2019) found similar results to this paper, after evaluating the antagonistic capacity of an undeclared *T. harzianum* strain (identified as a commercial strain), against *Rhizoctonia spp*., *Nakatea sigmoide* and *Sclerotium rolfsii*, which cause stem and pod rotting in rice, evidencing that *T. harzianum* colonizes a third of the medium in the presence of *S. rolfsii* when affected by the plant pathogen.

In another study, Pérez-Torres et al., 2018, when comparing *S. rolfsii* and *R. solani* control in dual culture, found that the same species of *Trichoderma* does not function in the same way and intensity against the two pathogens.

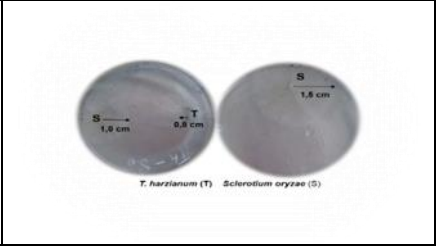
T. harzianum antagonism at 72 hours

At 72 hours, the *T. harzianum* antagonist reached a negative RGIP, which according to Bell et al. (1982) was grade 5; that is, when the pathogen grows over the antagonist and covers almost the entire surface of the culture medium. These results demonstrate that *T. harzianum* outgrows the pathogen's colony at 120 hours following *S. oryzae* colonizing (Table 2)

Martínez et al. (2013), in an article about different *Trichoderma* species and its control against *S. rolfsii*. They noted that the growth of the antagonist over the pathogen in dual culture does not ensure a high parasitic capacity, since their hyphae may share spaces and compete without parasitizing.

Table 2. *Trichoderma harzianum* and *Sclerotium oryzae* challenge

Indicators	Time (h)				
	24	48	72	96	120
Radial Growth Inhibition (%)	2.6	-2.8	-7.3	0	0
Grade Bell scale	4	4	5	5	5

Mycoparasitism	Coiling	Penetration	Unobserved lysis	Unobserved vacuolization	
Antibiosis					

(-): Presence of the action mechanism
Different scripts differ significantly for p≤0.05 ESẋ= 1.6

The *T. harzianum* Rifai antagonist against pathogen *Sclerotium oryzae* showed no antagonism by antibiosis at 24 hours after plating. Hence, there was no action of volatile and non-volatile secondary metabolites of *T. harzianum*, consequently, no inhibition of pathogen's radial growth was observed. These results did not match the reports of Duarte et al. (2017), which found that the *T. asperellum* strains evaluated after 24 h inhibited the growth of *S. rolfsii*, especially after 48 h, compared with the control. However, this effect was more evident at 72 h; the fungistatic action originated upon the hyphal contact involves the secretion of diffusible metabolites into the environment by the *Trichoderma* strains, which in this case was a different species from the one used in the experiment.

Pérez et al. (2017), in an assay of six *Trichoderma spp strains against Fusarium oxysporum* and *Botrytis cinerea*, noted that different culture media showed different results, both against the antagonist and the pathogens. The latter evidenced that the volatile compounds produced by a microorganism depend largely on the culture medium where they grow.

Pérez Torres et al. (2017) described the antibiotic effect observed at 24 h of challenge, with 14.3% inhibition of mycelial growth, when there was no interaction observed between the hyphae of the biocontrol agent and *Pyricularia grisea*. This response may be given by the presence of bioactive substances of volatile nature (non-diffusible) and non-volatile (diffusible) produced by *Trichoderma* strains. These results did not coincide with the ones observed in this research, considering that the responses of the antagonist and the pathogen were inversely proportional. the pathogen was capable of growing, and therefore take more space than the antagonist, in addition to outgrowing it. Following 120 hours, *T. harzianum* could outgrew the pathogen, though with slight action mechanisms, thus demonstrating that *T. harzianum* did not have antagonistic capacity against S. oryzae.

Hyphal interaction between the pathogen and the antagonist

The hyphal interaction between the pathogen and the antagonist took place at 72 hours, with the occurrence of mycoparasitism by coiling, and penetration, which were not expressed in the amount and magnitude expressed by the antagonist.

Coiling or strangling of the cytoplasmic content of the antagonist's hyphae over the pathogen's is one of *T. harzianum*'s control mechanisms, which manifests upon challenging with the pathogenic fungus, which can be associated with penetration. Though they may not be directly proportional, as they can occur separately and differently, this element can be observed through the microscope instantly (Figure 3).

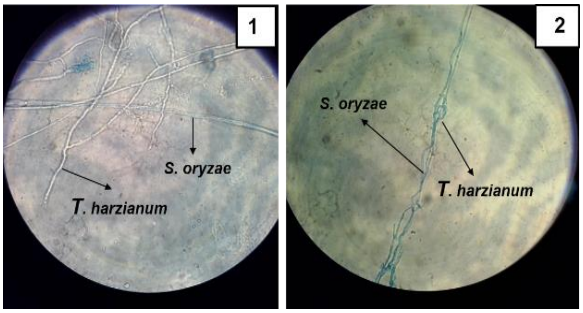


Fig. 3. Penetration (1) and coiling (2) of *T. harzianum* over *S. oryzae*

The mechanisms of vacuolization and cell lysis were absent between 72 and 120 hours during the experiment. It was closely related to the previously described mechanisms; if there is no coiling and penetration by the antagonist, the degradation and rupture of the cell wall of the pathogenic fungus's mycelium will not take place.

The study conducted by Garrido & Vilela (2019) on the antagonistic capacity of *Trichoderma harzianum* against *Rhizoctonia*, *Nakataea sigmoidea* and *Sclerotium rolfsii*, and the evaluation of the effect caused on these 14 native strain types of *Trichoderm* isolated from rice, showed that the greatest action of mycoparasitism was observed in *Rhizoctonia*, with frequent hyphal coiling and penetration. In *Nakataea*, only coiling was observed, whereas in *Sclerotium*, no action mechanisms were present, thus the competence over space and nutrition from which *Sclerotium* was capable of growing more in the culture medium was also observed in this study.

A research study done by Pérez Torres et al. (2017) demonstrated that the *T. harzianum* antagonistic strain A – 34 showed mycoparasitism by coiling, penetration, vacuolization, and lysis of elements that differ from the reports of *S. oryzae.*

Likewise, there is no correspondence with the findings of Reyes et al. (2008) and Osorio et al. (2016), after observing mycoparasitism by coiling, penetration, and vacuolization of *Trichoderma* isolates against the causal agent of the rice pod TIZON (*Rhizoctonia solani* Kühn).

These elements permit determining that the *T. harzianum* antagonist did not express the action mechanisms against the *S. oryzae* pathogen.

Conclusions

The antagonist did not affect mycelial growth of *S. oryzae* during the dual confrontation.

T. harzianum (strain A-34) showed no antagonistic activity against *Sclerotium oryzae*, with a negative radial growth inhibition percentage seen through antibiosis and the action mechanisms.

Author contribution statement

Leyanis de la Caridad Padilla Navarro: significant contribution in the conception, design, data collection and analysis, interpretation, and redaction of the manuscript.

Keyla de la Caridad Buil Martin: analysis of the results, design, and final review.

Manuel Rodríguez Saldaña: research planning, analysis of the results, redaction of the manuscript, and final review.

Daineris Hernández Torres: analysis of the results.

Conflict of interest statement

The authors declare the existence of no conflicts of interests.

Acknowledgments

The authors wish to thank everyone who offered their support to complete this research project.

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