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Compatibility study of *Trichoderma harzianum* Rifai and rice fungicides, and effects on three fungal plant pathogens

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ABSTRACT

This research took place at the Provincial Plant Sanitation Laboratory, in Camaguey, Cuba, between September 2013 and September 2015. The *in vitro* compatibility and antagonistic capacity of *Trichoderma harzianum* Rifai (strain A-34) on rice pathogens (*Bipolaris oryzae* Breda de Haan, *Sarocladium* oryzae (Sawada) w., Gams and D. Hawksworth and *Magnaporthe grisea* (Hebert Barr), was determined against pesticides used on rice. Assessment using traditional methods of microbiological isolation of mycelial growth, sporulation and conidial germination of the antagonist, to determine if the action mechanisms (antibiosis, competence, parasitism) against fungal pathogens, was made between 24 and 216 hours of application. A bifactorial design in dual culture was used for statistical analysis, along with scales for determination of microbial antagonistic capacity. Active ingredients tebuconazol + procloraz, trifloxistrobin+ ciproconazole, and epoxiconazole + kresoxim-methyl, affected mycelial growth of the antagonist. Moreover, the antagonist against active ingredients carbendazim, copper oxychloride, azoxystrobin and tebuconazo + triadimenol showed mycelial growth, sporulation and pathogen interaction, affecting their growth by means of coiling, penetration, granulation, and cell lysis, between 96 and 216 hours.

KEY WORDS/: *Trichoderma harzianum, Bipolaris oryzae, Sarocladium oryzae, Magnaporthe grisea, Oryza sativa*, compatibility, chemical fungicides, bio pesticides, rice

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INTRODUCTION

Rice is Cuban staple food, with an annual per capita consumption higher than the rest of the countries in the region. However, national production only suffices one third of the demand. The current emergency situation of world food production calls for analysis of the main causes affecting rice yields (Rivero et al., 2009).

Rice is cultivated all over Cuba, by both the state and private farmers. Recently, a more flexible approach has been implemented, that includes the average citizens who wish to plant rice and have the means to do it.

Some biological agents that have affected rice are *Pyricularia grisea* Sacc, *Bipolaris oryzae* Breda de Haan and *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksworth. The major methods for control rely on the use of more resistant varieties, and chemical controls. Today, biological methods are applied to preserve and keep the balance of the ecosystem. Biological controls include the use of fungi (*Trichoderma*) that can hyperparasite other fungi, and can be used to control several plant pathogens (Rodríguez and Harman, 1991).

Currently, biocontrols have acquired an important position within management practices of plant diseases caused by soil fungal pathogens, particularly the *Rhizoctonia, Sclerotium, Pythium, Phytophthora and Fusarium. Trichoderma* species have a good potential as antiparasitic controls, because they produce antifungal metabolites and hydrolytic enzymes with structural changes at the cell level; such as vacuolation, granulation, cytoplasmatic disintegration, and cell lysis (Harman, 2001).

This article evaluates the compatibility of *T. harzianum* with pesticides used on rice fields, as well as their antagonistic capacity against three fungal plant pathogens.

MATERIALS AND METHODS

The research was made at the Mycology Department of the Provincial Laboratory of Plant Sanitation (LPSV), located on 73° 80´ North latitude, and 135° 60´ West longitude, 98 m above sea level.

The antagonists and pathogens were cultured according the traditional microbiological isolation methods, whose samples were collected at the Ruta Invasora Grain Complex, in Vertientes. Discs of 5 mm diameter of each pathogen and the antagonist were placed on Petri dishes (70 mm diameter) containing contaminated and clean media, potato - dextrose - agar (PDA) medium, pH 5.5, and were incubated at 27±2°C, using dual culture, according to Rincón et al. (1992). Additionally, the cultures of the antagonist in pesticide-contaminated PDA medium, were made through microbiological isolation, with

5 mm discs from clean medium. The dosage for each product is shown in Table 2.

To assess the effect on mycelial growth and sporulation, the agar medium was contaminated with pesticides at desired concentrations (Pozo, 1987). The solution was prepared in sterile water, and the pesticide concentration was added; finally, it was poured in the medium. Petri dishes (70 mm) containing contaminated PDA medium were used. A disc of 5 mm diameter, taken from Trichoderma active growth area, was placed right in the middle of the dish, and it was incubated at 28 ± 1°C. Mycelial growth assessment was performed daily, for 9 days. The diameter of the colonies was measured to determine the effect of substances in relation with the witness. Sporulation was assessed nine days after.

A factorial design was used for the dual growth assay, to analyze the treatment factor: 1.Interaction T. harzianum, from PDA medium contaminated with pesticides - *Bipolaris oryzae*; 2.-Interaction *Trichoderma harzianum*, from contaminated medium – *Sarocladium oryzae*; 3.-Interaction Trichoderma harzianum, from contaminated medium – *Magnaporthe grisea*; 4. -Witness *Bipolaris oryzae*; 5. -witness *Sarocladium oryzae*; 6. -witness *Magnaporthe grisea*; 7.-witness *Trichoderma harzianum*- from uncontaminated medium - *Bipolaris oryzae*; 8.- witness *Trichoderma harzianum*, from uncontaminated medium - Sarocladium oryzae; 9. –witness *Trichoderma harzianum*, from uncontaminated medium - *Magnaporthe grisea*; 10.-witness *Trichoderma harzianum*, time factor (24 y 216 h interval), using a completely random design, with four replicas of treatment factors and time.

Table 1. Scale for determination of antagonistic capacity of microorganisms (Bell et al. 1982)

Degree	**Antagonistic capacity** (degree of antagonist´s growth)
1	It overgrows the pathogen and covers the medium surface.
2	It overgrows two-thirds of the culture medium surface.
3	It spreads over half the surface of the culture medium.
4	It covers a third of the culture medium.
5	No growth

Assessment was performed to action mechanisms, substrate competence, microparasitism, and titration.

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Substrate competence helped assess the pathogen´s radial growth diameter in all the treatments, using a ruler, for as long as the experiment lasted (24-216h). To determine the antagonistic capacity of the A-34 strain of *T. harzarium*, the five-degree scales suggested by Bell et al. (1982), was used. The radial growth percentage inhibition (RGPI) was assessed, using the formula suggested by Samaniego (1989), cited by Bernal *et al.* (2004). (RGPI) = [(R2-R1)/R2] x100. Where R1 is the radial growth of the treatment with interaction between the antagonist and the pathogen; and R2 is the witness radial growth.

Four samples were taken per treatment to assess microparasitism, at the site where the antagonist interacts with the pathogens. To determine the type or types of hyphal interaction (penetration, vacuolation, lysis and coiling), an optic microscope (40x magnification) was used.

Titration was assessed in four disc samples of 5.0 mm diameter each, weighing 1.0 g of biomass per treatment. The main solution was made in sterile distilled water with Twin, in Erlenmeyer. Then, 1.0 ml of the solution was added to 0.9 ml of sterile distilled water, with dilutions of 101 and 102 per spore count in Neubauer chamber.

Antibiosis of the A-34 strain of *T. harzianum* was used to assess the inhibition percent of mycelial growth (RGPI) in dual culture, at 24h.

Sensitivity of T. harzianum against the seven pesticides used was assessed using the scale suggested by Martínez and Figueroa (2007), which considers three levels of toxicity:

Degree 1 Compatible: less than 10% affectation of mycelial growth (ACM)

Degree 2 Moderately compatible: 10% - 30 % ACM

Degree 3 Non-compatible: more than 30% ACM

(Table 2) Fungicides and concentrations used in compatibility assays

Active ingredients	**Concentration mg i.a /l**
tebuconazol +triadimenol	140,150,160,170
tebuconazol + procloraz	150,160,170,180
azoxistrobin	100,110,120,130
trifloxistrobin+ cyproconazol	167,177,187,197
epoxiconazol + kresoxim-methyl	135,145,155, 165
carbendazim	305,315,325,335

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Copper oxychloride	433,443,453,463

RESULTS AND DISCUSSION

Effects of pesticides on mycelial growth of *T. harzianum*

In treatment 1 (tebuconazol+ triadimenol) (Table 3), *T. harzianum* was observed to have no mycelial growth at 24h. At 48h, an average growth of 1.0 mm in every concentration and replica in the fungicide study, was observed. The growth rate (GR) was 0.02 mm-h, with 95% RGPI. At 16h, the mycelial growth observed in all the doses ranged between 6.75 and 9.25 mm of growth, and between 0.03 and 0.04 mm-h, with 76 and 83% RGPI, respectively.

During the early hours of antagonist exposure against the active ingredient, mycelial growth was affected, and continued to be affected until 216h. It may be related to residues from the product (lasting 14 days), regardless of further growth produced, by eliminating the effect of the product on the medium, the fungus recovers, provided it did not undergo structural damage. The importance of this observation is that the moment to incorporate the antagonist in the culture and the time required between two applications can be defined.

Concerning tebuconazol + procloraz, trifloxistrobin+ ciproconazol, and epoxiconazol + kresoxim- methyl, they had a remarkable effect on mycelial growth of T. harzianum (strain A-34), in all the concentrations tested, with a RGPI of 100%.

At different concentrations, Carbendazim affected mycelial growth of the antagonist; at 24h, it was 100%; at 72h, growth was not inhibited, because the antagonist was able to cover the plate with radial growth of 30 mm. These results are different from reports by Reyes (2011), who found a fungistatic effect against isolates of *Trichoderma asperellum* Rifai.

The use of copper oxychloride against *T. harzianum* (A-34) failed to produce mycelial growth at 24h, though it increased at 48h, covering the disc completely. At 120h, no inhibition was observed, which corroborated findings by Morera (2009), who reported that *T. harzianum* and *Trichoderma viride* Rifai can withstand the effects of fungal mixtures well, and they can recover easily as soon as they get in contact with sub lethal doses of pesticides.

Table 3 Chemical compatibility of Trichoderma with pesticides applied to rice

Groups	Products	*All concentrations of T.*

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		harzianum (strain A-34)	
		Scale degree	**RGPI (%) 120 hours**
Fungicide	tebuconazol +triadimenol	3	88.06
	tebuconazol + procloraz	3	99.06
	azoxistrobin	3	77.62
	trifloxistrobin + cyproconazol	3	98.68
	epoxiconazol + kresoxim-methyl	3	99.5
	carbendazim	3	0
	Copper oxychloride	2	0
1-Compatible; 2-Moderately compatible; 3-Non compatible			E± = 0.04

Effect of pesticides on sporulation

Fungicides with no results means that no mycelial growth was observed; hence, sporulation is null.

Reyes (2007) reported conidium drying and constriction, cell wall lysis, and expulsion of cytoplasmatic contents of *Trichoderma* spp. against procloraz. In that case, it is combined with tebuconazol, so *T. harzianum* (A-34) response during and after contact with the product may be cause. The same effect occurred with epoxiconazol + kresoxim- methyl and trifloxistrobin + cyproconazol.

Sporulation of *T. harzianum* exposed to different fungicides was not affected in any of the cases, in comparison to the witness (Table 4), though most inhibited mycelial growth more than 45%.

All concentrations of *T. harzianum* had sporulation against tebuconazol + triadimenol, with values above 5.0 x 1008, whereas the witness reached 2.68x108. Therefore, no significant differences were observed in the concentrations, except between they and the control. It occurs because during the initial hours, mycelial growth was affected, but it was able to recover and degrade the active ingredient, speeding up the processes.

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In the PDA contaminated treatment, Azosxistrobina was slightly superior than the control, whereas mycelial growth was inhibited (60%). The previous result coincides with reports by Plant Health Care (Mexico), 2009, cited by Reyes (2007), for reported compatibility of Azoxistrobin. Although this fungicide showed the lowest percents of mycelial growth affectation, it affected sporulation and germination, concerning the witness. It was assumed in this study that compatibility differences were given because the species reported in the article and the species studied are different.

Table 4. ***Fungicidal effect on sporulation (conidios.mm-2) T. harzianum (strain A-34)***

Pesticides	Concentration	Titration	Control titration
tebuconazol +triadimenol	140 mg ia	8.20x108	2.68 x108
	150 mg ia	5.75x108	2.48 x108
	160 mg ia	9.12 x108	2.68 x108
	170 mg ia	5.65 x108	2.48 x108
azoxistrobin	100 mg ia	1.68 x108	2.15 x108
	110 mg ia	1.80 x108	2.19 x108
	120 mg ia	1.12 x108	2.54 x108
	130 mg ia	4.06x108	3.22 x108
carbendazim	305 mg ia	1.42 x108	2.01 x108
	315 mg ia	2.84x108	2.30 x108
	325 mg ia	1.30 x108	3.0 x108
	335 mg ia	1.62x108	2.63x108
Copper oxychloride	433 mg ia	2.18x108	2.54 x108
	443 mg ia	4.68 x108	3.0 x108
	453 mg ia	3.18 x108	3.22 x108
	463 mg ia	2,56 $x10^8$	2.63x108

Sporulation was similar for carbendazim and oxychloride, among the two concentrations, also coinciding with the control treatment, slightly superior to the PDA treatments contaminated. They showed adequate mycelial growth, when inhibition from 96 h on was 0, because the control treatment and the contaminated medium covered the disc fully at 72h.

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Conidium germination and viability

The Azoxistrobin treatment was the most affected, in terms of conidium germination (57.04%). It differed from reports by Reyes (2007) who observed that azoxistrobin did not affect conidiuml germination much. Also important is that the experiment was made with another *Trichoderma* species. Furthermore, there were significant differences among fungicides, but not in the concentrations.

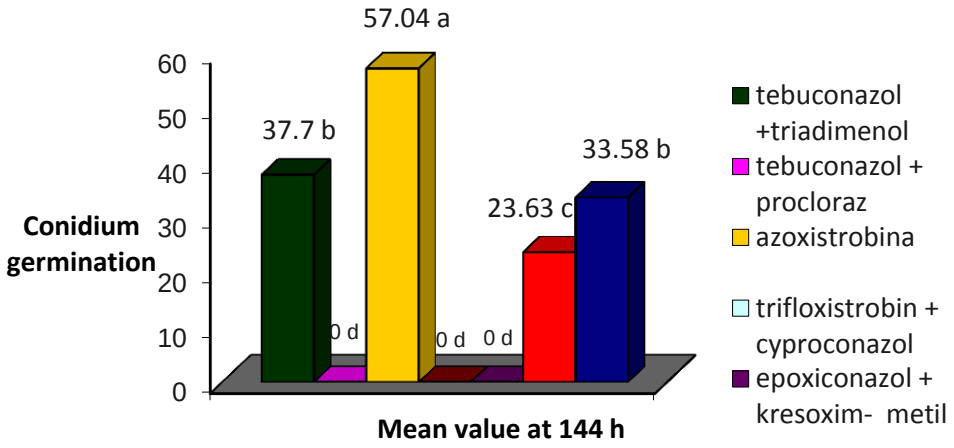

Figure 1 Evaluation of conidium germination at 144 hours

Dual culture

Significant differences were observed during the interaction of pathogens, but not in the concentrations. Tebuconazol + procloraz + cyproconazol, epoxiconazol + kresoxim- methyl showed no mycelial growth, it was 0. Tebuconazol + triadimenol, azoxistrobin, copper oxychloride and carbendazim, though, showed growth, with similar mean concentration values.

Antagonistic action of *Trichoderma harzianum* Rifai against *Bipolaris oryzae* Breda Haan to seven fungicides

At 24h and 48h, the pathogen kept low levels of inhibition against the antagonist and the control; only tebuconazol + procloraz, carbendazim and azoxistrobin showed significant pathogen inhibition, regarding the control, which was incremented until 216h. The highest values against copper oxychloride were 66.44%; and against carbendazim, 77%.

These results coincide with other results achieved by Agüero (2012) in dual challenging assays on non-contaminated PDA, between B. orizae and T. orizae

and *T. harzianum* (strain A-34), in which RGPI increased to 67.0%. As a result, there was limited radial growth of the pathogen interaction, which grew stronger as the exposure time increased due to antagonistic colonization, whereas radial growth of the witness grew faster.

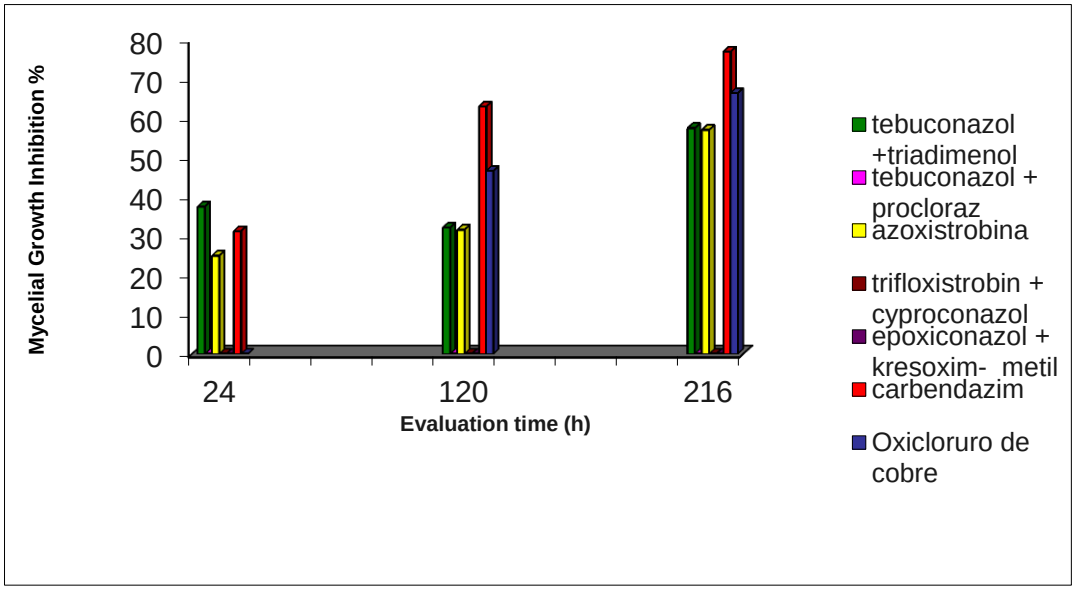

Figure 2. Inhibition of mycelial growth of B. oryzae against T. oryzae (strain A-34)

Trichoderma harzianum competence against *Bipolaris oryzae* (Breda de Haan) Shoemaker

According to *in vitro* tests, *T. harzianum* had elevated levels of competence over the substrate. It hyperparasitized colonies of the pathogen partially or totally, in almost all the fungicides, causing oversporulation in most cases.

These results coincide with Ghisalverti et al., (1990), and Agüero (2012) who noted the hyperparasitic potential of *Trichoderma* species, as strong competitors for substrate, with a very particular metabolic activity that confers them efficiency in destroying fungal structures.

Mycoparasitism of *Trichoderma harzianum* against *Bipolaris oryzae* Breda de Haan.

Hyphal interaction between the pathogen and the antagonist occurred at 72 h of challenging, mostly fungicides. Only tebuconazol + triadimenol initiated interaction at 96h. Mycoparasitism was manifested through coiling or strangling of the cytoplasmatic contents of the antagonist hyphae on the pathogen, and by penetration, causing granulation, vacuolation, and cell lysis. Santana *et al.*, (1995) found that the A-34 strains of *T. harzianum* evidenced

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mycoparasitism on *Sclerotium*. Lobato and Cañar (2009) also had similar results with *T. harzianum* (strain A-34) on rice pathogens *B. oryzae* and S. oryzae.

Figure 3. *T. harzianum* penetration into B. oryzae, an antagonist from the medium contaminated with carbendazim

Competence of *Trichoderma harzanium* Rifai against *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksworth

Trichoderma hyphae were able to colonize the disc containing S. oryzae at 144h. They stopped mycelial growth of the pathogenic fungus, proving that T. harzianum is an excellent competitor for a single substrate, nutrient, and space. Similar results were achieved by Sundara and Sakesna (1998), cited by Lobato and Cañar (2009) which concluded that this fungus has the capacity to trigger a fungistatic effect on the application site, halting the proliferation of hyphae from other species of fungi, particularly, plant pathogens. Those results also coincided with reports by Agüero (2012).

Mycoparasitism of *Trichoderma harzianum* Rifai against *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksworth

Throughout this period, colonies from the pathogen were observed to stop growth due to the antagonistic action of *T. harzianum*, from 120 to 216h, on the hyphal intersection of the antagonist and the pathogen. Then, parasitism took place, as a result of coiling and penetration into the four active ingredients that grew and challenged the dual assay (Figure 4). The results coincided with reports by Lobato and Cañar (2009), using rice pathogens *S. oryzae* and *B. oryzae*.

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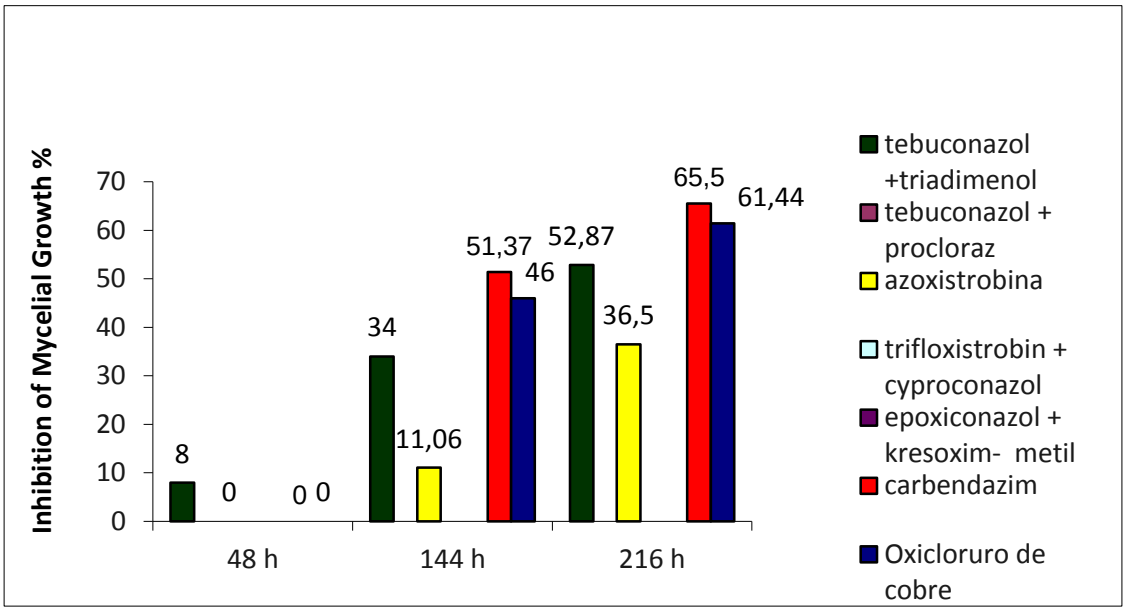

Figure 4. Inhibition of mycelial growth on dual T. harzianum– S. orizae interaction

It must be stressed that the mechanisms of vacuolation, granulation and lysis were present in all the pathogens, against all the fungicides. More regularly, more granulation was observed, especially at 192 and 216h.

Antagonistic action of *Trichoderma harzianum* Rifai against *Magnaporthe grisea* (Hebert) Barr, to seven fungicides

Regarding dual challenging to *M. grisea*, it must be highlighted that the assay was made only using carbendazim and copper oxychloride. No growth was observed for tebuconazol + procloraz, trifloxistrobin + cyproconazol and epoxiconazol + kresoxim- methyl.

At 24h, in all the cases, no pathogenic growth inhibition was observed, which meant that the antagonistic capacity was absent during the first hours of T. harzianum evaluation on the pathogen.

At 48h, pathogenic RGPI was observed for carbendazim 50 % and copper oxychloride 34.75 %. These values increased progressively as the interaction time was prolonged, beginning to interact at 216h, for carbendazim 77 % and copper oxychloride 78.37 %.

Therefore, *T. harzianum* proved high antagonistic activity on *M. grisea* when it settled on PDA discs contaminated with the above-mentioned active ingredients. It coincided with *in vitro* reports by Reyes *et al.* (2007), on rice

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pathogens; *R. solanis* (2007), on *R. solanis* and *P. grisea*; Lobato and Cañar, 2009, on *B. oryzae* and *S. oryzae*; and Reyes (2011), on *R. solanis*. They claimed that *Trichoderma* is effective as an antagonist against pathogens competing for a single substrate.

***Trichoderma harzianum* Rifai against *Magnaporthe grisea* (Hebert) Barr. competence**

Trichoderma hyphae could colonize the *M. grisea* disc at 96h, and pathogenic mycelial growth was stopped. It proved that *T. harzianum* is an excellent competitor for a single substrate, nutrient and space.

At 96h, the witness had a 4.75 cm growth in the colony, whereas pathogenic growth during interaction with the antagonist was 2.0 cm, with a 58% RGPI.

Mycoparasitism of *Trichoderma harzianum* Rifai against *Magnaporthe grisea* (Hebert) Barr.

Between 120 and 216h, the antagonistic microparasitic capacity of *Trichoderma* was observed through the microscope. Coiling of the antagonist hyphae was observed around the pathogen, as well as granulation, and cell lysis, even in the presence of contaminated PDA discs. Harman (2001) noted that these events are viable due to the enzymatic action that facilitates antagonist penetration and cause deformity, disintegration and death of the pathogenic hyphae. The mycoparasitic activity of T. harzianum against M. grisea was evidenced. This process had a series of chained events that ended in the destruction of the pathogenic hyphae, also reported by Harman (2006) and Vinale et al., (2008).

CONCLUSIONS

T. harzianum was observed to preserve its antagonistic capacity through mechanisms of antibiosis, competence for substrate, and mycoparasitism against *Magnaporthe grisea* (Hebert) Barr., *Sarocladium oryzae* (Sawada). W. Gams & D. Hawksworth, and *Bipolaris oryzae* Breda de Haan, in interaction with the four fungicides where mycelial growth was observed.

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