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Fungicide tolerance of *Trichoderma asperelloides* and *T. harzianum* strains

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ABSTRACT

Tolerance in isolations of *Trichoderma was developed* by exposing two strains of *T. harzianum* and three of *T. asperelloides* to increasing concentrations of chemical fungicides. This isolation of *Trichoderma* was exposed to three fungicides: Captan, Thiabendazol and the mixture Captan-Carboxin. Some selected lines of these strains reached tolerance to Captan and partial tolerance to the mixture Captan-Carboxin. The biological and genetic changes in these tolerant lines were monitored *by determining the* relative *growth rate* of the *fungus*, inhibition of *Fusarium* and by analyzing the genomic changes through UP-PCR. The results show that the tolerance to fungicides can be developed without affecting the parameters of biological activity in these lines of *Trichoderma* (growth and parasitism against *Fusarium*). Chemical tolerance to the fungicide was verified by means of changes at the DNA level (UP-PCR), mainly in the lines tolerant to Captan. This suggests that *Trichoderma* survives in environments with remnants of fungicide molecules.

Keywords: *Trichoderma*; Mutation; Chemical Fungicide; Biological Control; Tolerance

1. INTRODUCTION

A strategy of biological control of plant diseases caused by soil-borne plant pathogen fungi is the use of species of *Trichoderma*, these includes species of economic importance on industrial purposes for production of antibiotics and enzymes. In agriculture, these fungi, improves plant growth and development, has biological control activity against other fungi and nematodes [1-4]. It has been found that the persistent use of fungicides could weak the natural antagonistic activity [5]. However, *Trichoderma* has the capability of degradading xenobiotic compounds [6-8]. There are *Trichoderma* tolerant strains that can survive field concentrations of chemical fungicides. We now have several approaches that can be used to obtain *Trichoderma* strains resistant to chemical fungicides. Goldman *et al*. [9] and Mukherjee *et al.* [10] have sccesfully obtained *T. viride* and *T. pseudokoningii* strains tolerant to chemical fungicides. The resistance mechanism of some fungi to chemical fungicides is due to genetic mutations, which reduces the susceptibility to the fungicides and decreases their efficacy [9,11-13].

In order to study the consequences of fungicide resistance, were obtained selected fungicide tolerant lines of the strains of three *T. asperelloides* strains and two of *T. harzianum* by exposure to increasing concentrations of the fungicides Captan ((3aR,7aS)-2-[(trichloromethyl) sulfanyl]-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione), a mix of Captan/Carboxin ((3aR,7aS)-2-[(trichloromethyl) sulfanyl]-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione) /5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide) and Thiabendazol (4-(1H-benzimidazol-2-yl)-1,3-thiazole). Taking into account of a possible mutation caused by induction of fungicide resistance can also cause alterations in the fungal adaptation and fitness, antagonistic assays and growth evaluation were carried out in the selected tolerant lines and compared to the parental strains.

2. MATERIALS AND METHODS

2.1. Fungal Strains

All fungal strains used in these experiments were isolated in Colombian soils and are identified as *T. harzianum* strains T-7, T-53, *T. asperelloides* strains T-19, T-4, T-109 [14]. All strains demonstrated antagonist activity under *in vitro* conditions against *Fusarium oxysporum*, *Botrytis cinerea*, *Colletotrichum* sp., *Rhizocto-*

nia solani, and *Sclerotium rolfsii*.

2.2. Strategy for the Selection of Tolerant *Trichoderma* Lines to Chemical Fungicides

Before the selection experiments were started, the *Trichoderma* strains were grown in potato dextrose agar (PDA) supplemented with the chemical fungicides at increasing concentrations. The final concentrations used in the field are: Captan 1132.5 ppm, a mix 1:1 Captan-Carboxim 2000 ppm, and Thiabendazole 450 ppm. The objective was to determine the natural fungicide tolerance of the five *Trichoderma* strains. The selection of tolerant lines to chemical fungicides was performed by successive cultures of the *Trichoderma* strains in PDA supplemented with the correspondent fungicide at increasing concentrations. Five mm diameter disks from *Trichoderma* 10 days old cultures were placed on PDA with the chemical fungicides, and mycelial growth was measured on days 1, 2, 3, 4, 5, 7 and 11. *Trichoderma* lines displaying more than 20 mm of growth were selected to be grown under the following chemical fungicide concentration in subsequent rounds of selection. Strains that did grow 20 or more mm in diameter after 10 days of incubation, continue in the selection media; on the contrary, strains that grew poorly (less than 20 mm of diameter) or did not sporulate, were discarded. Tolerant strains were subjected to further selection experiments with increasing fungicide concentrations until the *Trichoderma* lines were able to sporulate.

To evaluate the tolerance of the selected *Trichoderma* lines to the chemical fungicides, they were grown in 30 ml of liquid medium (yeast extract 2.5%, glucose 2.5%, $NaNO_3$ 0.2%) in 125 ml flasks erlenmeyer supplemented with the fungicides, for four days at 28˚C, at 125 rpm. This experiment was performed twice, and in each one 2 replicates were set for each *Trichoderma* line.

2.3. Evaluation of the Antagonistic Activity and Growth of the Tolerant *Trichoderma* Selected Lines

The antagonistic activity of the selected tolerant *Trichoderma* strains was compared to the wild type strains by placing a 3 mm diameter disk from a *Fusarium oxysporum* 5 to 8 day old culture on PDA. After 24 h, a 3 mm diameter disk of the *Trichoderma* strain was placed 3 mm apart from the plant pathogen. Each treatment was done by triplicate, and incubated at 25 ± 1˚C under ligh*T.* The antagonistic activity of the *Trichoderma* strains was estimated according to two criteria,: the plant pathogen growth inhibition radius (IR) and the antagonism class system described by Bell *et al*. [15]. Means of growth rate and IR was analyzed by ANOVA and Fisher's least significant difference (LSD) test to determine statistically significant differences.

2.4. Identification of Molecular Characteristics of *Trichoderma* Fungicide Tolerant Selected Lines

The DNA of the *Trichoderma* fungicides tolerant strains was analyzed through universal primer PCR marker (UP-PCR), a multi-site amplification technique [16,17]. The amplifications patterns of these strains were compared to the wild type strain. DNA extraction was performed from 200 mg of lyophilized fungal mycelia according to the method described by [18]. PCR amplification mixture was compose of PCR buffer 1X, $MgCl_2$ 3 mM, dNTPs 0.2 mM, primer 1.6 µM, Taq DNA polymerase 1 U, 25 ng of DNA distilled water to a final volume of 25 µl. The following amplification program was used: initial denaturation at 94˚C during 2.5 min, followed by 30 cycles of 92˚C during 50 s, 53˚C during 90 s and 72˚C during 30 s, with a final extension at 72˚C during 3 min. UP primers used were L-45 (5' GTAAAA CGACGGCCAGT 3') and L-15 (5' GAGGGTGGCGG CTAG 3'). All amplification reactions were performed at least by duplicate. Amplification products were separated in 2% agarose, stained with ethidium bromide and visualized on a UV transilluminator. Additionally, a specific DNA fragment of the *β*-tubulin gene was amplified and used as target to diagnosed resistance to the fungicide Thiabendazole [19].

3. RESULTS

3.1. Selection of Tolerant *Trichoderma* Lines to Chemical Fungicides

After five rounds of selection, it was noticed that although 10 out of 15 *Trichoderma* lines used in the experiments accomplished the mycelial growth selection parameter, of at least 20 mm of colony radius in 10 days, the speed of growth in all cases was lower than the wild type strains (**Table 1**). Natural tolerance to the field dose of the chemical fungicide Captan (1132, 5 ppm) was achieved in all the *T. asperelloides* and *T. harzianum* strains evaluated in this study. In general, isolates of *T. harzianum* were less tolerant to the chemical fungicides than isolates of the *T. asperelloides* species.

At the end of the 9 rounds of selection with the chemical fungicides, tolerance to Captan varied between 176% and 207% of the dose recommended for field application. Isolates of *T. harzianum* could not develop tolerance to the fungicide Thiabendazole and the mixture Captan-Carboxim.

Table 1. Mean growth value of selected *Trichoderma asperelloides* and *T. harzianum* isolates exposed to several concentrations of the chemical fungicides Thiabendazole, Captan-Carboxin, and Captan compared to the wild type strains after 5 rounds of selection.

Strain	Treatment	Active ingredient concentration (ppm)	Radius of the *Trichoderma* colony after (hr)						
			24	48	72	96	120	168	240
T. harzianum T-7	Wild type	0	21.2	46.5	46.5	46.5	46.5	46.5	46.5
	Thiabendazole	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Captan-Carboxim	750	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Captan	1750	0.85	5.9	12.6	18.5	26.6	37.8	**40.5**
T. asperelloides T-19	Wild type	0	24.8	46.5	46.5	46.5	46.5	46.5	46.5
	Thiabendazole	20	2.6	7.6	10.1	13.0	14.2	18.8	**26.3**
	Captan-Carboxim	1500	0.5	3.1	5.9	9.0	12.7	20.1	**28.6**
	Captan	2000	3.1	8.7	13.9	20.1	21.0	24.8	**25.1**
T. harzianum T-53	Wild type	0	17.9	43.3	43.3	46.5	46.5	46.5	46.5
	Thiabendazole	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Captan-Carboxim	750	0.0	1.2	1.3	1.9	4.0	6.3	9.7
	Captan	1750	1.4	6.5	14.4	23.4	30.1	37.7	**38.3**
T. asperelloides T-84	Wild type	0	28.7	46.5	46.5	46.5	46.5	46.5	46.5
	Thiabendazole	20	3.4	6.8	8.9	11.5	15.5	20.8	**24.4**
	Captan-Carboxim	1500	0.7	1.4	5.9	8.5	11.2	13.9	**20.1**
	Captan	2000	2.6	14.8	24.2	29.8	37.2	43.1	**46.5**
T. asperelloides T-109	Wild type	0	24.7	46.5	46.5	46.5	46.5	46.5	46.5
	Thiabendazole	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Captan-Carboxim	750	0.0	5.2	13.7	20.4	24.6	31.3	**32.4**
	Captan	2000	2.9	6.4	13.8	25.1	32.1	39.3	**45.8**

Growth mean value in bold correspond to the *Trichoderma* isolates selected to continue in the fungicide tolerance selection experiments.

Table 2. Maximum concentrations tolerated by *Trichoderma asperelloides* and *T. harzianum* strains after multiple increasing exposures to the chemical fungicides Thiabendazole, Captan-Carboxin, and Captan, under laboratory conditions.

Strain	Active ingrediente	Maximum concentration tolerated (ppm)
T. harzianum T-7	Thiabendazole	0
	Captan-Carboxin	0
	Captan	2350
T. asperelloides T-19	Thiabendazole	20
	Captan-Carboxin	1500
	Captan	2350
T. harzianum T-53	Thiabendazole	0
	Captan-Carboxin	0
	Captan	2000
T. asperelloides T-84	Thiabendazole	20
	Captan-Carboxin	1500
	Captan	2350
T. asperelloides T-109	Thiabendazole	0
	Captan-Carboxin	1500
	Captan	2000

T. asperelloides isolates T-19, T-84, and T-109 were able to grow and to sporulate in the culture medium containing 75% of the dose recommended for field application (2000 ppm). In contrast, none of the evaluated strains were able to develop tolerance to the fungicide Thiabendazole at a concentration below 20 ppm. Selected tolerant strains cultured in liquid medium supplemented with chemical fungicides Captan and Captan-Carboxin (**Table 2**) do not shown differences from the wild type strains grown without fungicides, after four-days of culture (data not shown).

Analysis of the growth rate, (mm/hr) of the chemical fungicide tolerant *Trichoderma* lines compared to the wild type strains, show that this parameter was affected in 8 of the 10 tolerant selected lines. Statistical analysis indicate that the growth rate of six tolerant lines was lower than that of the wild type strains (*T. harzianum* T-7 Captan, *T. asperelloides* T-19 Thiabendazole, *T. asperelloides* T-84 Thiabendazol, *T. asperelloides* T-84 Captan, *T. asperelloides* T-84 Carboxin-Captan, and *T. asperelloides* T-109 Captan),. Also in two tolerant lines, growth rate was higher than the wild type strains (*T. harzianum* T-53 Captan and *T. asperelloides* T-109 Captan-Carboxin) (**Table 3**).

3.2. Antagonism Tests of Tolerant *Trichoderma* Lines to Chemical Fungicides

The antagonism test was performed with the plant pathogen *Fusarium oxysporum* and measured as the IR. It was observed that all tolerant *Trichoderma* strains kept their antagonism class 2 similar to the *Trichoderma* wild type, but strain *T. asperelloides* T-19 Thiabendazole shifted to class 3 of antagonism (**Table 3**). Comparison of the IR mean values displayed by the *Trichoderma* fungicide tolerant lines indicated that some lines have IR values that are significantly higher than the wild type strain, as in the case of *T. harzianum* T-7 selected with

Table 3. Mean growth rate of *Trichoderma* strains and antagonism against to *Fusarium oxysporum* caused by wild-type and selected fungicide tolerant lines of *Trichoderma asperelloides* and *T. harzianum.*

Trichoderma strain	Antagonism class[1]	Mean growth rate (mm/hour)[2]	Mean inhibition radius (mm)[2]
T. harzianum T-7 Wild type	2	0.98a	25.17b
T. harzianum T-7 Captan	2	0.77b	32.65a
T. asperelloides T-19 Wild type	2	0.74a	33.11b
T. asperelloides T-19 Thiabendazole	3	0.06b	6.38c
T. asperelloides T-19 Captan-Carboxin	2	0.76a	46.70a
T. asperelloides T-19 Captan	2	0.73a	39.45ab
T. harzianum T-53 Wild type	2	0.67b	36.51a
T. harzianum T-53 Captan	2	0.99a	31.31a
T. asperelloides T-84 Wild type	2	0.81a	42.45a
T. asperelloides T-84 Thiabendazole	2	0.73b	25.45c
T. asperelloides T-84 Captan-Carboxin	2	0.60c	32.91b
T. asperelloides T-84 Captan	2	0.72b	30.26bc
T. asperelloides T-109 Wild type	2	0.67b	39.26a
T. asperelloides T-109 Captan-Carboxin	2	0.71a	30.35b
T. asperelloides T-109 Captan	2	0.63c	26.88b

[1]Antagonism class determined according to Bell *et al.*, (1982) determined after 67 hours of culture on PDA; [2]Mean values followed by the same letter within each *Trichoderma* strain and column are not significative different (LSD, $\alpha = 0.05$).

Captan and strain *T. asperelloides* T-19 selected against Captan-Carboxin. While in the other cases, the IR was the same or significantly lower than the wild type strain (**Table 3**). Taking in account that one of the criteria used in the selection experiments was the ability of the tolerant lines to sporulate, the microscopic study performed indicates that all the selected *Trichoderma* lines kept this characteristic except for *T. asperelloides* T-19 exposed to Thiabendazol (data not shown).

3.3. Molecular Analysis

PCR analysis of the Captan-Carboxin lines and the wild type *Trichoderma* strains showed different amplification patterns such as deletion or addition of DNA bands. DNA amplified with primer UP-L45 indicated that the strains *T. asperelloides*, T-19 and T-84, selected with the fungicide mixture Captan-Carboxin contain the same genetic changes compared to the wild type strains, lost a of 1400 bp DNA band, while the bands of 1150, 500 and 450 bp were new in the fungicide treated lines (**Figure 1**).

Although the PCR diagnostic test designed to identify Thiabendazole susceptible/resistant genotypes indicated

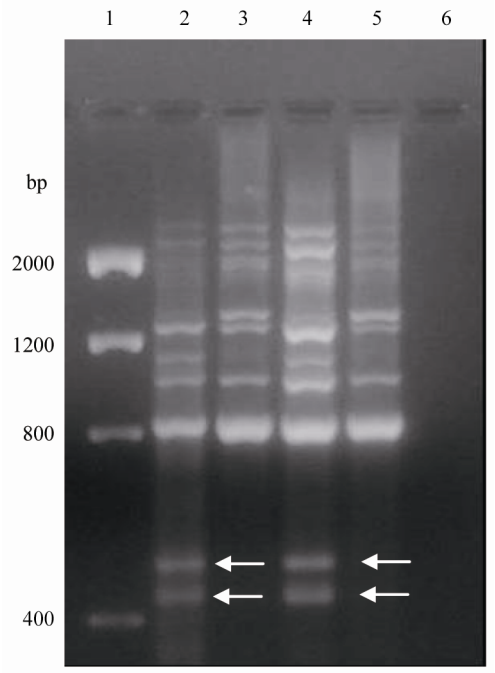

Figure 1. PCR analysis of *Trichoderma* strains tolerant to the fungicide mixture Captan-Carboxin with primer UP-L45. lane 1, molecular weight marker low DNA mass ladder; lane 2, tolerant *T. asperelloides* T-19; lane 3, *T. asperelloides* T-19 wild type; lane 4, tolerant *T. asperelloides* T-84; lane 5, *T. asperelloides* T-84 wild type; lane 6, negative control.

that there were no changes in the β-tubulin gene (**Figure 2(A)**), a change at the DNA level was observed when primer UP-L45 was used. This change is illustrated by the appearance of a new 400 bp band in both selected *Trichoderma* lines (**Figure 2(B)**). Treatment of the *Trichoderma* strains with the chemical fungicide Captan induced the largest changes at DNA level of the fungicides, primer UP-L45 was used for detection (**Figure 3**).

4. DISCUSSION

T. asperelloides and *T. harzianum* contain strains that could be of importance in biological control of plant pathogens [20-22]. *Trichoderma* strains used in this study were isolated from different geographical areas and from different sources. All of them were also naturally tolerant to the recommended concentration of the chemical fungicide Captan, and exposure of *Trichoderma* strains to increasing concentrations of this fungicide allowed for the selection of tolerant lines. Fungicide resistance is a stable, inheritable adjustment by a fungus to a fungicide, resulting in reduced sensitivity of the fungus to the fungicide. Resistant isolates are less affected or not inhibited at all by application of a fungicide [23]. The fungicide can in fact still can control sensitive isolates, causing natural resistant isolates to potentially

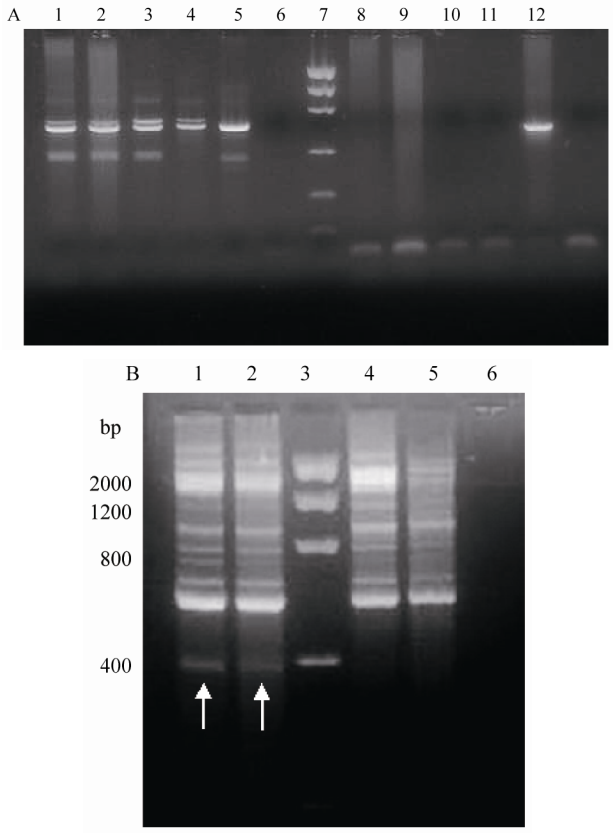

Figure 2. PCR analysis of *Trichoderma* strains exposed to the chemical fungicide Thiabendazole compared to the wild type strains. A. Resistance/susceptibility analysis. Bands in lines 1 to 6 were obtained with the primers designed to detect Thiabendazole susceptible genotypes. Bands obtained in lines 8 to 13 were obtained with the primers designed to detect Thiabendazole resistant genotypes. Lane 1, *T. asperelloides* T-19 wild type; lane 2, *T. asperelloides* T-84 wild type; lane 3, *T. asperelloides* T-19 selected with Thiabendazole; lane 4, *T. asperelloides* T-84 selected with Thiabendazole; lane 5, susceptible *Mycosphaerella fijiensis* strain (positive control); lane 6, negative control; lane 7, molecular weight marker low DNA mass ladder; lane 8, *T. asperelloides* T-19 wild type; lane 9, *T. asperelloides* T-84 wild type; lane 10, *T. asperelloides* T-19 selected with Thiabendazole; lane 11, *T. asperelloides* T-84 selected with Thiabendazole; lane 12, Thiabendazole resistant *M. fijiensis* strain (positive control); lane 13, negative control. B. PCR analysis with primer UP-15 of *Trichoderma* selected strains with the fungicide Thiabendazole. Lane 1, *T. asperelloides* T-19 wild type; lane 2, *T. asperelloides* T-84 wild type; lane 3, molecular weight low DNA mass ladder; lane 4, *T. asperelloides* T-19 selected with Thiabendazole; lane 5, *T. asperelloides* T-84 selected to Thiabendazole; lane 6, negative control.

may become dominant in populations under selection pressure of fungicide. This phenomenos happens in assays, evidencing the fact that *Trichoderma* has a natural ability to tolerate fungicides, which is called 'natural' or 'inherent resistance'. Resistance is as a response to repeated use of the fungicide, or to the repeated use of another chemically related fungicide and/or by a biochemical mechanism of antifungal action [24].

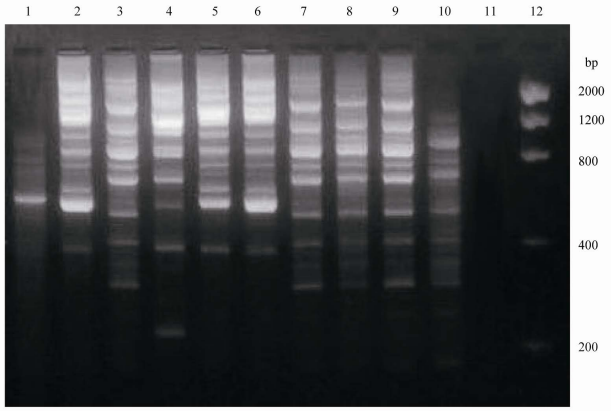

Figure 3. PCR analysis of *Trichoderma* strains exposed to the chemical fungicide Captan compared to the wild type strains with primer UP-L45. Lane 1, *T. harzianum* T-7 wild type; lane 2, *T. asperelloides* T-19 wild type; lane 3, *T. harzianum* T-53 wild type; lane 4, *T. asperelloides* T-84 wild type; lane 5, *T. asperelloides* T-109 wild typo; lane 6, tolerant *T. harzianum* T-7; lane 7, tolerant *T. asperelloides* T-19; lane 8, tolerant *T. harzianum* T-53; lane 9, tolerant *T. asperelloides* T-84; lane 10, tolerant *T. asperelloides* T-109; lane 11, negative control; lane 12, molecular weight marker low DNA mass ladder.

Ruocco *et al.* [25] explained that the ability of Trichoderma to withstand relatively high concentrations of a variety of synthetic and natural toxic compounds, including its own antibiotics, depends on efficient cell detoxification mechanisms supported by a complex system of membrane pumps. Now it is well know that the genome of *Trichoderma* includes ABC transporters (ATP-binding cassette (ABC) transporters), which are members of a protein superfamily that effluxes drugs from cells of target organisms. Thus transporters may provide a mechanism of protection against cytotoxic drugs and xenobiotic agents. The natural function of ABC transporters in plant pathogenic fungi may relate to transport of plant-defense compounds or fungal pathogenicity factors [26]. The ABC transporters may explain the natural tolerance of fungicides on *Trichoderma*, and their ability to successfully to survive in extreme environments.

Growth of *T. asperelloides* and *T. harzianum* strains in liquid medium with the fungicides Captan and Captan-Carboxin confirmed that the selected lines have developed a mechanism to tolerate the exposure to homogenous concentrations of the chemical fungicides. Tolerance to the fungicide mixture Captan-Carboxin was obtained in the treated lines of *T. asperelloides* strains T-19, T-84 and T-109, while some degree of tolerance to Thiabendazole was only obtained with the *T. asperelloides* strains T-19 and T-84. These data suggested de-

toxification mechanisms are restricted to particular strains, and are not present in all the specimens of a taxa.

In some cases, growth rate and IR of the *Trichoderma* tolerant lines were affected by the exposure to the chemical fungicides. The antagonism capacity under in vitro conditions was only negatively affected in one out of the 10 tolerant lines obtained. A similar phenomenon was found in Penicillium on Imazalil resistance and sensible strains on which was no difference in spore production and radial growth [27]. In two cases the antagonistic capacity was superior in tolerant lines (*T. asperelloides* T109 Captan/Carboxin and *T. harzianum* T53 Captan). Analogous results were obtained by Mukherjee *et al.* [10], with mutants of benomyl-tolerant strains of *T.* pseudokoningii, which were superior to the wild type in biocontrol potential on *S. rolfsii*. A correlation between fungicide resistance and antagonistic activity is suggested by Marra *et al.* [28], affirming that the upregulated expression of ABC transporter genes of *T.* atroviride during the three-way interaction with various plants and fungal pathogens, possibly supports both antagonistic activity and root colonization.

DNA changes were observed in *T. asperelloides* lines T-19 and T-84 treated with Thiabendazole (benzimidazole group) (**Figure 2(B)**). The results of the diagnostic test designed by Cañas (2004) indicated that there were not changes in the β-tubulin gene level. Nevertheless benzimidazole resistance was conferred by point mutations in the β-tubulin gene in most phytopathogenic fungi. However, exceptions have also been noticed through via site-directed mutagenesis, a mutation that confers benomyl tolerance to other fungi does not impart resistance in *T. viride* [29]. Kawchuk *et al.* [30] established that the amino acid sequences of the β-tubulin genes from several thiabendazole-resistant and sensitive isolates were identical in *Gibberella pulicaris*. This analysis confirmed that the β-tubulin gene was not linked to thiabendazole resistance. These results suggest that there must be other genomic regions involved in the resistance to benzimidazoles, but the exact molecular mechanism for this resistance still unknown.

Differences in the number of genetic changes observed in the *Trichoderma* strains treated with chemical fungicides could be due to their mode of action or to the approach used for tolerance development. It has been described that protectant fungicides such as Captan, induce mutations in several genes, contrary to systemic fungicides in which target a particular gene or gene product [9,11-13]. This coincides with the results, since high genetic changes observed in the Captan tolerant *Trichoderma* lines as compared to the wild type strains.

The results suggest that it is possible to develop *Trichoderma* tolerant lines to some chemical fungicides. Most importantly, the changes induced by this tolerance, in most cases, does not negatively affect the antagonistic activity of the biological control strains, and in some other cases, the growth rate and the IR are increased. The molecular study performed permitted us to recognize changes at the genomic level, which in most cases are not related to the loss of biological fitness of the fungal strains.

5. ACKNOWLEDGEMENTS

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