

ISOLATION AND CHARACTERIZATION OF CARBENDAZIM DEGRADING *Trichoderma harzianum* RIFAI MUTANTS

ITAMAR SOARES DE MELO*
CÉLIA MARIA MAGANHOTTO DE S. SILVA**
JANE L. FAULL***

Mutants of *Trichoderma harzianum* Rifai, obtained after ultraviolet (UV) light exposure, showed high resistant to the fungicide benomyl. A mutant (2B6) was capable of degrading carbendazim, other fungicide of the benzimidazole fungicide. This mutant degraded 41.5% of the molecule within five days. This and others mutants (2B1 and 2B2) presented variation in size and frequency of uni-nucleated and/or bi-nucleated spores compared to the wild type. Four primers generated RAPDs patterns that allowed the mutant to be differentiated from the wild-type. It is concluded that using UV mutagenization, it is feasible to obtain strains of *T. harzianum* with improved pesticide degradation ability.

KEY-WORDS: DEGRADATION; FUNGI; MICROORGANISMS; BIOTRANSFORMATION; PESTICIDES.

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- * Agronomist, Ph.D., Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Jaguariúna, SP, Brazil (e-mail: itamar@cnpma.embrapa.br).
** Biologist, Ph.D., EMBRAPA, Jaguariúna, SP, Brazil (e-mail: celia@cnpma.embrapa.br).
*** Biologist, Ph.D., Birkbeck College, London University, London, UK (e-mail: j.nicklin@bbk.ac.uk).

1 INTRODUCTION

Trichoderma spp. are common saprophytic fungi and they have been investigated as biological control agents against soil-borne plant pathogens (CHET *et al.*, 1979). Different application approaches have been used including integration of *Trichoderma* with reduced doses of chemical agents (DEV and DAWANDE, 2010). *Trichoderma* spp. also have considerable metabolic diversity. For example, *Trichoderma* spp. are capable of effectively degrading chitin and glucans (ELAD, CHET & HENIS, 1982), and they seem to be the best source of extracellular-cellulases that can solubilize highly ordered cellulose (RYU & MANDELS, 1980). The degradation of organic compounds other than cellulose by *T. harzianum* has also been reported. Katayama & Matsumura (1993) found a *T. harzianum* strain capable of degrading DDT, dieldrin, endosulfan, pentachloronitrobenzene and pentachlorophenol. Recently, a new strain of *T. viridae* associated with *Pseudomonas aeruginosa*, when applied in treated soil with monochrotophos and methyl parathion, efficiently increased the potential in degrading both pesticides (BALAMUYUGAN *et al.*, 2010). Searching for genetically modified *Trichoderma* strains with improved abilities will satisfy the growing needs to remediate organophosphate pesticide contaminated soil. TANG *et al.* (2009) used the REMI (restriction enzyme-mediated integration) technique to construct transformants of *T. atroviride* with improved capability of degrading organophosphate pesticide dichlorvos. In that work, the authors found 8 transformants that exhibited 30% higher in degradation rate than the parent isolate. Thus it may be possible to combine biological disease control with pesticide clean up in the rhizosphere.

Some strains of *Trichoderma harzianum* Rifai are rhizosphere competent (AHMAD & BAKER, 1987; MELO, FAUL & GRAEME-COOK, 1997). The rhizosphere maintains specific micro-environments that differ from bulk soil in the availability of nutrients. Therefore it has a higher and more active microbial biomass than the surrounding bulk soil. The rhizosphere may be used as vehicle for inoculation of pesticide-tolerant and pesticide degrading microorganisms *in situ* in combination with biological control ability.

The use of biological systems to bring about the timely remediation of man-made pollutants is the goal of soil bioremediation. Bioremediation utilizes the natural role of microorganisms in transformation, mineralization, or complexation by directing these capabilities toward organic and inorganic environmental pollutants.

The benzimidazole are important fungicides which have been produced on a large scale (DELP, 1995). Benomyl, carbendazim, methyl thiophanate, and thiophanate have been used extensively to control plant pathogenic fungi in several crops.

The aims of this study were to obtain *T. harzianum* mutants resistant to benomyl, to test their ability to increase the degradation rate of carbendazim, and to characterize the mutants morphologically and molecularly via Random Amplified Polymorphic DNA (RAPD).

2 MATERIAL E METHODS

2.1 FUNGUS AND MUTAGENIC TREATMENT

Trichoderma harzianum, strain TW5, was isolated from soybean rhizosphere soil, and has demonstrated antagonistic ability against *Verticillium dahliae* (MARTINS-CORDER & MELO, 1998). Mutants were induced by ultraviolet light irradiation of conidia at a survival level of 5%. The dose was 97.00 uw cm² x 10. After irradiation, the spores were plated into potato-dextrose-agar (PDA) supplemented with benomyl (500 µg.mL⁻¹). Resistant colonies to benomyl were transferred to test tubes containing Potato Dextrose Agar (PDA) amended with benomyl (100 µg mL⁻¹). The stability of resistance was checked by periodically plating colonies in PDA with and without the fungicide.

2.2 CHEMICALS

Analytical carbendazim-MBC (methyl benzimidazole-2-ylcarbamate) and benomyl (methyl 1-butyl-carbamoyl) benzimidazole-2-ylcarbamate were obtained from E.I. Du Pont.

2.3 NUTRIENT SOLUTIONS

PDA was used for maintenance of fungal cultures. For degradation experiments, various liquid media were previously tested, and PD-50% (potato 10 mL; dextrose 10 mg; distilled water to 1 L) was used in these experiments.

2.4 CHARACTERIZATION OF MUTANTS

The *in vitro* activity of benomyl against the mutants was tested on PDA to which aqueous suspensions of the fungicide were added to achieve a series of the required concentrations. All concentrations were based on 50% active ingredient of the commercial product. The plates were inoculated with a disc of PDA with abundant growth of *Trichoderma*. Colony diameter was measured in each of three replicates after 10 days of incubation at 28°C.

The morphology and number of conidia nuclei of selected mutants and the wild-type were determined by incubating the cultures at 28°C for 7 days. Once cultures sporulated they were fixed and stained as described by Tanaka, Murata e Kato (1979). Measurement of the conidia was carried out using an ocular micrometer attached to an Olympus-BHS/PM-10 AD microscope, at 10x magnification.

2.5 CHARACTERIZATION OF MUTANTS WITH RAPD TECHNIQUE

Mutant 2B6 and the wild-type were characterized using Random Amplified Polymorphic DNA (RAPD). Nucleic acids were extracted as previously described by Raeder & Broda (1985). DNA was amplified by RAPD technique (WILLIAMS *et al.*, 1990). The thermal cycler was programmed for an initial melt for 3 min at 95°C, followed by 40 cycles of 1 min at 94°C, fast ramp 37°C for 1.5 min and then to 72°C for 2 min. A final extension step of 7 min at 72°C was included.

2.6 LABORATORY STUDIES OF CARBENDAZIM DEGRADATION

Degradation was studied in cultures containing 100 mL of medium in 500 mL Erlenmeyer flasks, enriched with 100 µg.mL⁻¹ of MBC. The fungicide was dissolved in 0.5% acetone aqueous suspension. The *Trichoderma* strain 2B6 was incubated for up to 30 days at 28°C in an orbital shaker.

The quantitative determination of the fungitoxic residues, performed from the fifth day, was carried out by High Performance Liquid Chromatography (HPLC), after extraction and purification of the samples. The method was that described by Austin & Briggs (1976) and modified by Silva (1996). The samples were extracted with ethyl acetate-HCl 1N, concentrated and dispersed again in the mobile phase. After samples filtration, chromatographic analysis was conducted by using a cationic exchange column (Shim pack WCX1 40 x 6 mm) and the following conditions: column temperature: 40°C; mobile phase: ammonium phosphate 0.0125 M; flow: 0.2 mL.min⁻¹ and absorbance at 260 nm. Under these conditions, the retention time of MBC was 4 min 30 sec. Recovery of MBC from the liquid medium was 70-84%.

Data were subjected to analysis of variance and statistical difference between mean values determined by Tukey's test (GOMES, 1997).

3 RESULTS AND DISCUSSION

Three mutants (2B1, 2B2 and 2B6) were resistant to benomyl (Table 1). Benomyl at the concentration of 500 µg mL⁻¹ did not reduce spore germination and mutants had good spore production. The mycelial growth was completely normal in solid and liquid media. There was slight reduction in growth rates in the first 4 days of incubation, but after this time the growth was similar to that in culture medium without fungicide.

TABLE 1 - REDUCTION OF MYCELIAL GROWTH AND SPORULATION OF NEW MUTANTS OF *T. harzianum* RESISTANT TO BENOMYL, IN PDA AMENDED WITH 500 $\mu\text{g mL}^{-1}$ OF THE FUNGICIDE, AND MORPHOLOGICAL CHARACTERISTICS OF THESE MUTANTS AND WILD TYPE

Strains	Reduction of Mycelial Growth (%)				
	Days of incubation ^a		Spore production $\times 10^8/\text{mL}^a$	Size of conidia (μm) ^b	
	4	10		Length ^c	Width ^c
2B1	21.2	7.3	6.2 a	3.1 ± 0.2	2.7 ± 0.1
2B2	8.1	0.0	6.8 a	3.2 ± 0.2	2.7 ± 0.1
2B6	10.4	0.0	8.5 a	3.6 ± 0.4	3.3 ± 0.4
TW5*	100.0	100.0	-	2.9 ± 0.1	2.7 ± 0.1

^a Numbers followed by the same letter are not significantly different according to Tukey's test at $\alpha = 0,005$.

*The wild-type strain did not grow in medium with benomyl.

^b Values are means of 100 replications.

^c Values following the signals are standard desviations.

There have been few reports on the effects of benomyl on naturally occurring strains of *T. harzianum*. Pribela, Kovasc e Savillova (1976) reported that benomyl is highly active against *T. viride*. Baicu (1982) evaluated the high toxicity of benomyl on spore germination and mycelium of *T. viride*. In this study, the wild-type TW5 was highly sensitive to $1 \mu\text{g mL}^{-1}$ of benomyl.

Mutants of *T. harzianum* varied in regard to morphology and size and frequency of uni-nucleated and/or bi-nucleated conidia. Mutants had larger conidia than the wild-type and only the mutant 2B6 presented 9% of spores bi-nucleated. Mutants 2B1 and 2B2 produced green colonies that grew rapidly 6-9 cm in diameter after 7 days at 28°C in PDA amended with benomyl. Mutant 2B6 produced yellowish colonies that grew slightly slower than 2B1 and 2B2.

Four primers (three of them having 20 bases and one having 10 bases) generated distinct RAPD profile for the mutant and wild-type (Figure 1).

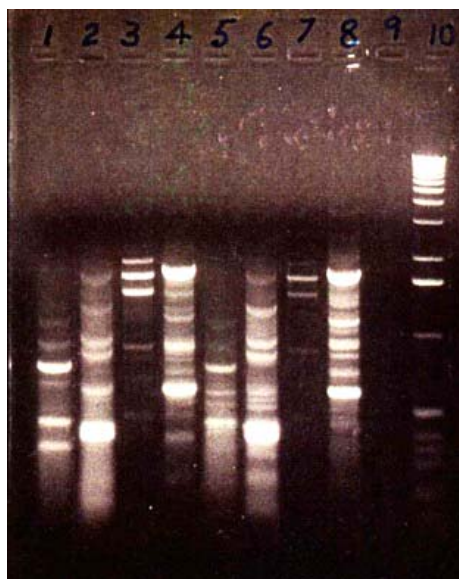


FIGURE 1 - RAPD PROFILE OF THE BENOMYL RESISTANT MUTANT (2B6) AND WILD-TYPE OF *T. harzianum* GENERATED BY THE PRIMERS P-1 (CGA CTG AAG TGA CCA AGC GC), P-2 (CAC CGC CCC AAA ATG GCC AG), P-3 (TTT GGG GCGG), AND P-6 (GTC CTC AGT CCC CCA ATC CC)

Number on the right indicates sizes (in kilobases) of the components of a 1-kb DNA ladder (Gibco-BRL). Strains numbers are presented above each lane. Numbers 1, 2, 3 and 4 correspond to the primers P-1, P-2, P-3 and P-6, respectively for the strain TW5. Numbers 5, 6, 7 and 8 are the same primers employed for the mutant 2B6 and number 9 is the control (without DNA).

Mutant 2B6, resistant to high concentrations of benomyl, had a high capacity to degrade MBC. In the first five days, this new strain degraded 41.5% of the product, reaching 66.4% in 30 days (Figure 2). Carbendazim was degraded with half-life of 37.8 days.

Studies on microbial degradation of benomyl and MBC in soil were carried out by Helweg (1977) who verified that MBC was a poor carbon source and suggested that the biodegradation of MBC is a cometabolic process.

Integration of biological with chemical controls has high potential for success. *T. harzianum* can be employed with benomyl or MBC at a low dosage to control plant pathogens. Fungicides can increase propagules susceptibility to mycoparasitic attack, and if the antagonist can also degrade the compound there should be a decreased risk of pesticide resistance developing in rhizosphere microorganisms.

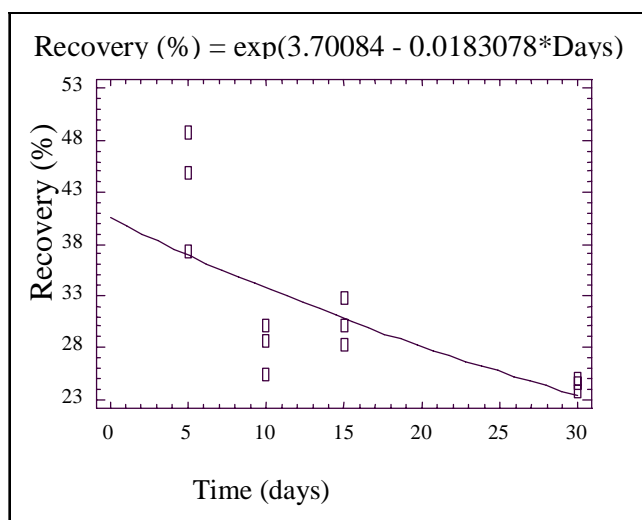


FIGURE 2 - DEGRADATION OF CARBENDAZIM (HALF-LIFE OF THE 37.8 DAYS) BY *T. harzianum*, MUTANT 2B6, IN LIQUID MEDIUM CONTAINING 100 µg mL⁻¹ OF MBC

4 CONCLUSION

In this research, it was demonstrated that a new *Trichoderma harzianum* mutant, obtained by UV irradiation, showed improved abilities of degrading the fungicide carbendazim.

RESUMO

ISOLAMENTO E CARACTERIZAÇÃO DE MUTANTES DE *Trichoderma harzianum* ENVOLVIDOS NA DEGRADAÇÃO DE CARBENDAZIM

Mutantes de *Trichoderma harzianum* Rifai, resistentes ao fungicida benomil foram obtidos por meio da irradiação com luz ultravioleta. Um mutante (2B6) foi capaz de degradar 41,5% de carbendazim, outro fungicida do grupo dos benzimidazóis. Esse e outros dois mutantes (2B1 e 2B2) apresentaram variação, tanto no tamanho quanto na frequência de conídios uni e binucleados quando comparados com a linhagem parental. Quatro "primers" geraram padrões de RAPD que permitiram diferenciar esses mutantes da linhagem parental. Conclui-se que é possível obter linhagens melhoradas de *T. harzianum* para degradação de pesticidas, utilizando-se de mutagenização com radiação ultravioleta.

PALAVRAS-CHAVE: DEGRADAÇÃO; FUNGOS; MICRO-ORGANISMOS; BIOTRANSFORMAÇÃO; PESTICIDAS.

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