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Use of *Trichoderma* spp. in biodegradation of Carbendazim

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ABSTRACT

Trichoderma spp. are most popular biocontrol agents (BCA) known worldwide for their great ability to combat different soil and foliar diseases of agricultural crops. The use of biocontrol agents in IPM strategies requires the effective strains compatible with pesticides which can be well utilized in an integrated pest management system and also suitable to reduce the residual effect of pesticides. Keeping this in view, the study was designed to evaluate to lerance levels and biodegradation of benzimidazole fungicide (Carbendazim) by *Trichoderma harzianum, Trichoderma viride* and *Trichoderma atroviride* at three concentrations 100, 150 and 200 ppm were determined. The maximum per cent inhibition of *T.harzianum*, *T.viride* and *T.atroviride* in carbendazim amended media were 60, 83 and 93 at with spore production of 2.0×10^6, 1.4×10^6 and 0.8×10^6spore/µl at 200 ppm. Biodegradation per cent of carbendazim after 5 days of incubation determined through HPLC analysis were 85% in *T.harzianum*, 47% in *T.viride* and 21% in *T.atroviride*, thus clearly indicating the biodegradation property of *Trichoderma* spp.

Key words: Biocontrol, Biodegradation, Carbendazim, HPLC-chromatography, *T. atroviride*, *Trichoderma harzianum*, *T. viride*

Trichoderma spp. are one among them known to have considerable metabolic diversity and unique role as biocontrol agents as well as organisms with enhanced chemical degradation capabilities. Some species of this genus are more active products of the enzymes that break down toxic substances and allow the cleaning of xenobiotic contaminants in soil (Sene *et al.* 2010). The degradation of organic compounds other than cellulose by *Trichoderma harzianum* has also been reported (Katayama and Matsumura 1993). Recently, a new strain of *Trichoderma viride* associated with *Pseudomonas aeroginosa*, when applied in treated soil with monocrotophos and methyl parathion, efficiently increased the potential in degrading both pesticides (Balamurugan *et al*. 2010). *Trichoderma* works through different mechanisms for the control of plant pathogens which includes mycoparasitism, competition of pathogenic fungi nutrients, secretion for antibiotics and fungal cell wall degrading enzymes (Harman *et al.* 2004, Sharma *et al*. 2014) and their products in the form of genes (Sharma *et al*. 2011, Harman *et al.* 2004). The genetically modified *T. harzianum* strain, T22 was able to tolerate concentrations up to 2 000 mg of cyanide per gram of soil. Similarly, (Tang *et al*. 2009) also showed that the improvised strain of *Trichoderma atroviride* T23 was able to degrade pesticides, viz. against dichlorides. Natural degradation of chemical compounds into their metabolite is carried by chemical reactions either by oxidation or hydrolysis. Searching for genetically modified *Trichoderma* strains with improved abilities will satisfy the growing needs to remediate contaminated soil. The objective of this study is to evaluate tolerance and biodegradation capacity of *Trichoderma* spp. against carbendazim.

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MATERIALS AND METHODS

The pure cultures of *T. harzianum* (Th3) (ITCC: 5593), *T.viride* (Tv9) (ITCC: 6315) and *T.atroviride* (Ta2) (ITCC:7445) were obtained from the Indian Type Culture Collection (ITCC) available in Division of Plant Pathology, ICAR- IARI, New Delhi and cultures were grown in potato dextrose agar (PDA) plates and stored at 4°C for further use.

To study the carbendazim tolerance levels of three isolates of *T.harzianum* (Th3), *T.viride* (Tv9) and *T.atroviride* (Ta2) triplet petri-plates were prepared for 100ppm, 150ppm and 200ppm using respective double strength media, stock solution and sterilized distilled water. The 5 mm discs were cut from 48 hrs old mother cultures placed in the centre of carbendazim amended petri-plates and unammended plates (control) and incubated at 28±2°C to check the growth after 5 days. Microscopic studies were undertaken to evaluate sporulation intensity of different species under test.

The 10 g samples of sterilized soil were added to 100 ml of mineral medium containing 1% D-glucose and 0.01%

Tween-80 ammended with three dosages of carbendazim, viz. 100, 150 and 200 ppm inoculated with fungal discs of *T. harzianum* (Th3), *T.viride* (Tv9) and *T.atroviride* (Ta2). The mixture was incubated at temperature 25°C, 200r/min for 8 days on rotary shaker. The experiment was conducted in triplicates. After each day the carbendazim containing media was taken and homogenized followed by adding 0.1 ml of hydrochloric acid. Homogenized sample was further extracted with 15 ml of n-hexane: acetone (80:20) using separating flask. The samples extracted were concentrated and dispersed again in the mobile phase during each day interval, i.e. day 2, day 3, day 4 and day 5, respectively.

The quantitative determination of the carbendazim residue was carried out by High Performance Liquid Chromatography (HPLC). 5 ml of aliquot was taken at different time intervals to check for the carbendazim degradation kinetics through HPLC 1200 series, Agilent Technologies 1200 capillary equipped with Zorbax (SB-18) capillary reverse phase column USA. The pressure of the column was upheld at 18.6 psi. The mobile phase in the column consisted of methanol and water with a ratio of 50:50 (v/v) and all analysis was performed at a flow rate of 0.8 ml/min. Injection of 5 ml extract sample wasused to perform HPLC quantitative analysis. The chromatograms in the form of peak areas were recorded and compared with standards to quantify sample concentrations.

The collected data were statistically computed using SPSS-16 software. Data were subjected to analyses of variance ANOVA and treatment means were compared by Graph Pad Prism, Inc. and found significant $P<0.05$.

RESULTS AND DISCUSSION

Screening of isolates of *Trichoderma harzianum* and *T. viride* against the commonly used pesticidesbenomyl, carbendazim, thiram, captan, mancozeb, iprodione, imidacloprid, chlorpyriphos, and pendamethalin by Sharma and Dureja (2004) clearly indicates the variation in sensitivity towards different groups of pesticides. This work was further extended to study the effects of carbendazim on the growth and changes in the morphological characteristicsof *T. harzianum* (Th3), *T.viride* (Tv9) and *T. atroviride* (Ta2) grown on carbendazim ammended PDA plates at a concentration of 100ppm, 150ppm, 200 ppm. It was observed that the colony diameter of *T. harzianum* (Th3) at 100,150 and 200 ppm of carbendazim ammended media was 5.34, 3.34 and 2.76cm, respectively. The growth inhibition per cent recorded was 24% at 100 ppm, 53% at 150 ppm and 60% at 200 ppm (Table 1). *T.viride* (Tv9) also showed similar trend of decrease in colony diameter from 2.1 cm at 100 ppm, 1.7 cm at 150 ppm and 1.08 cm at 200 ppm was recorded with the increase in per cent inhibition by 67, 73 at 100, 150 ppm and maximum of 83 at 200 ppm (Table 1). The colony diameter of *T.atroviride* (Ta2) at 100, 150 and 200 ppm doses of carbendazim was 4.9,1 and 0.5 cm respectively with the per cent inhibition of 31, 88 at 100, 150 ppm and 93 at 200 ppm (Table 1).

The decrease in the colony diameter was due to the effect of carbendazim. Impact of carbendazim on spore production in the *Trichoderma* spp. under test was also observed under the light microscope at 40 X. The production of spores was 3.93×10^6spore/µl in plates without carbendazim as compared to 3.2×10^6 spore/µl, 2.8×10^6spore/µl and 2.0×10^6spore/µlat 100ppm,150ppm and 200 ppm carbendazim dosage in Th3. Similar trend in spore count was found in Ta2 and Tv9 where it was 2.05×10^6spore/µl and 2.47×10^6spore/µl in controls and 1.4×10^6 and 0.8×10^6spore/µl at 200 ppm shown in Table 2. However, there was a significant decrease in colony diameter at 200 ppm, i.e 1.08 cm in *T.viride* (Tv9) and 0.5 cm in *T.atroviride* (Ta2) with per cent inhibition of 83 and 93 whereas, Th3 showed 60% inhibition. It is noteworthy that *T. harzianum*

Table 1 Growth performance of *T.harzianum* (Th3), *T.viride* (Tv9) and *T.atroviride* (Ta2) at different concentrations of carbendazim

Isolate code	ITCC accession number	Name of *Trichoderma* spp.	Diameter of colony (cm) at different ppm dosage*				Per cent Inhibition		
			100 ppm	150 ppm	200 ppm	Control	100 ppm	150 ppm	200 ppm
Th3	ITCC 5593	*Trichoderma harzianum*	5.34±0.05	3.34±0.03	2.76±0.1	7±0.09	23.71	52.28	60.57
Tv9	ITCC 6315	*Trichoderma viride*	2.1±0.1	1.08±0.1	1.08±0.1	6.42±0.0	67.28	73.50	83.17
Ta2	ITCC 7445	*Trichoderma atroviride*	4.9±0.06	0.5±0.02	0.5±0.02	7.2±0.1	31.90	88.50	93.03

*Figures in parentheses indicate mean fungal diameter with ± SE value. Total number of samples analyzed was N = 30 (3 isolates × 3 Replicates and 3 control).

Table 2 Spore count/µl of *T.harzianum* (Th3), *T.viride* (Tv9) and *T.atroviride* (Ta2) at different concentration of carbendazim

Isolate code	ITCC accession number	Name of *Trichoderma* spp.	Spore/µl at different ppm dosage*			
			100 ppm	150 ppm	200 ppm	Control
Th3	ITCC 5593	*Trichoderma harzianum*	3.2×10^6	2.8×10^6	2.0×10^6	3.93×10^6
Tv9	ITCC 6315	*Trichoderma viride*	$1.3x10^6$	1.2×10^6	1.47×10^6	2.05×10^6
Ta2	ITCC 7445	*Trichoderma atroviride*	2.1×10^6	1×10^6	0.8×10^6	2.47×10^6

*Figures in parentheses indicate mean fungal diameter with ± SE value. Total number of samples analyzed was N = 30 (3 isolates × 3 Replicates and 3 control).

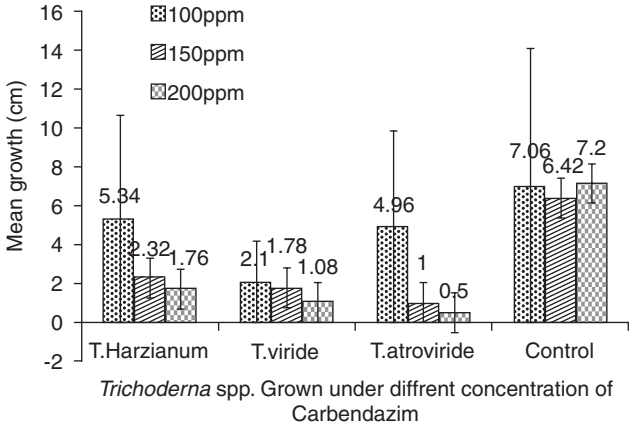

Fig 1 Growth performance of *T. harzianum*(Th3), *T.viride*(Tv9) and *T.atroviride*(Ta2) in different concentration of carbendazim. Values are means ±SE calculatedfrom three treatments of carbendazim (100 ppm,150 ppm and 200 ppm) measured in triplicate.

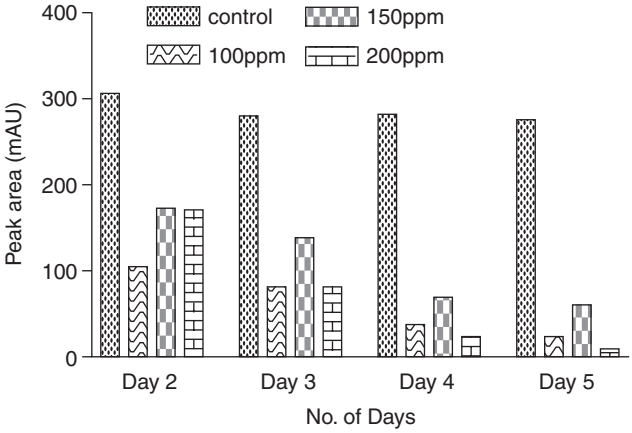

Fig 3 Carbendazim degradation characteristicsof *T.harzianum*, (Th3) grown on mineral media supplemented with three concentrations (100, 150 and 200 ppm) for 5 days through HPLC. Values are means of threereplicates ±SE with P≤0.05 according to Graph Pad Prism, Inc.

(Th3) in spite of reduction in colony diameter on third day at 200 ppm spore production (2.0×10^6spore/µl) was observed which depicts the tolerance level against carbendazim. These values are distinctly higher as compared to the reports made by Mohiddin *et al*. (2013) where 90% inhibition of colony growth was observed in *T.harzianum* and *T.virens* at 60 ppm and 40 ppm of carbendazim, respectively.

The metabolite detected through HPLC was carbendazim each day after shake incubation at 28^0C which decrease with incubation and growth of *Trichoderma* spp. (Fig 1). The reduction percentage recorded for day 2nd, 3rd, 4th and 5th (Th3) was 34, 55, 75 and 86 at 100 ppm, 80, 55, 60 and 81 at 150 ppm and 68,80,81, 84 at 200 ppm, respectively (Fig 3). In Tv9 reduction percentage for was 23, 43, 43, 44 at 100ppm, 33, 44, 43 and 47 at 150ppm and 35, 43, 44 and 47 at 200ppm at day2nd, 3rd, 4th and 5th of incubation. In case of Ta2 reduction percentage for day 2nd, 3rd, 4th and 5th was 14, 14, 15, 15 at 100ppm, 15, 15, 14, 20 at 150ppm and 14, 14, 16, 21 at 200ppm recorded. It was noteworthy that the reduction is more profound in Th3 strain of *Trichoderma harzianum* which showed maximum reduction percentage up to 84% at 5th day of treatment at 200ppm, whereas Tv9 strain of *Trichoderma viride* and Ta2 strain of *Trichoderma atroviride* showed decrease of 47% and 21% on 5thday of incubation with 200ppm dose of carbendazim. The values obtained are statistically significant and showed positive correlation with (P=0.04), r^2=0.70 for Th3, (P=0.01), r^2=0.78 for T2, (P=0.05), r^2=0.82 (Fig 2). Similarly, in a study performed by Harish *et al.* (2013) *Trichoderma harzianum* was able to grow in the presence of 100 ppm concentration of chlorpyrifos and utilize it as carbon source which was quite convincing that *Trichoderma harzianum* has a natural ability to tolerate fungicides whereas Manigandhan *et al*. (2013) observed that *T. viride* was effective in the hydrolysis of fenitrothion and fenitroxon taking them as a primary carbon source. Least degradation percent with *Trichoderma atroviride*

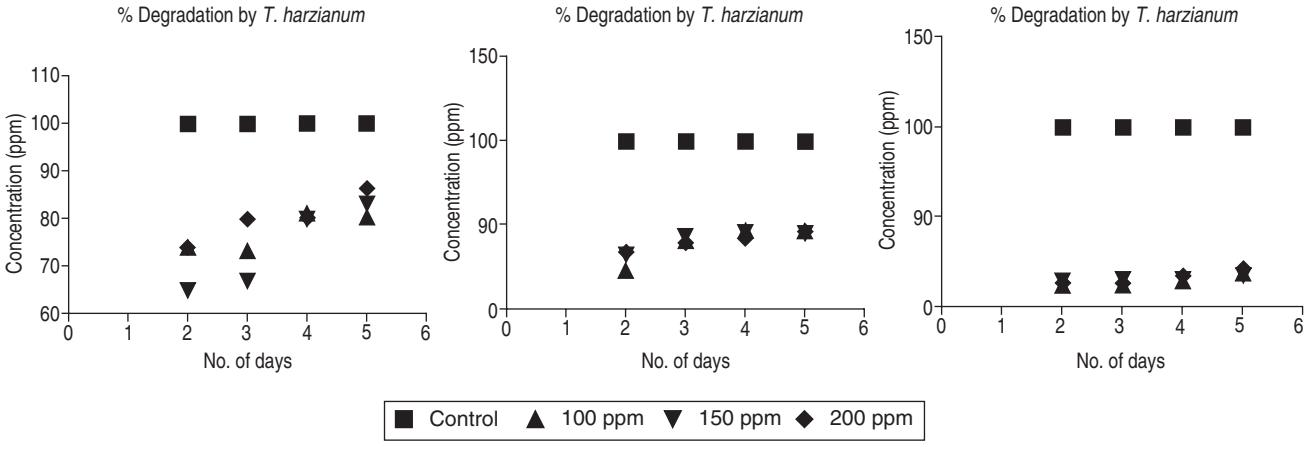

Fig 2 Correlation between the carbendazim dosage (ppm) and per cent degradation of fungicide by three *Trichoderma* spp. (A) *T.harzianum*; (Th3) (B)*T.viride* (Tv9) and (C)*T.atroviride*(Ta2). Thecorrelation analysis was performed between the mean values of per cent degradation with respect to control and different carbendazim concentrations (100ppm, 150ppmand200 ppm). Hence, the total number of sample analyzed was N = 4; p≤0.05 according to Graph Pad Prism, Inc.

(Ta2) may suggest its lower compatibility with the fungicide.

This study had shown that reduction of the carbendazim in soil under lab conditions by application of *Trichoderma* spp. is an added advantage of biocontrol agent by degrading the toxic residues in the soil and thus entering the food chains and food webs. Reduction in the levels of carbendazim withthe application of *T.harzianum* (Th3) followed by *T.viride* (Tv9) and *T.atroviride* (Ta2) indicated that selected isolates were able to degrade the fungicide. The implications of the observations obtained through this study leads to search fungicidal tolerance in biocontrol agents for the biodegradation of pesticides in field.

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