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Induction of UV - radiated mutant of *Trichorderma harzianum* Rifai and its antagonistic effect on *Sclerotinia sclerotiorum* (Lib) de Bary *in vitro*

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ABSTRACT: In the present investigation, carbendazim tolerance in *Trichorderma harzianum* was induced by exposing the culture to UV radiation for different periods (*viz.* 0, 2, 4, 6 and 8 h) and then the same were grown in PDA amended with different concentration of carbendazim *viz.* 0.01, 0.05, 0.1 and 1.0 per cent, respectively. In each concentration, colonies developed after 5 days were isolated, purified with serial dilution and sub-cultured thrice on PDA without carbendazim and tested their stability. These resulted in the development of 18 stable mutants, which could tolerate up to 0.1 per cent of carbendazim while wild *T. harzianum* (Th-w) failed to tolerate the same. Based on radial growth of above 18 stable mutants grown on fungicide free PDA, only three stable mutants were selected, which were designed as Th-M_1, Th-M_2 and Th-M_3. *In vitro* antagonistic activities of these three mutants tested against *Sclerotinia sclerotiorum*, the causal agent of white mold of French bean on PDA showed maximum reduction of radial growth of pathogen by Th-M_2. In another test, all the mutants arrested sclerotial production of *S. sclerotiorum*, significantly. However, the mutant Th-M_2 showed maximum reduction when it was inoculated seven days prior to inoculation with *S. sclerotiorum* in MSM (4%) as compared to wild *T. harzianum* (Th-w).

KEY WORDS: Carbendazim, mutant, *Sclerotinia sclerotiorum*, *T. harzianum*, UV- radiation, white mold

INTRODUCTION

White mold incited by *Sclerotinia sclerotiorum* (Lib) de Bary is a devastating disease of French bean, which can cause yield loss as high as 100 per cent (Purdy, 1979). Biocontrol offers solutions to many serious problems of modern agriculture and is an essential component in the development of sustainable agriculture. *Trichorderma harzianum* Rifai is a promising biocontrol agent of several plant pathogens. Unfortunately, this antagonist is sensitive to the agrochemicals. However, the possibility of increasing tolerance in *Trichorderma* to the agrochemicals through genetic manipulation and thereby improving their biocontrol efficacy has been reported by several workers (Lalithakumari *et al.*, 1996; Mukherjee *et al.*, 1997; Dutta & Chaterjee, 2004). Bavistin resistant mutant of *T. harzianum* have been reported by Viji *et al.*, (1993). They also reported that mutant of *T. harzianum* tolerant up to 16-μ M Bavistin. Rajappan (1997) and Hunjan *et al.* (2004) developed UV-radiated Bavistin-resistant mutant of *T. viride* with increased production of

biomass. In this context, the present study was undertaken to raise new biotypes of *T. harzianum* having higher tolerance to fungicide carbendazim, which increased parasitic activity against white mold of French bean pathogen *S. sclerotiorum*.

MATERIALS AND METHODS

Culture of *T. harzianum* was obtained from culture collection of department of Plant Pathology, Assam Agricultural University (AAU) and maintained on potato dextrose agar (PDA) medium. The pathogen *S. sclerotiorum* was isolated from white mold infected French bean variety 'Contender' from Horticultural Orchard, AAU, Jorhat and maintained on PDA. The experiment was conducted during the year 2003-04 in *Rabi* season.

Induction and isolation of UV-radiated carbendazim tolerant mutant of *T. harzianum*

Conidial suspension

Conidia of *T. harzianum* were obtained by growing the isolates on PDA medium. Conidial suspension of six-day-old culture of *T. harzianum* was prepared in 10 ml sterile distilled water. Centrifuged twice at 10,000 rpm and pallets of the suspension subsequently washed in 0.067 M phosphate butter (pH-7.0). After second washing, the pellets were resuspended in 4.0 ml of phosphate buffer and spore concentration were adjusted with sterile water to 4 x 10^6 conidia with haemocytometer.

UV irradiation and tolerance of the UV -mutant to carbendazim

Aliquotes of 10 ml of the conidial suspension were taken in sterile Petri-plates and exposed to UV radiation (with lid open) at 260 nm maintaining the 14 cm distance for different periods *viz.*, 0, 2, 4, 6 and 8 h. Molten PDA amended with 0.01 per cent carbendazim was added to 0.1 ml of the irradiated suspension and the plates were incubated in dark for 72 h. Colonies that appeared in the treated plates were isolated, purified with serial dilution technique are sub-cultured thrice on PDA without carbendazim to test their stability. Then the stable mutants were grown on PDA with carbendazim (0.05%) and the colonies developed after 72 h were again isolated, purified with serial dilution and sub-cultured thrice in PDA without carbendazim to test their stability after next higher concentration. The same procedures were repeated for 0.1 and 1.0 per cent carbendazim. The stable colonies produced were picked, transferred to PDA and incubated at 25 ± 1°C. Plates without fungicide served as control. Colony diameters were measured up to 72 h when the fungus completely covered the control plates. Then three stable mutants were selected on the basis of their higher radial growth. The phenotypic and morphological characters of mutants were studied after 72 h of incubation.

Antagonistic effect of carbendazim tolerant UV irradiated mutant of *T. harzianum* against *S. sclerotiorum*

The UV-irradiated mutant of *T. harzianum* (*i.e.* Th-M_1, Th-M_2 and Th-M_3) were evaluated and compared with wild *T. harzianum* (Th-w) for their antagonistic activity against *S. sclerotiorum* in dual culture. Seven replications were maintained for each treatment. The data were statistically analysed using by one-way ANOV A.

Ability of UV irradiated mutant of *T. harzianum* to suppress the sclerotial growth of *S. sclerotiorum* on MSM (4 %)

In vitro evaluation of the effect of wild and mutant *T. harzianum* on sclerotia production of *S. sclerotiorum* were carried out in MSM (4%) at different sequences of application (Table 4). Six replications were maintained using completely randomized design. Data were analysed by one-way ANOVA. The data were transformed to arcsine values before analysis.

RESULT AND DISCUSSION

Induction of carbendazim tolerance in *T. harzianum*

Conidial suspension of *T. harzianum* when exposed to UV-radiation for different periods *viz.*, 0, 2, 4, 6 and 8 h grown at different concentrations (0, 0.01, 0.05, 0.1 and 1.0%) of carbendazim, only

eighteen numbers of stable carbendazim tolerant colonies were developed. The colony growth of UV – irradiated *T. harzianum* was only after 2, 4 and 6 h up to 0.1 per cent concentration of carbendazim. While at 8 h there was zero colony growth (Table 1). However, maximum stable colonies were found after 4 h (10×10^6 cfu/ml) UV-irradiation of *T harzianum* that was followed by 6 h (5×10^6 cfu/ml) and 2 h (3×10^6 cfu/ml), respectively.

The growth of stable colonies was inversely proportionate to the concentrations of carbendazim. The stable colonies of *T. harzianum* obtained after UV-irradiation were designated Th_2M-Th_2M_3 (after 2 h), Th_4M_4-Th_4M_{13} (after 4 h) and T_6M_{14}-T_6M_{18} (after 6 h). These colonies were tested for viability with respect to their radial growth (mm) in fungicide free PDA medium for selection as the mutant. It also appeared that all stable mutants of *T. harzianum* irradiation at 4 h showed significantly higher radial growth as compared to 2 and 6 h UV irradiation at all period of incubation (Table 2). However, mutant of *T. harzianum* 48 h incubation showed higher radial growth as compared to 24 h of incubation, while at 72 h all the mutants completely covered Petri-plate including wild *T. harzianum*. The maximum radial growth at 48 h was recorded in Th_4-M_5 (86.75 mm), which was followed, by Th_4-M_{13} (84.25 mm) and Th_4-M_8 (82.75 mm), respectively as compared to wild *T. harzianum* (53.50 mm). Minimum growth was recorded at Th_2-M_1 and Th_2-M_3. Since these three mutants showed maximum radial growth were designated as Th-M_1 (Th_4-M_5), Th-M_2 (Th_4M_{13}) and Th-M_3 (Th_4-M_8) and the wild *T. harzianum* as (Th-w). The phenotypic and morphological characters were recorded after 72 h as given below.

Organism	Phenotypic character
Th-M	High sporulating colony with green aerial mycelium
Th-M_1	Initially colony with whitish mycelia later turn greenish, profuse sporulation and shows faster growth rate and yellow pigment appeared later
Th-M_3	Less sporulaton, pale green in colour, spores are densely formed in the margin, and sporeless marked zonation is visible

Table 1. Population of *T. harzianum* (x10^6 cfu/ml) in PDA media amended with carbendazim at different concentrations after exposing to UV-irradiation

Exposure of *T. harzianum* to UV-irradiation for different (h)	Population of *T. harzianum* (x10^6 cfu/ml) in PDA media amended with different concentrations of carbendazim (%)			
	0.00	0.01	0.05	0.10
0	56.00	29.00^c	8.00^c	0.00^d
2	54.00	39.00^b	25.00^b	3.00^c
4	56.00	47.00^a	38.00^a	10.00^a
6	56.00	27.00^c	14.00^c	5.00^b
8	57.00	23.00^d	10.00^d	0.00^d
SEM (±)	NS	1.06	0.89	0.47
CD (P = 0.05)	NS	2.27	1.91	1.01

Means within columns separated by Duncan's Multiple Range Test (P=0.05).
Means followed by the same letter shown in superscript are not significantly different.

Table 2. Radial growth of *T. harzianum* at fungicide free PDA medium after exposing to different hours of UV-irradiation

UV-irradiated *T. harzianum* isolates	Colony diameter of *T. harzianum* (mm) after different hours of incubation			Selected mutants
	24 h.	48 h.	72 h.	
Th_1M_1	15.00^{k}	37.75^{n}	54.00^{i}	
Th_2M_2	23.00^{h}	38.00^{mn}	56.00^{hi}	
Th_3M_3	18.50^{ij}	39.25^{m}	58.50^{h}	
Th_4M_4	28.25^{fg}	69.25^{g}	79.50^{d}	
Th_5M_5	43.75^{a}	86.75^{a}	90.00^{a}	*Th*-M_1
Th_6M_6	40.00^{bc}	72.25^{ef}	84.00^{c}	
Th_7M_7	33.00^{e}	73.25^{e}	86.25^{b}	
Th_8M_8	40.75^{b}	82.75^{c}	89.50^{a}	*Th*-M_3
Th_9M_9	32.50^{e}	78.75^{d}	87.50^{b}	
$Th_{10}M_{10}$	28.75^{f}	71.00^{f}	80.00^{d}	
$Th_{11}M_{11}$	35.25^{d}	73.25^{e}	83.25^{c}	
$Th_{12}M_{12}$	38.75^{c}	80.25^{d}	86.25^{b}	
$Th_{13}M_{13}$	42.75^{a}	84.25^{b}	90.00^{a}	*Th*-M_2
$Th_{14}M_{14}$	24.25^{h}	46.75^{kl}	62.75^{g}	
$Th_{15}M_{15}$	26.75^{g}	48.75^{jk}	78.00^{e}	
$Th_{16}M_{16}$	30.00^{f}	47.25^{k}	59.25^{g}	
$Th_{17}M_{17}$	33.75^{de}	49.25^{j}	60.75^{g}	
$Th_{18}M_{18}$	23.75^{h}	52.75^{i}	71.00^{f}	
Th wild	21.00^{i}	53.50^{i}	89.50^{a}	
SEM(±)	0.89	0.99	0.80	
CD (P=0.05)	1.78	1.99	1.59	

Numericals in underscript in Th denote hours of irradiation.
Means within columns are separated by Duncan's Multiple Range Test (P = 0.05).
Means followed by the same letter shown in superscript are not significantly different.

Antagonistic effect of carbendazim tolerant UV-radiated mutant of *T. harzianum* against *S. sclerotiorum*

In vitro evaluation of wild and UV radiated mutant of *T. harzianum* reduced radial growth of *S. sclerotiorum* significantly in dual culture. However, mutant Th-M_2 was found very effective in reducing the radial growth of the pathogen in all periods of incubation (Table 3), which was followed Th-M_1 and Th-M_3. Maximum per cent inhibition (91.53%) of radial growth of *S. sclerotiorum* was recorded when it was paired with Th-M_2, which was followed by Th-M_3 (64.01%). Both Th-M_1 and Th-w were less effective in inhibiting radial growth of *S. sclerotiorum*.

UV-radiated mutant of *T. harzianum* to suppress the sclerotial growth of *S. sclerotiorum* on MSM (4 %)

Sclerotial development of S. *sclerotiorum* was found to be arrested in all the treatment when mutant at *T. harzianum* inoculated along with *S. sclerotiorum* (Table 4). However, maximum inhibition of sclerotinia was observed when mutant

of *T. harzianum* (Th-M_2) preceded *S. sclerotiorum* inoculation by 7 days. It was followed by simultaneous inoculation of mutant *T. harzianum* (Th-M_2) and *S. sclerotiorum*. The inhibitory effect on sclerotial development was represented by both in decreasing the number of sclerotia as well as their total weight. Wild *T. harzianum* inoculation after 7 days of S. *sclerotiorum* shows higher sclerotial growth along with sclerotial weight (Table 4).

The UV - irradiated mutant posses higher tolerance to the systemic fungicide carbendazim and increases mycoparasitic potential. The mycoparasitic potential of the parental isolates against *S. sclerotiorum* as well as their biocontrol efficiency have been reported by several workers (Gohain Das *et al.*, 2002; AO *et al.*, 2002). Inductions of tolerance to benomyl fungicide in *Trichoderma* spp. through UV-radiation were reported by many workers (Ahmed and Bakar, 1987; Papavizas and Lewis, 1983). Abd El-Moity *et al.* (1982) reported that prolonged exposure of wild *T. harzianum* to fungicide (iprodione) at different concentrations induced new isolates of *T. harzianum*, which can grow well even at 500 µg a. i./ml concentration. Induction of carbendazim resistance in *Trichoderma* spp. through UV-radiation was reported by Viji *et al.* (1993) and Rajappan (1996). The development of stable mutant of *T. harzianum* resistant to carbendazim with different hours of exposure to UV-radiation appeared feasible. In the present study after different periods of UV-radiation, eighteen stable mutants of *T. harzianum* were obtained, which showed tolerance to carbendazim even at 0.1per cent while wild *T. harzianum* failed to grow at the same concentration.

This might be due to prolong exposure to UV-radiation. Abd-El-Moity *et al.* (1982) reported that this tolerance was due to results of "training" to the fungicides and different from their wild strains not only due to their tolerance to fungicides, but also in morphological characteristics, growth habit and sporulation.

The test on *in vitro* antagonistic activity in dual culture (Table 3) showed considerable differences between the three stable mutants and the wild *T. harzianum* in their ability to inhibit radial growth of *S. sclerotiorum*. This improved growth response of the mutants (Th-M_2) and (Th-M_3) could

Table 3. Radial growth and inhibition (%) of *S. sclerotiorum* in presence of wild and mutant *T. harzianum* in *in vitro*

Sl. no.	Treatment	Radial growth (mm) at different hours of incubation			Per cent inhibition at different hours of incubation		
		24	48	72	24	48	72
1.	*T. harzianum* (wild) + *S. sclerotiorum*	8.59	26.14^c	43.57^b	49.99 (45.00)^d	48.97 (44.41)^c	48.49 (44.13)^c
2.	Th-M_1 + *S. sclerotiorum*	7.87	27.00^b	42.29^c	54.17 (47.40)^c	45.92 (42.66)^d	49.98 (44.99)^c
3.	Th-M_2 + *S. sclerotiorum*	3.94	6.14^d	7.55^e	77.21 (62.10)^a	88.16 (69.93)^a	91.53 (73.05)^a
4.	Th-M_3 + *S. sclerotiorum*	6.70	19.14^c	30.43^d	61.00 (51.37)^b	63.16 (52.65)^b	64.01 (53.13)^b
5.	*S. sclerotiorum* alone	17.29	51.86^a	89.87^a	-	-	-
	SEM (±)	0.51	0.49	0.58	-	-	-
	CD (P=0.05)	1.01	0.97	1.18	-	-	-

be attributed to the production of higher amount of 1-3 glucanase which enables them to utilize substrate more efficiently than wild (Ahmed & Baker, 1987) also higher antagonistic capacity and direct mycoparasitic potential of mutant as compared to wild (Viji *et al.*, 1993). Efficacy of *S. sclerotiorum* to develop scleriotia in prior, after and simultaneous inoculation to wild and mutant of *T. harzianum* has significantly been reduced. It might be due to higher mycoparasitic ability of mutant and its ability to occupy the niche before colonification of *S. sclerotiorum* and thereby reducing the sclerotial growth (Papavizas and Lewis, 1983; Mukherjee *et al.*, 1997).

Table 4. Numbers of sclerotia, fresh and dry weight of sclerotia produced by *S. sclerotiorum* in presence of wild and mutant *T. harzianum* in MSM (4%)

Sl. no.	Treatment	Number of Sclerotia	Weight of Sclerotia (g)	
			Fresh	Dry
1.	*S. sclerotiorum* + MSM	220.00 (108.19)^{a}	4.10^{a}	2.00^{a}
2.	Application of *S. sclerotiorum* on MSM before 7 days of wild *T. harzianum* (Th-W)	102.66 (89.32)^{b}	3.16^{b}	0.99^{b}
3.	Application of *S. sclerotiorum* on MSM before 7 days of wild *T. harzianum* (Th-M_2)	60.00 (50.77)^{c}	1.90^{c}	0.64^{c}
4.	Application of *S. sclerotiorum* and wild *T. harzianum* (Th-W) on MSM simultaneously	36.00 (36.87)^{d}	1.70^{d}	0.40^{d}
5.	Application of *S. sclerotiorum* and wild *T. harzianum* (Th-M_2) on MSM simultaneously	18.00 (25.10)^{f}	1.30^{f}	0.12^{f}
6.	Application of *S. sclerotiorum* on MSM after 7 days of wild *T. harzianum* (Th-W)	26.00 (30.66)^{e}	1.56^{e}	0.20^{e}
7.	Application of *S. sclerotiorum* on MSM after 7 days of wild *T. harzianum* (Th-M_2)	10.00 (18.43)^{g}	1.00^{g}	0.09^{g}
	SEM (±)	2.46	0.02	0.08
	CD (P=0.05)	5.20	0.06	0.18

Means within columns separated by Duncan's Multiple Range Test P=0.05.

Means followed by the same letter shown in subscript are not significantly different.

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