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Evaluation of mutants and mother culture of *Trichoderma* spp. for production of enzyme

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ABSTRACT: *Out of seventeen isolates of Trichoderma, T1 and T7 isolates were selected for the development of mutants on the basis of highest antagonistic efficacy against Sclerotium rolfsii and Macrophomina phaseolina pathogen of groundnut and mungbean respectively. Conidial suspension with concentration of 10^7 cfu/ml was treated with ethylmethyl sulfonate with five concentrations @ 50, 75, 100, 125 and 150 µl/ml for 30 and 60 minutes. Selected single cell colonies were again treated with 0.1 and 0.2% colchicine. On the basis of highest antagonism of two mother cultures T1 and T7 the 13 mutants were selected for estimation of β-1,3 glucanase, β-1,4-endoglucanase and chitinase production. The highest chitinase activity was observed in M12 (3.22 U) whereas the activity of β-1,3 glucanase was observed in the range of 1.22 to 4.94 U and the highest activity of β-1,4-endoglucanase was observed in M13 (4.09 U).*

Keywords: *Chitinase, EMS, mutagens, mutants,*Trichoderma*, β-1,3 glucanase, β-1,4- endoglucanase*

INTRODUCTION

Strainal improvement by mutation is an age-old as a successful method. Therefore several approaches including chemical mutation, UV irradiation and their combinations were applied to obtain enhanced cellulose producing strains of *Trichoderma* (Kotchoni and Shonukan, 2002). Colchicine is known to be unsurpassed chemical agent, it has a strong mutagenic property. Colchicine is also known as a polyploidy inducer in microbes (Oenfelt and Klasterska, 1983). *Trichoderma* spp. is known to produce many extracellular hydrolytic enzymes *viz*., β-1,3 glucanase, chitinase, cellulose etc. by which they cause lysis in many plant pathogenic fungi. *Trichoderma* is also an effective biocontrol agent for protecting a number of crop plants from several soil borne plant pathogen (Mukhopadhyay *et al*., 1994). Certain mechanisms are known *viz*. mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilisation and sequestration of inorganic nutrients and inactivation on pathogens enzymes etc. (Harman, 2000). Sometime starvation condition could induce secretion of cell wall degrading enzymes (Ramot *et al*., 2000) whereas in other cell wall or cell wall components were required to trigger the enzyme (Elad *et al*., 1982). The ability of *Trichoderma harzianum* to produce extracellular â-1,3 glucanase, chitinase in to the medium supplemented with laminarin and chitin led several authors to postulate that the released enzymes were actively involved in microbiological control (El Katany *et al*., 2004). The aim of present study is to know the extent of enzyme producing activities of the mutants along with mother cultures.

MATERIALS AND METHODS

Conidiospores of 8 d old cultures were collected and incubated in 0.2M phosphate buffer of 8.0 P^H containing 50,75,100,125 and 150 ìl ethyl methane sulfonate (Sigma) per ml phosphate buffer for 30 and 60 min with the spore concentration of 10^7 cfu/ml placed on YMG agar medium. The plates were incubated at 28± 2^0C for two d. The colonies developed from single spore were selected and were inoculated in conical flasks containing 50ml Natick medium and incubated at 28±2 ^{0}C with shaking at 120rpm for 18hr. The conidia were treated with 0.1 and 0.2% (w/v) colchicine (Loba-Chemic ind) and incubated at 28^0C with shaking at 200rpm for 7 d. The 0.1 ml of dilution of the conidia were plated on medium containing 0.1% (v/v) Triton X-100 and

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incubated at 28 ^{0}C for six days. Accordingly among two mother cultures M_1 and M_7, five and eight mutants possessing high antagonistic activity were selected for further studies.

Quantification of enzyme: For preparation of mycelial powder, *Macrophomina phaseolina* was grown on potato dextrose broth for 10 d after which the mycelial mats were collected dried between two folds of blotting paper. Ten g of the mycelial mats were powdered with liquid nitrogen in pestle and mortar and suspended in 100 ml. distilled water and sterilized. The *Trichoderma* were grown on synthetic agar medium broth with 1 per cent mycelial powder of *M. phaseolina* prepared as mentioned above at pH 6.5. Twenty ml of medium was dispensed in 100 ml flasks. To each flask, 5 mm disc of 7 d old culture of *Trichoderma* was inoculated separately. After 10 d the culture was passed through Whatman filter paper No. 42. The culture filtrate, thus, collected was centrifuged at 50000 x g for 10 min. The supernatant was collected and stored in a refrigerator until use.

Chitinase assay

Preaparation of colloidal chitin: Twenty g unbleached crude chitin was successively washed for 24 hr with 1 N NaOH (400 ml) and then 1 N HCL (400 ml). The suspension was held at 4^{0}C during washing. The chitin was then rinsed with distilled water for 5 times and twice with 95 per cent ethanol. This treatment resulted into clean white flakes of chitin. Passed it through pre weighed filter paper, dried at 60^{0}C and determined the dry weight of chitin. Obtained clean chitin was moistened with acetone and dissolved in 50 ml of cold concentrated HCL by stirring for 20 min. in an ice bath, the thick syrup thus formed was filtered with suction through a thin glass wool pad in a Buchner funnel into 2 l. of stirred ice cold distilled water, precipitating the material as a fine colloidal suspension.

To determine chitinase activity, 1 ml of colloidal chitin (4 mg/ml) was added to 3 ml of 50 mM citrate buffer (pH 5.05) and incubated with 1 ml of culture filtrate of antagonists for 1 hour at 37^{0}C. After incubation the mixture was centrifuged and 0.5 ml of supernatant was taken in test tube. To it 0.1 ml of 0.8 M potassium tetraborate was added and heated vigorously in boiling water bath for 3 min. The test tubes were cooled in tap water and 3 ml of DMAB (pDimethylaminobenzaldehyde) reagent was added and absorbance was read at 544 nm. The amount of N acetyl-D-glucosamine present in 1 ml of supspension was determined by using a standard curve of N-acetyl-D-glucosamine (Reissing *et al.*, 1955).

ß-1, 3 glucanase assay: ß-1, 3 glucanase was assayed by estimating the glucose released from laminarin (Miller, 1959). The reaction mixture containing 20 g of crude enzyme was mixed in 400:l of 0.1M acetate buffer (pH 5.0) and 100 :l of 1% laminarin. The reaction was carried out at 37 ^{0}C in a water bath on shaker (100 rpm) for 6 hr interval for 24 hr. After incubation, 3ml of DNS reagent was added to the reaction mixture and boiled for 5 min in a boiling water bath followed by cooling under running tap water. Finally the absorbance was read at 640 nm in spectrophotometer and compared with the standard graph drawn by following the same procedure but using different concentration of glucose instead of culture filtrate. The quantity of reducing sugar was estimated and specific activity of ß–1, 3 glucanase was expressed as µ moles of glucose released per mg protein per hour.

ß-1, 4-endoglucanase assay: Enzymatic hydrolysis of carboxyl methyl cellulose (CMC) was assayed by dinitro salicylic acid method (Miller, 1959). The reaction mixture *i.e.* 0.5 ml of culture filtrate, 1 ml of 0.05 M citrate buffer (pH4.8), 0.5 ml of 1% CMC was incubated at 55 ^{0}C for 30 min. The reaction was stopped by boiling and the amount of reducing sugar released was estimated. The absorbance was read at 640 nm in spectrophotometer and compared with the standard graph drawn by using different concentrations of glucose instead of culture filtrate. The quantity of reducing sugar was estimated and specific activity of ß–1, 4-endoglucanase was expressed as release of µ mol glucose /ml of culture filtrate per min per mg protein.

RESULTS AND DISCUSSION

Conidiospores of *Trichoderma* isolates T_1 and T_7 treated with five ethylmethan sulfonate concentrations @ 50, 75,100,125 and 150 ìl/ml for 30 and 60 min. selected single cell colonies were again treated with 0.1 and 0.2% colchicine. On the basis of highest antagonism of 2 mother culture *i.e.* M_1 and M_7 and 13 mutants were selected for further studies and abbreviated as M_1 to M_{15} and the data for antagonism are presented in Table1. The procedure adapted supports the findings of Anonymous (1984) who observed the highest cellulose activity in local strain of *Trichoderma viride* 253 M16 mutant which was obtained after UV irradiation of parent species for 8 minutes. Oenfelt and Klasterska (1983) recorded that colchicines has a strong mutagenetic property and also known as a

polyploidy inducer in animal, plants and microbes. Mukherjee (1997) obtained 15 stable benomyl tolerant mutants of *T. viride* after exposing to N-methyl-n-nitro-N-nitrosoguanidine for 1 hr showing variation in production of antifungal metabolites.

The antagonistic ability (Table 1) revealed that all the 13 mutants were antagonist to both the pathogens *i.e. Sclerotium rolfsii* and *Macrophomina phaseolina* with 64.17-90.42% and 56.67-84.58% inhibition represents that the mother culture inhibited the pathogen to the extent of 76.67, 66.67% and 70.42, 66.25% against respective pathogens. M_{10}, M_6, M_3 and M_9 mutants inhibit the pathogen to the extent of 90.42, 90.00% and 84.58, 77.50% exhibiting highest ability against pathogens compaire to the mother cultures. The minimum efficiency was recorded by M_{14}, M_9 against *Sclerotium rolfsii*, while M_{14} and M_2 against *Macrophomina phaseolina*. It indicated that mutants had an ability to arrest the different pathogens with a varied type of efficiency as observed under present investigation. These finding supports the results of several workers *viz*. El-Bondkly, *et al*. (2010), Patil *et al*. (2009), Ashwani Tapwal *et al*. (2004) and Pan and Bhagat (2008) who observed that *Trichoderma* isolates have strong selectivity in their antagonistic potential towards a particular pathogen.

Estimation of ß-1, 3 glucanase, ß-1, 4-endoglucanase and Chitinase: All the fifteen mutants including two mother cultures produced enzymes in presence of mycelial powder of *M. phaseolina* as glucose supplimentive and varied among them to produce ß-1, 3 glucanase, ß-1, 4-endoglucanase and chitinase. It is revealed from the data (Table 2, Fig 1) that the highest chitinase activity was observed in M_{12} (3.22U) followed by M_{14} (2.80U). Minimum activity of chitinase was noticed in M_8 (0.56U). The activity of ß-1, 3 glucanase was observed in the range of 1.22 to 4.94U and the highest activity was observed in M_{11} (4.94U) followed by M_{12} (4.78U) and M_{15} (4.58U). Least activity was noticed in M_6 (1.22U) and M_2 (1.42U).

Highest ß-1, 4-endoglucanase activity was observed in M_{13} (4.09U) followed with M_{10} (4.01U) and M_6 (3.65U), whereas M_2 recorded minimum *i.e.* 0.36U followed with M_8 0.46U and M_5 0.56U, (Table 2 and Fig. 1). Vyas and Deshpande (1989) recorded high level of N-acetyl-D-glucosaminidase and chitinase production by *Myrothecium verrucaria* in a medium containing chitin as a sole carbon source. Kumar and Gupta (1999) observed the chitinase and ß–1,3 glucanase activities of *Trichoderma viride* biotypes and wild type, induced by the mycelial mats of *Macrophomina phaseolina* as carbon source substituted in the medium but did not induced the ß–1, 4-endoglucanase activities of the biotypes and wild type.

Bhagat and Pan (2008) evaluated ten isolates of *Trichoderma* spp. for their ability to produce ß–1,3 glucanase, chitinase and cellulase enzymes with different carbon sources, P^H and temperature levels

Table 1
Effect of *Trichoderma* mutants and mother cultures on radial mycelial growth of *Sclerotium rolfsii* and *Macrophomina phaseolina*

Mutants/ Mother cultures	*Sclerotium rolfsii*		*Macrophomina phaseolina*	
	Mycelial growth (mm)	*% inhibition*	*Mycelial growth (mm)*	*% inhibition*
M_1 (T_1)	26.67	66.67	23.67	70.42
M_2	14.67	81.67	32.00	60.00
M_3	18.00	77.50	12.33	84.58
M_4	18.67	76.67	18.67	76.67
M_5	20.00	75.00	34.67	56.67
M_6	8.00	90.00	22.67	71.67
M_7 (T_7)	18.67	76.67	27.00	66.25
M_8	21.00	73.75	22.33	72.08
M_9	28.67	64.17	18.00	77.50
M_{10}	7.67	90.42	19.00	76.25
M_{11}	16.00	80.00	21.00	73.75
M_{12}	16.00	80.00	19.67	75.42
M_{13}	24.00	70.00	30.00	62.50
M_{14}	28.67	64.17	33.33	58.33
M_{15}	17.00	78.75	21.00	73.75
Control	80.00		80.00	
SE (m) ±	0.76		0.88	
CD (P=0.01)	2.91		3.35	

Table 2
ß-1, 3 glucanase, ß-1, 4-endoglucanase and chitinase activities in *Trichoderma* mutants and mother cultures

Mutant/ Mother culture	*ß-1,3glucanase (µmol glucose /ml/min/mg protein)*	*ß-1,4-endoglucanase (µmolglucose/ml/ min/mg protein)*	*Chitinase (µmol N acetyl-D-glucosamine/ml/ in/mg protein)*
M_1(T_1)	2.28	1.44	1.26
M_2	1.42	0.36	0.88
M_3	1.55	3.03	0.90
M_4	3.43	1.68	1.30
M_5	3.33	0.56	1.53
M_6	1.22	3.65	1.11
M_7 (T_7)	4.13	2.01	1.22
M_8	1.98	0.46	0.56
M_9	3.24	2.75	1.15
M_{10}	3.02	4.01	2.39
M_{11}	4.94	2.20	2.54
M_{12}	4.78	3.46	3.22
M_{13}	3.66	4.09	2.22
M_{14}	4.49	1.48	2.80
M_{15}	4.58	2.64	2.03
SE (m)±	0.05	0.33	N.S.
CD (P = 0.01)	0.18	1.24	

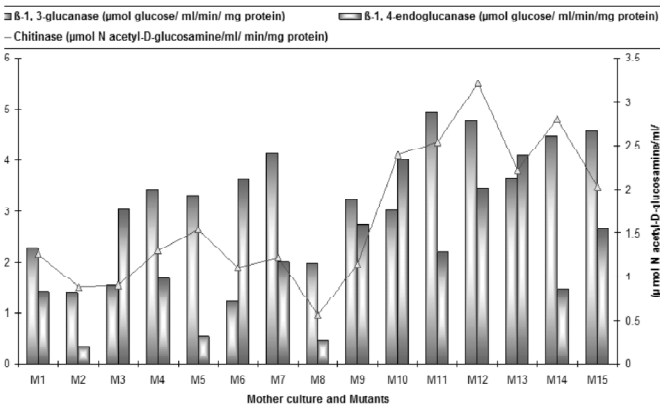

Fig. 10. ß-1, 3-glucanase, ß-1, 4-endoglucanase and chitinase activities in *Trichoderma* mutants and mother culture

and all the enzymes showed highest activity at 30°C, 3.0%, 4.0% and 0.75% glucan, chitin and cellulose as carbon source for respective enzymes. ß–1,3 glucanase was produced in media at P^H 5.5 and chitinase and cellulase at pH 5.0.

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