



Induction of mutant strains of *Trichoderma viride* 1433 for biocontrol of *Sclerotium rolfsii* Sacc. causing southern stem blight disease of Soybean (*Glycine max* (L.) Merrill)

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ABSTRACT

Twenty two mutants of Trichoderma viride 1433 were obtained after treatment with NTG which were further screened against Sclerotium rolfsii Sacc. by dual culture method. All the mutants indicated high sporulation count and better growth patterns as compared to wild type but only four mutants showed enhanced antagonistic activity against Sclerotium rolfsii in colony plate assay as compared with the wild type and control. Since mycoparasitism plays important role in antagonism mechanism of Trichoderma species, extracellular enzymatic activity of mutant strains was also assayed. The result of extracellular enzymatic activity showed that cellulase and β 1, 3-glucanase significantly increased in the mutant strains while protease activity was found suppressed in case of two mutant strains as compared to wild type. Among the mutant strains Tv m9 was found as most potent strain against Sclerotium rolfsii, therefore, this strain can be studied in glasshouse experiment against the above pathogen.

KEW WORDS: *Trichoderma viride* 1433, mutant strains, *Sclerotium rolfsii* Sacc.

INTRODUCTION

The pathogen *Sclerotium rolfsii* Sacc. is an important stem (sudden wilting or flagging on one or more shoots) disease pathogen and responsible for southern stem blight of Soybean (*Glycine max* (L.) Merrill). The pathogen is responsible for causing destructive diseases of many economically important crops including vegetables and field crops (Singh, 1974; Khan



and Kolte 1989). A wide host range of the pathogen has been recorded covering more than 500 species of cultivated and wild plants in tropical, subtropical and warm regions of the world (Aycock, 1966; Punja, 1985). The pathogen survives as clusters of brown, round sclerotia, which are about the size of mustard seeds, each with a diameter of 0.8-2.5 mm are often scattered on the surface of the soil or on the lower stem or collar of the dying plant. The sclerotia serve as the source of primary inoculum and usually, produced the superficial, white, fan-like fungal growth mat on the soil surface around the base or lower stem of the wilted plants as well as on fallen leaves and other organic debris.

The most common means to check the disease in plants is by using fungicides. Frequent use of these chemicals leads to environmental pollution. The increasing awareness of fungicide-related hazards has emphasized the need of adopting biological methods as an alternative disease control method. Species of the genus *Trichoderma* are well documented fungal biocontrol agents (Lewis and Papavizas, 1991; Haran *et al.*, 1996a; Haran *et al.*, 1996b; Elad, 2000; Howell, 2002; Joshi *et al.*, 2010; Hermosa *et al.*, 2012, Martinez-Medina *et al.*, 2016; Bastakoti *et al.*, 2017; Hyder *et al.*, 2017; Sridharan *et al.*, 2020). Extracellular enzymes produced by *Trichoderma* species are considered important determinants of the antagonistic ability of these fungi (Thrane *et al.*, 2000). The induction of chitinase, β -1, 3 glucanase and increase in total phenol content was also observed, suggesting their role in growth inhibition of pathogen during antagonism (Gajera *et al.*, 2012). Although wild strains of various *Trichoderma* spp. have shown considerable effect in the biocontrol of plant diseases, efforts have been made in the last few decades to develop more effective strains through mutagenesis (Kredics *et al.*, 2003; Papavizas *et al.*, 1982; Mandels *et al.*, 1971; Zaldivaret *et al.*, 2001). In view of the above, the present study was carried out to develop potent mutant strains of *Trichoderma viride* 1433 for enhanced antagonism against *Sclerotium rolfsii*.



MATERIALS AND METHODS

Fungal Isolates

The pure culture of *Trichoderma viride* 1433 was obtained from the culture collection of Institute of Microbial Technology (IMTECH), Chandigarh. A virulent strain of *Sclerotium rolfsii* was obtained from the Department of Mycology and Plant Pathology, Institute of Agriculture Science, Banaras Hindu University (BHU), Varanasi. The pathogenic and antagonistic strains were maintained on Potato Dextrose Agar medium (PDA; Merk) at $25 \pm 2^{\circ}\text{C}$ by regular subculturing.

Induction of mutant through N'-methyl-N'-nitro-N'-nitrosoguanidine (NTG) treatment

Mutagenesis of *T. viride* 1433 by the treatment with N'-methyl-N'-nitro-N'-nitrosoguanidine (NTG) was followed by the method of Chadegani and Ahmajian (1991) with some modifications. Conidial suspension of four-day old culture of *T. viride* 1433 was prepared in 5.0 ml sterile 0.1M sodium citrate buffer (pH 5.6), centrifuged twice at 10,000 rpm and subsequently washed with the same buffer. The spore concentration was adjusted to 1×10^5 spores/ml in sodium citrate buffer. A stock solution of NTG (1mg/ml) was prepared in sodium citrate buffer and the final concentration used was 50 $\mu\text{g/ml}$ of spore suspension. The NTG treated spore suspensions were incubated at 37°C in a shaking water bath in cool light for 10-90 min in order to achieve 5-10% viability. At selected intervals mutagenesis were stopped by passing entire sample through a 0.45 μm Millipore filter, washing the spores with 0.1M phosphate buffer (pH 6.0). The few pinhead colonies of treated spores that developed were picked-up and inoculated on minimal medium for colony forming units.

Selection of Mutants

The mutants obtained after the treatment with NTG was selected on PDA medium on the basis of colony growth, screening against *Sclerotium rolfsii* and sporulation count. These



mutants were compared with the parental isolate for the next seven to eight generations. About twenty two mutants showed increased growth rate and high sporulation count in comparison to wild type but only four mutants showed enhanced antagonistic activity against *Sclerotium rolfsii* in colony plate assay as compared with the wild type and control. The stable desired colonies of mutant *Trichoderma* strains were transferred on PDA slants and maintained at 25⁰C.

Colony growth inhibition assay

In *vitro* antagonistic activity of wild type and mutant strains of *Trichoderma viride* 1433 against *Sclerotium rolfsii* was studied in dual culture by followed the method described by Upadhyay and Rai (1987). The colony interactions were assayed as percent inhibition of the radial growth by the following formula (Fokkema, 1976); $R1 - R2 / R1 \times 100$, where, R1 denotes diameter of the radial growth of the pathogen towards opposite side and R2 denotes the radial growth of the pathogen towards the antagonist. The experiment was conducted in three replicates.

Comparative sporulation count of wild type and selected mutant strains

The estimation of spore production of the wild type and mutant strains of *T. viride* 1433 was done using the method described by El-Abyad *et al.* (1983). After incubating the inoculated Petri dishes at 25 ± 2°C for 7 days, one square cm block which was cut from the margin of the actively growing colony of isolates of wild type and mutant strains of *T. viride* 1433 was transferred to a test tube containing 2.5 ml of sterile distilled water. The tube was shaken continuously for 5 minutes. The density of spores was then counted using a haemocytometer under high power (×450) in a Nikon Trinocular Microscope (Model E-600).

Assay of enzyme activity of wild type and mutant strains of *T. viride* 1433

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) contained the following components (in grams per litre); MgSO₄.7H₂O, 0.2;



KH_2PO_4 , 0.9; KCl, 0.2; NH_4NO_3 , 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; MnSO_4 , 0.002 and ZnSO_4 , 0.002.

The medium was supplemented with the appropriate carbon source (Cellulase assay – Cellulose; 20g, β -1, 3 glucanase assay – Glucose; 20g, Protease assay – Peptone, 10g, Glucose; 20g). The pH was set to 6.3 with 50 ml phosphate buffer and autoclaved at 15 psi for 20 minutes. The medium was inoculated with a spore suspension to give a final concentration of 5×10^6 conidia per millilitre and placed on a rotator shaker at 150 rpm at 25°C for different time intervals. The cultures were harvested at fourth day of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4°C to get cell-free culture filtrate which were then used as enzyme source.

Cellulase Assay

Cellulase activity was assayed following the method of Miller (1959). The assay mixture contained 1 ml of 0.5% pure cellulose (Sigma Company) suspended in 50 mM phosphate buffer (pH 5.0) and 1 ml of culture filtrates of different *Trichoderma* strains. The reaction mixture was incubated for 30 min at 50°C. The blanks were made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit cellulase activity is defined as the amount of enzyme that catalyzed 1.0 mol of glucose per minute during the hydrolysis reaction.

β - 1, 3 Glucanase Assay

β - 1, 3 glucanase was assayed similarly by incubating 1ml 0.2% laminarin (w/v) in 50mM sodium acetate buffer (pH 4.8) with 1ml enzyme solution at 50°C for 1h and by determining the reducing sugars with DNS (Nelson, 1944). The amount of reducing sugars released was calculated from standard curve for glucose. One unit of β -1, 3 glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 μ mol of glucose equivalents per minute.



Protease Assay

Protease activity was determined by a modified Anson's Method (Yang and Huang, 1994). The substrate used (1% casein in 50mM phosphate buffer, pH 7.0) was denatured at 100°C for 15 min in a water bath and cooled at room temperature. The reaction mixture containing 1ml of substrate and 1ml of enzyme solution were incubated at 37°C for 20 min with shaking and was stopped with 3ml of 10% trichloroacetic acid (TCA). The absorbance of the liberated tyrosine in the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 280nm equivalent to 1µmol of tyrosine in one minute under the assay condition.

RESULTS

Generation of mutants through NTG treatments

The mutants of *Trichoderma viride* 1433 were generated to enhance biocontrol efficacy of antagonism against *Sclerotium rolfsii*. Treatment of spores with NTG was much stable and generated the isolates which had high significant levels of production in spores, colony growth antagonistic ability. Finally, four isolates were obtained after a series of 7 to 8 serial transfer on PDA media. Twenty two mutants that were obtained after treatment of NTG with better colony growth and higher sporulation count were given the name as Tv m1, Tv m2, Tv m3, Tv m4.....Tv m22.

In vitro screening of the wild type and mutant strains against *Sclerotium rolfsii*

All the four mutants showed significantly ($P < 0.05$) enhanced antagonistic activity against *Sclerotium rolfsii*. In comparison to wild type (Table 1). The mutant Tv m9 exhibited maximum inhibition of the pathogen (92.8%) which was followed by Tv m13 (84.8%), Tv m21 (77.8%) and Tv m6 (76.9%). The least inhibition in the growth of the pathogen was depicted by the wild type (73.0%).



Phenotype variation and sporulating ability of wild type and mutant strains of *T. viride* 1433

Table 2 reveals the phenotypic variation and sporulating ability of the wild type and mutant strains of *T. viride* 1433. Pigmentation on reverse side of the culture medium was recorded in case of wild type and two mutant strains namely Tv m9 and Tv m13. The other mutant strains did not show any pigmentation in the reverse. Maximum spores (364.7×10^5) were produced in case of the mutant Tv m9. This was followed by mutant Tv m13 (307.8×10^5), Tv m21 (285.4×10^5) and Tv m6 (263.4×10^5). The least number of spores (256.6×10^5) was produced by wild type. It is clear from the table that there is a great degree of variation among the isolates in their sporulating ability.

Extracellular enzymatic activities of the wild type and mutant strains of *T. viride* 1433

The results of extracellular enzymatic activities of the wild type and mutant strains of *T. viride* 1433 have been shown in Table 3. The highest cellulase activity was produced by the mutant Tv m9 (3.5U/ml) followed by Tv m13 (2.6U/ml), Tv m21 (2.3U/ml) and Tv m6 (1.9U/ml). The rate of enzyme production in case of wild type was recorded minimum (1.3U/ml) as compared to other mutant strains.

All the mutants significantly produced the higher β - 1, 3 glucanase activity than the wild type. The maximum enzymatic activity was produced by Tv m9 (44.8 μ g/ml) followed by Tv m13 (40.8 μ g/ml), Tv m21 (36.8 μ g/ml) and Tv m6 (34.6 μ g/ml). The least activity of β - 1, 3 glucanase (33.2 μ g/ml) was measured in case of wild type.

The maximum protease activity was produced by the mutant Tv m13 (363U/ml) followed by Tv m9 (346U/ml). The other mutant strains Tv m21 (309U/ml) and Tv m6 (294U/ml) showed lower enzymatic activity as compared to the wild type (321U/ml).



DISCUSSION

Development of *Trichoderma* mutants toward suppression of fungal plant pathogen is an important method in strain improvement, which yields effective and reliable strains for biological control. After development of mutants, assessing the efficacy through various techniques is equally important for the suppression of the pathogen (Nakkeeran *et al.*, 2005). Four mutants (designated as Tv m6, Tv m9, Tv m13 and Tv m21) were obtained that grew faster and sporulated better as compared to the wild type. These strains were generated as a result of mutation and not by selection due to fact that the mutant isolates were capable of growing and sporulating better even after 7 to 8 generations.

The result of the dual culture (Table 1) revealed that the highest mean inhibition values, above 90% were obtained due to Tv m9 followed by Tv m13. Antagonism of *Trichoderma* species against several pathogens has been reported (Chet and Baker, 1980; Papavizas, 1985; Elade, 2000; Howell, 2002). Formation of inhibition zone at the contact between *Trichoderma* and *Sclerotium rolfsii* in dual cultures could be explained on the basis of production of volatile and non volatile metabolites as well as the production of extracellular enzymes by *Trichoderma* (El-Katatny, *et al.*, 2001; Sridharan *et al.*, 2020). In the present study mycoparasitic activity by coiling of hyphae was observed. The mycoparasitic activity of the *Trichoderma* species might be as a result of production of cell wall degrading enzymes (Di Pietro, *et al.*, 1993; Schirmbock, *et al.*, 1994; Gajera *et al.*, 2012).

The extracellular enzymes play a vital role in mycoparasitism and degrade the pathogen cell wall. The mycolytic activity of *Trichoderma* species is an important mechanism proposed to explain their antagonistic activity against soil-borne plant pathogenic fungi (Ridout, *et al.*, 1986). The results (Table 3) showed that Tv m9 was efficient producer of extracellular enzymes such as cellulases, β -1, 3 glucanases and proteases. This might be one of the reasons for its biocontrol potentially. Hyper cellulase producing mutants were also



developed by Hao, *et al.*, (2006). The production of extracellular enzymes such as chitinases, β -1, 3 glucanases and proteases by *Trichoderma* species to degrade phytopathogen cell wall has been established (Elad, *et al.*, 1982; Haran, *et al.*, 1996a; Hjeljord and Tronsmo, 1998). As the cell wall of *Fusarium* species are composed of cellulose and β -1, 3 glucan (Bartinicki-Garcia, 1968), the enzymes, cellulase and β -1, 3 glucanases produced by *Trichoderma* might be involved in hydrolysis of *Sclerotium rolfii* cell wall during antagonism (Thrane, *et al.*, 1997). The involvement of glucanases in mycoparasitism has been demonstrated by Lorito *et al.*, (1994). Fungal cell wall also contains lipids and proteins (Hunsley and Burnett, 1970), therefore, antagonistic fungi synthesized proteases which might act on the host cell wall. The role of proteases in mycoparasitism has been reinforced with the isolation of new protease-overproducing strains of *T. harzianum* (Szekeres, *et al.*, 2004). Although, Tv m9 produced lesser protease than Tv m13 (Table 3), the biocontrol potential of Tv m9 is maximum. The result is consistent with Rey, *et al.*, (2001) who reported that *T. harzianum* mutant with low levels of protease activity was best biocontrol agent rather than the transformants with highest protease level. The reason was attributed to the high amounts of protein provoking toxicity in the transformants (Flores, *et al.*, 1997). Papavizas (1985) correlated the lytic activity of *Trichoderma* on cell walls of phytopathogenic fungi to the degree of biological control of these pathogens *in vivo*. These enzymes lysed the host cell wall and leads to leakage of protoplasmic contents which are in turn used as food material for the proliferation of antagonists (Tronsmo and Harman, 1993).

In conclusion, mutant Tv m9 was more effective biocontrol agents as compared to the other strain. Tv m9 produced higher extracellular enzymatic activities and possessed high competitive saprophytic ability (CSA). Therefore, the mutant Tv m9 may be a promising biocontrol agent against *Sclerotium rolfii*.



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Table 1. In vitro screening of the wild type and mutant strains of *T. viride* 1433 against *Sclerotium rolfsii* Sacc.

<i>Trichoderma strains</i>	<i>Per cent Inhibition</i> <i>(Sclerotium rolfsii Sacc.)</i>
<i>Tv m6</i>	76.9
<i>Tv m9</i>	92.8
<i>Tv m13</i>	84.8
<i>Tv m21</i>	77.8
<i>Tv 1433</i>	73.0

- Average of three replicates

Table 2. Phenotypic variation and sporulating ability of the parent and mutant strains of *T. viride* 1433

<i>Strains</i>	<i>Colony Morphology</i>	<i>Pigmentation</i>	<i>10⁵ spores per ml</i>
<i>Tv m6</i>	<i>Colony-green, compact mycelium. Sporulation is dark green at the centre and light green towards periphery and white coloured conidiogenous pustules appear in a scattered manner</i>	<i>None</i>	<i>263.4</i>
<i>Tv m9</i>	<i>Colony-dark green, highly ramified, compact tufted mycelium with dark green conidiogenous pustules</i>	<i>Yellow</i>	<i>364.7</i>
<i>Tv m13</i>	<i>Colony-dark green, mycelium is sparsed with white and pinhead like conidiogenous pustules at the centre</i>	<i>Yellow</i>	<i>307.8</i>



	<i>and periphery</i>		
<i>Tv m21</i>	<i>Colony light-green, mycelium is sparsed with dark green coloured conidiogenous fascicles</i>	<i>None</i>	<i>285.4</i>
<i>Tv 1433</i>	<i>Colony-light green, dense compact mycelium with dark green conidia</i>	<i>Yellow</i>	<i>256.6</i>

- Values are average of three replicates

Table 3. Assay of extracellular enzyme activities of the wild type and mutant strains of *T. viride* 1433

<i>Trichoderma strains</i>	<i>Cellulose activity (U/ml)</i>	<i>β- 1, 3 glucanase activity (μg/ml)</i>	<i>Protease activity (U/ml)</i>
<i>Tv m6</i>	<i>1.9ab±0.1</i>	<i>34.6b±0.3</i>	<i>294a±3.1</i>
<i>Tv m9</i>	<i>3.5d±0.2</i>	<i>44.8e±0.2</i>	<i>346d±3.7</i>
<i>Tv m13</i>	<i>2.6cd±0.3</i>	<i>40.8d±0.3</i>	<i>363e±2.4</i>
<i>Tv m21</i>	<i>2.3bc±0.2</i>	<i>36.8c±0.2</i>	<i>309b±2.0</i>
<i>Tv m1433</i>	<i>1.3a±0.1</i>	<i>33.2a±0.1</i>	<i>321c±2.9</i>

- Average of three replicates
 - Means in the column followed by same letter are not significantly different (P=0.05)