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Generation of PCNB resistant mutant of *Trichoderma harzianum* 4572 to enhance enzymatic production and biocontrol efficacy against *Sclerotium rolfsii* Sacc.

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The purpose of this study was to assess the biocontrol efficacy of the PCNB resistant mutant strains of *Trichoderma* species, extracellular enzymatic activity of mutant strains was also assayed. Twenty one mutants of *Trichoderma harzianum* 4572 were obtained after treatment with NTG and selection on PCNB amended medium, which were further screened agaisnt *Sclerotium rolfsii* by dual culture method. All the mutants indicated equally high tolerant capability of PCNB but only three mutants were shown enchanced antagonistic activity against *S. rolfsii* in colony plate assay as compared with the wild type and control. The result of extracellular enzymatic activity showed that chitinase and β 1, 3-glucanase significantly increased in the mutant strains as compared to wild type while protease activity was found suppressed in all the mutant strains. Among the mutant strains, Th mu6 was found as most potent strain against *S. rolfsii* causing Southern stem blight disease in soybean and therefore, this strain can be studied in glasshouse and field experiment to control the above disease.

Key words: *Trichoderma harzianum* 4572, mutant strains, extracellular enzymes, disease control., *Sclerotium rolfsii*

INTRODUCTION

Sclerotium rolfsii Sacc. is a ubiquitous soil-borne pathogen, 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). Control of the fungus is difficult as it does not produce asexual spores and overwinters as sclerotia on plant debris and in soil (Punja, 1988). Various methods of control have been investigated including genetic control (Branch and Csions, 1987; Smith *et al.,* 1989; Brenneman *et al.,* 1990; Besler *et.al.,* 1997), chemical control (Hagan *et al.,* 1988; Bowen *et al.,* 1992; Culbreath *et al.,* 1995), cultural practices (Gurkin and Jenkins, 1985) and biological control (Henis *et al.,* 1983; Elad *et al,* 1984; Benhamou and Chet, 1996), particularly with *Trichoderma* and *Gliocladium* species (Lewis and Papavizas, 1991; Haran *et al.,* 1996a; Haran *et al.,* 1996b; Elad, 2000; Hermosa *et al.,* 2000). There are several mechanisms involved in antagonism of *Trichoderma* species namely antibiosis, substrate competition, and mycoparasitism (Haran *et al.,* 1996a). Introduction of *T. viride* and *T. harzianum* significantly minimized the loss due to *Sclerotium rolfsii* on tomato, beans, cotton, potato and other vegetables in field experiment (Singh, 2001).

The soil-borne diseases of corps incited by species of *Sclerotium, Rhizoctonia* and *Fusarium* are sometimes difficult to be managed through one method of approach such as cultural practices or fungitoxicants or host plant resistance or bio-agents. The integration of chemical sublethal doses with some antagonistic fungi, such as *Trichoderma* spp., which are resistant to relatively high doses of chemicals, is one of the most attractive ways to enhance the antagonistic activity of antagonists and reducing the amount of fungicides (Chet. 1987; Khattabi *et al., 2001*; Upadhyay and Rai, 1988). Several biofungicides based on *Trichoderma* spp. have been commercialized in last few years. However, there is still considerable interest in finding more efficient mycoparistic fungi, especially within *Trichoderma* species, which differ considerably with respect to their biocontrol effectiveness (Elad *et al.,*

1982). Most of the work has been carried out on strains of *T. viride., T. virens* and *T. harzianum.* These strains have been extensively studied for their ability to produce extracellular enzymes such as chitinase. β-1-3 glucanase, and protease (De la Cruz *et al.,* 1992; Haran *et al.,* 1996b; Pitson *et al.,* 1993; Sivan and Chet, 1989; Flores *et al.,* 1997). The strains have been mutagenized and genetically modified to obtain an organism capable to improve production of antifungal and antagonistic potential of biocontrol agents to control a broad spectrum of phytopathogens (Rey *et al.,* 2001) and also for producting high levels of enzymes (Mandels and Andeotti, 1978; Szengyel *et al.,* 2000). However, despite the effort of many laboratories, no commercially efficient enzyme complex has been reported.

In view of the above facts, an attempt was made to generate a potent mutant of *T. harzianum* with increased tolerance capability to PCNB which could be explored in natural conditions either alone or integrating with the fungicide for better control of plant disease.

MATERIALS AND METHODS

The experiment was conducted in the Laboratory of Applied Mycology and Plant Pathology, Department of Botany, Banaras Hindu University, Varanasi.

Fungal Isolates

The pure culture of *Trichoderma harzianum* 4572 was obtained from the cultue collection of Institute of Microbial Technology (IMTECH), Chandigarh. A virulent strain of *Sclerotium rolfsii* (Sacc.) was obtained from the Department of Mycology and Plant Pathology, Institute of Agriculture Science, Banaras Hindu University (BHU), Varnasi. The pathogenic and antagonistic strains was maintained on Potato-Dextrose Agar medium (PDA; Merk) at 25±2°C by regular subculturings.

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

Mutagenesis of *T. harzianum* 4572 by the treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was followed by the method of Chadegani and Ahmadjian (1991) with some modifications. Conidial suspension of four days old culture of *T. harzianum* 4572 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 (1 mg/ml) was prepared in sodium citrate buffer and the final concentration used was 50 µg/ml of spore suspenstion. 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 0.1 M 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 sensitivity of wild-type cultures to PCNB was tested by amending the culture medium with increasing concentrations of the fungicide and incubating the inoculated plates at 28°C. Mycelial growth was marked after 24 hr and growth was measured following additional incubation. Once the initial sensitivity of the wild-type isolates was determined, the treated spores (0.1 ml) were spread onto PDA medium amended with 100 ppm PCNB that inhibited approximately 90% of the linear growth and was incubated at 28°C for 2-3 days. Cultures were checked daily for growth and the presence of fast-growing sectors, which were transferred to medium amended with a slightly higher concentration of the fungicide (initial increments of 20 to 25 ppm). If isolates grew well following several serial transrfers on PDA with increasing concentrations of fungicide, they were evaluated for stability of fungicide tolerance. Serial transfer to medium with increasing concentrations of the fungicide was continued until isolates were derived that were tolerant to fungicide concentrations.

These mutants were compared with the parental isolate for fungicidal tolerance and antagonistic activity against *S. rolfsii.* The stable desired colonies of mutant *Trichoderma* strains were transferred on PDA slants and maintained at 25°C.

Colony growth inhibition assay

In vitro antagonistic activity of wild type and mutant strains of *Trichoderma harzianum* 4572 against *S. rolfsii* was studied in dual culture by following the method described by Upadhyay and Rai (1987). The colony interactions were assayed as per cent inhibition of the radial growth by the following formula (Fokkema, 1976): R1 – R2/R1 × 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 opponent antagonist. The experiment was conducted in three replicates.

Inhibition of the sclerotial germination of S. rolfsii by culture filtrates of wild type and mutant strains of Trichoderma harzianum 4572

Two ml of filter sterilized cell free culture filtrates of wild type and mutant strains of *T. harzianum* 4572 were poured into the Petri dishes containing sterilized filter paper. Twenty sclerotia of *S. rolfsii* were put in each Petri dishes on the filter paper and incubated at 25 ± 2°C for 48 hrs. Control was set by pouring distilled water in the same volume in place of the culture filtrate. Per cent inhibition of sclerotial germination was calculated by using the formula : Per cent growth inhibition = C –T/C × 100, where C = number of sclerotial germination in control; T = number of sclerotial germination in the treatment.

Assay of enzyme activity of parent and mutant strains of Trichoderma harzianum

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) contained the following components (in grams per liter) : $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4$ $7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source (Chitinase assay–Colloidal chitin : 5 g; β-1,3 glucanase assay-Glucose; 20 g; Protease assay- Peptone; 10 g, Glucose: 20 g). The pH was set to 6.3 with 50 mM phosphate buffer and autoclaved at 15 psi for 20 min. The medium was inoculated with a spore suspension to give a final concentration of –5 × 10^6 conidia per milliliter and placed on a rotary shaker at 150 rpm at 25°C for different time intervals. The cultures were harvested at 1, 2, 4, 6, 7, 9 and 10 days respectively, and the culture filtrates 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.

Chitinase Assay

Chitinolytic activity was assayed by measuring the release of free N-acetylglucosamine (NAGA) from colloidal chitin following the method of Miller (1959). The amount of reducing sugar released was calculated from standard curves for GlcNAc and the activity of chitinase (A582, NAGA- U.h-1. ml-1) was defined as quantity of NAGA units released by 1 ml of enzyme solution per hour under the assay condition.

Glucanase Assay

β-1, 3-glucanase was assayed similarly by incubating 1 ml 0.2% laminarin (w/v) in 50mM sodium acetate buffer (pH = 4.8) with 1 ml enzyme solution at 50°C for 1 hr 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 μmol of glucose equivalents per min.

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 1 ml of substrate and 1 ml of enzyme solution were incubated at 37°C for 20 min with shaking and was stopped with 3 ml 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 280 nm equivalent to 1 μmol of tyrosine in one minute under the assay condition.

RESULTS

Generation of mutants through NTG treatments

The mutants of *Trichoderma harzianum* 4572 were generated to enhance the PCNB tolerant capability and efficacy of antagonism against *S. rolfsii.* Liner

growth of wild-type isolate was inhibited by approximately 95% ppm of PCNB in the medium. Treatment of spores with NTG was much stable and generated the isolates which had significant levels of tolerance to PCNB. Finally, three isolates were obtained after a series of 11 to 13 serial transfers on media with increasing concentrations of fungicide upto 200 ppm. While selecting mutants tolerant to PCNB, the starting fungicide concentration was 45 ppm, which was increased initially by 10 ppm and later by as much as 20 to 25 ppm over the previous concentration with each successive transfer. Tolerant isolates were capable of growing at fungicide concentrations lethal to wild-type isolates and were stable following culture on fungicide non-amended medium. Tolerance was found to be unaltered when isolates were retrieved from long-term storage cultures in the absence of the fungicide (PDA slants covered with paraffin oil).

Twenty one mutants that were obtained after treatment of NTG with higher tolerance capability of PCNB were given the name as Th mu1, Th mu2, Th mu3, ...Th mu21.

In vitro screening of wild type and mutant strains of Trichoderma harzianum 4572 against S. rolfsil

Different growth inhibitions of *S. rolfsii* by the wild type and mutant strains of *T. harzianum* 4572 were observed (Table 1). Maximum inhibition in radial growth of *S. rolfsii* was showed by the mutant Th mu6 (98.2%). The other mutant strains, except the mutant Th mu11 and Th mu19, either inefficient or showed lower antagonistic activity against *S. rolfsii* as compared to the control. The mutants, Th mu19 (90.6%) and Th mu11 (88.8%) showed enhanced antagonistic activity against *S. rolfsii* in comparison to the wild type (76.0%) but there was no significant difference (P = 0.05) between the two.

Table 1 : Screening of wild type and mutant strains of *Trichoderma harzianum* 4572 against *S. rolfsii* by dual culture method.

Trichoderma strains	Per cent inhibition*
Wild type	76.0±0.83
Th mu6	98.2±0.30
Th mu11	88.8±0.26
Th mu19	90.6±0.41

* Average of three replicates; ± SEM

Based on the above *in vitro* screening of antagonists against *S. rolfsii,* three most effective mutant strains, Th mu6, Th mu11 and Th mu19, were selected for further studies. The wild type strain was also used in the experiment for comparison.

Inhibition of sclerotial germination of S. rolfsii by culture filtrates of parent and mutant strains of Trichoderma harzianum 4572

The inhibitory effect of culture filtrates of the wild type and mutant strains of *T. harzianum* 4572 is presented in Table 2. As compared to the parent strain, all the tested mutants showed enhanced capability to reduce the sclerotial germination of *S. rolfsii.* Maximum inhibition was observed by the culture filtrate of Th mu6 (94.2%) followed by Th mu19 (86.6%). The wild type strain could only inhibit 70.6% of the sclerotial germination of *S. rolfsii.*

Table 2 : Effect of culture filtrates of the wild type and mutant strains of *Trichoderma harzianum* 4572 on per cent inhibition of sclerotial germination of *S. rolfsii*

Trichoderma strains	Per cent inhibition*
Wild type	70.6±0.05
Th mu6	94.2±0.25
Th mu11	80.8±0.20
Th mu19	86.6±0.52

* Average of three replicates; ± SEM

Phentypic variations and sporulating ability of parent and mutant strains of T. harzianum

The phenotypic variation and sporulating abilities of the wild type and mutant strains of *T. harzianum* 4572 is presented in Table 3. Pigmentation on the culture medium was recorded in case of wild type and two mutant strains Th mu6 and Th mu19. The other mutant strain has not shown any kind of pigmentation. Maximum spores (364.7×10^5) were counted in the case of mutant Th mu6. This was followed by the mutants Th mu19 (310.8×10^5) and Th mu11 (267.6×10^5). The wild type strain produced only 228.4×10^5 spores per milliliter. It is clear from the table that there is great degree of variation among the isolates in their sporulating ability.

Table 3 : Phenotypic variation and sporulating ability of the wild type and mutant strains of *T. harzianum* 4572

Strains	Colony morphology	Pigmentation	10^5 spores/ml*
Wild type	Colony-light green, dense compact mycelium with dark green conidia	Yellow	228.4
Th mu6	Colony-dark green, highly ramified, compact tufted mycelium with dark green condiogenous pustules	Yellow	364.7
Th mu11	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	None	267.6
Th mu19	Colony-dark green, mycelium is sparsed with white and pinhead like conidiogenous pustules at the centre and periphery	Yellow	310.8

* Values are average of three replicates

Enzymatic assay of mutants and wild type strain of T. harzianum

Chitinase assay

The mutant Th mu6 produced highest amount of chitinase as compared to wild type and other mutant strains at all sampling days. Maximum chitinase activity was measured at 7 day in case of all the antagonists and thereafter it gradually decreased. The rate of enzyme production in wild type was recorded minimum as compared to the other mutant strains (Fig 2).

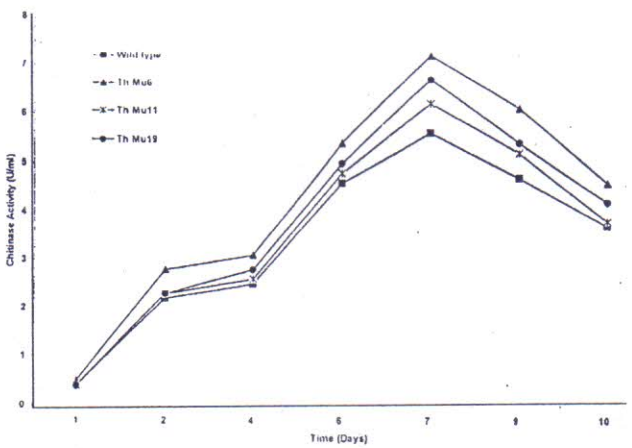

Fig. 1. Assay of chitinase activity of the parent and mutant strains of *Trichoderma harzianum* 4572 at different intervals (days)

β 1, 3-gulcanase assay

Maximum β 1, 3-glucanase activity was recorded after 4-day of inoculation of spores in case of all mutant and wild type strains (Fig. 3). The mutant Th mu6 produced highest amount of β 1, 3-glucanase (65.5 U/ml) followed by Th mu19 (54.6 U/ml) and Th mu11 (48.0 U/ml). Minimum enzymatic activity was recorded by wild type strain (44.9 U/ml) in all the sampling days.

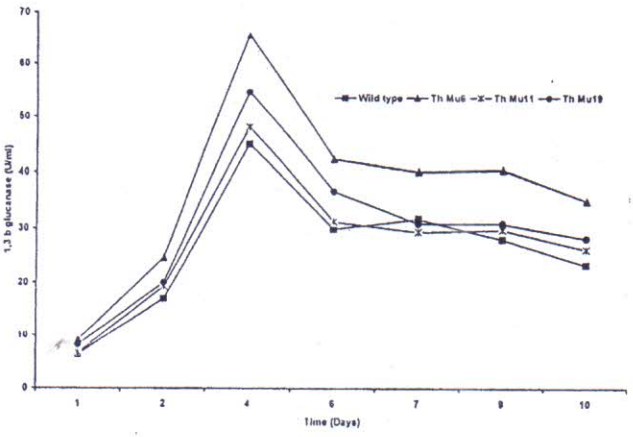

Fig. 2. Assay of *b-1, 3 glucauase* activity of the parent and mutant strains of *Trichoderma harzianum* 4572 at different intervals (days)

Protease assay

The highest protease activity was recorded in case

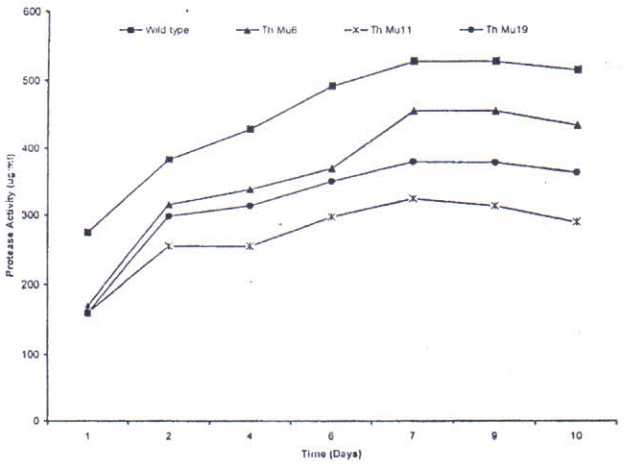

Fig. 3. Assay of protease activity of the parent and mutant strains of *Trichoderma harziamum* 4572 at different intervals (days)

of wild type strain (528 µg/ml) as compared to the mutant strains. Maximum protease activity was shown by all the antagonists at 9-day. The enzymatic activity for all mutants was almost constant between 7 to 9 days and thereafter it gradually decreased (Fig. 4). Amongst the mutants. Th mu11 produced highest amount of protease (485 µg/ml) followed by Th mu19 (456 µg/ml) and Th mu6 (404 µg/ml).

DISCUSSION

Development of *Trichoderma* mutants towards suppression of fungal plant pathogens is an important method in strain improvement, which yields effective and reliable strains for biological control. After development of mutants, assessing the bio-efficacy through various techniques is equally important for the suppression of the pathogen. In the present study, the *Trichoderma harzianum* 4572 was treated with mutagenic chemical NTG for improvement of its biocontrol efficacy and also for the tolerance of the fungicide PCNB at higher concentrations because an effective biocontrol agent required to be resistant to fungicides. PCNB tolerant mutants were desired for their integration with the fungicide to control the Southern stem blight of soybean in the glasshouse more effectively. After screening of several isolates appeared on the PCNB amended medium, finally three mutants were selected that were not only capable to tolerate 200 ppm concentration of PCNB but also grew and sporulated faster.

In the present study, twenty one different mutants of *T. harzianum* 4572 were screened for their higher tolerance capability to PCNB and Effective antagonism towards Southern stem rot of soybean caused by *S. rolfsii.* Based on the present sceening strategy, Th mu6 and Th mu11 and Th mu19 were grouped as effective strains because it resisted the PCNB upto 200 ppm concentration and also showed significantly highter antagonism towards *S. rolfsii* as compared to wild type. In most of the ineffective mutants and pathogen interaction, the pathogen was found to overgrow on the mutants.

The culture filtrate of mutant strains significantly reduced the selerotial germination of the *S. rolfsii* in comparison to wild type and control. The variation among the strains in reduction of selerotial germination might be due to varied concentrations of the metabolites, quality and quantity of the inhibitory substances present in the metabolites (Denis and Webster, 1971a, b; Skidmore, 1976; Upadhyay and Rai, 1987) and alteration in pH of the medium due to staling growth substances produced by the individual microorganisms (Bier, 1966; Bhatta and Vaughan, 1962). The same kind of result was also reported by Karthikeyan *et al.* (2006) with the culture filtrate of *T. viride 1* which inhibited the growth as well as sclerotial germinations of *S. rolfsii* to a greater extent than other isolates of *Trichoderma* species.

Isolation of mutants showing a small increase in several extracellular enzymes is a promising approach to improve biocontrol agents (Rey *et al.,* 2001). The production of extracellular β-1,3-glucanases, chitinases, and proteinase of the mutant and wild type strains was studied because the fungal cell wall is mainly composed of chitin, β-1, 3-glucan and protein (Peberdy, 1990) and hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens (Chet and Baker, 1981). Carsolio *et al.* (1999) isolated and characterized endochitinase enzymes from culture of *T. harzianum* and found that the enzyme performed *in vitro* antifungal activity against phytopathogens. Production of extracellular enzymes increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved

mycelium or isolated purified host fungal cell walls (Geremia *et al.,* 1993; De la Cruz *et al.,* 1995). Since the extracellular enzymes of the *Trichoderma* spp. are inducible, all of the above enzymes were grown separately with appropriate carbon source and the enzymatic activity was determined at different time intervals. It was found that the wild type and mutant strains exhibited considerable variability among each other with respect to their production of extracellular enzymes. The physiological age of the spore was seemed to be an important factor in rate of enzyme production. Chitinase, β-1, 3-glucanase and protease production rate by all mutants and wild type were highest at 7, 4 and 9 days. respectively, after inoculation of spore inoculum. Ulhoa and Peberdy (1991) found in their study on chitinase production using washed mycelium of *T. harzianum* strain that both mycelium harvested at 18 hr (exponentiai phase) and at 24 hr (early stationary pahse) showed similar production of extracellular chitinase. The enzyme β-1, 3-glucanase is commonly produced by fungi as a constitutive enzyme (Bull and Chester, 1966). The result depicted that the rate of enzyme production (chitinase and β-1, 3-glucanase) due to mutant strains were significantly higher than the wild type strain except in case of protease enzyme production where wild type strain was more effective in comparison to mutant strains. Rey *et al.* (2001) reported that *T. harzianum* mutant PFI with low levels of protease activity was best biocontrol agents in greenhouse 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). The enhanced activity of the chitinolytic and glucanolytic enzymes by the mutants suggests that these strains may be utilized for biocontrol agent in glasshouse and field experiment. In addition, these strains have proved to be very stable, showing little or no reversion and grow rapidly in both liquid and solid media.

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