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Management of southern stem blight of soybean by PCNB-resistant mutants of *Trichoderma harzianum* 4572 incited by *Sclerotium rolfsii*

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The purpose of this study was to assess the biocontrol efficacy of the mutant strains of *Trichoderma harzianum* 4572, alone and in combination with fungicide PCNB against *Sclerotium rolfsii*, causing Southern stem blight of Soybean (*Glycine max*), in the glasshouse and field conditions. Twenty one mutants of *Trichoderma harzianum* 4572 were obtained after treatment with NTG and selection on PCNB amended medium, which were further screened against *Sclerotium rolfsii* by dual culture method. All the mutants indicated equally high tolerant capability of PCNB but only three mutants were shown enhanced antagonistic activity against *S. rolfsii* in colony plate assay as compared to the wild type and control. In the glasshouse experiment, results showed that the mutant Th mu6 and Th mu19 controlled the disease effectively as compared to wild type and Th mu11. Disease control by the mutant strain Th mu6 was enhanced drastically when applied in combination with PCNB in both glasshouse and field conditions as compared to wild type and control.

Key words*: Trichoderma harzianum* 4572*,* mutant strains, stem blight, PCNB, disease control, *Sclerotium rolfsii*

Introduction

Sclerotium rolfsii Sacc., the causal organism of Southern stem blight of Soybean is widely distributed throughout the world including India. 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 Csinos, 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; Punja *et al*., 1986) and biological control (Henis *et al*., 1983; Elad *et al*., 1984; Benhamou and Chet, 1996), particularly with *Trichoderma* and

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Gliocladium species (Lewis and Papavizas, 1991; Haran *et al*., 1996a; Haran *et al*., 1996b; 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 crops 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). Abd-EI-Moity and associates (1982) combined iprodione-tolerant strain of *T. harzianum* with the fungicide and obtained significantly higher disease reduction compared with that obtained by the chemical alone. Khattabi *et al*. (2004) reported that antagonistic effect of *T. harzianum* against *S. rolfsii* on solid culture media was stimulated in the presence of nitrogen fertilizers. *T. viride* in combination of PCNB has been shown to provide good disease control and better yield in artificially inoculated fields of tomato (Wokocha, 1990). In a study by Csinos *et al.* (1983) it was found that *T. harzianum* alone did not decrease disease incidence of *S. rolfsii* but when it combined with pentachloronitrobenzene (PCNB) at 11.2 kg/ha, disease was reduced. Other treatments in their study containing PCNB in combination with various insecticides and nematicides also reduced disease and increased yields.

Several biofungicides based on *Trichoderma* spp. have been commercialized in last few years. However, there is still considerable interest in finding more efficient mycoparisitic 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 gulcanase, 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 metabolites and antagonistic potential of biocontrol agents to control a broad spectrum of phytopathogens (Rey *et al*., 2001) and also for producing high levels of enzymes (Mandels and Andreotti, 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 Southern stem blight of Soybean.

Materials and methods

The experiment was conducted in the laboratory and glasshouse of the Department of Botany and field experiment at Botanical Garden, Banaras Hindu University, Varanasi, India.

Fungal isolates

The pure culture of Trichoderma harzianum 4572 was obtained from the culture 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), Varanasi. The pathogenic and antagonistic strains was maintained on Potato- Dextrose Agar medium (PDA; Merk) at 25±2^{o}C by regular subculturings.

Induction of mutant through N'-methyl-N'-nitro-N'-nitrosoguanindine (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). Conidial suspension of six days old culture of *T. harzianum* 4572 was prepared in 5 ml sterile 0.1M sodium citrate buffer (pH 5.5), centrifuged twice at 10,000 rpm and subsequently washed with the same buffer. The pellets were re-suspended in 5.0 ml of sodium citrate buffer and spore concentration was adjusted to 1×10^{5} spores/ml. A stock solution of NTG (1 mg/ml) was prepared in sodium citrate buffer immediately before the treatment and the final concentration was used 50 µg/ml of spore suspension. The NTG treated spore suspensions was incubated at 37^{o}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 4 ml sample through a 0.45 µm Millipore filter, washing the spores with 0.1 M phosphate buffer. 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^{o}C. Mycelial growth was marked after 24 h 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^{o}C for 2-3 days. Cultures were checked daily for growth and the presence of fast-growing sectors, which was 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 transfers on PDA with increasing concentrations of fungicide, they were evaluated for stability of fungicide tolerance. The isolates were cultured on PDA without fungicide for 5 to 7 days, and then transferred back to PDA amended with the fungicide. Cultures continued to grow at the rate similar to what was observed prior to the transfer on non-amended medium, tolerance was assumed to be due to a spontaneous mutation rather than a physiological adaptation to the fungicide. Serial transfer to medium with increasing concentrations of the fungicide was continued until isolates were derived that was 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 ^{o}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). Agar block of 5mm size cut from the margin of freshly grown cultures of antagonists and *S. rolfsii* was placed at 3cm apart from each other in 9cm Petri plate containing 20ml PDA medium. The inoculated plates were incubated at 25±2^{o}C for 4 days. The control was made by inoculated *S. rolfsii* without any antagonist. 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 replications.

Formulation of wild type and mutant strains of T. harzianum 4572

Alginate pellets of wild type and mutant strains of *T. harzianum* 4572 were prepared for use in glasshouse experiments following the method of Lewis and Papavizas (1985). The spore suspension of *Trichoderma* species (final concentration 10^7 spores/ ml) was mixed with 750 ml sodium alginate (Fisher Scientific) solution (26·6 g L^{-1} water) and the mixture pumped drop wise into a calcium chloride solution (5 g L^{-1} water). The capsules thus formed was left in the $CaCl_2$ solution for 20 min and then filtered and washed with sterile water. This result in the encapsulation of spores within beads of alginate gel in diameter sizes 2-3 mm. The alginate capsules were induced to sporulate at 25±2 ^oC and conidia counted with a hemocytometer by shaking the capsules in distilled water with Tween 80 (0.05%). Finally, the capsules were air dried for 48 hs and kept at 5^oC until used. The resulting alginate pellets were dried under an airflow at room temperature and stored at 5°C until use.

Glasshouse experiments

The soil samples were collected from the agricultural field, Banaras Hindu University, and sterilized by autoclaving at 22 p.s.i. for 30 min. The pure inoculum of the *S. rolfsii* was mixed with strerilized soil at the ratio of 1 % (w/w) and kept at room temperature (30 ^oC) for 4 days for proper growth of the pathogen. The inoculum of *S. rolfsii* was prepared by inoculating five agar blocks of 5mm size into 250-mL flasks containing barley grains (100g) and water (30 mL) sterilized by autoclaving at 22 p.s.i. for 30 min, and incubating at 25±2 ^oC for 4 days for the active growth of the fungus.

The formulated wild type and mutant strains of *T. harzianum* 4572 was applied into the pathogen-infested soil at the ratio of 1 g of alginate beads per 100 g of soil after 4 days of inoculation of *S. rolfsii*. The soil mixture was then added to pots (25 × 15cm size) and the seeds of the susceptible variety (NRC7) were sown after 6 days of inoculation of antagonists at the rate of 10 seeds/pot. Before sowing, the seeds surface was sterilized by 0.1% aqueous solution of NaOCl for 1 min and washed thoroughly with sterilized distilled water for several times. For the integrated control experiment, the seeds were treated with 0.1% PCNB after surface sterilization and then sown into the treated pots. Three replications were maintained for each combination. Experiments included an inoculated control treatment consisting of soil and pathogen. Original moisture level (15%) was maintained throughout the experiment by adding tap water at frequent intervals. Emerging soybean plants were assessed regularly for symptoms of stem rot (yellowing and wilting plants) until a

maximum level of disease was reached in the inoculated control treatments; no further occurrence of infected plants became infected.

Disease control measure was assayed as per cent disease control by using the following formula:

$$\text{Percent disease control} = \frac{(\text{Percentage of disease in control} - \text{Percentage of disease in treatment}) \times 100}{\text{Percentage of disease in control}}$$

Field experiment

The field experiment was layed-out and divided into three plots with the size of 1.5 × 1.5 m and was prepared at the Botanical Garden, Banaras Hindu University, Varanasi, The studied was conducted between June and October, 2006. The pathogen and antagonist inocula were mixed with soil at 1% (w/w) and 200 g of this mixture and added to each plot. The initial inoculum density of mutant Th mu6, formulated as alginate beads in soil was 3×10^5 conidia/g of soil. The seeds of the susceptible variety (NRC 7) treated with 0.1% PCNB were sown at the rate of 30 seeds per plot. The plots were arranged in randomized complete block design with three replications. The pathogen infested plot without amendment with mutant strain sown with non-treated sterilized seeds served as control. One month after sowing, percentage of disease control in soybean plants was assayed.

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*. Linear growth of wild-type isolate was inhibited by approximately 95% at 50 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 selected 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 on the following culture of fungicide on the 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 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. rolfsii.*

Different growth inhibitions of *S. rolfsii* by the wild type and mutant strains of *T. harzianum* 4572 were observed (Fig. 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.

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 glasshouse experiment. The wild type strain was also used in the experiment for comparison.

Glasshouse experiments

Biological control of Southern stem blight of soybean caused by *S. rolfsii* was achieved by applying the wild type and mutant strains of *Trichoderma harzianum* 4572 to soil is presented in Fig. 2 Application of formulated antagonists in the form of alginate beads was applied in the soil after 4 days of inoculation of *S. rolfsii*. Susceptible variety of Soybean seeds (NRC7) was sown after 6 days of inoculation of antagonists. In case of integrated disease management, the seeds were treated with 0.1% PCNB prior to sowing. Southern stem blight disease reached to maximum level in the inoculated control treatments after 14 days in the experiment.

In the absence of PCNB, the mutant Th mu6 and Th mu19 significantly controlled (P= 0.05) the disease upto 76.5% and 63.1%, respectively as compared with the inoculated control plants. The mutant Th mu11 was found less efficient in controlling the disease as compared to other two mutants. The wild type strain showed lower efficiency in disease control (51.2%) as compared to all the three mutant strains. In the experiment, combination treatment of antagonists and PCNB resulted to increase in disease control drastically as compared with the inoculated control (Fig. 3). The mutant Th

mu6 significantly controlled the disease maximum (90.8%) followed by the mutant Th mu19 (78.4%). Wild type strain in this case also showed lower efficiency in reducing the disease and has achieved to 66.7% disease control.

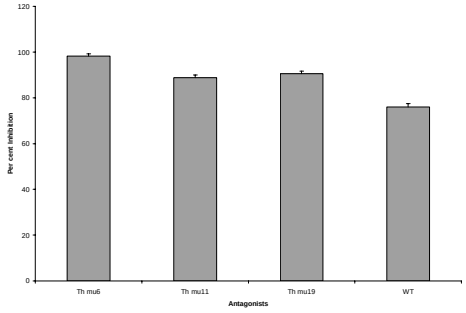

Fig. 1. Per cent growth inhibition of *Sclerotium rolfsii* by wild type and mutant strains of *Trichoderma harzianum* 4572.

Field experiment

In the field experiment, application of the *T. harzianum* 4572 mutant strain, Th mu6, formulated as alginate beads with integration of PCNB significantly controlled (P = 0.05) the stem blight disease of soybean as compared to control and wild type strain (Fig. 4).

Discussion

Development of *Trichoderma* mutants towards suppression of fungal plant pathogens is an important method in improvement of strain, 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 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 designed 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 in the PCNB amended medium, finally three mutants were selected that were not only capable to tolerate at the concentration of PCNB at 200 ppm but also grew and sporulated faster.

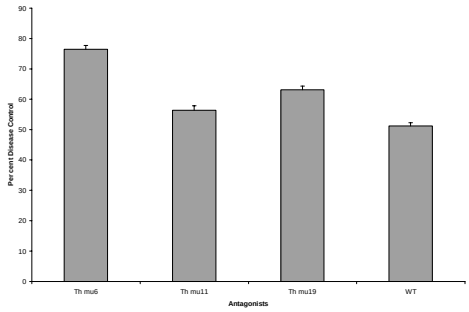

Fig. 2. Effect of wild type and mutant strains of *Trichoderma harzianum* 4572 on percent disease control of stem blight of soybean in glasshouse**.**

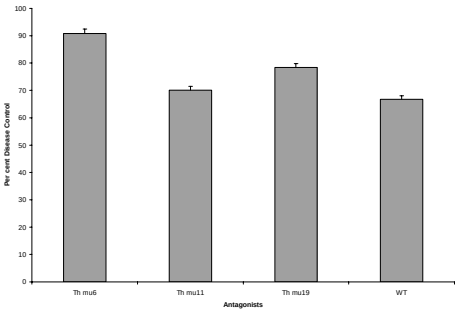

Fig. 3. Integrated effect of wild type and mutant strains of *Trichoderma harzianum* 4572 with PCNB on per cent disease control of stem blight of soybean in glasshouse.

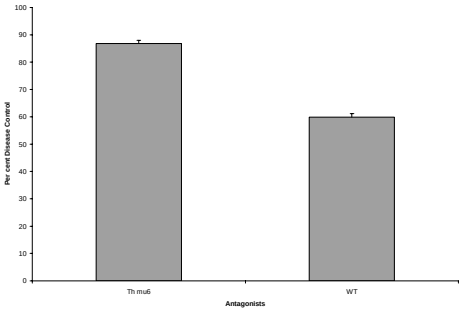

Fig. 4. Integrated effect of wild type and mutant strain, Th mu6, of *Trichoderma harzianum* 4572 on per cent disease control of stem blight of soybean in field.

In the present study, twenty one different mutants of *T. harzianum* 4572 was 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 screening 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 higher antagonism towards *S.*

rolfsii as compared to parent. In most of the ineffective mutants and pathogen interaction, the pathogen was found to overgrow on the mutants.

Carsolio *et al*. (1999) isolated and characterized endochitinase enzyme from culture of *T. harzianum* and found that the enzyme performed *in vitro* antifungal activity against phytopathogens. Since the cell walls of *S. rolfsii* are composed of β- 1,3-glucan and chitin (Bartnicki-Garcia, 1973), it was hardly assumed that endochitinase played an important role in the growth inhibition of the pathogen. Mycoparasitic activity of *Trichoderma* species against the phytopathogens might be as the result of production of antibiotics or cell wall-degrading enzymes. Recent evidence has shown that antibiotics and hydrolytic enzymes are not only produced together but act synergistically in mycoparasitic antagonism (Di Pietro *et al*., 1993 and Schirmböck *et al*., 1994). Bell *et al*. (1982) and Widyastuti *et al*. (1999) showed in paired cultures on agar that a biocontrol isolate which highly effective against one isolate of a pathogen could have performed minimal effect on other isolates of the same pathogen. More evidence supported that this might be related to the pathogen-specificity of antagonistic mechanisms such as antibiotic (Howell and Stipanovic, 1995) and cell wall degrading enzymes (Haran *et al*., 1996).

One of the major bottlenecks experienced in the biocontrol of plant diseases is the delivering of antagonist to the infection court. In this study, the application of mutant strains of *T. harzianum* 4572 as sodium alginate beads to the rhizosphere of soybean plant was highly effective both in the glasshouse and field experiments. In the glasshouse, wild type and mutants of *T. harzianum* 4572 were delivered through alginate beads at the rate of 1g alginate beads per 100g of soil. Among the mutants, Th mu6 and Th mu19 recorded significantly higher disease control as compared to the wild type and other mutant strain (Th mu11). The findings of Papavizas *et al*. (1982) indicated that several UV induced biotypes of *T. harzianum* was consistently more effective than parent in suppressing damping-off of peas and radish.

In the glasshouse, management of Southern stem rot of soybean by *T. harzianum* mutants on integration with PCNB has evidenced that Th mu6 and Th mu19 was aggressive with increased biocontrol capability against the *S. rolfsii*. The wild type strain was highly inferior compared to the mutants tested. In the field, the mutant strain Th mu6 controlled the disease upto 80.6% when integrated with 0.1% PCNB. The wild type and other mutant strains were not tested in field due to their inferior performance in glasshouse as compared to the mutant Th mu6.

The application of *T. harzianum* mutant (s) effectively controlled the disease, both in glasshouse and field, caused by *S. rolfsii* when combined with PCNB at 0.1% concentration. The dilution which the growth rate of the antagonist was least affected while the growth of the pathogen was severely

impeded. The results evidenced that the mutant Th mu6 was most aggressive with increased in biocontrol capability against the *S. rolfsii* as compared to the other two mutants. The wild type strain was highly inferior in comparison to the mutant strains tested. The reason behind the result was probably due to high potency of Th mu6 in producing extracellular enzymes as compared to the wild type and other two mutant strains. The lytic activity of several *Trichoderma* species on cell walls of phytopathogenic fungi has been correlatd with the degree of biological control of these pathogens *in vivo* (Papavizas, 1985). Clarkson *et al.* (2006) reported that *T. viride* in combination with Tebuconazole or compost enhanced control of *Allium* white rot incited by *Sclerotium cepivorum* and in some cases, eliminated the pathogen completely.

Base on the study, it was concluded that PCNB could possibly be successfully applied in combination with the mutant Th mu6 for the control of Southern stem blight of soybean incited by *S. rolfsii*. The fungicide significantly reduced the growth tempo of the pathogen while compared to the biological antagonist was not significantly reduced.

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