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Biological control of *Pythium aphanidermatum* causing damping-off of mustard by mutants of *Trichoderma viride* 1433

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The *in vitro* screening of wild type and mutant strains of *Trichoderma viride* 1433 was carried out against the pathogen *Pythium aphanidermatum* by dual culture method. Inhibition in the linear growth of *Pythium aphanidermatum* due to production of volatile and non-volatile metabolites was also recorded. The assay of competitive saprophytic ability (CSA) showed that the mutant Tv m6 has high CSA in comparison to wild type and other mutant strains. In the glasshouse experiment the biocontrol efficacy of mutant strains showed that the mutant strain Tv m6 controlled the disease by more than 85% in both the sterilized and natural soils. Therefore, it has been concluded that the mutant strain, Tv m6 may be used to control *Pythium aphanidermatum* causing damping-off of mustard.

Key words: *Trichoderma viride* 1433, mutant strains, CSA, biocontrol

Introduction

Mustard (*Brassica campestris* L.) is one of the most important oilseed crops of commercial value cultivated in India and several other countries. Being an important source of edible oil, mustard has been undoubtedly in focus for Indian oilseed industry. The production of mustard is affected by several diseases of fungal and viral origin. Among the fungal diseases damping-off incited by *Pythium aphanidermatum* is a common problem in the mustard growing areas, especially under the moist conditions that generally prevails during sowing. Fungicides are the most common means to check the disease in plants. Frequent and Intensified uses of these chemicals are hazardous to humans and environment (Cook and Baker, 1983) and 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, which is also ecology-conscious and eco-friendly. Species of the genus *Trichoderma*

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are well documented fungal biocontrol agents (Papavizas, 1985; Elad and Kapat, 1999; Howell, 2002). Growth inhibition of the phytopathogens by the *Trichoderma* metabolites has been well researched (Dennis and Webester, 1971a,b; Howell and Stipanovic, 1983; Ghisalberti and Sivasithamparam, 1991). Successful management of damping-off caused by *Pythium* species in various crops by application of *Trichoderma* has been previously reported (Sivan *et al.*, 1984; Lumsden and Locke, 1989; Kanjanamaneesathian, 2003; Le *et al*., 2003; Abdelzaher, 2004; Jayaraj *et al*., 2006).

In view of the above, the present study was carried out to control damping-off of mustard by the mutant strains of *Trichoderma viride* 1433 under glasshouse conditions. Attempts have also been made to investigate the production of volatile and non-volatile metabolites by the wild type and mutant strains of *T. viride* 1433.

Materials and methods

Microbial strains

A virulent strain of *Pythium aphanidermatum* and the wild type strain of *Trichoderma viride* 1433 (Tv-1433) were obtained from the Institute of Agriculture Science, Banaras Hindu University, Varanasi and Institute of Microbial Technology, Chandigarh, India, respectively. The mutant strains of *T. viride* 1433 were generated earlier by treatment with NTG (Khare and Upadhyay, 2009). The pathogenic and antagonistic strains was maintained on Potato-Dextrose Agar medium (PDA; Merck) at 25±2°C by regular subculturings.

Colony growth inhibition assay

In vitro antagonistic activity of wild type and mutant strains of *T. viride* 1433 against *P. aphanidermatum* 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.

Assay of production of volatile and non-volatile metabolites

Production of volatile metabolites from the wild type and mutant strains of *T. viride* 1433 against *P. aphanidermatum* was tested following the method of Dennis and Webster (1971b) as described by Eziashi *et al*. (2006). Bottoms of two Petri dishes containing PDA were individually inoculated with a disc of *P. aphanidermatum* and antagonistic strains (wild type and mutant strains of *T. viride* 1433). The bottoms were adjusted and attached by tape. Antagonists were inoculated 24 h earlier, since growth of *P. aphanidermatum* was more rapid. The control sets did not contain the antagonist. The cultures were incubated at 25°C. The studies were conducted in three replicates. The percent inhibition was obtained using the formula:

$$\text{Per cent inhibition} = \frac{C_2 - C_1}{C_2} \times 100$$

Where, C_2 means growth of *P. aphanidermatum* in control and C_1 means growth of *P. aphanidermatum* in treatment. Production of non-volatile metabolites was tested following the method of Lundberg and Unestan (1980) and Dennis and Webster (1971a) as described by Eziashi *et al*. (2006). Mycelial agar plugs (5 mm diameter) removed from the edge of a young culture of antagonists was transferred to the center of Petri dishes (9 mm diameter) containing PDA and a sterilized cellophane disc (9 cm diameter) adjusted on the medium surface. The strains were grown for 3 days. The cellophane containing the antagonist was removed, and on the same medium a disc of *P. aphanidermatum* was placed. The control treatments had *P. aphanidermatum* growing similarly on PDA medium where previously there was a cellophane disc without antagonist. The inhibition percent was obtained using the formula as described above.

Competitive saprophytic ability (CSA) of the wild type and mutant strains

Competitive saprophytic ability (CSA) of the wild type and mutant strains of *T. viride* 1433 was assayed by following the method of Ahmad and Baker (1987). The wild type and mutant strains of *T. viride* 1433 were grown on PDA medium. The desired number of conidia from the freshly grown cultures of wild type and mutant strains of *T. viride* 1433 was obtained as described by El-Abyad *et al*. (1983). The conidia harvested from the cultures were mixed with natural soil at the rate of 10^6 conidia/gram of soil. Twenty pieces of wheat straw (1 cm size) were buried in 500 ml conical flask containing the inoculum mixture of antagonists. Three replicates were maintained in each experiment. The control

was set without conidia. All the 20 pieces from each treatment including control were removed from the pots after six days. The wheat straw was washed thoroughly with tap water followed by surface disinfection in a mixture of 0.1% sodium hypochlorite solution and 5% ethanol for 5 min. Segments were plated on the *Trichoderma* selective medium (Elad *et al*., 1981) and incubated at 30°C for five days. Per cent colonization of wheat straw pieces by the wild type and mutant strains of *T. viride* 1433 was determined.

Glasshouse experiment

Preparation of P. aphanidermatum inoculum

For preparation of pathogen-inoculum, sand-maize medium was filled into glass bottles (600 g), sterilized and inoculated with mycelial discs (5 mm diameter) taken from one-week old culture of *P. aphanidermatum*. The bottles were incubated at 30°C for 10 days with frequent stirring. The content of the bottles were thoroughly mixed, and used as inoculum.

Seed treatment

Seeds of mustard (*Brassica campestris* L. 'Yellow Sarson') were treated with conidial suspensions of the wild type and mutant strains of *T. viride* 1433 (~10^6 conidia/ml) in water containing 3% solution of carboxymethylcellulose (CMC, Merck). The number of population of antagonists on the seed surface was counted by shaking 5 g of seeds in 50 ml of sterile tap water for 1 h in 250 ml Erlenmeyer flasks in a rotatory shaker at 200 rpm. Serial dilutions of the suspensions were plated on a *Trichoderma* selective medium (TSM).

Pot trials

Top soil from mustard field was collected, air dried, and mixed with 1 per cent (w/w) pure inoculum of the *P. aphanidermatum.* To perform the experiment in sterilized soil, the soil was first sterilized and then mixed with the inoculum. The pathogen infested soil (5 kg) was filled into pots (30 cm diameter) and incubated at room temperature (30 °C) for seven days. Mustard seeds (40 seeds) coated with wild type and mutant strains of *T. viride* 1433 were sown into the pots. Each experiment was conducted in three replicates. Seeds treated with CMC without antagonist served as control. The pots were transferred to glasshouse where disease was recorded after the 10th day of sowing by the method as described by Abdelzaher (2004). Pre-emergence damping off was determined as the difference in seedling emergence in

between healthy control soil and infested soil. Post emergence damping-off was determined from the number of diseased plants as a percentage of the emerged ones. The per cent disease control was calculated using the formula:

$$\text{Per cent disease control} = \frac{(\text{Per cent disease in control} - \text{Per cent disease in treatment})}{\text{Per cent disease in control}} \times 100$$

Results

Inhibition of colony growth of P. aphanidermatum by the wild type and mutant strains of T. viride 1433

All the four mutants showed significantly ($P < 0.05$) enhanced antagonistic activity against *P. aphanidermatum* in comparison to the wild type strain (Fig. 1). The mutant strain Tv m6 exhibited maximum inhibition of the pathogen (93.4%) which was followed by Tv m13 (87.6%), Tv m21 (79.2%) and Tv m9 (76.4%). The least inhibition in the growth of the pathogen was depicted by the wild type strain *T. viride* 1433 (72.0 %).

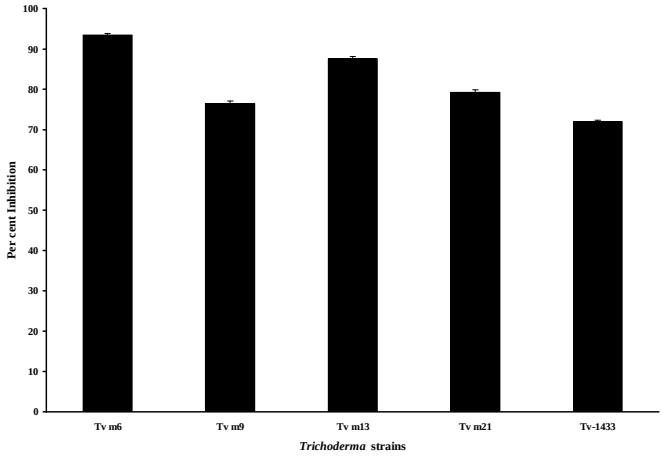

Fig. 1. *In vitro* screening of the wild type and mutant strains of *T. viride* 1433 against *P. aphanidermatum*.

Inhibition of colony growth of P. aphanidermatum by volatile and non-volatile metabolites from the wild type and mutant strains of T. viride 1433

The results of the production of volatile and non-volatile metabolites by the wild type and mutant strains of *T. viride* 1433 are presented in Fig. 2. The maximum inhibition of the radial growth of *P. aphanidermatum* was due to the

non-volatile metabolites of the mutant strain Tv m6 (68.1%), which was followed by Tv m13 (57.6%), Tv m21 (52.3%) and Tv m9 (46.4%). The wild type strain *T. viride* 1433 showed minimum inhibition in radial growth of the pathogen (37.6%) as compared to mutant strains. The inhibition of the pathogen due to volatile metabolites of tested antagonists was not as effective as non-volatile metabolites. The maximum inhibition of the radial growth of the pathogen due to volatile metabolites was recorded due to the mutant strain Tv m21 (32.2%), which was followed by Tv m13 (23.6%) and Tv m6 (19.5%). The least volatile metabolite producing mutant strain was Tv m9 where the inhibition of the pathogen was only 15.2%. All the mutants were more efficient than the wild type strain *T. viride* 1433, which inhibited the pathogen by 13.4% only.

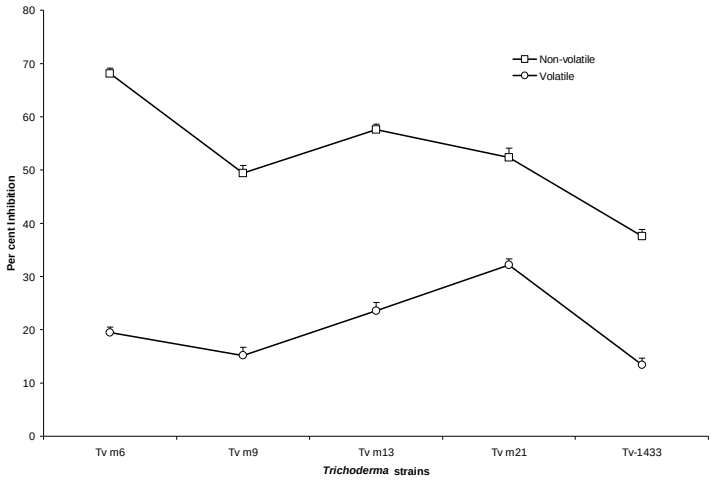

Fig. 2. Effect of non-volatile and volatile metabolites of parent and mutant strains of *Trichoderma* on percent inhibition of *P. aphanidermatum.*

Competitive saprophytic ability of the wild type and mutant strains of T. viride 1433

All the mutants significantly ($P<0.05$) colonized the wheat straw pieces higher than the wild type strain *T. viride* 1433 (Fig. 3). The highest per cent colonization was observed in case of the mutant strain Tv m6 (75.0 %), which was followed by Tv m13 (68.3%), Tv m21 (61.4%) and Tv m9 (56.6%). The colonization in case of wild type strain *T. viride* 1433 was recorded to 46.4%.

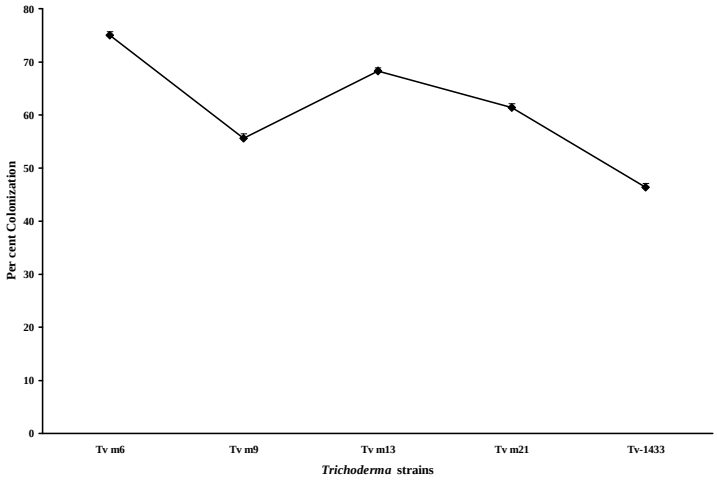

Fig. 3. Percent colonization of the wild type and mutant strains of *T. viride* 1433 on the wheat straw pieces.

Glasshouse experiment

The data reveals that mutant strains established better disease control as compared to the wild strain, *T. viride* 1433 in both sterilized and natural soils (Fig. 4 and 5, respectively). The maximum control of pre-emergence (87.7%) and post-emergence (89.5%) damping-off of mustard in the sterilized soil, infested with the *P. aphanidermatum*, were achieved through the seed treatment with the mutant strain Tv m6 followed by Tv m13, which controlled pre- and post-emergence damping-off by 77.2% and 83.6%, respectively. The least control of the disease was observed in case of the wild type strain *T. viride* 1433 (45.6 and 50.8%, respectively). In natural soils, infested with *P. aphanidermatum*, percent control of pre- and post-emergence damping-off of mustard was found 86.3% and 87.1%, respectively, when the seeds coated with the mutant strain Tv m6 were applied into the pots. The mutant Tv m13 controlled pre emergence and post emergence damping-off by 72.5% and 80.7%, respectively. The percent control of the disease was least due to the wild type strain *T. viride* 1433 (37.2 and 42.0%, respectively).

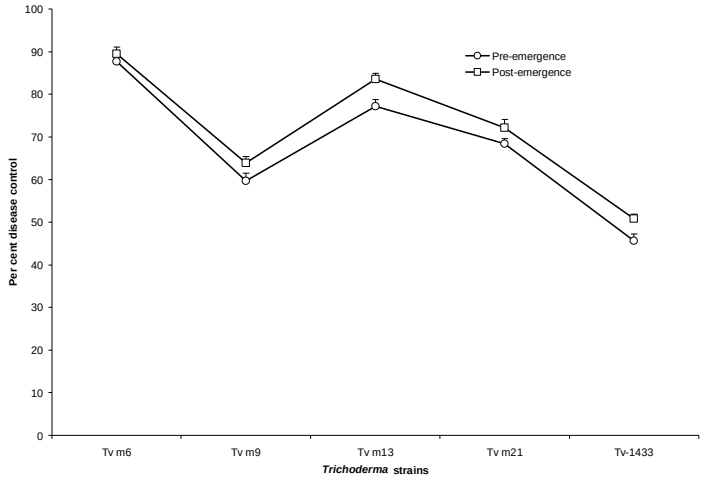

Fig. 4. Biological control of damping-off of mustard by the wild type and mutant strains of *T. viride*-1433 in sterilized soil under pot condition.

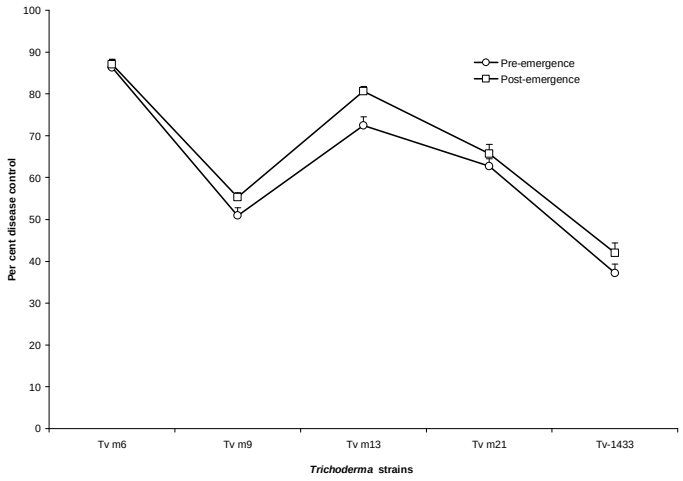

Fig. 5. Biological control of damping-off of mustard by the wild type and mutant strains of *T. viride*-1433 in natural soil under pot condition.

Discussion

The results of the dual culture (Fig. 1) revealed that the highest mean inhibition values, above 90% were obtained due to the mutant strain Tv m6 followed by Tv m13. The inhibition zone between the tested strains of *T. viride*-1433 and *P. aphanidermatum* was observed. The study suggests that some kind of antibiotics or toxic metabolite might be produced by these strains.

Antagonism of *Trichoderma* species against several phytopathogens has been reported (Chet and Baker, 1980; Papavizas, 1985; Elad, 2000; Howell, 2002). The interaction of the antagonists and the pathogen and occurrence of inhibition zone on agar media could be commonly considered as a result of the production of the antibiotics and competition for nutrients and space as was observed by Upadhyay and Rai (1987). El-Katatny *et al*. (2001) explained that inhibition zone in dual cultures is formed due to the production of volatile and non-volatile metabolites as well as the production of extracellular hydrolytic enzymes by *Trichoderma* species. It has been reported that antibiotics and hydrolytic enzymes are not only produced together but act synergistically in mycoparasitic antagonism (Di Pietro *et al*., 1993; Schirmböck *et al*., 1994). Many isolates of *Trichoderma* produced both volatile and non-volatile metabolites that adversely affect growth of different fungi (Dennis and Webester, 1971a, b; Claydon *et al*., 1987; Corley *et al*., 1994). A comparison between the inhibitory effects of volatile and non-volatile metabolites in the present study revealed that the non-volatile metabolites seemed to be more effective in the antagonism mechanism. The biocontrol efficacy of Tv m6 might be due to overproduction of non-volatile metabolite. Kucuk and Kvanc (2003) determined that non-volatile metabolites produced by *T. harzianum* had an inhibitory effect on the growth of *Fusarium*. Sivan *et al*. (1984) reported that *Trichoderma* produced inhibitory compounds against *P. aphanidermatum*. Mutants of *T. harzianum* with altered antibiotic production were found inhibitory to *Rhizoctonia solani*, *Pythium ultimum* and *Fusarium oxysporum* (Graeme-Cook and Faull, 1991). Metabolites produced from *T. viride* and *T. polysporum* reduced the growth of *Ceratocystis paradoxa*, the causal agent of black seed rot in oil palm (Eziashi *et al*., 2006). Howell (1998) reported that strains of *T. virens* with the best efficiency as biocontrol agents were able to produce gliovirin.

The results suggested that mutant strains produced volatile and non-volatile compounds and secondly, that such compounds might play role in the inhibition of colony growth of *P. aphanidermatum*. The biocontrol efficacy of the mutant Tv m6 might also be attributed to the production of non-volatile metabolites. The strains without CSA could not survive in the soil ecosystem (Nakkeeran *et al.*, 2005). Therefore CSA was included in the study. The result obtained in the present study (Fig. 3) revealed that the mutants have high CSA than the wild type strain. The maximum colonization was observed due to Tv m6 which was followed by Tv m13. Nakkeeran *et al*. (2005) reported that cellulase production in *Trichoderma* mutants had a positive correlation with CSA. As the mutant Tv m6 produced higher cellulase activity (Khare and

Upadhyay, 2009), it could utilize cellulose more efficiently and, therefore, possessed high value of CSA.

Trichoderma are well documented as effective biocontrol agents of plant diseases caused by soil borne fungi (Sivan *et al*., 1984). Pre- and post-emergence damping-off of wheat caused by *P. diclinum* was successfully controlled by *Gliocladium roseum* or *T. harzianum* (Abdelzaher, 2004). *Trichoderma* reduces damping off of cowpea caused by *Macrophomina phasolina* (Adekunle, 2006). Durman *et al*. (1999) used *Trichoderma* for the control of *Rhizoctonia solani* attacks on tomato plants in greenhouse. Biological control of soil borne plant pathogens can be achieved by seed treatment or soil application of antagonists. Hadar *et al.* (1979) investigated that the application of wheat bran preparation of *T. harzianum* to soils infested by *Rhizoctonia solani* reduced the incidence of disease in beans. Control of *Pythium* spp in tobacco, sugarbeet and cauliflower was achieved by *Trichoderma harzianum* through soil application (Mukhopadhyay *et al.*, 1986; Sawant and Mukhopadhyay, 1990). Application of conidia of *T. harzianum* or *T. koningii* to pea seeds reduced the incidence of preemergence damping-off induced by *Pythium* species (Lifshitz *et al*., 1986). Harman *et al.* (1980) reported the biocontrol of *Rhizoctonia solani* and *Pythium* species by coating radish and pea seeds with *Trichoderma harzianum*. Seed coating of biological antagonist like *T. harzianum* gave best results in reducing the disease as compared to other treatments (Das *et al*., 2002). *Pythium* spp. is usually effectively controlled by seed treatment because the fungus is active early in the season during seed germination.

In the present investigation, mustard seeds coated by conidia of wild type and mutant strains were sown in sterilized and natural soils infested by the *P. aphanidermatum*. The disease control potential of antagonists was observed more in sterilised soil as compared to the natural soil. The reason might be due to more competition in natural soils. The results evidenced that the mutant Tv m6 was most aggressive against *P. aphanidermatum* in both sterilized and natural soils followed by Tv m13. The wild strain *T. viride* 1433 was highly inferior compared to the mutant strains tested. Similarly, Singh and Upadhyay (2009) reported that NTG induced mutants of *T. harzianum* were highly effective against *Sclerotium rolfsii*. 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. The increased antagonistic potentiality of the mutant strains especially Tv m6 might be due to the increased production of non-volatile metabolites and high competitive saprophytic ability. The production of extracellular enzymes by

the mutant Tv m6 too played major role in the antagonism of *P. aphanidermatum* (Khare and Upadhyay, 2009).

In conclusion, mutant Tv m6 was found most effective biocontrol agent against *P. aphanidermatum* under glasshouse conditions as compared to the other strains. Therefore, the mutant strain Tv m6 may be a promising biocontrol agent against damping-off of mustard.

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