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Research Article

Studies on the development of hexaconazole-tolerant *Trichoderma asperellum*

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ABSTRACT: The generation of fungicide-tolerant *Trichoderma* strains is desirable for managing crop plant diseases during severe disease outbreaks as fungicides immediately suppress the fungal pathogens, and a fungicide-tolerant *Trichoderma* strain later suppresses the weakened pathogens caused due to fungicide exposure for a prolonged period of crop growth. Thus, in the present study, hexaconazole-tolerant *T. asperellum* was generated by induced mutagenesis using ethyl methyl sulfonate. Four hexaconazole-tolerant strains that did not show any growth defects were selected. Among them, two hexaconazole-tolerant strains, namely, *T. asperellum* strains T7 and T18, showed a threefold increased tolerance compared to their parental strain. Hexaconazole-tolerant *T. asperellum* strain 18 exhibited the maximum mycelial growth inhibition of *Macrophomina phaseolina* and improved black gram seed germination and vigour index compared to its parental strain. Further black gram's dry root rot disease incidence was effectively suppressed with the application of 0.01% hexaconazole. Thus, this study indicates that the combined application of hexaconazole-tolerant strains and the reduced dose of hexaconazole effectively suppressed black gram's dry root rot disease.

KEYWORDS: Biocontrol, induced mutagenesis, *Trichoderma*, black gram root rot

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INTRODUCTION

Trichoderma spp. are extensively used as a biocontrol agent for managing several soil-borne diseases of crop plants (Dutta *et al.*, 2023). In addition to biocontrol potential, certain *Trichoderma* spp. have been reported for their plant growth promotion by increasing seed germination, water absorption and root and plant growth (Contreras-Cornejo *et al.*, 2024; Song *et al.*, 2023). *Trichoderma* spp. can be isolated from agricultural soil, decaying wood, farm waste, and organic plant matter. They inhibit and suppress the phytopathogenic fungi through various mechanisms such as competition, antibiosis, mycoparasitism, lytic enzymes and inducing plant defense mechanisms (Guzmán-Guzmán *et al.*, 2023; Jayalakshmi *et al.*, 2021; Kashyap *et al.*, 2017). There are several *Trichoderma* spp. namely *T. harzianum, T. hamatum, T. koningii, T. pseudokoningii, T. longibrachiatum, T. viride, T. virens*, etc.

Various *Trichoderma* species were commercially produced on a large scale for plant protection and growth promotion of several crops in many countries, including the United States, India, Finland, Belgium, Colombia and Israel. They can overcome fungistatic effects and resist many toxic metabolites of soil microflora, microfauna, fungicides, herbi cides, etc. (Harman *et al.*, 2004). *T. asperellum* was found to effectively reduce the fusarial wilt of tomatoes in addition to enhancing plant growth promotion (Sehim *et al.*, 2023) and black root rot of strawberries (Elshahawy *et al.*, 2024). Several other *Trichoderma* spp. are also implicated in the biological control of various soil-borne diseases. *Trichoderma virens* reduced the sclerotial viability of *R. solani* by 90%

in soil (Howell, 1982). Gliotoxin-producing *T. virens* isolate showed a suppressive effect up to three subsequent plantings (replanting) (Lumsden & Locke, 1989).

Sudden outbreaks of diseases have recently become common. Chemical pesticides would be an immediate recommendation for managing the sudden outbreak of diseases. Though several fungal and bacterial biocontrol agents are being marketed, they are only effective when applied before disease development/outbreak. Under unprecedented disease outbreaks due to climatic changes, it is wise to use biocontrol agents along with a reduced dosage of chemical pesticides to get the following benefits: (i) immediate arresting/weakening of mycelial growth of fungal pathogens by fungicides, followed by suppression by *Trichoderma* in later stages of plant growth (ii) avoiding the development of fungicide resistant pathogenic strains, (iii) reducing environmental pollution, (iv) lowering the cost of chemical pesticides and inclusion of biocontrol agents in integrated disease management. Fungicide-resistant/tolerant strains of *Trichoderma* have recently been developed and successfully used in integrated plant disease management by applying a combination of fungicide-tolerant *Trichoderma* and a reduced dose of fungicide. Benomyl-tolerant *Trichoderma pseudokoningii* strains were produced by induced mutagenesis with chemical mutagen and gamma irradiation, and some of the benomyl-tolerant mutants were superior to the wild-type strain in the antibiosis and suppression of *Sclerotium rolfsii* causing ginger rhizome rot (Mukherjee *et al.*, 1999). Recently, two carbendazim-tolerant *T. asperellum* strains, developed by induced mutation using chemical mutagen, effectively suppressed dry root rot of chickpeas when applied along with half the recommended dose of carbendazim (Ramangouda *et al.*, 2023). Thus, induced mutation helps generate tolerant *Trichoderma* strains to desirable systemic fungicides. Hence, this study was designed to create a suitable hexaconazole-tolerant *T. asperellum* and to assess its effect in combination with a reduced dose of hexaconazole for managing black gram dry root rot as a model system.

MATERIALS AND METHODS

Fungal culture, growth media and conditions

Trichoderma asperellum and *Macrophomina phaseolina,* obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, were cultured on PDA medium at 25 and 35°C, respectively. Conidia of *T. asperellum* were harvested in sterile water containing 0.01% Tween 20, stored at 4°C for short-term storage, and in 20% glycerol at -80°C for long-term storage. Mycelia of *M. phaseolina* were stored on PDA slants at 4°C for short-term storage and in 20% glycerol at -80°C for long-term storage.

Assessment of IC_{50} and minimum inhibitory concentration of hexaconazole against *T. asperellum*

The IC_{50} (inhibitory concentration) of hexaconazole against *T. asperellum* was assessed using the poisoned food technique. Mycelial discs of *T. asperellum* were inoculated on rose Bengal agar medium/PDA medium amended with hexaconazole at various concentrations, namely, 0.05, 0.1, 0.25, 0.5, and 1 ppm. The media without hexaconazole served as the control. The mycelial growth on the hexaconazole-amended Rose Bengal medium was compared with that on the Rose Bengal agar medium without hexaconazole (control). The concentration at which 50% inhibition of mycelial growth occurred was recorded as the IC_{50} of hexaconazole. The minimum concentration of hexaconazole at which complete mycelial growth inhibition occurred was noted as the minimum inhibitory concentration.

Chemical mutagenesis of *T. asperellum* and selection of hexaconazole-tolerant mutant strains

Chemical mutagenesis of *T. asperellum* conidia with Ethyl Methane Sulfonate (EMS) was carried out. *T. asperellum* was cultured on a PDA medium and incubated at 30°C for 7 days or until full conidiation occurred. The conidia were collected in sterile water by rubbing the culture media with the glass L-rod to dislodge the conidia and stored in a 50 ml falcon tube. EMS was added to the final concentration of 40 mM and incubated for one hour in a shaker at 150 rpm. After the incubation period, the conidia were washed using sterile water three times to remove the residual EMS and were finally dissolved in one milliliter of sterile water. The number of conidia present in one milliliter of conidial suspension was counted using a hemocytometer. CA conidial stock solution containing 2000 conidia/ml was prepared. 100 microlitres of the conidial stock solution (200 conidia) were spread-plated onto Rose Bengal agar medium amended with 0.25 ppm (IC_{50}) and 1 ppm of hexaconazole (minimum inhibitory concentration of hexaconazole). Mutants were screened for tolerance to hexaconazole and characterization of the best hexaconazole-tolerant isolate was carried out further.

Assessment of growth parameters, biocontrol potential and plant growth promotion of hexaconazole-tolerant strains of *T. asperellum*

For assessing the growth rate of hexaconazole-tolerant strains, an 8 mm diameter mycelial disc of hexaconazole-tolerant strain was inoculated on the fresh PDA medium and incubated at room temperature. Mycelial growth was measured when the mycelial growth of any one of the *T. asperellum* strains grew and covered the entire Petri dish. The tolerant level of putative hexaconazole-tolerant strains was assessed by culturing them on PDA medium amended with different concentrations of hexaconazole (0.0, 0.25, 0.5 and 1.0 ppm), and the growth pattern was compared with parental

T. asperellum. A dual culture method was used to assess the antagonistic potential of hexaconazole-tolerant strains. An 8 mm diameter mycelial disc of hexaconazole-tolerant strain was inoculated 1 cm away from the periphery of the Petri dish, and a same-sized disc of *M. phaseolina* was similarly inoculated 1 cm away from the edge of the Petri dish on the opposite side. As a control, *M. phaseolina* alone was inoculated on a fresh PDA plate. The cultures were incubated at room temperature. The antagonistic activity was evaluated by measuring the radius growth of the *M. phaseolina* colony in the direction of the antagonist colony when the mycelial growth of the *M. phaseolina* colony in the control plate fully covered the entire plate. The percent growth inhibition was calculated using the formula:

Percent inhibition (I) = C - T/ C × 100

where, I – percent inhibition of mycelial growth; C - growth of the pathogen in control plates; T - Growth of the pathogen in dual culture plates.

The roll-towel method was used to assess the growth-promoting activity of hexaconazole-tolerant strains. The black gram variety VBN10 was chosen for this experiment. The conidial suspension (@10^6 conidia/ml) of hexaconazole-tolerant strains and parental strains *T. asperellum* was prepared in sterile water containing 0.5% carboxymethyl cellulose as a sticking agent. 10 ml of the conidial suspension was taken in the beaker, and 50 seeds were placed in each beaker and soaked for 2 h. Control (seeds soaked in water) was carried out simultaneously. Twenty-five seeds treated with each strain were placed on the germination paper at equal distances and rolled with polythene sheets. Seeds placed on germination paper were incubated for 7 days. Three replications were carried out for each treatment. The root length and shoot length of the individual seedlings were measured, and the percent of germination of the seeds was calculated. The seedling vigour index was calculated using the formula:

Vigour index = (Mean root length + mean shoot length) × %Germination.

Efficacy of hexaconazole-tolerant strains in the management of dry root rot of black gram

A pot culture experiment was conducted to test the effect of the hexaconazole-tolerant strains of *T. asperellum* on managing dry root rot of black gram plants. Conidial suspension (10^6/ml) of mutants and parental strains of T. *asperellum* was prepared in sterilized water containing 0.5% carboxymethyl cellulose as a sticking agent. The black gram seeds (cultivar VBN 10) were treated with a conidial suspension of mutant and parental *T. asperellum* strains. Treatments with control (seeds soaked in water) were carried out simultaneously. The treated seeds were sown (at 10 seeds per pot) in pots with a sterilized mixture of potting soil (soil, farmyard manure and coir pith) inoculated with *M. phaseolina* (5% w/w) mass-multiplied in sand-maize medium. Five plants per pot were maintained 10 days after sowing. Soil application of *T. asperellum* was done at 10^6 conidia per ml @100 ml per pot, along with 0.01% hexaconazole. Disease incidence was recorded 50 days after sowing. The disease incidence was calculated using the following formula:

PDI = (number of infected plants/total number of plants) *100

RESULTS AND DISCUSSION

Assessment of IC_{50} and minimum inhibitory concentration of hexaconazole against *T. asperellum*

To assess the IC_{50} and minimum inhibitory concentration of hexaconazole against *T. asperellum,* the Rose Bengal agar medium was amended with hexaconazole at different concentrations. The mycelial growth inhibition of *T. asperellum* was measured when its growth was completely covered on the Rose Bengal medium without hexaconazole (control). At 0.05 ppm concentration, the mycelial growth of *T. asperellum* was inhibited by 12% over control, and increasing the concentration to 0.25 ppm resulted in 56% mycelial growth inhibition and complete inhibition at 1 ppm of hexaconazole. Thus, IC_{50} and the minimum inhibitory concentration of hexaconazole against *T. asperellum* were 0.25 and 1 ppm, respectively (Table 1, Figure 1). Similar to the present study, Hu *et al.* (2023) reported that the EC_{50} value of hexaconazole was 0.30 µg/ml (0.3 ppm) against the mycelial growth of *Cladosporium fulvum* causing tomato leaf mold disease. Hexaconazole was found to be inhibitory to higher fungi such as ascomycetes and basidiomycetes, including soil-borne fungi (Yang *et al.*, 2022). It is an effective fungicide for the management of Ganoderma wilt of coconut.

Table 1. Assessment of IC_{50} and minimum inhibitory concentration of hexaconazole against *T. asperellum*

Concentration of hexaconazole (ppm) on Rose Bengal agar medium	Average mycelial growth of *T. asperellum* (mm)	Percent inhibition over control
Control (without hexaconazole)	83.66	
0.05	74.00	11.55
0.1	59.33	29.08
0.25	36.66	56.18
0.5	21.33	74.50
1	1.66	98.02
CD (p≤0.05)	**4.3**	

Values are means of three replications.

Development of hexaconazole-tolerant strains of *T. asperellum*

Several hexaconazole-tolerant strains were developed, and among them, four hexaconazole-tolerant strains, showing promising levels of hexaconazole tolerance and normal growth as the parental strain did, were selected for further studies. To confirm if there is any growth defect of hexaconazole-tolerant mutant strains of *T. asperellum,* their growth pattern was tested by growing them on Rose Bengal and PDA medium and comparing their growth pattern with parental *T. asperellum.* The growth of hexaconazole-tolerant strains was similar to that of the parental (commercial) *T. asperellum*.

The hexaconazole tolerance level was assessed by culturing the hexaconazole-tolerant strains at various concentrations of hexaconazole and compared with parental *T. asperellum*. Four stable hexaconazole-tolerant mutants were selected, and among them, two hexaconazole-tolerant strains, namely, tolerant strains 7 and 18, showed threefold levels of tolerance compared to the parental *T. asperellum* (Table 2, Figure 2). Similar to the present study, a few carbendazim-tolerant *T. asperellum* strains showing enhanced tolerance were generated by induced mutagenesis (Ramangouda *et al.*, 2023).

Assessment of biocontrol potential and plant growth promotion of hexaconazole-tolerant strains

To confirm if the hexaconazole-tolerant strains show any defects in their inhibitory effects against the mycelial growth of the pathogen, they were tested against the mycelial growth of *M. phaseolina* by following the dual culture technique. The results showed that all the hexaconazole-tolerant strains exhibited a similar level of inhibition against the growth of *M. phaseolina* as the parental strain did. *T. asperellum-tolerant* strain T18 showed the maximum level of mycelial growth inhibition by recording 47.78% inhibition (Table 3, Figure 3).

Further, these tolerant strains were assessed for their plant growth-promoting activity by the roll towel method using black gram seeds. Seed treatment with hexaconazole-tolerant strains T18 had good germination and vigour and was comparable with the parental strain. However, there is no significant difference in the vigor index among these strains (Table 4). Earlier studies also indicated that benomyl-tolerant *Trichoderma* strains, developed by induced mutation, were phenotypically different from their parental strain and produced higher levels of antibiotics than their parental strain (Mukherjee *et al.*, 1999). Similarly, carbendazim-tolerance *T. asperellum* showed a similar level of mycoparasitism and plant growth activity as its parental strain did (Ramangouda *et al.*, 2023).

Efficacy of hexaconazole-tolerant strains in the management of dry root rot of black gram

Seeds treated with *T. asperellum* tolerant strain T18, along with 0.01 % hexaconazole, showed significantly the minimum dry root rot disease incidence, resulting in the maximum reduction (80%) of disease incidence over control. Other tolerant strains showed similar levels of biocontrol efficacy in suppressing the dry root rot disease incidence (Table 5). Similar to the present study, the biocontrol activity

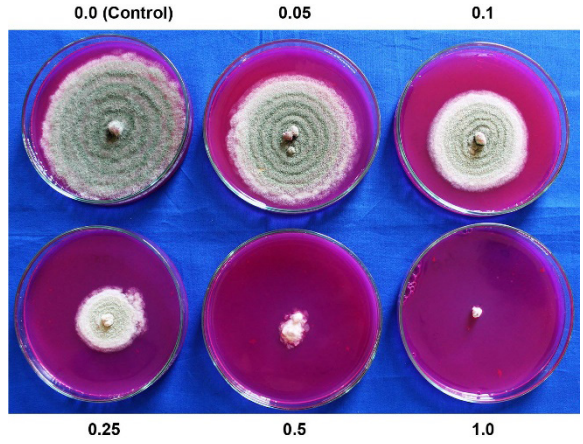


Figure 1. Assessment of IC_{50} and minimum inhibitory concentration of hexaconazole against *T. asperellum*.

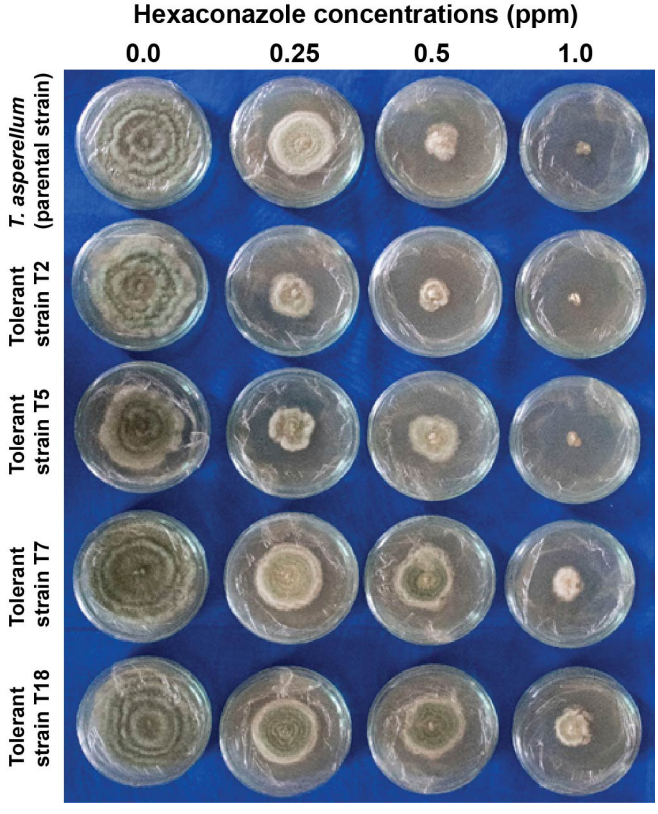


Figure 2. Development of hexaconazole-tolerant strains of *T. asperellum*.

Table 2. Mycelial growth of hexaconazole-tolerant mutant strains on hexaconazole-amended PDA

Strains	Hexaconazole concentration (ppm)			
	0.0	**0.25**	**0.5**	**1.0**
T.asperellum (Parental strain)	90.0	41.67	20.00	4.67
T.asperellum tolerant isolate T2	90.0	34.67	18.00	3.33
T.asperellum tolerant isolate T5	90.0	33.00	24.67	3.67
T.asperellum tolerant isolate T7	90.0	50.00	40.00	9.67
T.asperellum tolerant isolate T18	90.0	52.33	44.67	15.00
CD (P=0.05%)	N/A	5.1	4.2	1.7

Values are means of three replications.

Table 3. *In vitro* inhibitory efficacy of hexaconazole-tolerant mutant of *T. asperellum*

S. No.	***Trichoderma asperellum* isolates**	**Average mycelial growth of *M. phaseolina***	**Percent inhibition %**
1	*T. asperellum* (Parental strain)	52.5	41.67
2	*T. asperellum* tolerant isolate T2	55.5	38.33
3	*T. asperellum* tolerant isolate T5	51.0	43.33
4	*T. asperellum* tolerant isolate T7	51.5	42.78
5	*T. asperellum* tolerant isolate T18	47.0	47.78
6	Control	90.0	0.00
	CD (P=0.05%)	**6.7**	N/A

Values are means of three replications.

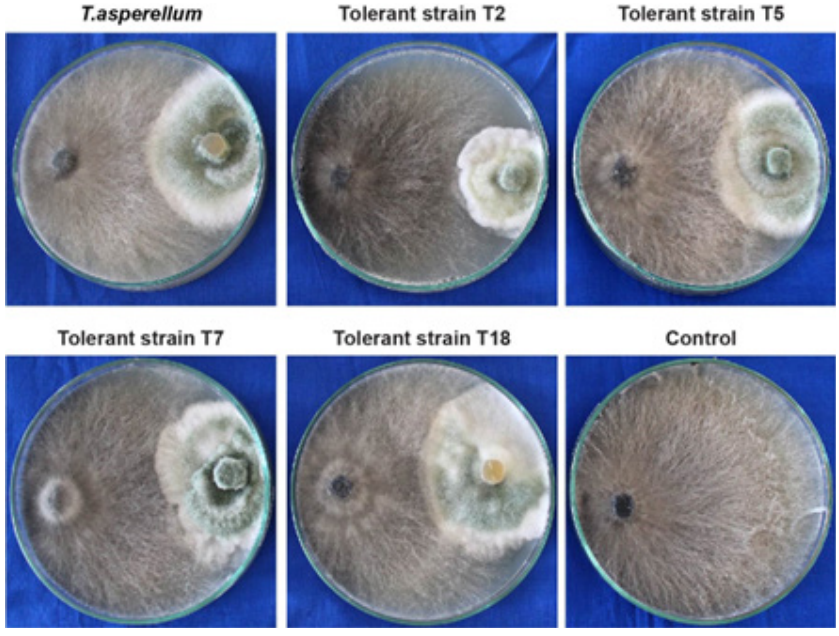


Figure 3. *In vitro* inhibitory efficacy of hexaconazole-tolerant mutant of *T. asperellum.*

of the carbendazim-tolerant strain of *T. asperellum* with a half-dose of carbendazim significantly controlled chickpea dry root rot, resulting in extremely low dry root rot disease incidence (Ramangouda *et al.*, 2023). Benomyl-tolerant *Trichoderma* strains effectively reduced the mycelial growth of *Sclerotium rolfsii* in zinger rhizome (Mukherjee *et al.*, 1999).

Table 4. Effect of hexaconazole-tolerant mutant of *T. asperellum* in growth promotion of black gram

Strains	Germination (%)	Root length (cm)	Shoot length (cm)	Vigor index
T.asperellum (Parental strain)	86.66 (68.91)	9.05	16.32	2203.68
T. asperellum tolerant isolate T2	85.33 (67.81)	8.66	17.60	2238.00
T. asperellum tolerant isolate T5	81.33 (64.61)	6.51	16.75	1882.84
T. asperellum tolerant isolate T7	77.33 (61.71)	6.90	14.70	1802.67
T. asperellum tolerant isolate T18	88.00 (69.91)	10.98	17.72	2524.33
Control	74.66 (59.88)	7.77	15.67	1752.24
CD (P=0.05%)	**NS**	**1.54**	**1.5**	**356.75**

Values are means of three replications. Values in the parentheses are arc sin transformed values.

Table 5. Effect of hexaconazole-tolerant mutant strains of *Trichoderma asperellum* + 0.01 % hexaconazole in the management of dry root rot of black gram

Strains	Disease incidence (%)	% reduction over control
T.asperellum (Parental strain)	25.00 (29.68)	60.00
T. asperellum tolerant strain T2	29.17 (32.47)	53.33
T. asperellum tolerant strain T5	20.83 (26.89)	66.67
T. asperellum tolerant strain T7	25.00 (29.68)	60.00
T. asperellum tolerant strain T18	12.50 (18.79)	80.00
Control (path inoculated)	62.50 (52.66)	-
Control (path uninoculated)	0.00	-
CD (P=0.05%)	**10.506**	**N/A**

Values are means of three replications. Values in the parentheses are arc sin transformed values.

CONCLUSION

The application of hexaconazole-tolerant *T. asperellum* combined with a reduced dose of hexaconazole effectively suppressed the incidence of dry root rot disease in black gram.

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