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Induction and evaluation of benomyl-tolerant mutants of *Trichoderma viride* for biological control of *Botrytis* grey mould of chickpea

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ABSTRACT : Benomyl-tolerance is absent in the natural population of *Trichoderma* spp. used in biocontrol of plant pathogenic fungi. In the present investigation, we have obtained stable benomyl-tolerant mutants of *Trichoderma viride* isolate T-15 for use in integrated biocontrol of *Botrytis* grey mould of chickpea. Some of the mutants are hyper-producers of antifungal substances in culture filtrates, and are equally effective as the wild type strain in disease control potential. The usefulness of these mutants in biocontrol programme has been discussed.

Keywords : *Botrytis cinerea, Trichoderma,* chickpea, biocontrol, benomyl, fungicide tolerance

Grey mould, caused by *Botrytis cinerea* Pers. ex Fr. is a devastating disease of several crop plants worldwide (Maude, 1980). In chickpea it can cause 70-100% yield loss in Northern India, under favourable conditions (Grewal & Laha, 1983). In the absence of resistant cultivars, growers rely heavily on chemical fungicides to combat the disease. Fungicides of the groups dicarboximides (like vinclozolin) and methyl benzimidazole carbamates (MBCs, like carbendazim) are recommended, but the fungicides alone cannot give protection if the climatic conditions favour infection (Haware & McDonald, 1992). Development of resistance of the pathogen to these fungicides is quite common, which reduces the effective period of use. Existence of cross-resistance further aggravates the problem. Application of fungicides combined or alternated with biocontrol agents like *Trichoderma* spp. has been successful in managing *B. cinerea* in several fruit and vegetable crops (Elad, 1994; Elad and Zimand, 1991; Elad *et al.,* 1994; Gullino *et al.,* 1985; Tronsmo, 1991).

Recently, we have isolated a *Trichoderma viride* Pers. ex Fr. (isolate no. T-15) from chickpea rhizosphere which is highly effective against *Botrytis* grey mould (BGM) of chickpea in the growth room (Mukherjee and Haware, 1993) and in the field (Haware *et al.,* 1996). This isolate was originally highly sensitive to vinclozolin and benomyl. In our previous studies, we obtained vinclozolin-tolerant isolates of *T. viride* by selecting on the fungicide amended medium (Mukherjee *et al.,* 1995). But tolerance to benomyl could not be induced using this method, indicating the absence of benomyl tolerance in natural population of this fungus. Integration of benzimidazoles with natural isolates of *Trichoderma* was therefore not possible (Elad, 1994).

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Use of benomyl-tolerant strains of *Trichoderma* spp. has several advantages. Apart from enabling combination with MBC fungicides, benomyl-tolerance can also be used as a selective marker for tracking and monitoring the population of the introduced antagonist over time and space. Several benomyl-tolerant strains (mutants) of *Trichoderma* spp. are also reported to have improved biocontrol potential (Ahmad and Baker, 1988; Papavizas and Lewis, 1983; Papavizas *et al.,* 1982). In the present investigation, we tried to obtain benomyl-tolerant strains of *T. viride* isolate T-15 through chemical mutagenesis and we report here on the antagonistic and biocontrol potential of these mutants on BGM of chickpea.

MATERIALS AND METHODS

Fungal isolates

T. viride isolate no. T-15 was isolated from chickpea rhizosphere (Mukherjee and Haware, 1993). A vinclozolin-tolerant isolate of *T. viride* (isolate no. T-15.4) included for comparison in the growth room was obtained from our previous studies (Mukherjee *et al.,* 1995). *B. cinerea* was taken from ICRISAT culture collection. The cultures were multiplied and maintained on potato dextrose agar (PDA) at 25±1°C.

Induction and isolation of benomyl-tolerant mutants of *T. viride*

For induction of benomyl tolerance, *T. viride* conidia were exposed to N-methyl-n'-nitro-N-nitrosoguanidine (100 mg L^{-1} in 0.05 M Tris buffer pH 6) for 1 h. The conidia were washed thrice in sterile distilled water and plated on PDA containing 10 mg L^{-1} benomyl (E.I. du Pont de Nemours & Co., Wilmington), 500 mg L^{-1} streptomycin and 150 mg L^{-1} rose bengal. The plates were incubated at 25±1°C and the colonies that developed (after 5 d) were isolated, purified and sub-cultured 7 times on PDA without benomyl to test their stability. The stable mutants were grown on PDA with 50 mg L^{-1} benomyl, and the colony diameter was recorded after 7 d. The mutants showing high degree of tolerance to benomyl were selected for further studies.

Antagonism in dual culture

The selected mutants were evaluated for their ability to overgrow and lyse the colonies of *B. cinerea* in dual culture as described previously (Mukherjee *et al.,* 1995).

Antibiosis

Ability of *Trichoderma* mutants to produce antifungal metabolites in liquid culture was studied using poisoned food technique. The antagonist isolates were grown in potato dextrose broth (PDB, pH 6) for 10 d without shaking. The culture filtrate was passed through bacteria-proof filter and amended (10% v/v) with PDA. Plates were centrally inoculated with *B. cinerea* culture and incubated at 25±1°C. Observation on colony diameter was recorded after 4 d and per cent inhibition calculated.

Disease control potential

Ability of the wild type (T-15), vinclozolin-tolerant (T-15.4) and three benomyl-tolerant mutants (T-15.M-3, T-15.M-11 and T.15.M-14) of *T. viride* to suppress BGM of chickpea was evaluated in growth room as described previously (Mukherjee *et al.,* 1995), except that *Trichoderma* was applied at a lower concentration (10^6 conidia ml^{-1}) for better comparison. The spore suspension was sprayed on 10-day old chickpea seedlings of CV H-208. The disease was scored on a 1-9 point scale (1 = no infection and 9 = plants killed) after 10 d.

Experimental design and statistical analysis

All the experiments were carried-out in completely randomised design with 3 replicates, and the statistical analysis was performed using NCSS programme on a personal computer.

RESULTS AND DISCUSSION

Fifteen stable benomyl-tolerant isolates of *T. viride* were obtained after exposing to nitrosoguanidine followed by selection on 10 mg L^{-1} benomyl. The mutants were designated as T-

Table 1. Effect of benomyl (50 mg L^{-1}) on the growth of *Trichoderma viride* isolates (7 d)

Isolates	Colony diameter (mm)[a]
T-15	0
T-15.M-1	59.3
T-15.M-2	48.0
T-15.M-3	65.7
T-15.M-4	51.0
T-15.M-5	68.3
T-15.M-6	31.3
T-15.M-7	31.7
T-15.M-8	51.3
T-15.M-9	44.3
T-15.M-10	48.0
T-15.M-11	58.3
T-15.M-12	44.7
T-15.M-13	50.7
T-15.M-14	74.7
T-15.M-15	47.0
CD ($P \leq 0.05$)	5.7

[a]Mean of 3 replications

15.M-1, T-15.M-2, etc. When grown on 50 mg L^{-1} benomyl, T-15.M-14 attained maximum growth (74.7 mm colony diameter in 7 d). This was followed by T-15.M-5, T-15.M-3, T-15.M-1 and T-15.M-11 (Table 1). These 5 mutants were selected for further evaluation. All the 5 mutants and the wild type isolate were equally effective in antagonising the pathogen in dual culture; all could overgrow and lyse the pathogen in 5 d. However, there was significant difference in the ability of the isolates to produce antifungal metabolites in liquid culture. Four mutants (T-15.M-3, T-15.M-5, T-15.M-11 and T-15.M-14) showed increased ability to suppress the growth of *B. cinerea* through the production of fungitoxic metabolites, while one mutant (T-15.M-1) exhibited reduced potential (Table 2).

In growth room trial on disease control, all the test isolates (one wild type, one vinclozolin-tolerant, and three benomyl-tolerant isolates) were equally effective in suppressing BGM of chickpea (Table 3). The trial was repeated to confirm the results. The disease rating on plants sprayed with *B. cinerea* alone was 6.7 compared to 3.3-4.3 in cases where the plants were sprayed with both *Trichoderma* and *B. cinerea.* The two mutants (T-15.M-1 and T-15.M-5) were not evaluated in the growth room because they sporulated very poorly in PDB.

Induced mutation is one of the most widely used tools to improve antagonistic fungi for biocontrol properties like fungicide tolerance, survival ability, antagonistic and biocontrol poten-

Table 2. Effect of culture filtrate of *Trichoderma viride* isolates on the growth of *Botrytis cinerea* (4 d)

Isolate	Colony diameter[a] (mm)	Inhibition (%)
T-15	29.0	53.2
T-15.M-1	45.0	27.4
T-15.M-3	18.7	69.8
T-15.M-5	23.3	62.4
T-15.M-11	20.7	66.6
T-15.M-14	23.3	62.4
Control	62.0	-
CD ($P \leq 0.05$)	4.5	-

[a]Mean of 3 replications

Table 3. Suppression of Botrytis grey mould through application of *Trichoderma* isolates to chickpea seedlings in growth room (10 d)

Treatments	Disease rating[a]
BC + T-15	4.3
BC + T-15.4	3.3
BC + T-15.M-3	4.0
BC + T-15.M-11	3.7
BC + T-15.M-14	3.7
BC (Control)	6.7
CD ($P \leq 0.05$)	1.4

[a]Mean of 3 replications.

The disease was scored on a 1-9 point scale where 1 = no infection and 9 = plants killed.

Botrytis cinerea (BC) was applied at 4×10^5 conidia ml^{-1}

Trichoderma (T) was applied at 10^6 conidia ml^{-1}.

tial, and ability to colonise plant parts (Baker, 1991; Mukherjee and Mukhopadhyay, 1993; Papavizas, 1987). In the present investigation, we have been able to generate stable benomyl-tolerant mutants of *T. viride* which produce more antifungal substances, and are equally effective as the wild type strain in disease control potential of BGM of chickpea under controlled conditions. After further verification, it will be possible to undertake field trials on an integrated control programme involving MBC fungicides and *T. viride* for the management of BGM. Use of these mutants would also make it possible to monitor the population of the introduced antagonist over time and space, because benomyl tolerance, unlike vinclozolin tolerance is a stable selective marker which is absent in natural population of *Trichoderma.*

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