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Evaluation of four *Trichoderma* spp. for the biological control of Black bundle disease (*Cephalosporium acremonium*) in maize

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ABSTRACT

Four *Trichoderma* species with known biocontrol activity (*T. harzianum, T. hamantum, T. viride* and *T. virens*) were tested for their efficacy against *Cephalosporium acremonium*, a causal organism of Black bundle disease in maize. Interactions between the fungi were assessed *in vitro* to study mutual antagonisms. Among the four bio-agents, *T. hamantum* proved to be promising which showed maximum inhibition (51.3%) followed by *T. virens* (45.2%), *T. harzianum* (44.6%) and *T. viride* (20.3%). *T. hamantum* was also found to be hyperparasite on *C. acremonium* whereas, *T. harzianum* and *T. viride* showed mycelial deformation. The volatile toxicants released by all the *Trichoderma* spp. were inferior in inhibiting the growth of *C. acremonium* when compared to the control as evident by inverted plate technique. The results of *in vivo* experiments confirmed the results from the *in vitro* study and showed that all the four bio-agents significantly reduced the disease incidence of Black bundle disease.

Introduction

Cephalosporium acremonium Corda is an Ascomycetes fungus which is known to cause Black-bundle disease in maize [1, 2]. The disease is common in wet heavy soils in hot regions. When the plant becomes infected it shows wilting like symptoms of the uppermost leaves and brown vascular bundles in the lower portion of the stem. Reddish-purple coloured leaves and stalks, barren stalks, multiple ears per node (more than single ear per node) and excessive tillering are the other symptoms reported [1]. Black bundle disease kills the plants during flowering [1]. Although the disease was discovered long ago [1], no effective management strategies have been developed in managing the Black bundle disease.

Biological control method was used because of various factors like, it is considered to be the best alternative to chemical control methods, being ecofriendly and effective especially against soil borne pathogens [2]. The critical peruse in to the literature showed that *Trichoderma* spp. are proved to be antagonistic in nature and act as biocontrol agents on a broad spectrum of soil borne fungi [4] and various species of *Trichoderma* have provided varying levels of control of a number of important soil borne plant pathogens [5]. *T. harzianum* consistently reduced fungal populations of *Phytophthora capsici* in the plant growth substrate over time [6]. Different *Trichoderma* strains that were used to inoculate pea have shown increased growth of plant biomass and also reduced the disease incidence of *Pythium ultimum* [7]. Two isolates of *Trichoderma viride* and one isolate of *Trichoderma pseudokoningii* degraded up to 80% of the sclerotia of four isolates of *Sclerotium cepivorum* in a silty clay soil, and also degraded up to 60% of the sclerotia in three other soil types [8]. A seed dressing and soil application formulations from the isolates of *T. viride*, *T. virens* and *T. harzianum* were evaluated individually and in combination in pot and field experiments and were found to be superior to other formulations in reducing disease incidence and increasing seed germination and shoot and root lengths in mung bean [9]. Application of *Trichoderma* sp. significantly reduced the number of *Fusarium udum* propagules and wilt incidence [10]. The compatible microbial consortia *of Pseudomonas aeruginosa*, *Trichoderma harzianum* and *Bacillus subtilis* triggered larger defence responses in pea than the microbes alone and provided better protection against *Sclerotinia* rot [11]. *Trichoderma* spp. acted as mycoparasites, producing antibiotics and had enzyme systems capable of attacking a wide range of plant pathogens [12]. *Trichoderma* spp., that are common saprophytic fungi, found in almost any soil and rhizospheric microflora, have been investigated as potential biocontrol agents because of their ability to reduce the incidence of diseases caused by soil borne phytopathogenic fungi [3], although some have been occasionally recorded as plant pathogens [13]. Hence, in the present investigation an effort was made to investigate the efficacy of four *Trichoderma* spp. against *C. acremonium* by conducting *in vitro* and *in vivo* experiments.

Materials and methods

Isolates

Among the four *Trichoderma* species were used in the present study, two species (*T. virens* MTCC-794 and *T. hamantum* MTCC-3113), were procured from the Microbial Type Culture Collection, Institute of Microbial Technology (CSIR), Chandigarh, India. The other two species (*T. harzianum* and *T. viride*) were isolated from the rhizosphere soil of healthy maize plants. *C. acremonium* was isolated from infected maize stalk tissue by culturing on potato-dextrose agar (PDA) medium. All the cultures were maintained on PDA medium and stored at 4 °C until further use.

Dual culture technique

The antagonistic property of each *Trichoderma* sp. against *C. acremonium* was evaluated by the dual culture technique described by Morton and Stroube [14]. A 5-mm diameter mycelial disc from a 7-days-old actively growing culture of all *Trichoderma* spp. and *C. acremonium* were taken from the margin and placed juxtaposed at an equal distance from the periphery. Three replicates were maintained for each isolates.

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Control plates (without a bio-agent) were also maintained in triplicate. All the plates were incubated at 25 °C±1 °C for 7 days. The radial growth was recorded after 7 days of incubation and relative growth was calculated [15] using the following equation;

$I = [(C-T)/C] \times 100$ ---------- {1}

Where, I = inhibition of radial mycelial growth.

C = radial growth measurement of the pathogen of the control plate.

T = radial growth of the pathogen in the presence of the bio-agent.

Scanning Electron Microscopic observation

Scanning Electron microscopic study was carried out at Central Sericulture Research and Training Institute, Mysore, India to study the hyperparasitic mode of action of the *Trichoderma* spp. on *C. acremonium*. The slides were prepared from samples obtained from 14-days-old dual culture plates and sliced into 3 × 3 mm pieces with a thin slice of the gel at the base, and fixed for 2 hrs in 2.5% glutaraldehyde prepared in 0.2 M cacodylate buffer (pH 7.2), post-fixed in 2% osmium tetroxide in the same buffer for 2 hrs and dehydrated in a graded ethanol-acetone series. The dehydration started in 50% ethanol, passed through 70% and 90% ethanol; then two changes each through 100% ethanol, 3:1, 1:1 and 1:3 ethanol-acetone combinations, and thereafter through pure acetone, keeping the tissues for 15 min at each stage. The specimens were then critically dried in a critical point drier (EMS – 850, USA) using CO_2 as transition fluid. The dried samples were mounted on copper stubs using double-sided tape and coated with about 20 nm gold particles in a sputter coater (EMS – 550, USA). Specimens were observed with a JEOL 100 CX-II electron microscope fitted with a scanning attachment (ASID-4D, Japan) at 20 kV.

Volatile toxicity

To evaluate the effect of volatile toxicity of the four *Trichoderma* spp. on colony growth of *C. acremonium*, the inverted plate technique as proposed by Dennis and Webster [16] was followed. The 5-mm diameter mycelial discs of 7-days-old cultures of the *Trichoderma* spp. were placed centrally in a petri dish containing PDA, and incubated at 25 °±1 °C for 48 hrs. After incubation, the lid was removed and replaced by a bottom plate containing a centrally placed 5-mm diameter disc of *C. acremonium*. The dish was sealed with parafilm tape and incubated further without disturbing the plates. Simultaneously, control plates were also maintained containing only *C. acremonium* cultures. All plates were maintained in triplicate and were incubated at 25°±1 °C for 7 days. Radial growth of the mycelium was recorded and percentage inhibition was calculated using equation 1.

In vivo experiment

In vivo pot experiment was conducted to evaluate the efficacy of the four *Trichoderma* spp. on the management of Black bundle disease. Earthenware pots of 20 kg capacity were filled with a mixture of sterilized soil and sand (3:1 ratio). Treated pots contained each of the dual inoculum of *Trichoderma* sp. and *C. acremonium* (CA) (*T.harzianum* + CA, *T. hamantum* + CA, *T.viride* + CA and *T.virens* + CA). Fourteen-days-old dual cultures of each combination were mixed thoroughly in sterile soil-sand before sowing the maize seeds. The control pots contained only the soil-sand as negative control; the *C. acremonium* soil mixture was maintained as positive control. All treatments were maintained in triplicate. Seed of the Black bundle disease susceptible maize variety (Renuka G-25) was obtained from Agriculture Research Station, Arabhavi, Belgaum District, Karnataka State, India. Initially, seeds were sown in the pots at the rate of 6 seeds per pot and after germination thinning was carried to 3 seedlings per pot to reduce crowding in the pots. The pots were fertilized with 1.0 grams of Nitrogen, 1.5 grams of Phosphorus and 0.5 grams of Potash per pot in two doses at different growth phase of the plants. The plants were watered regularly, and were monitored during 90 days for disease symptoms. The disease incidence was calculated by using the following equation.

$$\text{Percentage of disease incidence} = \frac{\text{Total no. of plants showing disease symptoms}}{\text{Total no. of plants observed (sown in pots)}} \times 100$$

---------{2}

Estimation of dry weight

After 90 days of growth, all plants were uprooted, taking care not to damage the roots. The root systems were washed in running tap water until the adhering soil particles were removed. Roots and stems, were separated and oven-dried at 72° C for 48 h. The dry weights of roots and shoots were recorded, separately.

Statistical analysis

The data from all tests/experiments were statistically analysed statistically by using analysis of variance (ANOVA). The Tukey HSD test was used for multiple comparisons. Statistical significance was determined at $p \leq 0.05$.

Results

Dual culture technique

It is clearly evident from the results of dual culture technique that, all the four *Trichoderma* spp. were able to inhibit the growth of *C. acremonium* compared with the control (Figs. 1 and 2). The inhibition was strongest for *T. hamantum* (51.3%) followed by *T. virens* (45.2%), *T. harzianum* (44.64%) and *T. viride* (20.34%). The percentage inhibition of the four bio-agents differed signigicantly ($p \leq 0.05$) from the control. Within the *Trichoderma* treatments, *T. hamantum* differed statistically ($p \leq 0.05$) from the other three bio-agents. At first, a zone of inhibition was observed after 3-4 days of incubation in all the dual-culture plates. After further incubation, only *T. hamantum* and *T. virens* were able to overgrow on the colony of *C. acremonium*, showing hyperparasitic properties (Fig. 2).

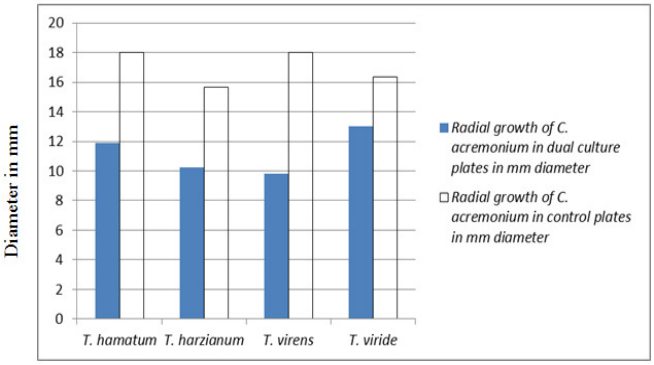
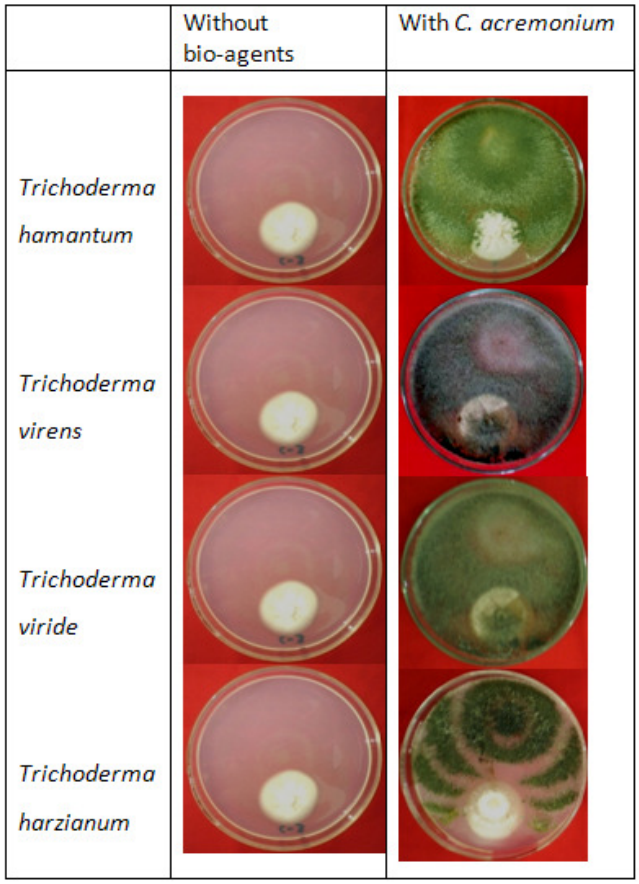


Figure 1: Mycelial growth inhibition of *C. acremonium* in dual cultures with *Trichoderma* species after 7 days of incubation

Electron microscope scans

The electron microscope scans clearly reveal that *T. hamantum* exhibited hyperparasitism by overgrowing the colony of *C. acremonium* and coiling around the pathogen mycelium exhibiting mycoparasitic nature (Figs. 3-A and 3B). *T. harzianum* and *T. viride* did not show any hyperparasitism on *C. acremonium,* but mycelial and conidial deformations were observed, which may be due to the enzymes released by these two bio-agents (Figs. 4-A and 4B).

Figure 2: Effect of four Trichoderma species on the mycelial growth of C. acremonium after 12–14 days of incubation



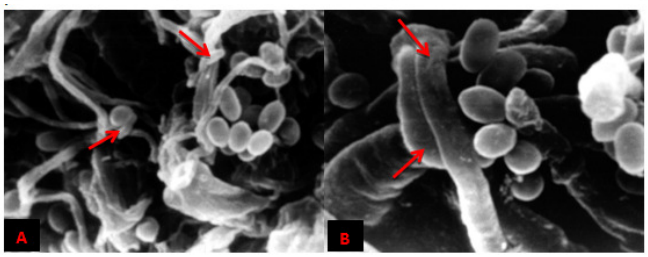


Figure 3: Electron microscopic photographs showing hyphae of Trichoderma hamantum coiling (arrows) around the hyphae of Cephalosporium acremonium. A. at ×3000 magnification, B. at ×5000 magnification

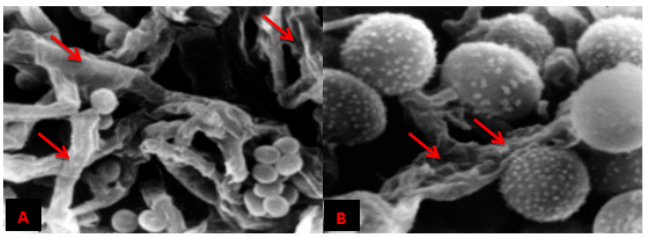


Figure 4: Electron microscopic photographs showing mycelial deformation (arrows) of *Cephalosporium acremonium* interacting with A. *Trichoderma harzianum* at ×3000 magnification, and B. *Trichoderma viride* at ×10,000 magnification

Volatile toxicity test

The results from the volatile toxicity test reveal that compared with the control, the four bio-agents were not significantly ($p \leq 0.05$) inhibited the growth of *C. acremonium*. The inhibition by *T. virens* was only 5.5% and by *T. harzianum* it was 1.3%. Between the four treatments, there was no statistical difference either. The results, suggest that there was no action of volatile organic metabolites on the test pathogen (Figs. 5 and 6).

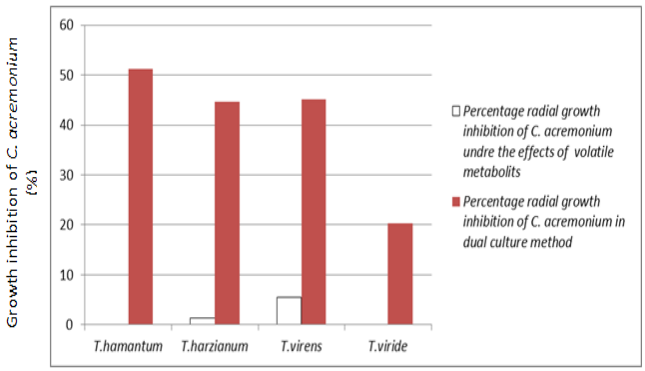


Figure 5: Effect of volatile toxicity of four *Trichoderma* species on growth inhibition of *C. acremonium* using the inverted plate technique compared with the dual culture technique

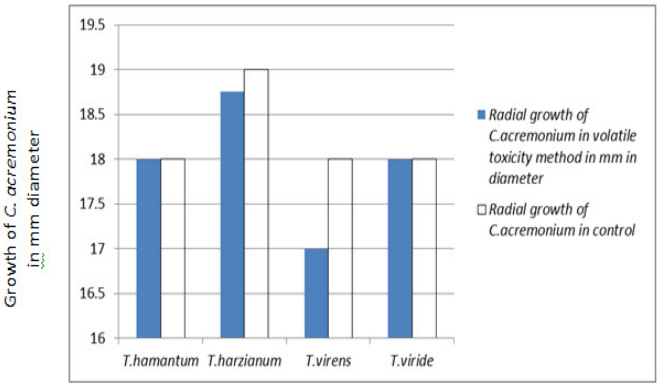


Figure 6: Mycelial growth inhibition of *C. acremonium* in the volatile toxicity test after 7 days of incubation

Table 1. Effect of dual inoculation with different *Trichoderma* species and *C. acremonium* (CA) on % disease incidence, shoot length and biomass dry weight of maize plants.

Treatment	Disease incidence (%)	Shoot length (cm)	Total dry weight of plant (g)
T. hamantum + CA	00.0a*	136.3b	130e
T. harzianum + CA	16.7b	146.4c	77b
T.virens + CA	16.7b	154.3d	87.5c
T.viride + CA	00.0a	132.7b	55a
Control	66.7c	118.0a	117.5d

*Data in the same column, followed by a different letter are statistically different ($p \leq 0.05$), following the Tukey HSD test.

In vivo experiment

The results of the dual inoculation experiment with each *Trichoderma* spp. and *C. acremonium* showed that, the four *Trichoderma* spp. proved to be effective in controlling Black bundle disease. The symptoms of the disease did not become evident until 80 days and symptoms appeared only when the plants attained maturity. Among the four bio-agents investigated, *T. hamantum* and *T. viride* were able to completely control the disease, resulting in better growth of the plants, whereas, the pots inoculated with *T. harzianum* and *T. virens* showed 16.7 % disease incidence (Table 1). This clearly suggests that the *Trichoderma* spp. investigated were effective in reducing the disease incidence significantly ($p \leq 0.05$) when

compared with the control. Furthermore, only *T. hamantum* proved to be significantly ($p \leq 0.05$) effective in increasing total plant biomass when compared with the three other species of *Trichoderma* and the control (Table 1).

Discussion

The present study corroborates earlier reports that *Trichoderma* spp. have the ability to reduce soil borne plant diseases. The results of the dual culture experiment carried out *in vitro* clearly show that *T. hamantum* and *T. viride* were able to inhibit the growth of *C. acremonium,* forming an inhibition zone due to an aversion effect. This was in agreement with reports that *Trichoderma* spp. are known to produce inhibitory enzymes that are considered to be responsible for forming an inhibition zone [3, 17-20]. Different isolates of *Trichoderma* have various strategies for fungal antagonism and also have an indirect effect on plant health. Selection of biocontrol agents as well as understanding the mechanisms involved in the antagonistic effect of *Trichoderma* sp. on the management of plant diseases are important in designing effective and safe biocontrol strategies. Harman [21] reported that *Trichoderma* sp. inhibit the fungal growth by three mechanisms: (1) competition (for space and nutrients), (2) parasitism (deriving nutrients from the host) and (3) antibiosis (production of an inhibition metabolite or antibiotic). From our results it becomes evident that *T. hamantum, T. harzianum* and *T. viride* have shown mycoparasitic properties, as is evident from the electron microscopic scans. The bio-agents were able to overgrow and hyperparasitize on *C. acremonium* by coiling and disruption of hyphal cells. Similar observations were made by Elad *et al.* [22], where they observed coiling and penetration of hyphae of *R. solani* and *S. rolfsii* by *T. hamantum* and *T. harzianum*, and formation of appressorium-like structures and hooks. The disruption of hyphal cells may be due to the enzymatic activity of the bio-agents [23-27]. In the volatile toxicity test no statistically significant ($p \leq 0.05$) reduction in mycelial radial growth of *C. acremonium* was observed in any of the plates when compared with the control, suggesting that the volatile compounds produced by the bio-agents were not effective, though earlier evidence suggests that volatile antibiotics produced by *Trichoderma* sp. inhibited the growth of many fungi [16, 28-30]. This needs to be further validated by conducting experiments to unravel the mechanisms involved, as *C. acremonium* is known to produce certain metabolites [31, 32].

The results from the *in vivo* experiment confirm the result of our *in vitro* experiment, which show reduced disease incidence in the pots inoculated with combinations of each *Trichoderma* species and *C. acremonium.* This confirms earlier reports that, *Trichoderma* spp. reduces the incidence of soil borne plant pathogenic fungi under natural condition and that the inhibitory action of *Trichoderma* spp. against soil borne fungal pathogens may be due to the presence of many kinds of compounds that are released by *Trichoderma* spp. into the rhizosphere, causing interaction with and inducing resistance in plants [3, 18, 33-41]. It was also demonstrated that the gene expression profile of the host plants depends on the *Trichoderma* isolate colonizing the plant [42] and *Trichoderma* treated maize has an average yield increase of approximately 5%, as we have observed in case of *T. hamantum* treated plants, but there are significant varietal differences, with some maize lines giving a neutral or even a negative growth responses [21]. Further work needs to be done to understand the mechanism of inhibitory effect of *Trichoderma* spp. against *C. acremonium in vivo.*

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