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Antifungal potential of *Trichoderma* species against *Macrophomina phaseolina*

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Antifungal potential of different strains of *Trichoderma* species against growth and development of *Macrophomina phaseolina* were assessed in this study. In dual culture all strains of *Trichoderma* showed inhibitory effects on *M. phaseolina* and they were ranked according to the degree of inhibition. Different concentrations of culture filtrates of *Trichoderma* on colony growth of *M. phaseolina* were also studied. It was found that *T. harzianum*-1 showed maximum inhibition of *M. phaseolina* at 40 % concentration of culture filtrate followed by other screened culture filtrates of *Trichoderma* strains. Moreover, the study of non-volatile and volatile metabolites from selected *Trichoderma* species showed that *T. harzianum*-1 released both metabolites in high proportion and showed maximum inhibitory effect on mycelial growth of *M. phaseolina.*

Keywords: Biocontrol, *Trichoderma*, *Macrophominaphaseolina*, antifungal metabolites.

Introduction

Macrophominaphaseolina (Tassi) Goidanich (Syn. *Rhizoctonia bataticola* (Taubenhaus J. Butler) is an important cosmopolitan fungal pathogen and causes several diseases in crop plants. In India as well as in tropical countries plants are severely infected by this pathogen (Malaguti, 1990). The fungal pathogen has wide host range which infected more than 500 plant species (Dhingra and Sinclair, 1978). Fungi of the genus *Trichoderma* are important biocontrol agents (BCAs) of several soil borne phytopathogens (Shanmugaiah*et al.,* 2009).

Several modes of action have been demonstrated by *Trichoderma*. Direct effects of the biocontrol agent over the pathogen include inhibition by antimicrobial compounds (antibiosis), competition for colonization sites and nutrients, degradation of pathogenicity factors and parasitism. Indirect mechanisms include improvement of plant nutrition and damage compensation,

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changes in the root system anatomy, microbial changes in the rhizosphere and activation of plant defence mechanisms. Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. This includes antibiotic compounds and extracellular enzymes that damage plant pathogens, as well as compounds that may enhance *Trichoderma* in maintaining a favorable balance as a portion of the biota (Benítez*et al*., 2004; Harman *et al*., 2004).

The aim of this study was to evaluate the possible role of antifungal metabolites from *Trichoderma* on growth and development of *M. phaseolina*.

Materials and methods

Trichoderma-M.phaseolina interaction

In vitro screening of *Trichoderma*species for their antagonistic potential was done by inoculating 5 mm agar block of *Trichoderma* against *M. phaseolina* on PDA medium, 3 cm apart from each other. The inoculated plates were incubated at 25 ± 2^0C and the observations were made after 6 days. The assessment of the colony interaction in dual culture was done by following the method described by Upadhyay and Rai (1987). The parameter used for the assessment was percent inhibition of radial growth of the pathogens by using the formula: 100 × (R1 – R2)/R1 (Fokkema, 1976), where R1 denotes the radial growth of the pathogen towards the opposite side and R2 denotes the radial growth of the pathogen towards the antagonist.

Effect of culture filtrates of the Trichoderma on M. phaseolina

The selected *Trichoderma* spp. and *M. phaseolina* were grown on PDA medium in Petri dishes at 25 ± 2^0C for 4 days. Two equal size blocks (5 mm each) of *Trichoderma* species, cut from the actively growing margins of 4 day old cultures, were inoculated separately into 250 ml Erlenmeyer flasks each containing 100 ml sterilized Potato Dextrose broth in triplicates. After 10 days of incubation at 25 ± 2^0C, the static culture were filtered firstly through Whatman filter paper number 44 and finally through Seitz filter (G 4) attached to vacuum pump to obtain cell free culture filtrates. Two, four and eight ml culture filtrates of each *Trichoderma* spp. were poured into empty sterilized plates in three replicates separately which was immediately followed by pouring 18, 16 and 12 ml of autoclaved and cooled PDA medium, so as to make the final concentrations of the culture filtrates 10, 20 and 40 %, respectively. The control set was prepared by using sterilized distilled water

mixed in the same ratio in the medium. Five mm agar blocks of actively growing colonies from 5 days old culture of *M. phaseolina* were cut from the margin of the colony and inoculated at the center of Petri-plate separately containing PDA medium and the culture filtrate. The control set was made by pouring 20 ml PDA medium only in sterilized Petri-plates. The inoculated Petri-plates were incubated at 25±2^{o}C and measurement of the radial colony growth was done after 4 days of incubation. The percent inhibition in the radial growth of the colony was calculated by the following formula:

Per cent growth inhibition = (C - T) / C × 100, where C = Growth in control and T= Growth in treatment.

Bioassay of antifungal metabolites of Trichoderma

Non-volatile metabolites on the growth of M. phaseolina

Effect of non-volatile metabolites on the radial growth of *M. Phaseolina* was assayed by following the well-in-agar method. Monax flasks (500 ml) containing 100ml of Wendling's medium (pH 4.0) were inoculated with 5 mm discs cut from the margin of vigorously growing culture of selected *Tichoderma* species. The flasks were incubated in a shaker (150 rpm) at 25^{o}C. After 6 days of incubation, the samples of culture liquid were filtered through Whatman number 1 paper to remove hyphal fragments. The filtrate was centrifuged at 12000 rpm for 15 minutes to remove spores. 20µl of these culture filtrates were placed in wells made on the four corners of PDA plate. In control set, wells made on PDA plate were filled with distilled water. A 5 mm disc cut from the margins of 3-day old cultures of *M. phaseolina* was then placed in center of the Petri plate. The plates were incubated at 25^{o}C for 24 h. The test plates and control plates were set up in triplicate. The colony diameter of *M. phaseolina* was measured after 2, 3 and 4 days of incubation and compared to that on the control plates. The per cent inhibition of the growth of *M. phaseolina*was calculated by using formula: $(C_2-C_1)/ C_2\times100$ (Edington *et al.,* 1971) where, C_2 means growth of *M.phaseolina* in control and C_1 means growth of *M. phaseolina* in treatment.

Volatile metabolites on the growth of M. phaseolina

Production of volatile metabolites was assayed by following the method of Dennis and Webster (1971b) as described by Eziashi *et al*. (2006). The selected *Trichoderma* strains were grown on PDA medium in Petri dishes for 6 days at 25±2^{0}C. After this, the lid of each dish was replaced by a bottom

containing PDA medium inoculated with *M.phaseolina*. The two dishes were taped together with adhesive tape. The lids of control plates, which had not been inoculated with any of the *Trichoderma* strain, were also replaced in the same way. The test plates and control plates were set up in triplicate. The colony diameter of *M. phaseolina* was measured after 2, 3 and 4 days of incubation. The per cent inhibition of the growth of *M. phaseolina* was calculated by using formula as described earlier.

Results

The culture of all eleven *Trichoderma* strains and the pathogen were maintained on PDA at 25±2^{o}C. Results from the antagonist-pathogen interaction showed that all the *Trichoderma* strains taken were able to inhibit the growth of *M. phaseolina* and possessed varied degree of zone of inhibition (Table 1). These *Trichoderma* strains were ranked according to degree of interactions (Siddiqui, 2001). *Trichoderma harzianum*-1 firstly produced zone of inhibition but later overgrew on the pathogen *M. phaseolina* and showed strong mycoparasitism (Fig. 1b). In comparison to other *Trichoderma* strains studied during present investigation. Interestingly in case of *T.virens*-2 and *M. phaseolina* interaction, a clear cut zone of inhibition was produced and no further growth of both the colonies was observed (Fig. 1a).

Table 2 showed the effect of different concentrations of culture filtrates of *Trichoderma* strains on the per cent inhibition of radial growth of *M. phaseolina*. Three concentrations of the culture filtrates such as 10 %, 20 % and 40 % were used in the study. The level of inhibition in the radial growth of *M. phaseolina* varied at each concentrations of culture filtrates of all the *Trichoderma* used. Maximum inhibition of radial growth of *M. phaseolina* by all strains was found at 40 per cent concentration of the culture filtrate as compared to 10 and 20 per cent concentrations of the culture filtrates. It was evident from the observation that maximum per cent of inhibition of *M. phaseolina* was found at 40 per cent concentration of *T. harzianum*-1 (42.2%) which was followed by *T .virens*-2 (39.4%).

Non-volatile metabolite test showed that all the selected strains of *Trichoderma* were able to inhibit the growth of *M. phaseolina*. The per cent inhibition varied from 22 to 76% (Fig.2a). Figure 2a showed that mycelial growth of *M. phaseolina* was significantly inhibited by non-volatiles released by *T. harzianum*-1 (76%), while least inhibition by *T. viride*-12 (22%).

Effect of volatile metabolites on the mycelial growth of *M. phaseolina* is presented in Fig2b. All the selected strains of *Trichoderma* inhibited the growth of *M. phaseolina.* The inhibition varied from 13 to 74 %. It indicated that mycelial growth of *M. phaseolina* was maximally inhibited by the volatile

metabolites released by *T. harzianum*-1 (74%) than the other strains used in the study (Fig 2b).

Table 1. Colony interaction in between *Trichoderma* and *M. phaseolina*

***Trichoderma* strains**	**Inhbition %**	**Zone of inhibition (mm)/ Degree of interaction***
T. harzianum-1	74.8	B
T. harzianum-2	66.3	A
T. virens-56	58.5	E
T. virens-2	68.3	D
T. atroviride	38.9	E
T. pseudokonningii-3	42.6	E
T. pseudokonningii-6	62.8	E
T. hamatum	28.2	C
T. konningii-4	62.1	E
T. konningii-7	57.2	E
T. viride-12	64.6	E

*A. A zone of inhibition was produced and no further growth was observed.
B. A zone of inhibition was produced. *T.harzianum*-1 later overgrew the zone of inhibition, and colonies of both organisms intermingled.
C. Growth of *T. hamatum* inhibited but later overgrew and met the colony of *M. phaseolina*. No further growth was observed.
D. Colonies of *T. virens*-2 and *M. phaseolina* met each other. No further growth was observed.
E. Colonies of *Trichoderma* strains and *M. phaseolina* intermingled.

Table 2. Effect of culture filtrates of the *Trichoderma* on *M. phaseolina*

***Trichoderma* strains**	**Concentrations (%)**		
	10	20	40
T. harzianum-1	32.4 ± 1.1	36.2 ± 1.2	42.2± .35
T. harzianum-2	28.6 ± .73	30.1 ± .70	33.5 ± .88
T. virens-56	21.2 ± .81	24.8 ± 1.4	29.3 ± 1.0
T. virens-2	30.2 ± 1.2	34.5 ± 1.6	39.4 ± 1.3
T. atroviride	18.4 ± .60	21.5± .55	25.8± .87
T. pseudokonningii-3	19.2± .20	22.3 ± .78	26.4± 1.5
T. pseudokonningii-6	24.5± 1.3	27.2± 1.3	30.8 ± 1.6
T. hamatum	10.3 ± .47	16.1 ± .62	20.5 ± .96
T. konningii-4	22.8 ± .85	26.2± .66	30.2 ± 1.5
T. konningii-7	20.7± .92	22.6 ± 1.1	27.2 ± 1.2
T. viride-12	26.4± 1.2	28.6±1.3	32.3±.92

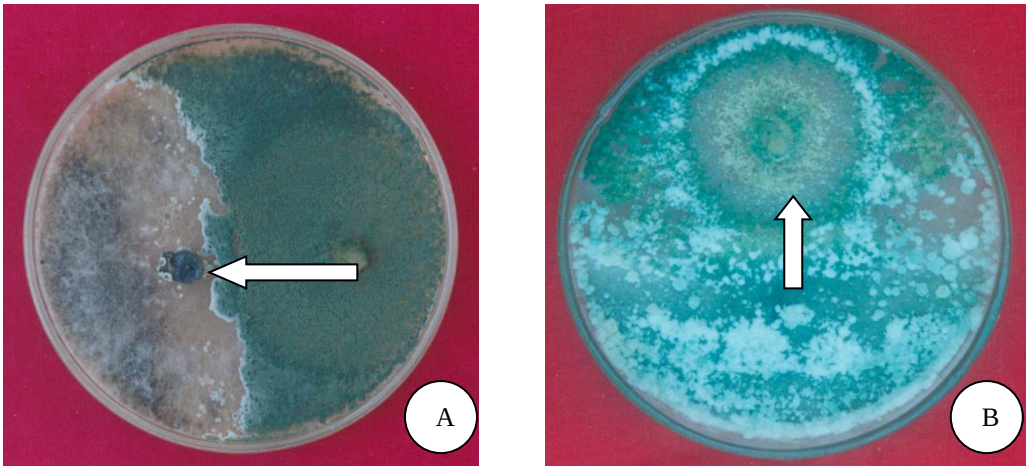

Fig.1A. Colony interaction in between *T. virens*-2 and *M. phaseolina* on agar plate, arrow showing the clear zone indicating presence of non-volatile metabolite released by *T.virens*-21B. *T. harzianum*-1 mycoparasitic over *M. phaseolina*

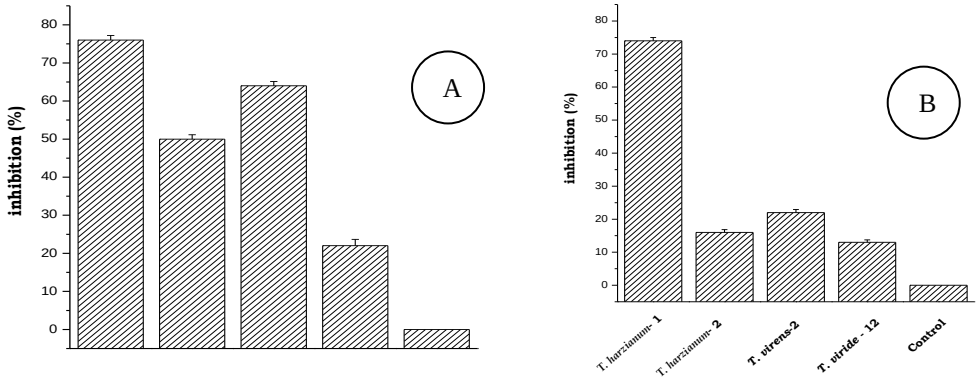

Fig. 2A. Growth of *M. phaseolina* in the presence of non-volatile released by *Trichoderma* 2B. Growth of *M. phaseolina* in the presence of volatile released by *Trichoderma*

Discussion

It is noticeable that a microbial biocontrol agent offers harmless to the animals and human beings, cheaper than chemicals and highly effective. There is no risk of the pathogens develop resistance, fungicide residues in food and ground water (Rajendiran *et al*., 2010). Regarding *M. phaseolina*, a number of reports demonstrated that some fungi, in particular *Trichoderma* spp. could be effectively used for the suppression of this pathogen (Aly*et al.*, 2001, 2007).

The marked inhibitory effect of different *Trichoderma* strains on the growth and development of *M. phaseolina* can be seen by the antagonist–pathogen interaction performed in dual culture test. Two component screening

(dual culture) is exclusively related to interaction studies and potential antagonists are typically ranked according to their ability to inhibit the growth of the pathogen expressed by an inhibition zone (Skidmore and Dikinson 1976). From the result of *in vitro* experiment, it is evident that eleven different strains of *Trichoderma* species were screened against *M. phaseolina*, possessed varying degree of inhibition of radial colony growth. Only *T. harzianum*-1, *T. virens*-2 and *T. viride*-12 were found more effective in comparison to other strains (Table 1). Observing the zone of inhibition at the point of contact of pathogen and the antagonist and measuring their colony diameter after inoculation, served as an indicator of their *in vitro*biocontrol activity (Anand and Reddy, 2009).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 antibiotics, cell wall degrading enzymes and competition for nutrients and space (Upadhyay and Rai, 1987; El-Katatny, 2001).

Culture filtrates have been used in the present study to demonstrate the possible presence and role of fungal metabolites in the process of antagonist behavior of *Trichoderma* species (El-Hasan*et al.,* 2008). Three concentrations of culture filtrates such as 10 per cent, 20 per cent and 40 per cent were used in this study. It was evident from the observation that maximum % of inhibition of *M. phaseolina* was found at 40 per cent concentration of *T. harzianum*-1(42.2%) which was followed by *T. virens*-2 (Table. 2). Inhibition of radial growth of *M. phaseolina* by all strains was found to be maximum at 40 per cent concentration as compared to 10 and 20 per cent concentration of the culture filtrates. It was reported that production of metabolites from different *Trichoderma* strains depend on ecological factors, and so the strains show varying effect on pathogens (Papavizas, 1985). Metabolites produced from *T. viride* and *T. polysporum* reduced the growth of *Ceratocystisparadoxa*, the causal agent of black seed rot in oil palm (Eziashi*et al.,* 2006). *Trichoderma* species produces both volatile and non-volatile metabolites that adversely affect growth of different fungi (Dennis and Webster, 1971a, 1971b; Horvath *et al.,* 1995). Biological activity of antagonist fungi and bacteria may partially be associated with production of antibiotics (Etabarian *et al.,* 2000). On the basis of above two screening method, only 3 *Trichoderma* species that were found effective were selected for non-volatile and volatile metabolite test. Non-Volatile antibiotic test showed that all the selected strains of *Trichoderma* inhibited the growth of *M. phaseolina*. The mycelial growth of *M. phaseolina* was significantly inhibited by *T. harzianum*-1(76%) while least inhibition was recorded with *T.viride*-12 (Fig. 2A). Volatile antibiotic test showed that all the selected strains of *Trichoderma* except the *T. viride*-12 inhibited the growth of

M. phaseolina(Fig. 2B). The conclusion of this study suggest, firstly, that *T. harzianum*-1produces non-volatile and volatile compounds both in high proportions and secondly, that such compounds may play role in the inhibitory effects observed on colony growth of *M. phaseolina*. Our result also explains that significant success in bio-control is achieved under *in vitro* conditions.

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