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IN *Vitro* SCREENING OF *Trichoderma* SPECIES AGAINST *Macrophomina phaseolina* (TASSI) GOIDANICH FOR ANTAGONISM

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ABSTRACT

In *Vitro* study of antagonism was performed by dual culture method between the test pathogen, *Macrophomina phaseolina* and twelve strains of six *Trichoderma* species. Maximum inhibition of radial colony growth of *M. phaseolina* was observed due to *T. harzianum* IVRI (88.8%). The least effective antagonist for inhibition of radial colony growth of the pathogen was recorded due to *T. virens* 3067 (14.2%). Based on the results obtained for the colony interaction between different strains of *Trichoderma* species and the test pathogen, five potent strains of *Trichoderma* species were selected for further in *vitro* study. These were, *T. harzianum* IVRI, *T. harzianum* BHU, *T. viride* 1, *T. pseudokoningii* NBRI and *T. virens* BHU. The effect of different concentrations of culture filtrates of the different strains of *Trichoderma* species on per cent inhibition of radial growth of *M. phaseolina* was performed. Maximum inhibition of radial growth of *M. phaseolina* was observed in case of *T. harzianum* IVRI (42.2%), while minimum inhibition of radial growth was observed by *T. virens* BHU (31.2%) at 40% concentration.

KEYWORDS : *In vitro* Screening, Culture filtrate, *Trichoderma*, *Macrophomina phaseolina*, Antagonism

The species of genus *Trichoderma* have been reported as most potential biocontrol agents against several soil-borne plant pathogens (Lewis and Papavizas, 1991; Haran et al., 1996a; Haran et al., 1996b; Elad, 2000; Hermosa et al., 2000; Joshi et al., 2010; Hermosa et al., 2012) due to their ability to successfully antagonize other fungi. Establishment of the *Trichoderma* and other biocontrol agents in the soil ecosystem has greatly affected by numerous biotic (nature of the target organism and of the host plant, presence of predators, parasites or antagonistic microorganisms among the resident micro flora) and abiotic (nature of the soil or substrate, humidity, availability of nutrients, temperature, radiations, salinity and pH) factors (Dandurand and Knudsen, 1993; Eastburn and Butler, 1988a, b; Knudsen and Bin, 1990). There are several mechanisms involved in antagonism of *Trichoderma* species namely antibiosis, enzyme secretion, substrate competition, hyphal interactions and mycoparasitism (Haran et al., 1996b). In order to solve the national and global problems of environmental hazards due to application of chemicals for disease control, antagonistic microbes have been considered as prospective agents for the purpose (Cook, 1985). Chemicals are necessary for control of different diseases but its adverse effect on human and animal health, environmental contamination, phytotoxicity, development of resistance against pathogens, and their high cost (Mulder, 1979; Mukherjee and Garg, 1983) make their application difficult to be continued in future.

Pesticides were originally based on toxic heavy metals such as arsenic, mercury, lead or copper. Modern pesticides being organic compounds, with a high degree of specificity towards their target organism, also exert effects on non-target beneficial organisms and consequently may be hazardous threat to the environment. It is also possible that long-term effects of these compounds might be subtly detrimental to soil fertility. Moreover, the practical usefulness of these measures in the tropics is rather doubtful, as it is being coherent to the development of resistant mutants of the pathogen concomitant with the prevalence of new pathological races. These fears have led biotechnologists to examine alternatives to chemical pesticides as a means of controlling agricultural pests. A relative alternative for such environmental pollution is to include integration of biological control for control of mites, nematodes, plant pathogens and weeds (Tauber and Baker, 1988). Therefore, biological control of plant diseases has received significant attention, since it promises to offer a more sustainable food supply. Moreover, a successful biological management strategy of a crop disease can offer a marketable products at considerably lower cost compared to conventional measures (Chung, 1994).

The pathogen *Macrophomina phaseolina* (Tassi) Goidanich (Syn. *Rhizoctonia bataticola* (Taubenhaus E. J. Butler) is an important root and foliar disease pathogen and responsible for root rot disease of blackgram. The fungus can infect the root and lower stem over 500 plant species in

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the arid and semi-arid areas of the world (Dhingra and Sinclair, 1978). The pathogen survives as microsclerotia in the soil and on infected plant debris. The microsclerotia serve as the source of primary inoculum and have been found to persist within the soil up to 3 years under adverse conditions such as low soil nutrient levels and temperature above 30°C. In the present study, twelve strains of six *Trichoderma* species were used as antagonists to screen out the effective antagonistic strains against *Macrophomina phaseolina* (Tassi) Goidanich, the causal agent of root rot disease of blackgram.

MATERIALS AND METHODS

Source of the Pathogen

A virulent strain of *Macrophomina phaseolina* (Tassi) Goidanich was obtained from the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. The culture was maintained on Potato-Dextrose Agar medium at 25±2°C by regular subculturings.

Source of the *Trichoderma* species

The pure culture of different strains of *Trichoderma* species were obtained from Laboratory of Applied Mycology and Plant Pathology, Department of Botany, Banaras Hindu University, Varanasi where the cultures were maintained from the collection centres of Institute of Microbial Technology (Chandigarh), National Botanical Research Institute (Lucknow), Indian Agricultural Research Institute (New Delhi), Indian Vegetable Research Institute (Varanasi). Local species/strains of *Trichoderma* were isolated from soils of various locations from and around Banaras Hindu University Campus, Varanasi, on the *Trichoderma* Selective Medium. The cultures were maintained on PDA by periodically subculturing and were stored in a refrigerator at 4°C.

Performance of Pathogencity Test

Seed of the susceptible variety of blackgram (T 9) was surface sterilized with 0.1% NaOCl solution for 1 min, washed thoroughly with sterilized distilled water and sown in earthenware pots (15 × 25 cm size) containing sterilized garden soil. The pathogencity test of *M. phaseolina* was performed in pure sand inoculum of the test pathogen mixed with sterilized garden soil in the ratio of 1:9. The sand inoculum of the test pathogen was prepared in sand + 3% maize meal (Upadhyay and Rai, 1987). The seedlings (12-day old) were transferred to inoculum of soil mixture. The moisture level of the soil was maintained at 15-20%. The pots were kept in polyhouse and process of disease development was observed. Infected roots were collected, washed and cut into small pieces. They were treated with 0.1% NaOCl for 1 min, rewashed with sterilized distilled water and transferred on Potato Dextrose Agar (PDA) in slants. After 7 days, the isolated organism was examined, compared with the original stock cultures and their identity was confirmed.

***In vitro* screening of *Trichoderma* species against *Macrophomina phaseolina* for antagonism**

Colony Interactions

The colony interaction was studied in dual culture following the method described by Upadhyay and Rai (1987). Five mm agar blocks of freshly grown culture of *M. phaseolina* from the margin of the colony were placed separately with different strains of *Trichoderma* species over PDA medium 3 cm apart in paired combinations. The inoculated plates were incubated at 25±2°C for 6 days. The control sets were single or dually inoculated cultures of the same fungus. The colony interactions were assayed as per cent inhibition of the radial growth of the pathogen following the formula: R_1 R_2/ R_1 100 (Fokkema, 1976), where, R_1 denotes the radial growth of the pathogen towards the opposite side and R_2 denotes the radial growth of the pathogen towards the test antagonist.

Inhibitory effect of the culture filtrates of selected ***Trichoderma* species on radial growth of *M. phaseolina***

Based on the results obtained in colony interaction between different strains of *Trichoderma* species and the test pathogen, five potent strains of *Trichoderma* species were selected for further study. These were as follows: *T. harzianum* IVRI, *T. harzianum* BHU, *T. viride* 1, *T. pseudokoningii* NBRI and *T. virens* BHU.

The selected antagonists and the test pathogen were grown on PDA medium in Petri dishes at 25 2°C for 4 days. Two blocks of the equal size (5 mm each) cut from the margins of actively growing cultures of *Trichoderma* species, were inoculated separately into 250 ml Erlenmeyer flask each containing 100 ml sterilized potato dextrose broth in triplicate. The flasks were then incubated at 25 2°C for 10 days after which the culture filtrates were filtered through Whatman filter paper no. 44 and finally through a Seitz filter (G 4) attached to vacuum pump to obtain filter sterilized cell free culture filtrates.

Two, four and eight ml culture filtrates of each *Trichoderma* species were poured into empty sterilized plates in three replicates which was immediately followed by pouring 18, 16 and 12 ml of autoclaved and cooled PDA medium, so as to make the final concentration of culture filtrates 10, 20 and 40%, respectively. Five mm agar blocks of actively growing 5-day old culture of the test pathogen were cut from the margin and inoculated at the center of Petri-plate separately containing potato dextrose agar (PDA) medium and the culture filtrate of *Trichoderma* species. The control set was made by pouring 20 ml PDA medium only in sterilized Petri plates. The inoculated Petri plates were incubated at 25°C and measurement of the radial colony growth was done after 4 days of incubation. The percent inhibition in the radial colony growth was calculated by using the formula: Per cent growth inhibition = C T /C ×100, where, C = Radial growth in control set; T = Radial growth in treated set.

RESULTS

Colony interactions

The results of the colony interaction between the test pathogens and different strains of *Trichoderma* species is presented in Table 1. Twelve strains of six *Trichoderma* species were screened for their antagonistic activity against *M. phaseolina* in vitro by dual culture technique (Figures 1).

Maximum inhibition of radial growth of *M. phaseolina* was observed due to *T. harzianum* IVRI (88.8%) followed by *T. harzianum* BHU (78.5) and *T. viride* 1 (76.3%). The least effective antagonist for inhibition of radial growth of the pathogen was recorded due to *T. viride* 1433 (19.0%) and *T. virens* 3067 (14.2%). Other strains, except few, were also effective in inhibiting the radial growth of *M. phaseolina* but less efficiently as compared to the above three strains (Table 1). The mean difference in per cent inhibition of mycelial growth between the antagonists and pathogen were found statistically significant (P = 0.05).

Based on the results obtained for the colony interaction between different strains of *Trichoderma* species and the test pathogen, five potent strains of *Trichoderma* species were selected for further study. These were as follows: *T. harzianum* IVRI, *T. harzianum* BHU, *T. viride* 1, *T. pseudokoningii* NBRI and *T. virens* BHU.

Effect of Culture Filtrates

The effect of different concentrations of culture filtrates of the above selected strains of *Trichoderma* species on per cent inhibition of radial growth of *M. phaseolina* was presented in Table 2. Three concentrations

Table 1 : In *vitro* screening of *Trichoderma* species against test pathogen (*Macrophomina phaseolina*)

Antagonists	Test Pathogen (% inhibition)* (*Macrophomina phaseolina*)
T. harzianum BHU	78.5± 0.51
T. harzianum NBRI	63.0± 0.41
T. harzianum IVRI	**88.8± 0.63**
T. viride 2109	59.8± 0.52
T. viride 1433	19.0± 0.61
T. viride 1	76.3± 0.55
T. koningi NBRI	52.3± 0.64
T. pseudokoningi NBRI	76.0± 0.57
T. virens BHU	71.0± 0.61
T. virens 3067	14.2± 0.33
T. virens 2194	36.6± 0.48
T. atroviride BHU	68.3± 0.79

Table 2. Effect of Culture Filtrate of Selected Strains of *Trichoderma* Species on the Per Cent Inhibition of Radial Growth of *Macrophomina phaseolina*

***Trichoderma* species**	***Macrophomina phaseolina* (Concentration in per cent*)**		
	10	**20**	**40**
T. harzianum IVRI	32.1± 0.1	34.8± 0.5	42.2±0.1
T. harzianum BHU	30.2± 0.5	34.6± 0.1	40.3± 0.4
T. viride 1	23.4± 0.5	30.6± 0.2	37.6± 0.1
T. pseudokoningi NBRI	22.6± 0.2	28.2± 0.1	34.1± 0.5
T. virens BHU	20.4± 0.5	26.3±0.3	31.2± 0.2

*Values are average of three replicates ± SEM

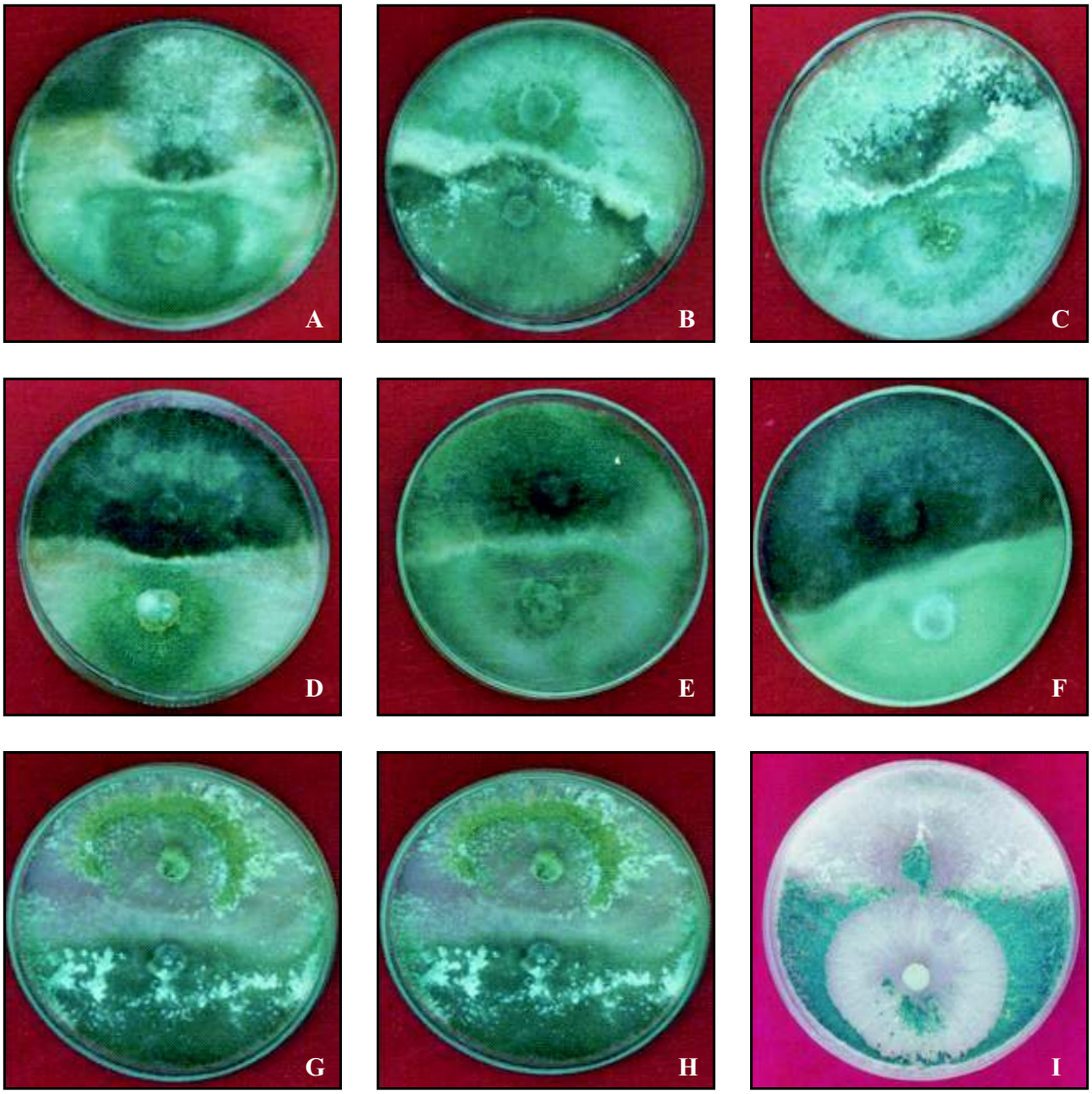

Figure 1. In *vitro* Screening of Different Strains of *Trichoderma* Species Against *Macrophomina phaseolina*

A. *T. harzianum* NBRI B. *T. virens* BHU C. *T. harzianum* BHU
D. *T. pseudokoningii* NBRI E. *T. harzianum* IVRI F. *T. koningii*
G. *T. viride* 1 H. *T. virens* 3067 I. *T. atroviride* BHU

of culture filtrates such as 10%, 20% and 40% were used in the present study. The level of inhibition in radial growth of the test pathogen was increased with increasing the concentrations of the culture filtrate. Maximum inhibition in radial colony growth of *M. phaseolina* was observed at 40% concentration in case of *T. harzianum* IVRI (42.2%) which was followed by *T. harzianum* BHU (40.3%) and T. viride 1 (37.6%). While, the least effective antagonist was observed due to *T. virens* BHU.

DISCUSSION

In in *vitro* experiment, all the strains of *Trichoderma* species, except *T. viride* 1433 and *T. virens* 3067, showed varied degree of inhibition on the radial growth of the *M. phaseolina*. The maximum inhibition of the growth of *M. phaseolina* was observed due to *T. harzianum* IVRI (Table 1). The in vitro antagonistic effect of *Trichoderma* species was reported long back (Dennis and Wells et al., 1972; Chet et al., 1978).

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 and competition for nutrients and space as observed by Upadhyay and Rai, 1987. The growth inhibition of a fungus in vitro by another cannot be expected to be same in field soil because of different ecological factors. However, In *vitro* studies might indicate the potentiality of antagonism of a fungus in soil. Such studies are important for screening the effective antagonists against soil-borne pathogens (Bell et al., 1982).

The effect of culture filtrates of the potent strains of *Trichoderma* species showed varied degree of inhibitory effect on *M. phaseolina* depending on their concentration and toxicity produced by them. In present study maximum inhibition in radial colony growth of *M. phaseolina* was recorded with *T. harzianum* IVRI at all the tested concentration, therefore, this strain can be used to control the pathogen after testing in *vivo* experiment.

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