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In-Vitro Efficacy of *Trichoderma viride* Against *Sclerotium rolfsii* and *Macrophomina phaseolina*

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Abstract

The fungal pathogen causes serious widespread losses to agricultural crops worldwide. Therefore, economy of countries may worsen especially of developing countries. In addition, harmful chemical pesticides which are being used today for increasing crop production creates very serious health hazardous problems to human beings and ecosystem as a whole. The antagonistic potential of *Trichoderma* species which has been long known to control various soil-borne fungal pathogens in biological way may be utilized. The faster growth rates with which it competes with fungal pathogen mainly brings upon their antagonistic characteristics. An investigation was carried out in laboratory condition towards biological efficacy of *T. viride* on potato dextrose agar (PDA) medium for the bio-control of soil-borne plant pathogens *Sclerotium rolfsii* and *Macrophomina phaseolina* in *in-vitro* condition. The dual culture technique was followed in which *T. viride* showed significant antifungal activities towards both the pathogens. *T. viride* significantly inhibited the mycelial radial growth of *S. rolfsii* by 75% and *M. phaseolina* by 71.42%. The results showed variable mycelial growth rate for all fungal isolates which was determined after 6 days of incubation in which *T. viride* showed minimum of 4.00 days to completely cover the petri-plates and *S. rolfsii* showed 4.33 days whereas *M. phaseolina* showed 6.33 days. Thus, *T. viride* showed encouraging results regarding their biopesticidal and biofungicidal potential against plant pathogens which may be endorsed to substitute harmful chemical supplements that exists in modern day agricultural practices.

Keywords: antifungal, biocontrol, dual culture, mycelial growth, potato dextrose medium, soil-borne pathogen

Introduction

Sclerotium rolfsii (Sacc.) is considered to be ruinous against several crops worldwide with an extensive host range, which includes more than 500 species in 100 families worldwide (Agrios, 2005; Cilliers *et al.,* 2000). The pathogen *S. rolfsii* may cause damping-off or stem rot on host plant with the help of sclerotial germination, which may measure 1-3 mm with mustard-like appearance formed on surfaces of the affected plant parts (Koike, 2004). The pathogen may exploit host plant internally as well as externally for their growth with white cottony-like mycelium formation. When this fungus first starts demonstrating its symptoms in soil then eradication becomes nearly impossible even with the help of chemical fungicides. Besides, unavailing use of chemicals it may prove ineffective and costly to growers.

Another important pathogen is *Macrophomina phaseolina* (Tassi.) Goid. And it is distributed worldwide causing seedling blight or charcoal root-rot under favorable condition on more than 500 cultivated and non-cultivated species of crops, which may be of economic importance (Diourte *et al.,* 1995; Hall, 1991). According to Mihail (1992) the fungus *M. phaseolina* (Tassi). have been assigned with various other synonyms which may include *Macrophoma conchoci* Swada, *Macrophomina phaseoli* (Maubl.) Ashby and *Rhizoctonia bataticola* (Taub.) and *Sclerotium bataticola* (Taub.). The fungus *M. phaseolina* is soil-borne and may survive as sclerotia (Meyer, 1973). The fungus exists mainly in the regions where the climates is arid, tropical and subtropical (Manici *et al.,* 1995).

Nowadays unfortunately there are hundreds of chemical substitutes available for present day agriculture systems to protect several species of crops from various kinds of diseases. In view of this growers are even opting for broad spectrum chemicals for faster results and some are using air service for spraying their fields in the notion of confirmed production and to gain certain monetary benefits. By contrast these chemical substitutes are far more devastating to environment and economy than we all are anticipating it today. So, biological control which, comprises of employment of various micro-organisms to control plant pathogens is seemed to be very beneficial as it may be economically as well as environmentally utile and safer option for modern agriculture practice today, which is nearly dependent on several harmful chemical pesticides. Amongst various biological antagonists *Trichoderma* species since long have been extensively used and investigated for their biocontrol ability as they suppresses pathogens by various mechanism such as competition (Baker and Cook, 1974; Harman *et al.,* 2004; Reino *et al.,* 2008). Henis *et al.* (1982) reported mycoparasitism (penetration and infection) of

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Trichoderma spp. against *Sclerotium rolfsii*, where chlamydospores were abundantly produced in contrast to conidia within the infected fungal sclerotia. Even Majumdar *et al.* (1996) showed antagonistic property of *Trichoderma* isolates towards against *M. phaseolina*. Among *Trichoderma* species as fungal antagonists, *Trichoderma viride* is considered to be one of the important antagonists that are being considered to eradicate widest range pathogens biologically. Thus, appropriate formulation of fungal antagonist seems to be an alternative way to decrease burden of excessive and harmful use of chemical pesticides.

Hence, the present study was undertaken to investigate biocontrol efficacy of *T. viride* in *in-vitro* condition against pathogen *S. rolfsii* and *M. phaseolina* by dual culture technique.

Material and methods

Collection of fungal isolates and culture maintenance

For initial screening of *Trichoderma* isolate (*in vitro*) for using its capacity as bio-controller, the fungi *S. rolfsii* was isolated from roots of groundnut plants showing yellowing and stems rot symptoms from the fields of Pune district, Maharashtra, India (18°32'N; 73°51'E). The infected plant material was brought to laboratory and was washed, cut into pieces of 3 mm segments and were surface sterilized for two minutes with 0.05% sodium hypochlorite solution and rinsed with three changes of sterile water. The segments were dried with tissue paper fungus was maintained in potato dextrose agar plates for seven days (28°C) impregnated with streptomycin. Plates of PDA (90 mm) were inoculated with mycelia plugs of *S. rolfsii* and incubated at 28°C for 2-3 weeks until mature sclerotia formed. The sclerotia were collected until needed and stored in test tubes at room temperature. Pure culture was obtained by sub-culturing with the help of single hyphal tip method and was maintained on potato dextrose agar slants in incubator at 28°C temperature. Later on the fungus was identified as *Sclerotium rolfsii* (KD-1) species, identified by Dr. Sanjay K. Singh (Scientist), Division of Mycology and Plant Pathology, ARI, 77, Pune, Maharashtra, India. The fungi *Macrophomina phaseolina* was kindly provided by Agharkar Research Institute, Pune, Maharashtra, India. Plugs of one week old *M. phaseolina* were inoculated in PDA and incubated at 28°C for 2-3 weeks until needed. Pure cultures of *M. phaseolina* were maintained on PDA slants in test tubes and kept in the refrigerator until required.

Antagonists

T. viride isolates were obtained from Department of Microbiology, Agricultural College of Pune, Maharashtra, India. Single colonies of the isolate were sub-cultured in PDA and stored in refrigerators to maintain their genetic purity.

Potato Dextrose Agar (PDA) medium

For preparation of potato dextrose agar (PDA) medium potatoes (220 g) were washed, peeled and diced. It was placed in casserole after addition of 1000 ml distilled water. Potatoes were boiled until they became soft enough to be eaten but not overcooked. Strain the decoction through cheese cloth (or thin layer of cotton) in the funnel; collect the liquid in the beaker. Restore the volume of decoction to 1000 ml by adding fresh distilled water and put back in the casserole. Potatoes were discarded. Dextrose (20 g) and agar (18 g) powder were added to the casserole. The medium was boiled with occasional stirring until the agar completely dissolved. The medium was transferred into test tubes and/or petri plates and/or into 250 ml Erlenmeyer flasks (plugged with cotton wool). Again aluminum foil was used to loosely cover. The medium was used to maintain cultures of pathogens (*S. rolfsii* and *M. phaseolina*), antagonistic isolates (*T. viride*) and produce sclerotia of *S. rolfsii*.

Sterilization

The whole volume of media was sterilized before inoculation. Sterilization was accomplished by heating, chemicals or ultraviolet radiation, also with steam.

Water and culture media were sterilized by heating in autoclave (steam pressure sterilizer), the pressure most commonly used was 15 pounds per square inch in excess of the atmospheric pressure, which gave a temperature of 121°C, usually maintained for 15-20 minutes. After sterilization, the tubes or bottles filled with proper amount of medium were still hot. Therefore, they were placed in an inclined position to make the slants. When the medium solidified on cooling, the test tubes were put in an upright position in an incubator and kept at 34-35°C for one day. The medium, in which bacteria, molds or yeast developed were discarded, since such organisms deprives the mycelium of food and oxygen and may also produce toxic substances that damage the mycelium and retards its growth.

Mycelial growth rate

Mycelial growth rate of *Trichoderma viride* was determined (Elad *et al.,* 1981) as follows: Petri dishes (90 mm diameter) containing potato dextrose agar (PDA) was centrally inoculated with 5 mm of agar plugs from 7-day-old PDA culture of *T. viride* to determine the average linear growth. The plates were incubated at 28°C in oven under continuous light and inspected for three successive days. Observation of radial mycelia growth at right angles was recorded every 24 hours during that period. The colony diameter was measured as the mean of two perpendicular diameters measured at the third day minus the diameter at first day. The average linear growth rate (ALG) of assay was recorded as per (Elad *et al.,* 1981) daily by measuring colony diameter calculated by using formula:

Average Linear Growth (ALG) (mm/day) = [C3-C1]/T

where:

C1: colony diameter in mm after one day of incubation;

C3: colony diameter in mm after three days of incubation;

T: the difference in time (day).

The used experimental design was a completely randomized block with three replicates (plates) for each treatment.

Percent growth inhibition

The percent growth inhibition was calculated using the formula as per Edington, (1971):

$I(\%) = CT/C \times 100$

where:

I: Percent growth inhibition;

C: radial growth in control;

T: radial growth in treatment.

In vitro antifungal activities of *Trichoderma* isolates against major pathogens (*S. rolfsii* and *M. phaseolina*) of groundnut plant in dual culture interaction.

The *Trichoderma* isolates were evaluated for their *in vitro* antifungal activities against two fungal pathogens of groundnut in dual culture. The antifungal activity against the facultative soil-borne fungi *S. rolfsii*, which was isolated locally from groundnut plants and *M. phaseolina*, obtained from Agharkar Research Institute, was determined in dual culture method. A 5 mm diameter mycelia block 7-day-old, removed from margin of young culture of *Trichoderma* isolate and of pathogen colonies, placed 3 cm apart near the edge on the surface of PDA medium. Three replicates of petri plates were incubated at 28°C, under continuous light, and inspected daily for approximately 7-8 consecutive days for mycoparatisim. The fungal colony was arranged in a manner by which it would meet 2-3 days after inoculation. The area of interaction of mycoparasite and the host was measured every 24 hours after contact (Dennis and Webster, 1971 a, 1971 b). A similar dual culture interaction was carried out for *Trichoderma* against *M. phaseolina*. The experiment was conducted as completely randomized design with three replicates in Petri plates.

Statistical analysis

The experiment was laid in complete randomized block design (CRBD) with three replicates. Data were expressed as mean value of three replicates. The values are mean ± SD. All the calculations were made by using a Microsoft Excel 2007 for data analysis.

Results

The antifungal activities of Trichoderma isolate

The antifungal activity of *T. viride* was determined by testing their effect on radial growth rate.

Inhibition of radial growth

The antifungal activity of *T. viride* was determined by testing their effect on radial growth with dual culture method. The *Trichoderma* isolate inhibited the radial growth of *S. rolfsii* by 75% and *M. phaseolina* by 71.42% (Tab. 1). *Trichoderma viride* showed significant inhibition of the test fungi *S. rolfsii* and *M. phaseolina*.

Mycelial growth rate (Average Linear Growth)

The average mycelial growth rate for three isolates and growth of isolate in dual culture are presented in (Tab. 1). The highest colony diameter was 85.33 mm/day for *Trichoderma viride* followed by *Sclerotium rolfsii* 73.33 mm/day and *Macrophomina phaseolina* 70.33 mm/day. The efficacies of *Trichoderma* isolate to inhibit the growth of *S. rolfsii* and *M. phaseolina* in dual culture method was determined on PDA medium. Results are presented in (Tab. 1). The percent inhibition by *T. viride* for *S. rolfsii* was 75% while for *M. phaseolina* the value was 71.42%.

The growth rate of fungal isolates in vitro

The results showed statistically significant difference in growth rate between the different isolates of the *T. viride*, *S. rolfsii* and *M. phaseolina*. *T. viride* showed a minimum of 4.00 days for the complete coverage of the medium, whereas *S. rolfsii* took 4.33 days and *M. phaseolina* 6.33 days, respectively. The *T. viride*'s minimum time to complete coverage gives an edge over both *S. rolfsii* and *M. phaseolina* in the completion for food and space.

The fungal isolates *T. viride*, *S. rolfsii* and *M. phaseolina* exhibited colony diameter of about 24.5%, 20.5% and 11.5% respectively (Fig. 1). The bioagent *T. viride* inhibited growth of *S. rolfsii* by 75% and *M. phaseolina* by 71.42% in dual culture method over control (Tab. 1). The maximum inhibition was observed by bioagent *T. viride*,

Tab. 1. Growth rate and dual culture technique using *T. viride, S. rolfsii* and *M. phaseolina* after 6 days of incubation *in vitro*

S. No.	Treatments	Medium	Mycelial growth*	
			Colony diameter (mm/day)*	Inhibition over control (%)
1	*T. viride alone* (*Tv*)	PDA	85.33±3.30	-
2	*S. rolfsii alone* (*Sr*)	PDA	73.33±4.19	-
3	*M. phaseolina alone* (*Mp*)	PDA	70.33±2.05	-
4	Sr + Tv	PDA	-	75 %
5	Mp + Tv	PDA	-	71.42 %

* = Each value is mean of triplicate; ± = Standard deviation

Abbreviations used: Tv = *Trichoderma viride*; Sr = *Sclerotium rolfsii*; Mp = *Macrophomina phaseolina*; PDA = Potato dextrose agar

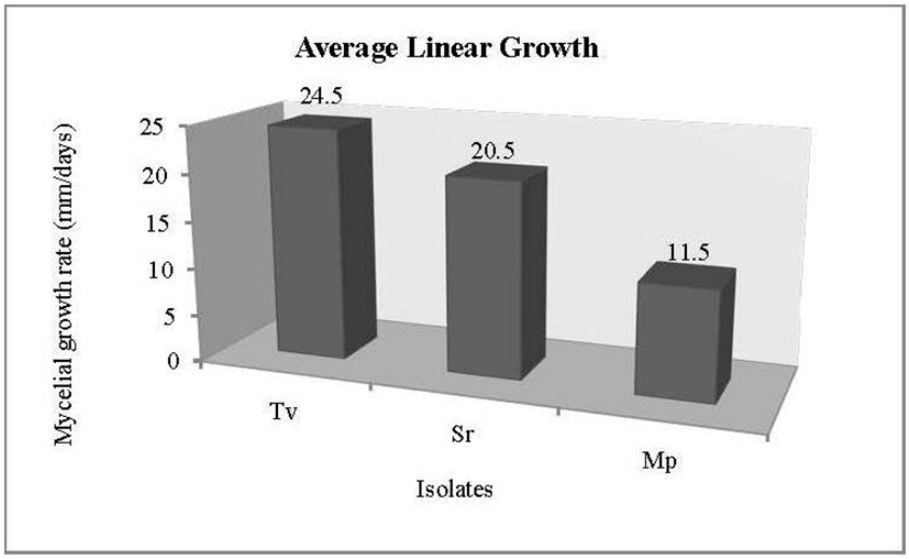

Fig. 1. Mycelial growth rate of *T. viride*, *S. rolfsii* and *M. phaseolina* (mm/day) growing on PDA medium incubated at 28°C after 6 days

in case of *T. viride* + *S. rolfsii* as compared to *T. viride* + *M. phaseolina*. The antagonistic activity of *T. viride* against *S. rolfsii* and *M. phaseolina* was most likely due to mechanism of antibiosis and/or competition.

The growth rate of *T. viride, S. rolfsii* and *M. phaseolina* after 6 days of *in vitro* incubation showed 4 days for *T. viride* and *S. rolfsii,* whereas for *M. phaseolina* it took 3.66 days (Tab. 2, Fig. 2). The present study observed almost similar findings to those of Inbar *et al.,* (1996) who also reported a successful inhibition of *Sclerotinia sclerotiorum* by *Trichoderma harzianum*.

Discussion

Sclerotium rolfsii is the causal fungal pathogen that causes stem-rot on groundnut plants. The pathogen was collected from infected root and stem of groundnut from the fields of Pune district, Maharashtra, India. The pathogen *Macrophomina phaseolina* that causes charcoal root-rot was obtained from Agharkar Research Institute, Pune, Maharashtra, India. *Trichoderma* isolates were obtained from Department of Microbiology, Agricultural College of Pune, Maharashtra, India. The information in Tab. 1 illustrates the growth of fungal pathogen, individually and in combination with *Trichoderma* isolate. All three fungal isolates showed variance in their growth on potato dextrose medium (PDA). The antagonistic growths of *Trichoderma* isolate on both *S. rolfsii* and *M. phaseolina* have been illustrated in Tab. 1.

Tab. 2. Growth rate of *T. viride, S. rolfsii* and *M. phaseolina* after 6 days of incubation *in vitro*

S. No.	Treatments	Mean days for the complete coverage of the medium (no.)*	Medium
1.	*T. viride* alone (*Tv*)	4.00±0.81	PDA
2.	*S. rolfsii* alone (*Sr*)	4.33±1.24	PDA
3.	*M. phaseolina* (Mp)	6.33±1.24	PDA

* = Each value is mean of triplicate; ± = Standard deviation
Abbreviations used: Tv = *Trichoderma viride*; Sr = *Sclerotium rolfsii*; Mp = *Macrophomina phaseolina*; PDA = Potato dextrose agar

It is well documented that *Trichoderma spp.* is mycoparasite (Elad and Chet, 1983) and has been widely used as antagonistic fungal agents. Several *Trichoderma* species are considered to be stoutly antagonistic to other phytopathogenic fungi (Coley-Smith, 1991; Harman, 1996). Their different mechanism of parasitism to other phytopathogenic fungi occurs by various mechanisms such as competition, antibiosis, mycoparasitism, induced resistance and inactivation of pathogen's enzyme. The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanism for their antagonistic activity against phytopathogenic fungi (Chet, 1990; Cruz, 1995). Results obtains in present investigation suggests competition by *T. viride* against pathogen being one of the main reason for biological control. Already the *Trichoderma* species are known to produce a number of antibiotics such as trichodermin, trichodermol, harzianum A and harzianolide (Claydon, 1991; Dickinson, 1995), which may help in reducing ill effects of harmful pathogens. Thus, *T. virde* was evaluated for their efficacy against pathogens *S. rolfsii* and *M. phaseolina.*

The present investigation reveals the rate of growth being faster by *T. viride* singly or in combination with pathogens in artificially controlled environment. This quick rate of growth may be helpful and beneficial to *Trichoderma* isolate as compared to plant pathogenic fungi *in vitro* in the competition for space and nutrients.

In our investigation we have showed that *T. viride* prevented infestation supposed to be incurred by pathogens *S. rolfsii* (75%) and *M. phaseolina* (71.42%) in controlled condition. In the present experiment *Trichoderma* isolate grew towards *S. rolfsii* and *M. phaseolina* colony and

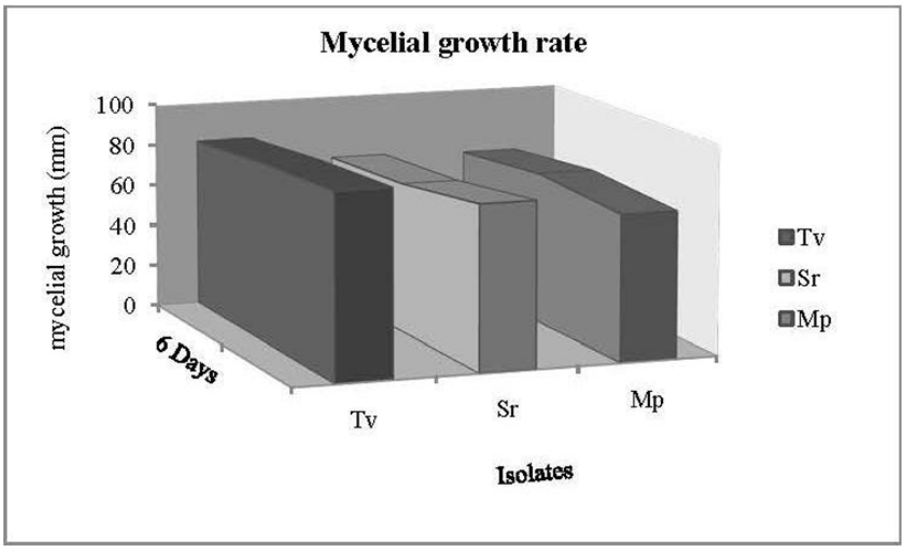

Fig. 2. Mycelial average Linear Growth (ALG) rate of *T. viride*, *S. rolfsii* and *M. phaseolina* isolates (mm/day) growing on PDA medium incubated at 28°C

provided blockade to pathogen. Inbar *et al.* (1996) have reported similar type of observation for *Trichoderma harzianum* and *Sclerotinia sclerotiorum*. Antagonistic effects of *Trichoderma* against *S. rolfsii* have already been reported by various other researchers on different hosts (Chet, 1987; Elad *et al.,* 1983; Harman *et al.,* 1980; Papavizas, 1985; Prasun, 1997). The above result is in agreement with Karthikeyan *et al.* (2006) who demonstrated culture filtrates of *T. viride* inhibiting the growth of the pathogen as well as sclerotial germination to a greater extent. In dual culture assay, Henis *et al.* (1983) observed *T. viride* pq 1 inhibited the growth of *S. rolfsii*, parasitized and lysed the mycelium of *S. rolfsii*. In another observation degradation of chitin in the cell walls of *S. rolfsii* by Chitinases of *Trichoderma* spp., facilitates their penetration of *S. rolfsii* mycelium (Harman *et al.,* 1996).

Khan and Gupta (1998) have demonstrated that *T. polysporum* was stimulatory for radial growth of *M. phaseolina* on PDA. Aly *et al.* (2001) observed the *in vitro* antagonism of *Trichoderma* spp. and *Penicillum* spp. against *M. phaseolina.* Antagonistic ability of *Trichoderma* isolates was found to be highly variable (Chet *et al.,* 1979). Ganesan (2004) and Ganesan and Sekar (2004 a, 2004 b) have reported the control of *S. rolfsii* by employing *T. harzianum*. Chakraborty and Purkayastha (1984) and Deshwal *et al.* (2003) reported that culture filtrates of *Bradyrhizobial* strains significantly helped the growth inhibition of *M. phaseolina.*

Hence, the present demonstration of *T. viride* as an antagonist against fungal pathogen shows their efficacy as a biological control agent.

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