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Effect of Extracellular Metabolites of *Trichoderma pseudokoningii* on Radial Growth of *Fusarium oxysporum*, *Colletotrichum capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*

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A B S T R A C T

The aim of the present experiment was to observe the effect of extracellular metabolites of the biocontrol agent *Trichoderma pseudokoningii* on the radial growth of four selected pathogens viz. *Fusarium oxysporum*, *Colletotrichum capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* in *in vitro* conditions. The cell free culture filtrate of the bioagent was added @ 0, 5, 10, 15 and 20% (v/v) concentrations against the pathogens. Maximum inhibition in the radial growth of the pathogens was found when they were applied @ 20% concentrations of the culture filtrates. The treatments tested were not so much significantly different as compared to control where the difference was markedly visible as well as at highest concentration applied. Among the pathogens tested, highest control in the radial growth was found against *C. capsici* i.e. 65.01% (mean of three replications) followed by *S. sclerotiorum* (62.82%), *R. solani* (50.22%) and *F. oxysporum* (34.53%) proving that the extracellular metabolites released by the biocontrol agent have an effect on the growth of the pathogens.

Introduction

Trichoderma has long been considered as one of the most promising biocontrol agent for several plant pathogens. Numerous authors have reported the use of *Trichoderma* as a biocontrol agent against many soil borne plant pathogens. Now days the indiscriminate use of many different pesticides has led to a serious concern from the environmental point of view. There are numerous reports on the non-target effect of soil chemical treatment on the biocontrol agents *Trichoderma* and *Gliocladium* spp. (Munnecke, 1972; Rodriguez kabana and Curl, 1980; Cook and Baker, 1983). The biocontrol potential of *Trichoderma* has been indicated in a no. of reports (Kehri and Chandra, 1991; Elad *et al.*, 1986; Jeyarajan *et al.*, 1991). Based on many reports a dominating idea prevails that anything biological is always safe from the environmental point of view and so is found in case of biological control of plant pathogens *in vitro* as well as *in vivo*. *Trichoderma* spp. has been recognized as a source of various cell wall degrading enzymes and secondary metabolites (Vinale, 2008; Anita *et al.*, 2012). *Trichoderma* is able to secret 40 different secondary metabolites that may contribute to their mycoparasitism and antibiotic action

(Sivasithamparam and Ghisalberti, 1998). The extracellular enzymes secreted by *Trichoderma* such as chitinolytic and glucanolytic enzymes degrades the cell wall and play a role in biological control through mycoparasitism (Ratnakumari *et al.,* 2011). The culture filtrates when found effective against pathogens can be developed into biopesticides with desirable properties and can be effectively employed for management of different pathogens. *Trichoderma* strains are reported to be effective as biocontrol agents due to their high reproductive capacity, ability to survive under highly infavourable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms (Benitez *et al.,* 2004).

Materials and Methods

Isolation of *Trichoderma* spp.

Trichoderma spp. was isolated following the method given by Mishra *et al.,* (2011). One gram of the soil sample was taken and added to 1ml of sterilized distilled water to make a dilution of 10^{-1}. This suspension was then subjected to serial dilutions and a dilution of 10^{-5} was attained. One milliliter of each dilution *viz*., 10^{-3} to 10^{-4} was poured on to *Trichoderma* Specific Medium (TSM) and purified by single spore method. They were identified on the basis of their morphological characters. Cultures were identified according to conidiophore, shape of the phialides and emergence of phialophores and phialospores. The purified and identified cultures of *Trichoderma* spp. were maintained on Potato Dextrose Agar (PDA) medium and stored at 4°C for further use. From the isolated *Trichoderma* spp., *Trichoderma pseudokoningii* was used to study its bioefficacy against different pathogens.

Isolation of pathogens

The pathogens viz., *Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium oxysporum* and *Colletotrichum capsici* were isolated from the diseased samples collected from the field and nursery plots. All the pathogens were isolated using a single same protocol for all i.e. pure culture isolation and later on using hyphal tip culture method.

The diseased samples from the field and nursery were brought to the laboratory and washed thoroughly with clean water. Then the diseased portions were separated from the sample and are rinsed with distilled water before being taken to the inoculation chamber.

The diseased specimens were cut into small pieces and then surface sterilized with 0.5% sodium hypochlorite ($NaOCl_2$) for 2 minutes which were subsequently rinsed well with three changes of sterile distilled water. The sterilized pieces were then transferred to potato dextrose agar medium and incubated at 28±1°C. The pure culture of the pathogens was then obtained by isolating the fungus by hyphal tip culture.

Extraction of extracellular metabolites from *Trichoderma pseudokoningii*

The extraction of extracellular metabolites of *Trichoderma psuedokoningii* was done on Czapek dox broth medium (Distilled water: 1000ml, Sucrose: 30 g, Dipotassium phosphate: 1g Potassium chloride: 0.5g, Sodium nitrate: 3 g, Magnesium sulphate: 0.5g, Ferrous sulphate: 0.01 g, pH: 7.3 ± 0.2). Fifty (50) ml of CDB medium were prepared on 100 ml conical flasks.

Then 1 ml of spore suspension of *T. pseudokoningii* (10^5 cfu/ml) was inoculated in the medium from 7 day old culture. The inoculated broth culture was then filtered

through Whatman no. 1 and10. The filtrate was then centrifuged at 10,000 rpm at 4°C for 15 minutes. Then the culture filtrate was passed through Whatman no. 42 for sterilization.

Preparation of different concentrations of the CDA medium

Different concentrations of CDA (Distilled water: 1000ml, Sucrose: 30 g, Dipotassium phosphate: 1g, Potassium chloride: 0.5g, Sodium nitrate: 2 g, Magnesium sulphate - 0.5g, Ferrous sulphate: 0.01 g, Agar-Agar: 15 g, pH: 7.3 ± 0.2) medium amended with *T. pseudokoningii* culture filtrate were prepared at doses 0%, 5%, 10%, 15% and 20% (v/v) cocncentration.

In each petriplate 20 ml media amended with required doses of *T. pseudokoningii* culture filtrate was poured. Pathogen inoculated on CDA medium without any culture filtrate was kept as control.

Screening of *T. pesudokoningii* isolate against the pathogens

The petriplates poured with amended media were inoculated with 5mm mycelial disc of each pathogen cut with the cork borer. For each concentration three replications were made for each of the pathogen. Then the plates were incubated at 28±1°C. Periodical observation at 24 hours was made and data were recorded by measuring the colony diameter of the treated plates as well as the control. The mycelial growth inhibition was calculated by the following formula:-

$$\text{Mycelial growth inhibition (\%)} = \frac{(DC - DT)}{DC} \times 100$$

Where, DC = fungal colony diameter in control set and DT = Fungal colony diameter in treatment set.

Results and Discussion

Isolation of *Trichoderma* isolate

The biocontrol agent *T. pseudokoningii* was isolated in TSM (*Trichoderma* selective medium). The isolate which was recognized as *T. pseudokoningii* produced typical symptoms i.e. typical morphological as well as cultural characters. The characteristic of *T. pseudokoningii* such as colony appearance and sporulation pattern were examined after growing on PDA medium.

Microscopic observations of conidiophores and conidia were made and measurements were taken after slide preparation of the culture in clean grease free slide stained with lactophenol- cotton blue. On Potato dextrose agar medium *T. pseudokoningii* formed white and transparent mycelia with little or no conidia production at first. Later on the conidia formed by *T. pseudokoningii* were pale green in colour and measured around 2.3 × 2.6 µm. The phialides of *T. pseudokoningii* arised singly and laterally and the complete reproductive structure was observed as single.

Isolation of pathogens

For isolation of the pathogens at first the diseased specimens were collected based upon the typical symptoms observed that are produced by the pathogens. Specific disease symptoms such as sheath blight of rice, root rot, anthracnose and white mold of French Bean were collected to get the specific causal agent. Then each of the pathogen was isolated following hyphal tip culture method to get the pure culture. The characteristics of the pathogens were then studied for their confirmation. Pathogens such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *Colletotrichum capsici* were isolated and were studied in the experiment against *T. pseudokoningii*.

Effect of extracellular metabolites (cell free culture filtrate) of *T. pseudokoningii* by dual culture method with the pathogens

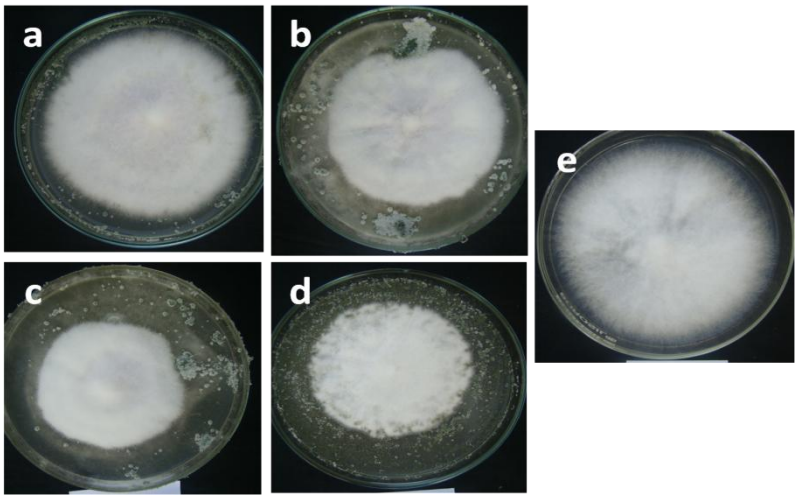
In obvious way it was found that *Trichoderma* has a dominating role over many plant pathogens including the pathogens used in the current experiment. Satisfactory result was found when the cell free culture filtrates of *T. pseudokoningii* were added to the pathogens at different rates of concentrations. The best effect of culture filtrate was observed in *Colletotrichum capsici* where higher mycelia growth inhibition was found in nearly all the concentrations. The mycelia growth inhibition of *C. capsici* was found to be 65.54%, 69.63%, 55.08% and 65.01% at 5%, 10%, 15% and 20% concentrations respectively. At 0% percent concentration the plate was fully covered by the pathogen. The second best effect of the culture filtrates was found against the pathogen *Sclerotinia sclerotiorum* where the mycelial growth inhibition was 0%, 45.72%, 64.15%, 58.67% and 62.82% at 0, 5, 10, 15 and 20% concentration respectively whereas at 5% concentration 41.10%, at 10% 55.62, at 15% 47.55 and at 20% 50.22% reduction in mycelia growth was observed against the pathogen *R. solani*. *Fusarium oxysporum* showed the lowest reduction in mycelial growth when grown in the media amended plate. The growth inhibition was observed at 0%, 31.57%, 36.10%, 36.25% and 34.53% at 0, 5, 10, 15 and 20% concentration. However, an inhibitory effect of the bioagent was found against the pathogen when the media was treated with cell free culture filtrate (Table 1).

Table.1 Effect of cell free culture filtrate of *T. pseudokoningii* on growth inhibition of different pathogens

Concentration of *T. pseudokoningii* culture filtrate (%)	Percent mycelial growth inhibition of pathogens (%)*			
	R.solani	***S. sclerotiorum***	***F. oxysporum***	***C. capsici***
0.0	0.00	0.00	0.00	0.00
5.0	41.10	45.72	31.57	65.54
10.0	55.62	64.15	35.76	69.63
15.0	47.55	58.67	36.25	55.08
20.0	50.22	62.82	34.53	65.01

Plate.1 Pure culture *T. pseudokoningii*

Plate.2 Radial growth of of *Colletotrichum capsici* at different concentration of culture filtrate of *T. pseudokoningii*



a. 5%, b. 10%, c. 15%, d. 20%, d. Control

Thus, the finding of these experiments is in agreement with many workers who have also reported the inhibitory effect of culture filtrate of *Trichoderma* upon several plant pathogens. Mishra., 2011 reported that more than 50% growth inhibition was found at 10% cell free culture filtrate of *T. viride* against pathogens like *R. solani, S. rolfsii, M. phaseolina* and *C. capsici* while at 20% concentration 100 % mycelial growth inhibition was observed which suggest the inhibitory action of cell free culture filtrate of *Trichoderma* as found in the present experiment. But Bokhari and Parveen, 2012 found that culture filtrates of *T. harzianum* and *T. viride* caused reduction in the growth of *Fusarium solani* by 21.3 and 17.3 % only respectively. Anita *et al.,* 2012 reported that presence of certain compounds such as N-phenylethylenediamine, phenol, pthalic acid, diallylamine and propanal in *Trichoderma* spp. perhaps serves the antagonist for survival function by competing against the pathogen. Prasad and kumar, 2011 also reported that the antagonist *Trichoderma* produced non-volatile anti-fungal antibiotics and observed radial growth inhibition of *R. solani*. The undiluted culture filtrates of *T. harzianum* Rifai and *T. pseudokoiningii* Rifai showed varying degree of inhibition of spore germination. *T. pseudokoningii* culture filtrate had a moderate to strong inhibitory effect on mycelia of the rot pathogens (Odebode, 2006). So by looking into several reports it can be said that experiments conducted by studying the extracellular metabolites of *Trichoderma* spp. gives positive results with a view point of controlling the growth of pathogens in *in vitro* conditions.

This is an era which demands more precautionary measures right from a single beneficial insect to human beings due to the toxic nature of chemicals which are released in one or a several ways into our environment. So new methods and technologies should be developed for utilizing the different metabolites released by the biocontrol agents and their subsequent injection into crop protection measures in nurseries as well as in field levels.

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