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In vitro evaluation of *Trichoderma* species for antagonistic activity, fungicide tolerance and competitive saprophytic ability

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Abstract

Soil microbe interactions directly or indirectly affect plant health and soil quality. Plant growth-promoting and bio-control microorganisms have emerged as safe alternatives to chemical pesticides. *Trichoderma* species are known to exhibit antagonistic activity against a number of plant pathogenic organisms. The present study aims to understand antagonistic, pesticide tolerance and rhizosphere competence of three *Trichoderma* species namely *Trichoderma harzianum*, *T. koningii*and *T. pseudokoningiiin vitro*. *Trichoderma* sp. were subjected against root wilt and rot causing pathogens namely *Fusarium oxysporum* and *Sclerotiumrolfsii*via dual culture and exhibited effective antagonistic activity. Dithane M-45 75% W. P. fungicide at different concentrations viz. 50,100,150,200,250,300 ppm was tested by poison food method and *Trichoderma harzianum* and *T. koningii*exhibited tolerance to fungicide at 300 ppm. Fungal cultures, 2 x 10^6spore suspensions were analysed for saprophytic ability. 40 ml of *Trichoderma* sp. conidial suspensions and colonization of paddy straw segments at 1cm apart from the bottom of the sterile plastic cup filled with 200 g of autoclaved potting medium up to 7-cm length was determined at 10 and 21 days of incubation. *Trichoderma harzianum* and *T. koningii*exhibited good saprophytic and colonization ability. Both *T.harzianum* and *T. koningii* was isolated at 7cm depth with colonization frequency of 100% and 88% respectively whereas *T. pseudokoningii* colonized till 4cm depth with isolation frequency of 38% at 21 days of incubation. Most of the *Trichoderma* species show effective *in vitro* antagonistic ability but success in field depends on colonization efficiency. Thus present study details on applicability and necessary modifications for field triumphs of biological agents.

Key words – Bio – control – Colonization – Fungicide – Saprophytic ability

Introduction

Trichoderma sp. are habitual in soil and root ecosystems and have been demonstrated as parasites of several soil borne phytopathogens, plant growth enhancers and also inducers of defense responses (Chang et al. 1986, Windham et al. 1986, Lorito et al. 1994, Harman et al. 2004, Vinale et al. 2009, Ha 2010). A number of commercially available compounds against numerous fungal pathogens involve in the use of *Trichoderma* spp. (Jash 2006). Most of the *Trichoderma* sp. interact with phytopathogens includes competition, myco-parasitism (Papavizas 1985) and antibiosis i.e. production of cell wall degrading enzymes and secondary metabolites (Sivan et al.1984).

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The potentiality of *Trichoderma* sp.to parasitize destructive plant pathogens have beckoned attention of agricultural scientists, farmers, policy makers worldwide and a vast information on biological control of plant pathogens by *Trichoderma* have accumulated in the recent past (Weindling 1932, Bliss 1951, Rifai 1969, Samuel 1996, Mukherjee et al. 2013, Jaklitsch 2014, Bissettet al. 2015).

Increase in world population demands for high productivity and this necessitated indiscriminate application of chemical fertilizers and in effect has led in the development of resistance of pathogens and pests. Their persistent use affected environment and lead in quest for an alternative approach for eco-friendly management for sustainable production (Denholm & Rowland 1992, Leroux et al. 2002).

Prospects of biological control by the genus *Trichoderma* as a promising bio-control agent, has been described (Morsy et al. 2009, Sabalpara et al. 2009). However the effectiveness of different isolates of *Trichoderma* showed considerable *in vitro* and *in vivo* variations stressing on the selection of successful isolates against particular pathogens (Biswas & Das 1999, Ramezani 2008).

Trichoderma sp. growth and functional attributes have been vastly studied on artificial culture media but their correlation between various growth responses on agar and in soil is vague (Cook & Baker 1983). The lack in the understanding of the ecology of these bio-control agents and also the variability and complexity in environment and field soil might have limited *Trichoderma* sp. as successful bio-control agents in field conditions (Lewis &Papavizas 1991). The present objective was to understand the saprophytic competency of *Trichoderma* spp. namely *T.harzianum*, *T.koningii* and *T.pseudokoningii in vitro*.

Materials & Methods

Isolation and Purification of *Trichoderma* sp.

Trichoderma sp. namely *T. harzianum*, *T. koningii* and *T. pseudokoningii*were obtained from Forest Pathology Department, Kerala Forest Research Institute (KFRI), Peechi, Thrissur, Kerala. *Trichoderma* cultures were purified on antibiotic amended Potato Dextrose Agar medium (PDA) and incubated for 5-7 days at 25±2^{o}C.The cultures were identified morphologically referring standard manuals (Barnett 1972, Gams &Bisset 1998).

In vitro antagonism by dual culture technique

In vitro antagonism of *Trichoderma* sp. were studied against root wilt and rot causing pathogens namely *Fusarium oxysporum* and *Sclerotium rolfsii* via dual culture method (Gopalakrishnan et al. 2011) on PDA medium.Fungal cultures were obtained from Forest Pathology Department, Kerala Forest Research Institute (KFRI), sub-cultured on antibiotic amended PDA medium and characterized morphologically referring standard manuals (Barnett 1972, Leslie &Summerell 2006). Seven mm diameter discs of selected fungal pathogens and *Trichoderma* sp.were taken from the actively growing edge of five-day-old cultures using a cork borer. Antagonistic activity was evaluated by inoculating the pathogen at one side of the Petri plate and *Trichoderma* sp.at opposite side of the same plate by leaving 3-4 cm gap. The control plates were inoculated with the pathogens and the antagonist separately. The plates were incubated for ten days and observed for dual culture activity. The percentage inhibition of radial growth of fungal plant pathogens was calculated using formula given by Vincent (1947).

$$\text{Percentage of Inhibition} = \frac{R_1 - R_2}{R_1} \times 100$$

Where,
R1= Radial growth of fungal pathogens in control

R2= Radial growth of fungal pathogen in dual culture

In vitro fungicide resistance of *Trichoderma* sp. by Poisoned Food Method

Poison food method was used to test the *in vitro* efficacy of *Trichoderma* sp. against Dithane M-45 (a. i. Mancozeb [dithiocarbamates group of compounds] 75% W. P.) fungicide at different concentrations viz. 50,100,150,200,250,300 ppm (Nene &Thapliyal 1993). Different concentrations of fungicides were supplemented in a conical flask containing 100 ml molten PDA medium. The flasks containing poisoned medium were well shaken to get uniform mixture of fungicide and 10-15 ml of medium were poured in each sterilized petri-dishes. Seven mm discs of fungal mycelium were inoculated at the center of fungicide amended PDA plates and incubated at 25 ± 2°C for 5- 7 days. The plates without the fungicide served as control. After 7 days of incubation the radial growth of the mycelium was measured. The percent growth inhibition of the pathogens over control was calculated using formula given by Vincent (1947).

$$\text{Percentage of Inhibition} = \frac{R_1 - R_2}{R_1} \times 100$$

Where,
R1= Radial growth of fungal pathogens in control
R2= Radial growth of fungal pathogen in dual culture

In vitro Competitive Saprophytic Ability

Preparation of fungal inoculum

Trichoderma sp. were cultured in PDA medium for 7 days at 25±2^{o}C. Freshly grown cultures were inoculated in Potato Dextrose Broth (PDB) were kept at incubator shaker for 14 days at 25±2^{o}C. Mycelial mat was separated out by filtering through Whatsman No.1 filter paper, dried, grounded using mortar and pestle and centrifuged at 10000 rpm for 15 min to remove hyphal debris. Conidial suspensions thus obtained were then suspended in sterile distilled water and the concentration was adjusted to 2×10^6.

Testing saprophytic competency

Saprophytic ability of the *Trichoderma* sp. was tested by the Cambridge method (Garret 1970). Freshly harvested paddy straws were cut in to 1-cm long segments and autoclaved. Sterile plastic cups procured from the market were perforated at the bottom and plugged with sterile cotton pads. These cups were filled with 200 g autoclaved potting medium up to 7-cm length of the cup and placed with paddy straw segments at 1cm apart from the bottom of the cup. Eighteen autoclaved straw pieces were placed in a radial fashion and sterile potting medium was over laid up to 8-cm length of the cup. 40 ml of conidial suspensions were poured over the potting medium separately and each set was placed in an individual plastic tray containing sterile distilled water. The cups were not watered from the top but the potting medium in the cup was allowed to imbibe water only through capillary action from the holes at the bottom of the cup. Colonization of paddy straw segments by *Trichoderma* sp. was determined at 10 and 21 days of incubation.

Isolation of *Trichoderma* sp.

Paddy straw segments removed after regular intervals of incubation were washed in slow running tap water, then twice in sterile distilled water and placed on antibiotic amended PDA medium at 25±2^{o}C for 14 days. The fungal colonies developing from these segments were identified and compared with the characteristics of the original colony culture. Percent colonization by *Trichoderma* sp. at different depth levels at given time was determined.

Results

Antagonistic activity

Dual culture analysis of *Trichoderma* sp. against the pathogens: *Fusariumoxysporum* and *Sclerotiumrolfsii* resulted good antagonistic activity though the percent of inhibition varied among the species. *T. harzianum* exhibited greater inhibitory activity (>66%) against both the pathogens followed by *T. koningii* (>64%) and *T. pseudokoningii* (>61%) (Table 1).

Table 1 Antagonistic activity of *Trchoderma* sp. against root disease causing fungal pathogens

Sl. No.	***Trichoderma* sp.**	**Percent growth inhibition of fungal species against root wilt and rot pathogens**	
		Fusarium oxysporum	***Sclerotiumrolfsii***
1	*T. harzianum*	66.63±0.64[1]	67.14±0.28[1]
2	*T. koningii*	64.56±0.44[1]	65.18±0.68[1]
3	*T. pseudokoningii*	61.88±0.66[1]	61.28±0.18[1]

[1] Percent inhibition mean value and standard deviation

Fungicide tolerance

Trichoderma sp. were subjected for their efficacy to tolerate fungicide Dithane M-45 at different concentrations (50,100,150,200,250,300ppm) respectively. Many *Trichoderma* species has an innate resistance to many fungicides but resistance levels vary with the fungicide. The study showed variable tolerance to different fungicide concentrations by *Trichoderma* sp. where *T. harzianum* exhibited 100% tolerance followed by *T. koningii* 88% at 300 ppm on the other hand *T. pseudokoningii* exhibited 28% tolerance at 250 ppm and 0% tolerance at 300 ppm (Fig. 1).

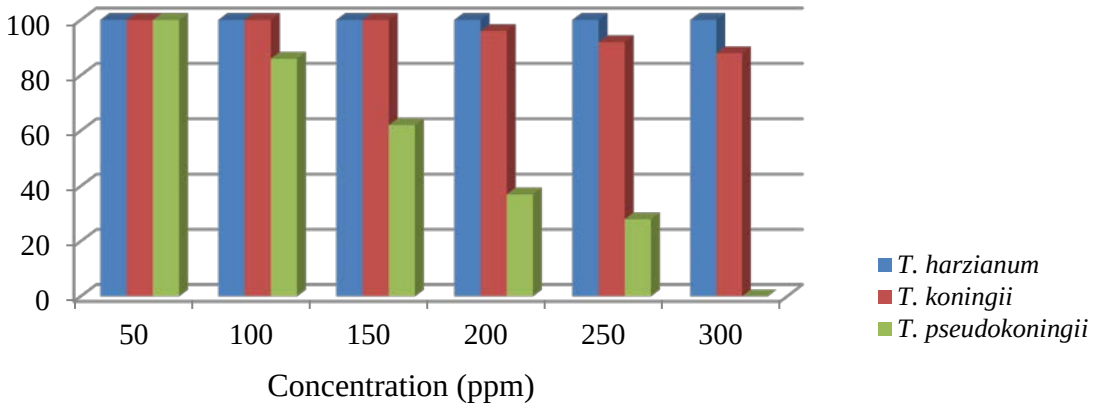

Fig. 1 – Effect of Fungicide on *In vitro* Growth of *Trichoderma* sp.

Competitive saprophytic ability

Paddy straw segments infested with *Trichoderma* sp. were subjected for saprophytic and colonizing ability at various levels of depths after 10 and 21 days of incubation (Fig. 2). Isolation of fungi from dead plant materials inhumed in soil provides information on the ability of fungi recovered as potential saprophytes (Garret 1970). *Trichoderma harzianum* and *T. koningii*were found to exhibit good saprophytic and colonizing ability.

Saprophytic and Colonization ability

Saprophytic ability of *Trichoderma* sp. was analysed after 10 and 21 days of incubation. The current research effort was to assess the competency of potential rhizosphere inhabitant *in vitro*. The study demanded certain criteria viz. quantitative analysis of *Trichoderma* sp. densities at each depth level, use of raw soil to maintain ecological conditions, no watering in order to prevent the

possible leaching out of spores. The results showed *T. harzianum*and *T. koningii*colonised at 6 and 5 cm depth respectively where as*T. pseudokoningii*was isolated at a depth of 3cm after 10 days of incubation. At 21 days of incubation, *T. harzianum*and *T. koningii*was isolated at 7 cm depth where as*T. pseudokoningii*was able to colonise only up to 4cm depth (Fig. 3).

Fig. 2 – Competitive saprophytic and colonizing ability of *Trichoderma* sp.

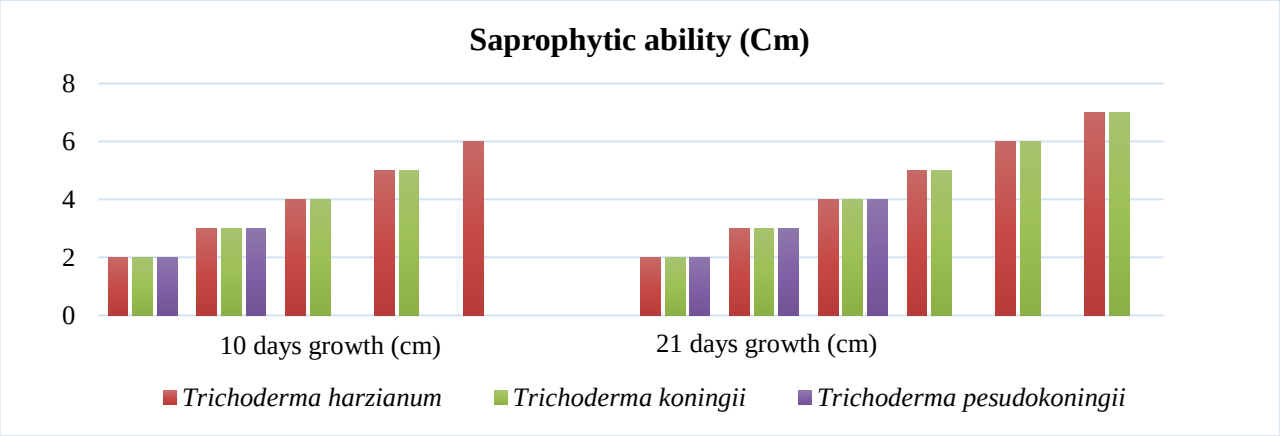

Fig. 3 – Saprophytic ability of *Trichoderma* sp. at various levels of depth at 10 and 21 days of incubation

Similarly colonization frequency of paddy straw segments by *Trichoderma* sp. was also enumerated at various levels of depths. *T. harzianum* exhibited resulted in 100% and 88% colonization frequency for *T. harzianum* and *T. koningii* respectively at a depth level of 7cm whereas *T. Pseudokoningii* showed 38% colonization frequency at 4 cm depth after 21 days of incubation (Fig. 4). *Trichoderma* sp. tested in this experiment showed variation in the competency among the species and also the time and magnitude of their colonization. *T. harzianum* followed by *T. koningii* exhibited better saprophytic activity even at 10 days of incubation whereas *T. pseudokoningii* failed to colonize beyond 4 cm depth even after 21 days of incubation. The

colonization frequency of the species in the given days also showed variations for *T. harzianum*followed by *T. koningii*as efficient colonizer their by stressing detailed analyses for field triumphs. Thus competitive saprophytic ability is relevant in determining the success of biocontrol agents which are affected by a number of biotic and abiotic factors.

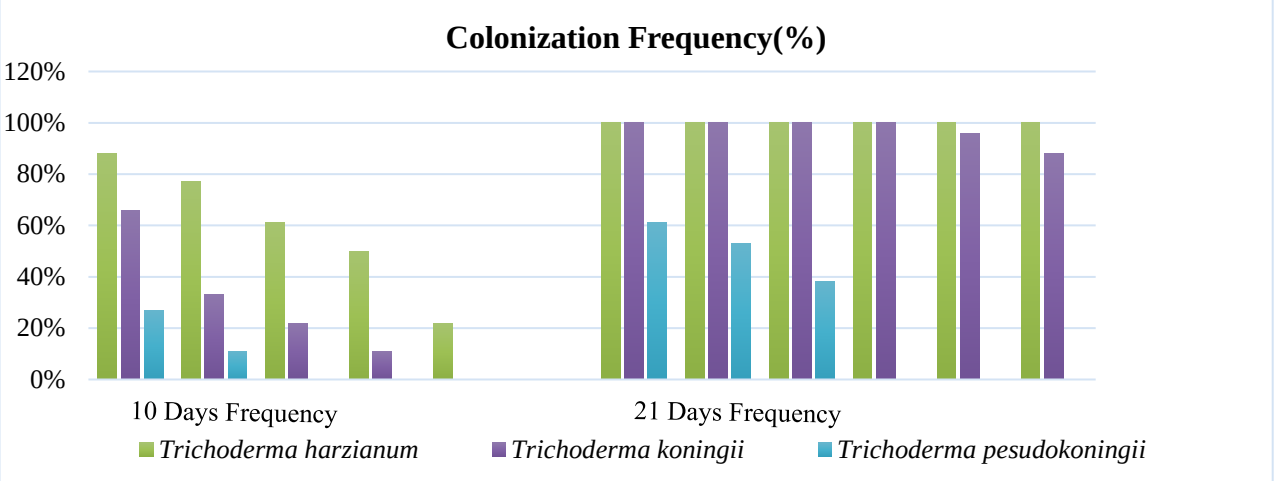

Fig. 4 – Colonization frequency of *Trichoderma* sp. at 10 and 21 days of incubation

Discussion

Trichoderma sp. is widely isolated soil fungi and has gained great significance as bio-control and plant growth enhancer (Papavizas 1985, Sreenivasaprasad&Manibhushanrao 1990, Ha 2010). A number of species of *Trichoderma* in the management of various phyto-pathogenic fungi *in vitro* have been reported by various workers (Chet et al. 1997, Dubey 2002, Dubey 2003, Poddar et al. 2004). Nonetheless, *in vitro* successes of *Trichoderma* sp. are not always positively antagonistic *in vivo* (Campanile et al. 2007) stresses on the ability to survive and colonize the applied soil conditions influenced by nutrient requirements and temperature has been reported by various researchers (Danielson & Davey 1973, Tronsmo& Dennis 1977). Success of *Trichoderma* sp. as a bio-control agent implies the ability to cope up with the biotic and abiotic conditions and thereby minimising the excess application of chemical pesticides. Integrated approach asks for the possible compatibility of *Trichoderma* strains to the chemicals (Kredicset al. 2003) as their combined application has attracted much consideration in order of synergistic approach in the management of soil-borne pathogens (Locke et al. 1985).

Present study, in dual culture resulted *Trichoderma* sp. active against root pathogens *Fusarium oxysporum* and *Sclerotiumrolfsii*. The ability of *Trichoderma* sp. to inhibit mycelial growth of various soil borne pathogens namely *Fusarium* sp., *Rhizoctonia* sp. *Sclerotium* sp., *Phytophthora* sp., *Macrophominaphaseolina* etc. have been reported by various authors (Kirik&Steblyuk 1974, Henis et al. 1983, Patale&Mukadam 2011, Kakde&Chavan 2011). Also, the evaluation to tolerate the fungicide Dithane M-45 resulted in variant tolerance capability of three *Trichoderma* sp. Dithane M-45 was found safe to incorporate with *T. harzianum* (Parabet al. 2009, Saxena et al. 2014) and *T. koningii* at prescribed concentrations but was found to be negatively correlated with *T. pseudokoningii*. Studies with fungicides Benomyl, Topsin-M and Carbendazim for *T. pseudokoningii* suppressed growth of the fungi but for *T. harzianum*, *T.longibrachiatum*and *T. viride* prescribed concentrations of the fungicides found to be tolerating (Khan&Shahzad 2007). Fungicide composition and dosage is critical in determining compatibility of bio-agent in field application (Monte 2001).

In order to minimise chemical pollution an integrated strategy whereby fungicide compatible bio-agent proves to be effective in the management of pathogens can be practised (De Cal et al. 1994) again delimited by rhizosphere survivability. A number of preliminary studies based on population densities of microbes in rhizosphere and non-rhizosphere have been done to evaluate the

rhizosphere competence of microbes with respect to plant species (Papavizas 1967, Wells et al. 1972, Newman & Bowen 1974, Chao et al. 1986). *T. harzianum* and *T. koningii*exhibited higher colonization frequency indicating potentiality of these species at rhizopshere regions. The ability of *Trichoderma* sp. can be attributed to the enzymatic degradation of cellulose on or near the root surface but has not been justified (Garret 1970, Foster et al. 1983). Although bio-control agents exhibit good antagonistic activity but lacks rhizosphere and rhizoplane competency which are also influenced by biotic factors (Papavizas 1967). The current work highlighted on the fact that different antagonistic evaluating strategies need to be carried out thereby an integrated approach with minimal chemical application and higher rhizosphere competent strains can be selected as potential biological agents.

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