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Effect of different environmental conditions on growth and sporulation of some *Trichoderma* species

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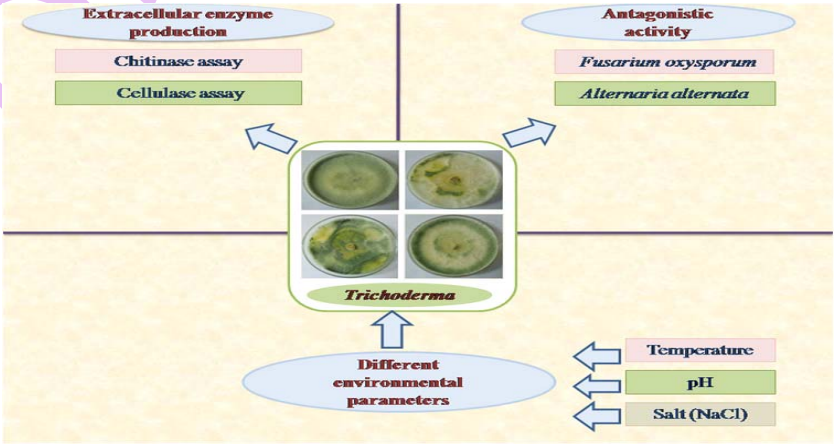
Abstract

Aim: To evaluate the effect of the different environmental conditions (temperature, pH and salt) on growth and sporulation of some *Trichoderma* species.

Methodology: In the present study, *Trichoderma* spp. were isolated from rhizospheric soil of various cultivated plants and identified as *Trichoderma harzianum, T. viride, T. hamatum* and *T. asperellum* on the basis of morphological and conidial characteristics. These identified *Trichoderma* spp. were tested *in vitro* for their antagonistic activity against *Fusarium oxysporum* and *Alternaria alternata*, enzyme production and tolerance to different environmental conditions such as salt, temperature and pH.

Results: The results obtained revealed that out of the four tested *Trichoderma* species, maximum inhibition of the growth and spore production of both the pathogens were found by *T. harzianum*, with the greatest percent inhibition was observed for *F. oxysporum* (68.5%) and A. alternata (52.93%) in dual cultures. The inhibition rate was also significantly correlated with the culture filtrate of all the isolates. Significant chitinase and cellulase acttivities of all *Trichoderma* isolates have been recorded during plate assay. *T. harzianum* exhibited a higher diameter of clear zone than the other *Trichoderma* spp. on media containing chitin and cellulose. Thus, they showed a better cellulolytic and chitinolytic actitivties as compared to other isolates. However, all these isolates responded distinctly to different environmental parameters. The most favourable temperature for growth and sporulation of *T. harzianum* and *T. viride* was found in between 25-40°C, where as for *T. asperellum* and *T. hamatum*, it was 25-35°C. Similarly, the most favourable range of pH was in between 4.6-7.6 for all the four spp. of Trichoderma. None of the applied NaCl concentrations except 1000 µM significantly inhibited mycelial growth of all tested *Trichoderma* species.

Interpretation: On the basis of this study, it can be concluded that *T. harzianum* is a potential candidate as a biocontrol agent against various fungal pathogens in agriculture system under different environmental stresses.



Introduction

Trichoderma spp. are free living fungi that commonly occur in all types of soil and other natural habitats (Siddique *et al*., 2010). *Trichoderma* spp. grow rapidly in culture media and produce numerous green and white conidia (Joshi *et al*., 2012). *Trichoderma* spp. is great interest because of their ability to produce cellulases, chitinases, glucanases, xylanases and protease enzymes (Srivastava, 2014), as well as their role in biological control of plant pathogens (Benitez *et al*., 2004). Further, many strains are strong opportunistic invaders, fast growing, prolific producers of spores and serve as a source of powerful antibiotics commercially as well (Al-Saeedi and Al- Ani, 2014). Several species of genus *Trichoderma* have also been recognised for their growth promotion abilities (Sharma *et al*., 2012) and many strains have been commercially registered for use in plant protection (Hermosa *et al*., 2012). There are number of efficient biocontrol agents within the genus, which includes *T. harzianum, T. viride, T. hamatum, T. atroviride, T. virens, T. asperellum, T. asperelloides, T. roseum, T. koningii* and *T. gamsii.*

When planning the application of antagonistic *Trichoderma* strains for the purpose of biological control, it is important to consider various parameters affecting their growth and sporulation. There are several biotic and abiotic factors that affect the growth, development and antagonistic property of *Trichoderma*. The most important abiotic factors are temperature, pH, moisture, inoculum concentration, and host susceptibility. *Trichoderma* strains are of great importance as biocontrol strains and should have better stress tolerance level than the plant pathogens against which they are to be used for biological control (Kredics *et al.,* 2004). Several abiotic factors such as temperature, heavy metals, water relations, salt, pH and even the pesticides have been already reported to deteriorate the antagonistic properties of *Trichoderma* species against the plant pathogenic fungi (Dłużniewska, 2003). However, there have been few attempts *in vitro* on the abiotic stresses which limit the growth of this antagonistic organism (Longa *et al*., 2008). The knowledge on these factors may lead to a better understanding of the population dynamics of *Trichoderma* in soil and other habitats.

Although, there is extensive literature available on the enzymes and biological control potential of *Trichoderma*, however, studies on their ecological requirements in relation to their growth and sporulation is meagre. In spite of enormous research on *Trichoderma* spp. as a biocontrol agent (Asad *et al*., 2014), their physiological and environmental requirements have not been studied in depth. In the present study, isolated *Trichoderma* spp. were screened for *in vitro* antagonism against fungal phytopathogens (*Fusarium oxysporum* and *Alternaria alternata*) by dual plate, as well as culture filtrate assay. The isolates were further tested for their chitinase and cellulase production ability. A study was also carried out to assess the effect of pH, temperature and salt on mycelial growth and sporulation of *T. harzianum, T.viride, T. hamatum* and *T. asperellum* at different days of incubation.

Materials and Methods

Isolation of *Trichoderma* species : *Trichoderma* spp. were isolated from soil samples of cultivated plants collected from Varanasi district, Uttar Pradesh, India, by serial dilution technique on *Trichoderma* selective medium and incubated at 28°C for 4–6 days. Morphologically distinct colonies were selected and purified following sub-culturing. Out of eight isolates of *Trichoderma*, four isolates were identified upto species level on the basis of morphological and cultural characteristics by using the key of Rifai (1969), Bisset (1984), Harman *et al*. (1998). The identified isolates were *T. viride, T. asperellum, T. harzianum* and *T. hamatum*. All these *Trichoderma* spp. were selected for further studies.

Efficacy of *Trichoderma* spp. on growth of *Fusarium oxysporum* and *Alternaria alternata*

Dual plate assay : Growth inhibition of these two pathogens by *Trichoderma* spp. were carried out on PDA medium using dual culture technique (Morton and Stroube, 1955). Five millimeter diameter mycelial plugs of each *Trichoderma* spp. and both pathogenic fungi were placed on the opposite side of the plate at equal distance from the periphery and incubated at 28±2°C for 7 days (Evans *et al.,* 2003). In control experiment, *Trichoderma* spp. were replaced with sterile agar plugs. Growth of pathogens in both the test, as well as in control experiments was recorded. Data were obtained for the percentage inhibition of radial growth [100x(C-T)/C], where C is the radial growth of the pathogen in control and T is the radial growth of the pathogen in dual culture with antagonist (Garrett, 1956).

Culture filtrate assay : One hundred milliliter (100ml) of potato dextrose broth was dispensed into separate 250 ml flasks and inoculated with 5mm-diameter discs from the edge of 7-day-old culture of each *Trichoderma* spp. maintained on the nutrient medium. Each flask was inoculated with three discs and the set up was incubated at 28±2ºC for 7 days. Culture filtrates were harvested by filtering through Whatmann No.1 filter papers and finally through Millipore filter (0.45µm) to obtain sterile culture filtrate. The pH of the culture filtrate was adjusted to 5.6 by using 0.1N HCl or 0.1N NaOH before use. Twenty five percent concentration of the culture filtrate was mixed with cooled PDA before plating. The medium devoid of culture filtrate served as control. Petridishes were inoculated separately with a 9 mm agar disc of *F. oxysporum* and *A. alternata* cut from actively growing colony of 5-day-old culture, and incubated at 28±2ºC. The radial growth of *F. oxysporum* and *A. alternata* was measured after 24 hrs intervals. The percent inhibition of radial colony growth was calculated.

Cellulase assay : *Trichoderma* spp. were grown on yeast extract peptone agar medium (yeast extract 0.1 g, peptone 0.5 g, agar 16

g, congo red 0.2%, distilled water 1000 ml) supplemented with 0.5% Na-carboxymethyl cellulose. A clear zone surrounding the colonies (cm) which indicated cellulolytic activity was observed after 5 days. The experiment was done by using completely randomized design with three replicates.

Chitinase assay : Medium for chitinase assay consisted of a basal medium comprising (per litre) of 4.5 gm of colloidal chitin, 0.3 gm of $MgSO_4.7H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 0.15 g of bromocresol purple (Agrawal and Kotasthane, 2009).The pH of the medium was adjusted to 4.7 and then autoclaved at 121°C for 15 min. After cooling, the medium was poured into Petri plates and allowed to solidify. The fresh culture plugs of *Trichoderma* spp. were inoculated into the medium and incubated at 25-27°C for 2-3 days and observed for the formation of coloured. Chitinase activity was identified due to the formation of purple coloured zone. Colour intensity and diameter of purple coloured zone was taken as criteria to determine chitinase activity after 3 days of incubation.

Effect of temperature : The ability of *Trichoderma* spp. to grow at restrictive temperature was assessed by growing the cultures on PDA plates at different temperature viz., 15, 20, 25, 30.....45°C. For measuring the radial growth rate, *Trichoderma* spp. was inoculated in triplicates at the centre of 90 mm potato dextrose agar plates. Inoculum was aseptically punched with a cork borer in the form of 5 mm mycelial discs from the margin of colonies. The plates were incubated at different temperature and the radial growth was measured (in mm) everyday upto 6 days of inoculation.

Effect of pH : Different pH used for the study was 4.1, 4.6, 5.1, 5.6, 6.1, 6.6, 7.1, 7.6, 8.1 and 8.6. One hundred ml PDA media were prepared in triplicates and its pH was adjusted by adding HCl or NaOH before autoclaving. Disc of fungal culture was inoculated on the plates and measurement of the radial growth and sporulation were recorded similarly as described above.

Effect of NaCl : The direct effect of NaCl was tested on growth of *Trichoderma* spp. cultured on potato dextrose agar (PDA) medium. PDA was amended with NaCl at 200 µM, 400 µM, 600 µM, 800 µM and 1000 µM concentrations. The diameter of colonies was measured everyday up to 6 days after inoculation with a 5 mm diameter plug of *Trichoderma* spp.

Statistical analysis : Statistical analysis was performed by SPSS ver. 16 software via analysis of variance (one-way ANOVA) followed by Duncan's multiple range test at $P \leq 0.05$ significance level. Data were expressed as mean ± standard deviation of at least three replicates.

Results and Discussion

Results of the colony interactions clearly demonstrate that *Trichoderma* clearly inhibited the radial growth of *F. oxysporum and A. Alternata*. The maximum inhibition of *F. oxysporum* was by *T. harzianum* (68.5%) followed by *T. viride* (62.7%) (Fig.1A). However, least inhibition of *F. oxysporum* was done by *T. asperellum* (52.2%). Where as in case of *A. alternata*, maximum and least inhibition was done by *T. harzianum* (52.9%) and *T. asperellum* (36.3%) respectively. The degree of inhibition varied from one species to another. Maximum inhibition of both the pathogen was observed with *T. harzianum*. Formation of inhibition zone at the contact between *Trichoderma* spp. and the pathogens in dual cultures could be explained on the basis of production of volatile and non-volatile metabolites (such as terpenes, pyrones, polyketides, etc.), as well as production of extracellular hydrolytic enzymes by *Trichoderma* (Tapwal *et al*., 2011; Rao *et al*., 2015; Garkoti *et al*., 2014). Motesharrei and Salimi (2014) reported that *T. harzianum* was more capable to influence the growth of *Fusarium* in dual culture through production of volatile and non volatile inhibitors under conrolled conditions. Highest percent inhibition was also observed by *T. harzianum* against different pathogens such as *Curvularia lunata*, *Fusarium oxysporum*, *Alternaria alternata*, *Colletotrichum gloeosporioides* and *Rhizoctonia solani* (Tapwal *et al*., 2015).

Maximum inhibition of both the pathogens was observed by culture filtrate of *T. harzianum*, which was followed by *T. viride* and *T. hamatum*. Least inhibition was observed in case of *T. asperellum* (Fig. 1B). The culture filtrates of strains of *Trichoderma* species were found effective to inhibit the growth of pathogen at varied degrees. Maximum inhibition was due to culture filtrate of *T. harzianum*. Metabolites extracted from liquid culture filtrates also depicted almost same trend of superiority as mentioned in dual culture *i.e.,* the same isolate further proved its better potentiality when compared with the rest. The effect of culture filtrate of *Trichoderma* on the pathogens might be due to the production of antibiotics, volatile and non volatile substances (Shi *et al*., 2012). Non volatile substances produced by *T. harzianum* are inhibitory to *F. oxysporum* f. sp. *ciceris* causing chickpea wilt (Dubey and Suresh, 2006).

It was observed that *T. harzianum* and *T. viride* exhibited a higher diameter of clear zone than other *Trichoderma* spp. on media containing cellulose and chitin. The least cellulase and chitinase activity was produced by *T. asperellum* (Fig. 1C). Lunge and Patil (2012) isolated many strains of *Trichoderma* and reported that out of all the isolates, highest purple color zone formation was shown by *T. harzianum* on chitin containing medium. Elad *et al*. (1982) reported that the isolates of *T. harzianum,* which were found to differ in their ability to attack *Sclerotium rolfsii*, *Rhizoctonia solanii* and *P. aphanidermatum*, also differed in the levels of mycolytic enzymes produced by them. Kumar *et al*. (2014) observed the cellulase enzyme activity of *T. viride* with highest zone of clearance (24.2 mm). As the cell wall of *F. oxysporum* and *A. alternata* is composed of chitin, the enzymes-chitinase produced by *Trichoderma* might be involved in hydrolysis of cell wall of pathogens during antagonism (Marcello *et al*., 2010). The fragments of cell wall of pathogenic fungi in turn induces the production of further enzymes and trigger a cascade of physiological changes, stimulating rapid and

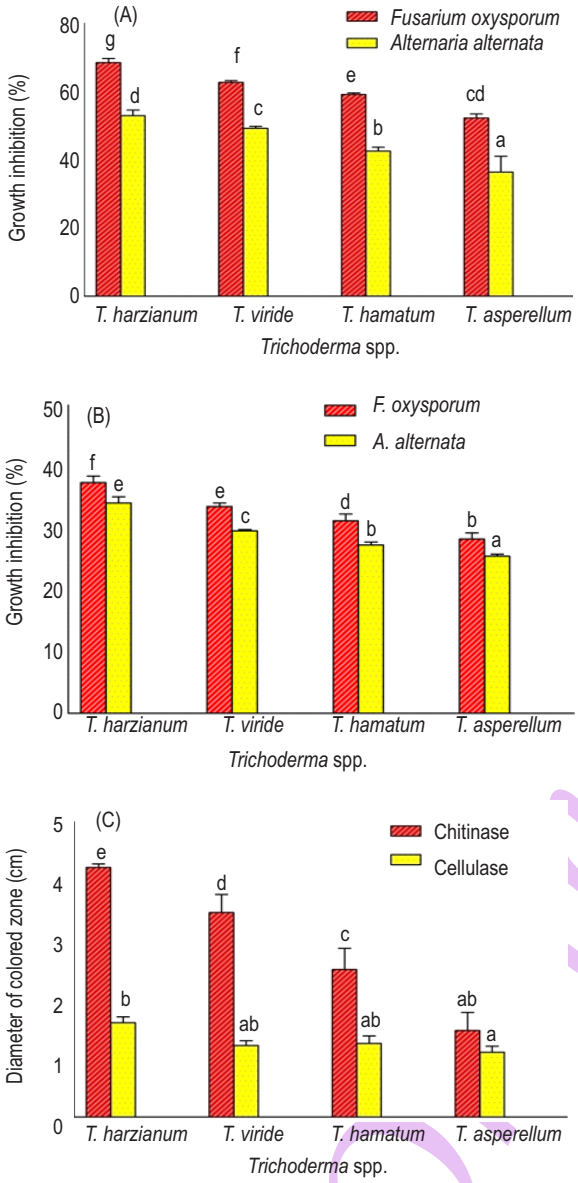


Fig. 1 : Growth inhibition of *F. oxysporum* and *A. alternata* by (A) Dual culture, (B) Culture filtrate, (C) Chitinase and cellulase production by different species of *Trichoderma*. Values are mean ± SD of three replicates. Significant at $p \leq 0.05$

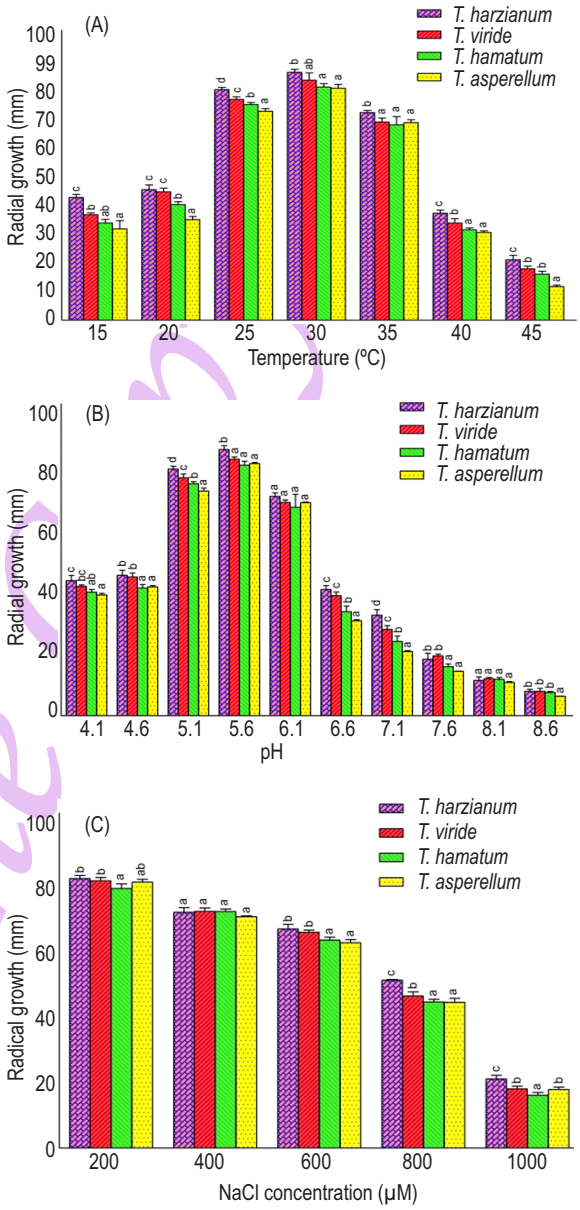


Fig. 2 : Effect on the growth of *Trichoderma* spp. (A) Temperature, (B) pH and (C) NaCl. Values are mean ± SD of three replicates. Significant at $p \leq 0.05$

directed growth of *Trichoderma* species (Zeilinger *et al*., 1999; Schubert *et al*., 2008).

Temperature plays an important role in expressing the activity of any biological system; it has great influence on radial growth and sporulation of *Trichoderma*. Temperature significantly affected the radial growth and sporulation of *Trichoderma* spp. *T. viride* and *T. harzianum* showed a high range of temperature tolerance. It grew and sporulated well in between temperature 20 to 40°C. *T. asperellum and T. hamatum* grew and sporulated well between temperatures (25-35°C). But at low temperature (15°C), the growth was slow and all the *Trichoderma* spp. failed to sporulate even after 7 days of incubation. Similar trend was also observed at high temperature for all the four spp. of *Trichoderma*.

Growth was slow above 40°C and ceased at 45°C. In addition, many morphological variations were apparent at various temperatures. At 40°C, they produced abnormal compact colonies with irregular margin and scanty sporulation, while at 45°C they failed to sporulate even after 7 days of incubation. Maximum sporulation of the isolates was observed at 30°C which declined above 35°C (Fig. 2A). Conidial maturation stage, dependent on the media used for growth, is crucial for determining the thermotolerance level of the isolates (Kim *et al.,* 2010). High temperature tolerance of *T. harzianum* is due to the increase in accumulation of trehalose, mannose and raffinose. The role of these sugars in fungal survival and as stabilising agents of cell structures and cellular proteins under heat stress conditions is already well established (Poosapati *et al.,* 2014). Singh *et al*. (2014) studied the effect of varying pH, temperature and agitation on the growth of *Trichoderma* species and found that *T. harzianum* grew well between 25-40°C. In natural soil, temperature may exceed maximum for growth and it may be a limiting factor that determines the distribution of *Trichoderma* species in soil.

The pH values of nutrient medium belong to the most important parameters affecting *Trichoderma* growth and sporulation because it is known to determine mineral availability and that influences metabolic rates and enzyme activity. Optimum growth and sporulation of all the four species of *Trichoderma* was recorded between pH 4.1 to 8.6. Significant variation in growth and sporulation was observed among all the four spp. of *Trichoderma* at all the tested pH values (Fig. 2B). Growth and sporulation of all the isolates decreased significantly with either decrease or increase in the pH below 4.6 or above 7.6. At pH 4.6, the growth was still good but sporulation was poor. At pH 8.6, the growth was completely ceased. At pH values in between 5.1-5.6 the colonies were larger than 7.3 cm in diameter, whereas for higher pH, the diameter did not exceed 6.1 cm. With increasing time, all the species showed significant increase in growth and sporulation at all the pH levels, except at pH 8.1and 8.6 where increase was not significant. Chet and Baker (1980) reported that the acidic pH levels *in vitro* enhanced the growth of *T. harzianum* and stimulated its conidiophore formation and conidial germination. *Trichoderma* spp. prefer and grow well in soils having acidic pH and high organic matter (Upadhyay and Rai, 1979). Optimum pH of *Trichoderma* species was reported between 5-6 (Ghidiyal and Pandey, 2008; Aanuoluwa *et al*., 2015). Ali *et al*. (2015) studied the effect of different pH on growth and sporulation of indigenous *Trichoderma* spp. and observed reduction in colony diameter, growth rate and sporulation of different isolates at pH 8 as compared to pH 4. Effects of different pH on mycelial growth of *Trichoderma* strains reveal useful information about the applicability of biocontrol strains in agricultural soils with certain pH-relations (Kredics *et al*., 2003).

Salt stress is an important abiotic factor that highly impacts the microbial ecosystem of soil and limiting crop productivity (Poosapati *et al*., 2014). All the four spp. of *Trichoderma* showed a high range of NaCl tolerance. The highest tolerance was shown by *T. harzianum* followed by *T. viride.* It was observed that at 200 µM and 400 µM concentrations of NaCl, the growth and sporulation was good. Growth was supported well but sporulation was poor at 600 and 800 µM concentration. At 1000 µM concentration, the growth and sporulation was poor in all the isolates (Fig. 2C). Soil salinity constitutes an environmental factor which limits *Trichoderma* capacities. At all the tested concentration of salt, *T. harzianum* was able to grow but sporulation was inhibited at high concentration of salt. Presence of sodium chloride in the medium modifies the morphological aspects of *T. harzianum* and its antagonistic capacity (Regragui, 2005). *Trichoderma* species have been shown to develop extrusion systems to keep levels of intracellular sodium below toxic concentration to cell (Gunde-Cimerman *et al*., 2009). *T. harzianum, T. viride* and *T. koningii* isolated from the soil samples of glacier sites of Indian Himalaya could tolerate high range of salt concentration (Ghidiyal and Pandey, 2008). Furthermore, Harman *et al*. (2010) reported that high concentration of salt can be used to reduce the occurrence of fungal/bacterial contamination in stocks of salt tolerant species of *Trichoderma*. *T. harzianum* is a promising antagonist and will be included in more comprehensive future research of their antagonistic effects against *F. oxysporum* and *A. alternata*, as well as its ability to grow well against different environmental stresses.

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