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RESEARCH ARTICLE

Isolation of cold tolerant antifungal strains of *Trichoderma* sp. from Northern Hilly Zones of Chhattisgarh

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ABSTRACT

Three species of *Trichoderma viz., Trichoderma viride, Trichoderma harzianum* and *Trichoderma konengii* have been isolated from the soil samples collected from forest sites in higher altitudes of Northern Hilly Zones of Chhattisgarh in Mainpat, Dist-Surguja, Chhattisgarh. The species could grow between 4 to 42^0 C temperatures and 3 to 13 pH on agar plates, the optimum requirement being 26^0 C and 5.5 Ph, respectively. Further incubation of the agar plates showing normal growth of *Trichoderma* species at 4^0 C, induced heavy sporulation in three weeks of time. Induction of sporulation on exposure to low temperature appeared to be a strategy for survival of these species in extreme cold environment experiencing sub zero temperatures. Antifungal activities were demonstrated between *Trichoderma* species and phytopathogenic fungi in dual cultures. The antifungal metabolites produced by *Trichoderma* species, diffusible as well as volatile, caused abnormalities in fungal structures of pathogenic fungi. Plant growth promotion abilities of *Trichoderma* species was also demonstrated through a plant based bioassay in greenhouse. The study is important for documentation of microbial diversity of Northern Hilly Zones of Chhattisgarh in Mainpat, Dist-Surguja, Chhattisgarh and determination of the associated biotechnological applications.

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INTRODUCTION

Trichoderma species are free living fungi that occur in nearly all the soils and other natural habitats. They can be easily isolated from soil and decomposing organic matter (Grondona *et al.,* 1997). Most of the *Trichoderma* species grow rapidly in artificial culture media and produce large number of green or white conidia from conidiagenous cells. Their abundance in soil under diversified clamatic conditions is mainly due to their ability to degrade a variety of organic substrates in soil, their metabolic versatility and their resistance to microbial inhibitors (Kullnig-Gradinger *et al.,* 2002).

Certain strains of *Trichoderma* species including *Trichoderma viride* are known to be restricted to the areas identified by low temperature, *Trichoderma harzianum* is mostly found in warmer climate and strains of *Trichoderma hamatum* and *Trichoderma konengii* are widely distributed in areas of diverse climatic conditions (Fu-Qiang *et al.,* 2004). Diversity in various ecosystems, including forest and mountains and molecular taxonomy of *Trichoderma* species have received importance in the recent literature (Woo *et al.,* 2006).

There is considerable interest in manipulating the soil microbial community to achieve the biological control of soil borne plant pathogens (Cook and Baker, 1983). A successful bio-control system is one, which is easy and economical to produce, safe, stable in the environment and easily applied

during the conventional agricultural practices (Tronsmo and Hjelijord, 1998).

Several species of the genus *Trichoderma* received attention mainly due to their importance in biological control of soil borne plant pathogens. Antibiosis, mycoparasitism and competition for nutrients are the mechanisms involved in biological control. Recent studies have shown that they are opportunistic, avirulent plant symbionts, as well as the parasites of other fungi. Many species of the genus *Trichoderma* have also been recognized for their plant growth promotion abilities (Vizcaino *et al.*, 2005). In the present study, three species of *Trichoderma* isolated from soil samples collected from forest sites have been investigated *in vitro* for growth characters and bio-control properties. Bioassay was also performed to demonstrate the effect of these species on plant growth.

MATERIAL AND METHODS

Study site :

Soil samples were collected from various locations in high altitudes (beyond 1079 m above mean sea level) of Northern Hilly Zones of Chhattisgarh in Mainpat, Dist-Surguja, Chhattisgarh.

Isolation and growth characters of fungi :

Serial dilutions of soil samples were made for isolation of fungi. Appropriate dilutions were plated (pour plate method) using potato dextrose agar (PDA), sabouraud maltose and malt extract agar. The agar plates were incubated at 26 ^{0}C for 7 days. Morphologically distinct colonies were subjected to purification following sub-culturing. The pure cultures were maintained on PDA slants at 4 ^{0}C in a refrigerator. Based on colony morphology and microscopic observations (smears made in lactophenol cotton blue) the frequently occurring species of genus *Trichoderma* were selected for further experiments. For colony morphology and microscopic observations, fresh cultures were grown on PDA at 26 ^{0}C for 5 days. After taking observations on growth characters, the plates were kept in refrigerator. The temperature and pH requirements of the *Trichoderma* sp. were determined by incubating the fungal isolates at different temperatures (4, 7, 14, 21, 28, 35 and 42^{0}C) for 7 days and by inoculating the fungal isolates on PDA plates set at different pH levels, *i.e.*, 3.0-13.0 with an interval of 0.5 units. For salt tolerance, the cultures were inoculated on PDA plates supplemented with 2.0, 5.0 and 7.0% salt, respectively.

Plate assays for evaluation of biocontrol properties :

Three phytopathogenic fungi *viz., Fusarium oxysporum, Alternaria alternata* and *Cladosporium oxysporum* were selected for bio-control experiments. These fungi were isolated from the same study locations and are known to cause minor or major disease symptoms in a range of plant species. Dual cultures were performed for determination of production of antifungal metabolites, both diffusible and volatiles. For determination of diffusible metabolites individual *Trichoderma* species and phytopathogenic test fungus were grown on PDA plates. 5 mm disc of both of the fungi (*Trichoderma* species and phytopathogenic test fungus) were placed PDA agar plate about 2.0-2.5 cm away from each other. The plates were incubated in inverted position at 26^0 C for 7 days. Inhibition of the pathogenic fungal growth was measured by using the formula :

$$\frac{R_1 - R_2}{R_1} \times 100$$

where, R_1 control value represents the largest distance grown by the test fungus in the direction of maximum radius, R_2 represents the distance between the inoculums of pathogen and *Trichoderma* species. For determination of production of volatile substances dual cultures were performed in sealed plates. 5 mm disc of *Trichoderma* species and test fungus were placed in centre of two separate PDA plates of same size and sealed with parafilm and incubated at 26^0 C for 7 days. Control was taken without *Trichoderma* species in the bottom plates. Observation was recorded after one week and per cent inhibition was calculated using formula :

$$\frac{r_1 - r_2}{r_1} \times 100$$

where, r_1 was the radial growth of pathogen without *Trichoderma* species, r_2 represents the radial growth of pathogen inoculated with *Trichoderma* species. All the experiments were conducted in triplicates. Fungal smears were prepared in lactophenol cotton blue taking the growth from the inhibition area. Fungal smears were also prepared from the control plates to make the comparison.

Bioassay for evaluation of plant growth promotion ability of *Trichoderma* sp. :

Chickpea (*Cicer arietinum* L.) was chosen as a test species for conducting the bioassay under greenhouse conditions. The four treatments under consideration were: 1-control (seeds without any inoculum) and 2, 3 and 4 seeds inoculated with *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma konengii*, respectively. *Trichoderma* inoculum (for each species, individually) was grown on PDA plates (incubated 26 ^{0}C for one week). Three mycelial discs (5 mm) of *Trichoderma* sp. were cut from the agar plates and used as inoculum for one seed at the time of sowing. Seeds were grown in trays (32x26x6 cm). The trays were kept inside the greenhouse (26 ^{0}C) of the Department. Each treatment was taken in triplicate. After 28 days of growth, 10 plants from each treatment were selected randomly and fresh weight of roots and shoots were taken. Dry weight was taken after

drying the roots and shoots in oven at 65^0 C for 48 hours. Analysis of rhizosphere soil was done for determining the effect of inoculation on rhizosphere microflora. Soil samples (in triplicate) were collected from all the four treatments and serial dilution technique was carried out for bacteria and fungi on tryptone yeast extract and PDA, respectively. Enumerations were made after 5 days of incubation at 26^0 C.

RESULTS AND DISCUSSION

The frequently occurring fungal species in soil samples of glacier sites in high altitudes of Indian Himalaya mainly belonged to the genera *Alternaria, Aspergillus, Chrysosporium, Cladosporium, Epicoccum, Fusarium, Gangronella, Myrothecium, Paecilomyces, Phoma, Phytophthora* and *Trichoderma* (Pandey *et al.,* 2008). *Trichoderma* was observed as one of the most frequently occurring genera in dilution agar plates throughout these experiments. The species were often observed covering the entire area of the culture plates, not allowing the other species to grow. The colony morphology and microscopic features of three species of *Trichoderma* are presented in Table 1. Based on these characters, the *Trichoderma* spp. were identified as *Trichoderma viride, Trichoderma harzianum* and *Trichoderma konengii*. These species could grow between 4 to 42^{0}C temperature and 3 to 13 pH on agar plates. The optimum temperature and pH requirement of these species was observed to be 26^{0}C and 5.5, respectively. It was interesting to notice that all the three species showing mycelial growth with moderate sporulation after 5 days of incubation at 26^{0}C resulted in heavy sporulation in about three weeks when shifted to 4^{0}C. Induction of heavy sporulation due to low temperature might be a strategy of these species for their survival under low temperature environments including the sub-zero temperatures.

In plate based experiments, all the three species of *Trichoderma* showed antagonistic interactions against the three test phytopathogens, *Fusarium oxysporum, Alternaria alternata* and *Cladosporium oxysporum*. Table 2 presents the results on the effect of diffusible and volatile metabolites produced by *Trichoderma* species in terms of reduced radial growth of the test fungus. The inhibition of test pathogens due to production of diffusible metabolites ranged from 56.88 to 73.90%, 53.33 to 64.16% and 53.33 to 63.56% by *Trichoderma viride, Trichoderma harzianum* and *Trichoderma konengii*, respectively. While inhibition due to production of volatile metabolites ranged from 41.43 to 71.66%, 47.22 to 60.15% and 39.63 to 63.56%, by *Trichoderma viride, Trichoderma harzianum* and *Trichoderma konengii*, respectively. Microscopic observations revealed that diffusible as well as volatile substances induced morphological abnormalities in fungal structures. Deformation in mycelial, hyphal, or conidial structure was common in all the test fungi. In general, the diffusible substances appeared to be more effective as compared to volatiles. Induction of deformities was probably due to the ability of *Trichoderma* species to develop direct interaction with pathogens and to produce antimicrobial substances as the mycoparasitism involves physical contact and synthesis of hydrolytic enzymes, toxic compounds, or antibiotics (Benitez *et al.,* 2004).

Studies have been conducted on effect of low temperature on spore germination and germ tube growth of *Trichoderma* strains. In earlier studies, performed on cold tolerant species of *Trichoderma*, out of 360 *Trichoderma* strains investigated, 14 (identified as strains of *T. aureoviride, T. harzianum* and *T. viride*) could grow well at 5^{0}C, all the cold tolerant strains produced appresoria and antagonized the plant pathogens, *Rhizoctonia solani* and *Fusarium oxysporum*, in dual culture tests performed at 10^{0}C (Antal *et al.,* 2000). Species of *Trichoderma* are known for their competence for rapid colonization of plant roots. Due to their nature of colonizing in presence of healthy roots they have evolved numerous mechanisms for attacking other fungi and for enhancing plant

Table 1 : Growth characters of three *Trichoderma* species

Characters	*T. viride*	*T. harzianum*	*T. konengii*
Colony morphology	Dark green colony with yellow tint & cushion and needle shaped structures	Dark green colony with yellow tint & cushion and needle shaped structures conidia round, conidiophores branched	White colony with green tint and cushion shaped structures
Microscopic features	Conidia round, conidiophores branched	Conidia round, conidiophores branched	Conidia oval, conidiophores branched

Table 2 : Biocontrol related properties of *Trichoderma* species against pathogenic test fungi

Pathogenic test fungi	Per cent inhibition in radial growth (cm) due to production of					
	Diffusible metabolites			Volatile metabolities		
	T. viride	*T. harzianum*	*T. konengii*	*T. viride*	*T. harzianum*	*T. konengii*
Fusarium oxysporum	73.90	60.97	63.56	41.43	47.22	39.63
Alternaria alternata	62.78	64.16	60.15	45.67	55.55	63.56
Cladosporium oxysporum	56.88	53.33	53.33	71.66	60.15	46.66

and root growth (Harman 2006). Knowledge of the prevalence of environmental conditions, both climatic and edaphic, in the habitat of a given organism may be useful for exploitation of the potential applications associated with the organism. The habitat of the species of *Trichoderma* in present study was forest locations mainly dominated by species of *Abies, Rhododendron* and *Betula*. Dominance of *Trichoderma* species in tea gardens and temperate forest locations of Indian Himalayan Region has been reported by Pandey *et al.* (2001).

Observation taken on chickpea (*Cicer arietinum* L.) based bioassay were indicative of plant growth promotion abilities of the *Trichoderma* species. Biomass of root and shoot of chickpea in inoculated plant was higher after 28 days of growth as compared to control, statistically significant ($p<00.5$) in most cases. Maximum benefit was observed in case of *T. harzianum*. The inoculated *Trichoderma* species colonized the rhizosphere of test plant species and increased the plant biomass (Fig. 1 and 2). Further, *Trichoderma* species inoculation stimulated the bacterial and suppressed the fungal population in the rhizosphere of test plant, *i.e.*, chickpea, indicative of associated antifungal properties.

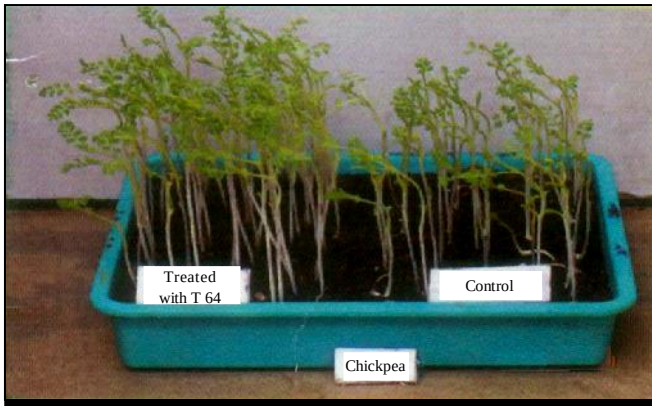

Fig. 1 : Plant Growth Promotion Ability of *Trichoderma* sp. of Gram (*Cicer arietinum* L.)

Fig. 2 : Plant Growth Promotion Ability of *Trichoderma* sp. of Gram (*Cicer arietinum* L.)

There is considerable interest in manipulating the soil microbial community to achieve the biological control of soil borne plant pathogens (Cook and Baker, 1983). Biocontrol is primarily linked to a sustained increase in active propagules of the antagonist. Root rot is the most serious disease-affecting chickpea (*Cicer arietinum* L.) the disease is caused by *Rhizoctonia solani* and is soil borne. *Trichoderma* sp. is widely used for the biological control of *Rhizoctonia solani* infection in root rot of chickpea and other vegetables. *Trichoderma* species have got attention due to their role in biological control. Mycoparasitism has been proposed as the major mechanism supporting the antagonistic activity of *Trichoderma* species. Strains of *Trichoderma* species inhibit pathogens through production of antifungal antibiotics and or/hydrolytic enzymes. Ability for plant growth promotion and induced resistance in plants by *Trichoderma* species has also been reported (Monte, 2001; Vizacaino *et al.*, 2005; Harman, 2006).

Biological control is one of the key components of integrated pest management that envisage the conservation and augmentation of naturally occurring bioagents such as parasitoids, predators, entomopathogens and antagonistic fungi and bacteria. Biofungicides include in a broader sense fungicides of biological origin *i.e.* botanical and microbial. The use of microbial fungicides as one of the major components of IPM is gaining acceptance, as these are generally specific, apparently harmless to the beneficial insects, animals and human beings with no residue problems and environmental hazards. Microbial fungicides are made of microbes such as eco-friendly fungi (Prakash *et al.*, 1999).

The present study is important in view of the documentation of soil microbial diversity in Northern Hilly Zones of Chhattisgarh in Mainpat, Dist-Surguja, Chhattisgarh. It is mainly based on the isolation of three most frequently occurring species of *Trichoderma* and demonstration of their biocontrol abilities. Isolation and screening of cold tolerant strains of *Trichoderma* species is important in order to select the biocontrol agents for application in colder regions.

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