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Characterization of native rice specific isolates of *Trichoderma* and evaluation of its effect on sheath blight pathogen *Rhizoctonia solani*

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Abstract

Trichoderma species is one of the most important antagonistic fungi commonly found in soil and is effective against many soil borne plant pathogens. It has been reported to improve plant growth and development in addition to its biocontrol effects on the pathogens. Sheath blight is one of the most economic important disease of rice causing significant yield loss. Biocontrol using *Trichoderma* spp. is reported to be a safe and effective alternate strategy to the use of fungicides which are harmful to the environment and consumers alike. About 4 native isolates of *Trichoderma* spp. were isolated from rhizospheric soil collected from different rice growing areas in India including the states of India viz., Telangana, Karnataka, Chhattisgarh, Jharkhand and Madhya Pradesh. The isolates were characterized both morphologically and by molecular means using ITS sequencing of *Trichoderma* specific 18s rRNAs.(NCBI ID2146474-IIRRCK1 to IIRRCK4) Accordingly the isolates were identified and the most potential isolate identified from the results was found to be *Trichoderma asperellum* based on the query score and similarity percentage. Results from the studies on the bio-efficacy of the isolates indicated that *T. asperellum* isolate IIRRCK1 was found to be the most effective in improving plant growth and high inhibition of *R.solani* (51.8%) under *in vitro* conditions.

Key words: *Trichoderma sp., Rice, Sheath blight, Rhizoctonia solani*

Introduction

Trichoderma is a genus of asexually reproducing fungi that are often the most frequently isolated soil fungi, from all temperate and tropical soils. *Trichoderma* strains may have one or all mechanisms of action according to species and strain. *Trichoderma* can offer several advantages over synthetic chemicals i.e., they are environment friendly, do not leave toxic residues, do not harm non-target friendly micro/macro flora and fauna and finally promote natural defense, growth and health of the host plants in addition to their biocontrol ability (Tahia et al., 2004).The mode of action of *Trichoderma* spp. include antibiosis, hyper-parasitic activity against pathogens and induction of systemic resistance in the host plants. *Trichoderma* strains produce a great variety of lytic enzymes most of which play a great role in their biocontrol activity against a broad spectrum of fungal pathogens *viz.,* species of *Rhizoctonia*, *Fusarium*, *Alternaria*, *Ustilago*, *Venturia* and *Colletotrichum* and the Oomycetes like *Pythium* and *Phytophthora* which lack chitin in their cell walls (Sriram et al., 2004). *Trichoderma* produces secondary metabolites and some of them are involved in the antibiosis activities against the pathogens. The secondary metabolites are classified into i) volatile antibiotics ii) water-soluble compounds, i.e. heptelidic acid or koningic acid and iii) peptaibols (Susanne et al., 2006). For successful; deployment, *Trichoderma* strains should be carefully chosen according to their mechanism of action and adaptation to the specific environment.

Rice (*Oryza sativa*) is India's most important cereal crops and is the staple food for more than 60 % of the world's population. Intense cultivation and adoption of semi-dwarf, early maturing and fertilizer responsive high-yielding rice varieties, coupled with increased fertilizer application have brought in the risk of increased incidence of pest and disease. Sheath blight disease caused by the fungus *Rhizoctonia solani* Khun, a soil borne saprophytic fungi, is one of the most destructive diseases of rice next to blast (Singh *et al*., 2003). The disease was first reported in Japan in 1910, later from all over the world (Prasad and Eizenga, 2008). The disease is severely endemic in areas where temperature and relative humidity are high and cultivation is intensive. The disease can cause yield losses up to 50

per cent in advanced stages and even adversely affects the quality of straw thereby limiting its value as fodder (Rajan, 1987). Results from several investigations have indicated that *Trichoderma* spp. are effective for control of sheath blight disease either single or in combination (Jasmine et al., 2005).

In the present investigation *Trichoderma* spp. isolated from rhizosphere soils of rice were characterized and evaluated for their efficacy on rice growth promotion and against *R. solani* in vitro and in vivo conditions.

Material and Methods:

Materials: *Trichoderma* isolates were collected from rhizosphere soils of farmer's rice fields during the monitoring trials and in the nearby fields around Hyderabad. The dilution-plate method was used to isolate *Trichoderma* from soil as described by Moubasher and Abdel-Hafez (1978) using *Trichoderma* medium E (Papavizas *et al.*, 1982). The cultures were maintained in PDA slants under refrigerated conditions. Morphological characterization was done as per the key characters of *Trichoderma* as described by Rifai (1969) and Lieckfeldt et al., (1999).

DNA extraction ITS analysis: The DNA extraction was done as per the standard cTAB (cetyl trimethyl ammonium bromide) protocol (Moeller et al., 1992: White at al., 1990). Mycelium disc was collected individually from the isolates and ground to a fine powder using liquid nitrogen. The powdered mycelium was mixed with 2x (CTAB) buffer and incubated at 65°C for 30 min, followed by extraction with chloroform phenol and iso-amylalochol (25:24:1) micture. The tubes were centrifuged and resulting pellets were washed with 0.1 ml of 70% ethanol, followed by air drying of the pellet. A region of nuclear rDNA, containing 18S ribosomal RNA gene (partial sequence); internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (complete sequence); and 28S ribosomal RNA gene (partial sequence) was amplified by PCR using the primer combinations ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and LR3R (5'-GGTCCGTGTTTCAAGAC-3') in 25 µl volume, in an automated temperature-cycling device (Eppendroff) was adopted (White *et al.*, 1990). PCR conditions was 1 min denaturation at 94°C: 30 cycles of 1 min denaturation at 94°C: 1 min primer annealing at 50°C: 90 s extension at 74°C: and final extension period of 7 min at 74°C. The desired amplified product was around 1200bp . The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, India.) DNA sequences were aligned with Clustal W and NCBI Blast N was used for identification of the 4 isolates that were isolated from the soil samples.

Studies on the efficacy of *T. asperellum* on *R. solani:* The antagonistic potential of the different isolates of *T. asperellum* on *R. solani* was tested under in vitro conditions. Petri plates containing PDA was inoculated at the with 5mm mycelial discs of *T. asperellum* was inoculated at 1cm away from the edge of the plate opposite to each other. After two days, a disc of Petri plates inoculated with pathogen at one end alone served as control. The Petri plates were incubated at room temperature for 3-4 days and radial growth afterwards were measured and recorded. Three replications were maintained per treatment. The antagonists found effective against these pathogens under *in vitro* conditions were further evaluated in the glass house on susceptible rice cultivar, TN-1. The plants were raised in plastic pots of 15 cm diameter and about 4 hills per pot were maintained in the glass house at a temperature of 27 ± 2^0 C and RH of 75-90%. Three different application methodologies, i.e., soil application, seed treatment and seed treatment followed by soil application were used to assess the biocontrol potential of the antagonist in suppressing rice sheath blight disease caused by *R. solani*. The antagonist, *T. asperellum* IIRRCK1 was applied in different ways i.e., as seed treatment followed by inoculation of *R. solani* on 30DAS, applications of T. *asperellum* IIRRCK1 as seed treatment followed by soil application on 25 DAS and followed by inoculation of *R. solani* on 30 DAS. *R. solani* was inoculated by placing the colonized shoot pieces of *T. angustata* between the tillers in the central region of the rice hills, 5-10 cm above the water line.

The severity of sheath blight disease was assessed 20 days after pathogen inoculation according to relative lesion height (RLH) method as suggested by Sharma *et al*. (1990) and the disease incidence was recorded using 0-9 grade scale. The disease progression in terms of vertical disease spread (cm) was measured with the help of scale from 3rd day to 20th day after inoculation of *R. solani*. The observations were taken on 3rd, 5th, 10th, 15th and 20th day to calculate PDI on respective days and to analyze the disease progression in the presence of the respective antagonists to know their potency to check the disease.

Results and Discussion:

Characterization of *Trichoderma* isolates

Four different isolates of *Trichoderma* spp. were isolated from the soils collected from different regions as given in the table (Table 1).

Table 1: Geographical location colony characters, conidial initiation growth and percent inhibition of *R. solani* over control of the isolates of *Trichoderma* spp. collected from the rice-rhizosphere soils of different regions in India.

Isolate No	Isolates	Location	Colour of the colony	Conidiation Initiation	Percent inhibition of R. solani over control in vitro in 5 days
1	*T. asperellum*	Hyderabad	Light green	46 hrs	**51.8 (46.03)**
2	*T. asperellum*	Hazribagh	Dark green	36 hrs	38.2 (38.04)
3	*T. asperellum*	Raipur	Light green	7 days	37.0 (36.54)
4	*T. asperellum*	Rewa	Dark green	72 hrs	35.2 (33.04)
CD					0.652
SE(±m)					0.274
CV					0.871

The isolates were characterized based on the colour of the colony, initiation of conidia in the media. The isolates varied in the initiation of conidiation ranging from about 36 hours (*T. asperellum* HZB) to 7 days (*T. asperellum* RPR). Conidial colour change was observed from white to varying shades of green (Table 1). In the microscopic studies of *T. viride* and *T. asperellum* similar type of arrangement of conidiophores and phialides were observed in all the 6 isolates. The isolates were characterized by highly branched divergent and dendritic conidiophores. Divergent phialides were typically arranged in whorls of 3-5 and held at 90° with respect to the hyphae from which they arose, or solitary. Those in whorls were typically flask-shaped, enlarged in the middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base. Terminal phialides were arranged in a whorl or solitary, were typically cylindrical or at least not conspicuously swollen in the middle and longer than the sub-terminal phialides. Majority of these above characteristics resembled with *T. asperellum* and *T. viride.*

In order to arrive at a conclusion on the identity of the species, 18sRNA sequencing and subsequent blast with the NCBI repository was carried out (Table 2). Several reports have been published on isolation and identification of *Trichoderma* in Saudi Arabia (Molan, 2009; Hussein and Yousef, 2011).

Table 2: Molecular characterization of *Trichoderma* strains using ITS regions sequencing

Isolate Code	Accession Number	Similarity %	Given (anamorph) name
Isolate-1	KF723005.1	99%	*Trichoderma. asperellum*
Isolate-2	KF723005.1	97%	*Trichoderma. asperellum*
Isolate-3	KF723005.1	100%	*Trichoderma. asperellum*
Isolate-4	KF723005.1	93%	*Trichoderma. asperellum*

Based on the query score and similarity percentage, the isolates were identified the isolates were identified as *T. asperellum*. Four isolates were sequenced; the length of the amplified fragments was 1200 bp (Figure- X). The sequencing results have been blasted against gene bank. Four isolates showed similarity ranging from 100% to 93% with the sequence results of *Trichoderma asperellum* and *Trichoderma viride*. This complexity comes from the fact that many of these species types are overlapping and therefore, two closely related organisms become attributed to different species recognized based on incomparable criteria. The isolates 1, 2, 3 and 4 showed similarity percentages of 99%, 97%, 100% and 93% respectively with *T.asperellum (*Fig. 1). The ITS sequences of all the four isolates of T. asperellum IIRRCK1 to IIRRCK4 were submitted to NCBI with the reference ID 2146474 .

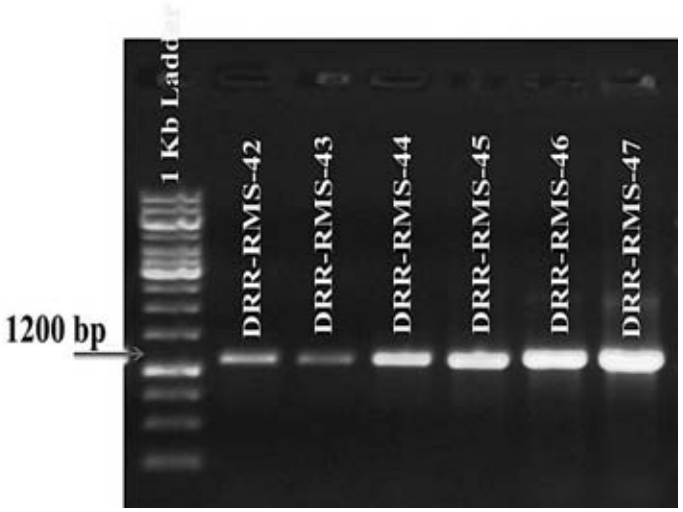

Figure 1: Gel picture representing the molecular characterization of Trichoderma isolates using ITS regions

Lanes: DRR-RMS-42- Isolate-1, DRR-RMS-43- Isolate -2, DRR-RMS-44- Isolate -3, DRR-RMS-45- Isolate -4

Biocontrol efficacy of *T. asperellum* IIRRCK1 against *R.solani*

All the four isolates of *T. asperellum* inhibited the radial growth of *R. solani* when inoculated in the plates; three days post inoculation of *R. solani* (Table 1, Fig. 2). Maximum growth suppression was recorded by the isolate 1 named as *T. asperellum* IIRRCK1 (51.8% over control), followed by the other three isolates. Due to its maximum antagonistic potential, the isolate *T. asperellum* IIRRCK1 was used in the study to estimate the progress of sheath blight disease in potted rice plants.

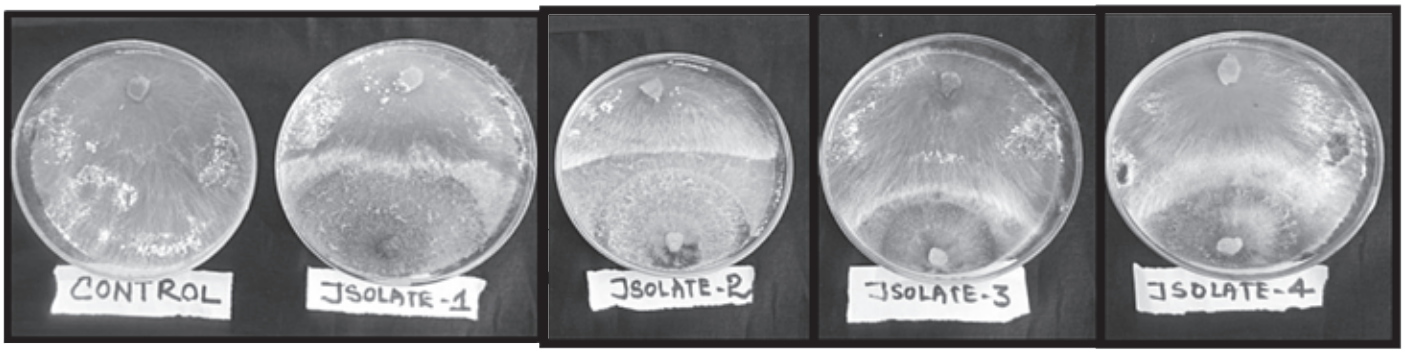

Figure 2: Effect of different isolates of T. asperellum on R. solani under in vitro conditions.

Progress of sheath blight disease in the presence of *T. asperellum* IIRRCK1

Results from the pot culture studies on the biocontrol efficiency of *T. asperellum* IIRRCK1 on sheath blight disease in rice (Table 3) indicated that the biocontrol agent was able to suppress the rate of development of sheath blight disease during the entire period of observation. Seed treatment with *T. asperellum* IIRRCK1 was more effective in suppressing the development of the disease (63.97 and 58.27 % over control) when compared to soil application alone (33.69 and 27.94 % over control). This indicated that *T. asperellum* IIRRCK1 could establish in the plants more effectively when the seeds are treated with the fungus. This may be due to the endophytic nature of this particular isolate of *T. asperellum* which shall be investigated. It has been reported by several workers that the endophytic *Trichoderma* strains have the ability to induce disease resistance and also improve the overall development of the host plants ((Bae et al., 2011: Chen et al., 2016: Elad et al., 1983). Further analysis of the data (Table 3) indicated that with progress of time, the biocontrol effect of *T. asperellum* IIRRCK1 got reduced suggesting that the antagonistic fungus population needs to be augmented with the second dose of soil application.

Table 3: Effect of indigenous isolate *T. asperellum* IIRR1 *in the* progress of Sheath blight disease of rice under pot culture conditions in the rice variety TN1.

Treatments	No. of days after pathogen inoculation									
	3		5		10		15		20	
	Mean	Percent reduction over control	Mean	Percent reduction over control	Mean	Percent reduction over control	Mean	Percent reduction over control	Mean	Percent reduction over control
Ta St *fb Rs* on 30DAS	7.4 (15.66)	63.97	13 (21.18)	51.43	20.52 (26.92)	34.75	22.95 (28.57)	33.95	22.95 (28.60)	46.25
Ta ST *fb Ta* SA on 25 DAS 25DAS *fb Rs* on 30 DAS	8.57 (17.01)	58.27	14.77 (22.57)	44.82	20.9 (27.19)	33.54	21.1 (27.43)	39.28	21.1 (27.36)	50.58
Ta SA on 25DAS *fb Rs* on 30DAS	13.65 (21.67)	33.69	21.07 (28.49)	21.29	23.22 (28.81)	26.16	28.67 (32.36)	17.49	35.05 (36.25)	17.91
Rs on 30DAS *fb Ta* SA on 35 DAS	14.8 (22.63)	27.94	16.92 (24.28)	36.79	22.25 (28.11)	28.61	24.32 (29.53)	30.01	27.47 (29.65)	35.66
Rs on 30DAS (Control)	20.54 (26.96)	0.00	26.77 (31.14)	0.00	31.45 (34.06)	0.00	34.75 (36.09)	0.00	42.7 (40.78)	0.00
CD	*0.20*		*0.12*		*1.05*		*1.15*		*0.05*	
CV	*0.77*		*0.40*		*2.90*		*2.99*		*0.12*	
SE(m)	*0.06*		*0.04*		*0.35*		*0.38*		*0.01*	

However this needs to be standardized. Similar observations in the need for augmentation of the biocontrol inocula were reported by several workers (Kumari et al., 2016: Lenka *et al*. 2012: Prasad and Reddykumar, 2011).

Conclusions

The isolate of *T. asperellum* IIRRCK1 can significantly inhibit *R. solani* and suppress the sheath blight disease severity in rice. Further studies on the standardiszation of the dose and time of application and the suitable formulation for precise and sustained release of *T. asperellum* IIRRCK1 is under progress in this laboratory.

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