Microbiological Research 214 (2018) 83–90

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Novel *Trichoderma* strains. isolated from tree barks as potential biocontrol agents and biofertilizers for direct seeded rice

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ARTICLE INFO

Keywords:
Trichoderma erinaceum
Biocontrol
Biofertilizer
Stress enzymes
Sheath blight
Nutrient uptake
Direct seeded rice

ABSTRACT

This study is the first time report of utilization of *Trichoderma* spp. isolated from different tree barks from Odisha state of India for rice crop health management and higher productivity. Six isolates of *Trichoderma* spp. were identified based on the morphological characteristics and species determination was performed by molecular assays. One of the isolated strains determined as *Trichoderma erinaceum* outperformed others. *Trichoderma erinaceum* controlled three soil borne plant pathogens i.e. *Rhizoctonia solani, Sclerotium rolfsii* and *Sclerotium oryzae* effectively under controlled condition and *R. solani* and *Helminthosporium oryzae* under filed condition. Seed treatments with the formulated isolates improved the germination rate of rice and enhanced vigour. These parameters along with higher chlorophyll content could be related to higher yield observed in two rice varieties; *Karuna and Shabhagidhan*. Among the six isolates tested, *Trichoderma erinaceum* treatment recorded highest yield. Significantly higher expression of some stress related enzymes was observed in *Trichoderma* treated plants which helped in better crop growth both under biotic and abiotic stresses. These isolates helped both the varieties to accumulate more nutrients This study proves that *Trichoderma erinaceum* obtained from tree bark may be incorporated in integrated rice crop management both as biocontrol agent and biofertilizer.

1. Introduction

Trichoderma spp., are the most widely used microorganisms with disease biocontrol and plant growth promoting activity (Druzhinina et al., 2011; Druzhinina and Kubicek, 2013). These fungi are mycoparasites and produce a plethora of antimicrobial secondary metabolites including phytohormones (Harman et al., 2004; Harman, 2006; Howell, 2006; Shoresh et al., 2010). Phytohormones produced by *Trichoderma* spp. promote plant (root and shoot) growth (Harman et al., 2004; Shoresh et al., 2010) and these fungi mobilize plant nutrients for better crop yield (Mastouri et al., 2010, 2012). They are presently marketed as biopesticides, biofertilizers, growth and yield enhancers as well as nutrient solubilizers and organic matter decomposers (Woo et al., 2014). An interesting aspect of *Trichoderma* mediated biocontrol is their ability to colonize roots and induce systemic resistance against invading fungi, bacteria, viruses and even insects, at a site away from *Trichoderma* inoculation (Segarra et al., 2007; Contreras-Cornejo et al., 2011; Salas-Marina et al., 2011). *Trichoderma* spp. are generally isolated from soil or rhizosphere for their use in biocontrol, and there are only a few reports on the evaluation of *Trichoderma* as biocontrol agents isolated from above-ground habitats (*e.g.* Mukherjee et al., 2014). Rice provides food security to more than half of the global population and to about 85% population of India (Ghose et al., 2013). The major constrain of rice production is different biotic stresses which reduces yield considerably. Rice suffers from several fungal diseases like brown spot, sheath blight, blast, seedling blight, false smut *etc*. Of these, brown spot and blast are major foliar diseases and sheath blight and seedling blight being major soil borne diseases. To manage these diseases huge amount of fungicides are being used which are not only hazardous for the farmers but also for the environment. In order to manage these diseases in an ecofriendly way it is needed to search for alternative management practices. Use of biocontrol agents (BCA) especially *Trichoderma* based BCA have been good commercial success in different crops other than rice. But unfortunately the most of the BCA present in the Indian market

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https://doi.org/10.1016/j.micres.2018.05.015
Received 19 January 2018; Received in revised form 20 April 2018; Accepted 17 May 2018
Available online 23 May 2018
0944-5013/ © 2018 Published by Elsevier GmbH.

Table 1
Details of *Trichoderma* isolates used in the present study.

Strain Designation	Source of collection	Place of collection	GPS Location	Species identified	NCBI Accession Numbers.
CRRI-T1	bark of a *Litchi chinensis*	NRRI, Cuttack	85°92′E, 20°45′N	*Trichoderma harzianum*	KX853519.1
CRRI-T2	bark of a *Cassia tora*	42-Mouza(Barala), Cuttack	86°92′E, 20°44′N	*Trichoderma erinaceum*	KR014407.1
CRRI-T3	bark of a *Cassia tora*	42-Mouza(Barala), Cuttack	86°58′E, 20°45′N	*Trichoderma atroviride*	KR014408.1
CRRI-T5	bark of a *Mangifera indica*	Manitri, Jagatasinghapur	88°12′E, 20°45′N	*Trichoderma atroviride*	KX853518.1
CRRI-T9	bark of a *Mangifera indica*	Barala, Cuttack	86°02′E, 20°44′N	*Trichoderma atroviride*	KX863696.1
CRRI-T13	bark of a *Mangifera indica*	Barada (Kishan Nagar) Cuttack	86°92′E, 20°45′N	*Trichoderma atroviride*	KX863695.1

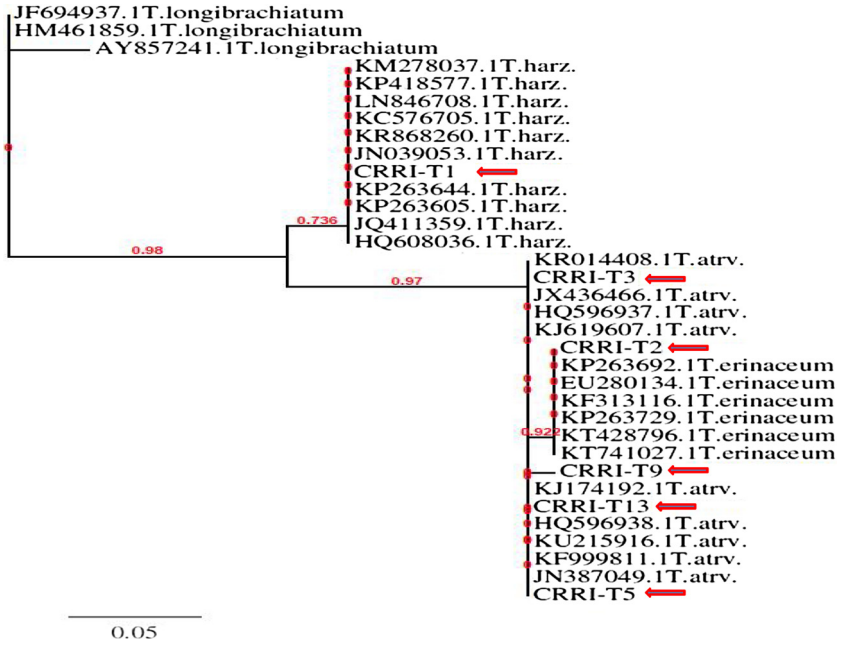

Fig. 1. Phylogeny of the isolated *Trichoderma* spp. (CRRI-T1 to CRRI-T13) used for the present study.

Table 2
Confrontation assay showing the inhibition of pathogen growth by different *Trichoderma* isolates in PDA medium.

Treatment Name	Percentage of inhibition		
	Rhizoctonia solani	*Sclerotium oryzae*	*Sclerotium rolfsii*
CRRIT1	99.57^{A}	58.57^{B}	22.83^{B}
CRRIT2	100.00^{A}	67.38^{A}	35.48^{A}
CRRIT3	100.00^{A}	62.75^{AB}	32.98^{A}
CRRIT5	90.77^{B}	46.24^{C}	4.35^{D}
CRRIT9	80.25^{C}	30.61^{D}	15.75^{C}
CRRIT13	80.26^{C}	30.08^{D}	16.30^{C}
CV (%)	1.46	4.08	6.07
Tukey's HSD at 5%	3.801	5.705	3.6646

Means with same letter are not significantly different at $p \leq 0.05$.

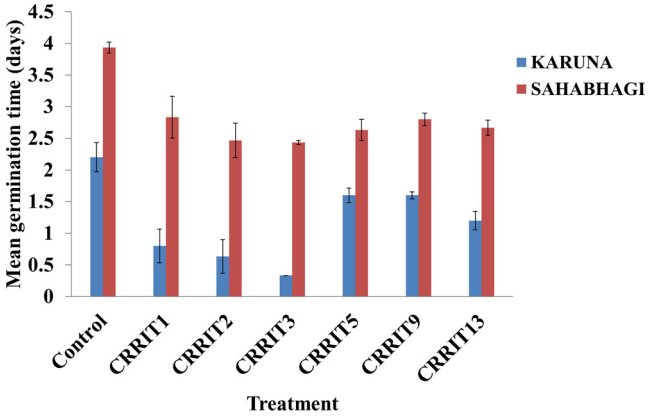

Fig. 2. Mean germination time in rice varieties treated with *Trichoderma* and control condition. (± = standard error).

originated from the same source *i.e.* with *Trichoderma viride* collected from soil (Mukherjee et al., 2013). In a recent study it has been reported that native *Trichoderma* isolates exhibit better performance (Mukherjee et al., 2014). Even though *Trichoderma* spp. has proved effective in management of diseases in a wide variety of crops, their applications in rice have been limited to laboratory condition only. It is generally thought that *Trichoderma* spp., being strictly aerobic, may not perform in paddy field, especially in flooded rice fields.

One of the best approaches to find effective BCA for application to soil or seed material is to seek them from those locations where the pathogen is supposed to cause disease but isn't (Cook, 1985). The main habitat of *Trichoderma* is classically viewed as soil or plant rhizosphere, even though the maximum diversity of these species occurs aboveground *e.g.*, on tree bark and wild mushrooms (Druzhinina et al., 2011). *Trichoderma* spp. isolated from tree bark mainly is being used for the production of enzymes for decomposition of agro-wastes. This is the first attempt to study the utilization of *Trichoderma* spp. isolated from bark of trees in Odisha state of India, as a potential biocontrol and biofertilization agent in rice. In this study, in addition to biocontrol properties we investigated changes in some stress related enzymes, plant nutrient uptake and crop growth parameters on use of *Trichoderma* spp. formulation as seed treatment under field condition.

Table 3
Effect of *Trichoderma* treatment on seedling Vigour index of rice Varieties.

Treatment Name	Seedling Length (c.m.)		Seedling Dry weight(g)		Vigour index-1		Vigour index-2	
	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan
Control	22.10^{B}	23.67^{C}	0.10^{D}	0.12^{D}	2210.00^{B}	2366.67^{C}	9.67^{D}	12.33^{D}
CRRIT1	24.70^{AB}	30.03^{AB}	0.11^{C}	0.14^{CD}	2470.00^{AB}	3003.33^{AB}	11.33^{C}	14.00^{CD}
CRRIT2	27.40^{A}	32.70^{A}	0.14^{A}	0.17^{A}	2740.00^{A}	3270.00^{A}	14.00^{A}	17.33^{A}
CRRIT3	26.53^{A}	29.63^{AB}	0.14^{AB}	0.16^{AB}	2653.33^{A}	2963.33^{AB}	13.67^{AB}	16.00^{AB}
CRRIT5	25.90^{A}	31.80^{AB}	0.15^{A}	0.17^{AB}	2590.00^{A}	3180.00^{AB}	15.00^{A}	16.67^{AB}
CRRIT9	24.70^{AB}	31.20^{AB}	0.12^{BC}	0.16^{AB}	2470.00^{AB}	3120.00^{AB}	12.33^{BC}	16.00^{AB}
CRRIT13	24.30^{AB}	27.90^{BC}	0.12^{C}	0.15^{BC}	2430.00^{AB}	2790.00^{BC}	12.00^{C}	15.33^{BC}
CV (%)	4.37	5.45	3.75	4.49	4.37	5.45	3.75	4.49
Tukey's HSD at 5%	3.1359	4.6064	0.0135	0.0197	313.59	460.64	1.3471	1.9719

Means with same letter are not significantly different at $p \leq 0.05$.

Table 4
Chlorophyll Content in rice varieties due to the treatment with different *Trichoderma* isolates.

Treatment Name	Chla (mg/g of fresh leaf)		Chlb (mg/g of fresh leaf)		Chla/Chlb (mg/g of fresh leaf)		Total Chlorophyll (mg/g of fresh leaf)	
	Karuna	Sahabhagidhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan
Control	3.86^{G}	1.25^{G}	0.88^{G}	0.14^{G}	4.41^{A}	8.92^{A}	4.73^{G}	1.39^{G}
CRRIT1	7.41^{F}	9.77^{F}	2.01^{F}	3.05^{E}	3.69^{BC}	3.21^{C}	9.42^{F}	12.81^{F}
CRRIT2	12.10^{B}	14.19^{B}	3.41^{B}	4.31^{B}	3.55^{D}	3.29^{C}	15.51^{B}	18.49^{B}
CRRIT3	11.70^{C}	13.36^{C}	3.15^{C}	4.03^{C}	3.72^{B}	3.31^{C}	14.84^{C}	17.39^{C}
CRRIT5	10.04^{D}	11.92^{D}	2.75^{D}	3.53^{D}	3.65^{C}	3.38^{C}	12.80^{D}	15.45^{D}
CRRIT9	8.08^{E}	10.75^{E}	2.47^{E}	2.68^{F}	3.27^{E}	4.01^{B}	10.54^{E}	13.42^{E}
CRRIT13	13.42^{A}	19.84^{A}	3.76^{A}	6.12^{A}	3.57^{D}	3.24^{C}	17.18^{A}	25.96^{A}
CV (%)	0.10	0.24	0.40	0.39	0.67	2.95	0.06	0.12
Tukey's HSD at 5%	0.0263	0.0805	0.0301	0.0384	0.0705	0.3531	0.0215	0.0496

Means with same letter are not significantly different at $p \leq 0.05$.

2. Materials and methods

2.1. Fungal isolates

Six *Trichoderma* isolates (*i.e.* CRRIT-1, CRRIT-2, CRRIT-3, CRRIT-5, CRRIT-9, and CRRIT-13) were collected from bark of different trees (Table 1) or from parasitized wild mushrooms at Cuttack, Odisha, India. The isolation and purification was made as described by Mukherjee et al., 2014).The fungi were collected with a sterile cotton swab and the conidial suspension was prepared in sterile double distilled water. The suspension was poured on Potato Dextrose Agar (PDA) plates after serial dilution with dilution factor 1:1000 and the isolated colonies were further purified by repeated serial dilution and plating. The isolates were maintained as glycerol stock in −80 °C deep freezer as well as life cultures.

The pathogen cultures used in the present study were *Rhizoctonia solani* CRRI-RS-8 (MTCC-12232) causing sheath blight of rice, *Sclerotium oryzae* CRRI-S.O (MTCC-12230) causing seedling blight of rice and *Sclerotium rolfsii* causing foot rot of rice. Field evaluation of *Trichoderma* treated seeds of rice was also performed against brown spot and sheath blight diseases.

2.2. Morphological & molecular characterization

Morphological identification of *Trichoderma* spp. was done based on colony and microscopic characteristics (Gams and Bissett, 1998) according to ISTH (International Sub-commission on *Trichoderma* and *Hypocera*) after incubation for four days at 27 °C on PDA medium. Genomic DNA was isolated from fresh mycelia and washed thoroughly with autoclaved distilled water and then blotted to dry. Total genomic DNA from the mycelia was isolated by using standard SDS (Sodium Dodecyl Sulphate) method (Mukherjee et al., 2014). The molecular characterization of all the fungal isolates were based on the sequences of Internal Transcribed Spacer (ITS) regions, Translation Elongation Factor 1 (TEF1) regions and RNA Polymerase B-larger subunit-II (RPB-II) regions as per standard methods (http://www.isth. info/tools/blast/markers.php). The species were identified by BLASTN search on the NCBI site and the identity confirmed by comparing the sequences with authentic sequences from GenBank, and a phylogenetic tree constructed on http://www.phylogeny.fr

2.3. Confrontation assays

Ability of the *Trichoderma* isolates to antagonize the isolated pathogens were assessed by using confrontation assay on PDA plates by simultaneous inoculation of both *Trichoderma* and the pathogen near the edge of the plate, placed opposite to each other. Plates inoculated with pathogens only were used as control. The percentage of mycelial growth inhibition was calculated according to Hajieghrari (2010.

Percentage of inhibition = [{(R1-R2)/R1}] × 100

Where, R1 = Radial growth of the pathogen in control plate, R2 = Radial growth of the pathogen in test plate

2.4. Greenhouse experiments

Validation of isolates as biocontrol agents and growth promoters was performed in a greenhouse experiment. Seeds were treated and placed in sterile autoclaved soils filled in 12 inch × 12 inch pots (each pot having 5 kg of autoclaved soil) and incubated under ambient conditions (25° C). Seeds were treated with respective *Trichoderma* talc based formulations (Mukherjee et al., 2014) (10 g/Kg of seeds, conidia 10^{7}cfu/g in 0.5% aqueous carboxy-methyl cellulose) and for control treatment, seeds were sown without *Trichoderma* application.

2.4.1. Germination and early vigour

The germinability of treated seeds (25 seeds in each replication) in green house was determined every day to calculate mean germination

Table 5
Plant growth promotion in different rice varieties due to *Trichoderma* treatment as indicated by various Agronomical Parameters.

Treatment Name	DryRoot Weight (g)		DryShoot Weight(g)		FreshRoot Weight(g)		FreshShoot Weight (g)		Root Length(cm)		Shoot Length(cm)		No.of Tiller/Hill		Yield/Hill (g)		Yield/Plot (g)	
	Karuna	Sahabhagidhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagi dhan	Karuna	Sahabhagidhan	Karuna	Sahabhagi dhan
Control	0.23^{D}	0.15^{E}	0.34^{D}	0.64^{E}	0.41^{C}	0.57^{E}	1.47^{F}	1.85^{D}	10.30^{C}	8.66^{E}	30.43^{E}	35.54^{E}	13.25^{E}	12.25^{C}	20.89^{E}	24.33^{E}	940.04^{F}	1144.79^{F}
CRRIT1	0.26^{D}	0.27^{D}	0.52^{D}	0.84^{D}	0.49^{C}	0.94^{D}	1.83^{E}	2.78^{C}	10.85^{C}	9.96^{D}	35.43^{D}	40.01^{D}	16.25^{D}	18.50^{B}	27.31^{D}	32.56^{D}	1229.09^{E}	1677.89^{E}
CRRIT2	**0.66^{A}**	**0.64^{A}**	**3.16^{A}**	**2.15^{A}**	**1.39^{A}**	**2.08^{A}**	**8.81^{A}**	**5.71^{A}**	**17.43^{A}**	**14.68^{A}**	**67.53^{A}**	**48.05^{A}**	**22.50^{A}**	**23.50^{A}**	**39.31^{A}**	**45.74^{A}**	**1828.81^{A}**	**2141.38^{A}**
CRRIT3	0.63^{A}	0.58^{B}	2.45^{B}	1.96^{B}	1.17^{B}	1.82^{B}	7.21^{B}	5.48^{A}	15.50^{B}	13.67^{B}	65.25^{A}	46.69^{AB}	20.75^{B}	22.00^{A}	34.78^{B}	44.16^{AB}	1614.47^{B}	2063.84^{B}
CRRIT5	0.55^{B}	0.54^{B}	0.99^{C}	2.03^{B}	1.16^{B}	1.73^{B}	3.24^{C}	5.47^{A}	15.13^{B}	12.82^{C}	44.17^{B}	44.85^{B}	19.75^{B}	22.75^{A}	33.35^{B}	43.23^{B}	1500.78^{C}	1962.74^{C}
CRRIT9	0.49^{BC}	0.35^{C}	0.88^{C}	1.31^{C}	1.03^{B}	1.49^{C}	2.63^{D}	3.84^{B}	14.18^{B}	10.34^{D}	40.37^{C}	42.28^{C}	18.25^{C}	20.00^{B}	30.54^{C}	41.44^{C}	1374.23^{D}	1754.57^{D}
CRRIT13	0.47^{C}	0.35^{C}	0.87^{C}	1.31^{C}	1.03^{B}	1.49^{C}	2.71^{D}	3.82^{B}	14.04^{B}	10.18^{D}	39.28^{C}	42.17^{CD}	18.25^{C}	20.00^{B}	30.19^{C}	41.40^{C}	1358.33^{D}	1751.55^{D}
CV (%)	9.61	6.84	10.05	5.05	10.88	5.90	3.44	5.04	7.53	3.32	5.53	3.51	4.70	5.28	3.34	3.06	3.57	1.82
Tukey's HSD at 5%	0.1055	0.0656	0.309	0.1727	0.2428	0.1995	0.3202	0.4871	2.4489	0.8908	5.9489	3.5089	2.0235	2.4497	2.4114	2.7858	117.4	75.726

Means with same letter are not significantly different at $p \leq 0.05$. Bold values indicate the highest values.

time till 8th days of sowing. On 8th day, germination was calculated as the ratio of the number of normal seedlings to the total number of seeds and expressed as percentage. On day eight of germination, ten normal seedlings were selected randomly in each treatment from all replications for measurement of shoot and root lengths. Seedlings used for recording seedling length from each replication were subsequently weighed after removing the cotyledons and the fresh weight was expressed in gram per 10 seedlings. These seedlings were subsequently dried in an oven at 55 ± 1 °C for 72 h and the dry weight was expressed in mg per 10 seedlings. Vigour of the coated seeds was assessed based on germination percentage, seedling length and seedling dry weight. Vigour index was calculated by using the formula given by Abdul-Baki and Anderson (1973)

Vigour index −I = Germination (%) × Seedling length (cm)

Vigour index −II = Germination (%) × Seedling dry weight (mg)

2.4.2. Determination of chlorophyll content

Chlorophyll content of the *Trichoderma* treated plants grown in greenhouse was determined following the procedure of Porra (2002). Chopped fresh leaf tissue of 0.1 g was transferred to a capped measuring tube containing 25 ml of 80% acetone and kept inside a refrigerator at 4 °C for 48 h. Measurements were made using a spectrophotometer (CARY 100 Bio UV–vis; Double Beam Spectrophotometer, USA) at a wavelength of 663 nm and 645 nm to calculate chlorophyll a and chlorophyll *b* content, respectively.

2.5. Growth promotion under field conditions

In-vivo experiment (in 1 m × 1 m plot) was carried out to check the effect of seed treatment on natural incidence of brown spot (60 DAS) and sheath blight diseases (75 DAS) in sick beds and for evaluation of growth promotion by taking two direct seeded rice varieties in two consecutive *Kharif* seasons (*i.e.* "Karuna" and "Sahabhagidhan") with 4 replications each. Seeds were treated with respective *Trichoderma* talc based formulations (@10 g/Kg of seeds, conidia 10^{7}cfu/g in 0.5% aqueous carboxy-methyl cellulose). Non-treated seeds sown in field served as control. The disease was recorded in 10 random plants from each replication and averaged. Observations on health of plant (*i.e.* shoot length, root length, fresh shoot weight, dry shoot weight, fresh root weight, dry root weight) was recorded after 45 days and grain weight, yield/hill and yield/plot were recorded after harvesting.

2.6. Plant stress related enzyme analysis

Expression of stress related enzymes (*i.e.* catalase, peroxidase and superoxide dismutase) produced by the plants treated with different isolates of *Trichoderma* in the field conditions were analyzed according to Nounjana et al. (2012).

2.7. NPK analysis

The total nitrogen, phosphorous and potassium (NPK) content of the plants treated with different isolates of *Trichoderma* in the field conditions were estimated by methods of Keeney and Bremner (1965), Olsen et al. (1954) and Hanway and Heidel (1952), respectively.

2.8. Statistical analysis

Statistical analysis were performed by using the Statistical Analysis Software (SAS) of Indian Agricultural Statistics Research Institute (IASRI), New Delhi through the portal www.iasri.res.in/sscnars/-. Seed germination percentage data were ARCSINE transformed. The other seed quality parameters were analyzed without any transformation. All the data were subjected to one-way classified analysis of variance

Table 6
Expression of Stress related enzymes in the rice Varieties.

Treatment Name	Expression of Catalase (in unit/min/gm)				Expression of Peroxidase (in unit/min/gm)				Expression of Superoxide dismutase (in unit/min/gm)			
	Karuna		Sahabhagidhan		Karuna		Sahabhagidhan		Karuna		Sahabhagidhan	
	ROOT	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT	SHOOT	ROOT	SHOOT
SHOOT	5.00^{E}	7.00^{E}	5.00^{E}	6.00^{E}	0.56^{D}	0.46^{D}	0.51^{E}	0.42^{D}	3.81^{C}	6.14^{E}	4.48^{E}	4.47^{E}
CRRIT1	10.33^{D}	15.33^{D}	12.00^{D}	11.33^{D}	0.73^{CD}	0.78^{CD}	0.67^{D}	0.69^{C}	6.59^{B}	8.45^{D}	5.37^{D}	6.32^{D}
CRRIT2	**19.17^{A}**	**27.83^{A}**	**22.17^{A}**	**25.50^{A}**	**1.47^{A}**	**1.84^{A}**	**1.97^{A}**	**1.19^{A}**	**10.60^{A}**	**12.86^{A}**	**11.41^{A}**	**12.85^{A}**
CRRIT3	18.17^{A}	25.70^{B}	20.73^{A}	24.50^{AB}	1.14^{B}	1.59^{AB}	1.83^{AB}	1.06^{A}	10.09^{A}	12.05^{AB}	10.81^{AB}	12.14^{AB}
CRRIT5	17.00^{B}	25.00^{B}	20.67^{B}	23.50^{B}	1.07^{B}	1.40^{B}	1.74^{B}	1.12^{A}	7.37^{B}	9.56^{C}	8.41^{C}	8.52^{C}
CRRIT9	15.33^{C}	20.50^{C}	17.00^{C}	15.90^{C}	0.95^{BC}	1.00^{C}	1.12^{C}	0.88^{B}	9.52^{A}	11.11^{BE}	10.12^{B}	11.85^{B}
CRRIT13	14.67^{C}	20.38^{C}	16.67^{C}	15.50^{C}	0.93^{BC}	0.99^{C}	1.10^{C}	0.86^{B}	9.54^{A}	11.95^{AB}	10.20^{B}	12.29^{AB}
CV (%)	3.03	1.91	1.71	2.45	9.89	9.78	4.14	5.26	7.11	3.31	2.97	3.26
Tukey's HSD at 5%	1.2341	1.1037	0.7986	1.2204	0.2761	0.3215	0.1512	0.1333	1.6685	0.9737	0.7364	0.9106

Means with same letter are not significantly different at $p \leq 0.05$. Bold values indicate the highest values.

(ANOVA) and means of treatments were compared based on Tukey's honestly significant difference test (HSD) at 0.05 probability level using SAS. All the experiments repeated at least three times.

3. Results and discussion

3.1. Isolation and characterization of Trichoderma strains

On the basis of data of confrontation assay six promising isolates of *Trichoderma* spp. were selected for further study. Based on the morphological characteristics and molecular identification, isolates were identified as *Trichoderma harzianum* (CRRI-T1), *Trichoderma erinaceum* (CRRI-T2), *Trichoderma atroviride* (CRRI-T3, CRRI-T5, CRRIT-9 and CRRIT-13) respectively (Fig. 1 and S-1Fig. 1). The ITS sequence data from all the 6 isolates have been deposited with GenBank viz. Accession number. KX853519.1, KR014407.1, KR014408.1, KX853518.1, KX863696.1, KX863695.1, respectively (Table 1). It was interesting to note that out of 6 isolates 4 belonged to *T. atroviride* which indicates probable abundance of the species in the studied area. However, it needs detailed investigation using more numbers of isolates.

3.2. Confrontation assay

All six *Trichoderma* isolates showed varying degrees of antagonistic responses against three soilborne plant pathogens *i.e.*, *Rhizoctonia solani, Sclerotium rolfsii and Sclerotium oryzae*. Growth of *R. solani, S. oryzae and S. rolfsii* were inhibited by 80.25–100.00%, 4.35–35.48% and 30.08–67.38%, respectively for all six isolates. Among the six *Trichoderma* isolates CRRIT-2 (*T. erinaceaum*) was able to completely overgrow the *R. solani* within 3 days followed by other three isolates. CRRIT-2 and CRRIT-3 growth was significantly faster in dual culture against *S. oryzae* and it overgrew at least 62% of the medium surface within 3 days, but other isolates could not colonize beyond 60% of the medium surface (Table 2). However, all the isolates were poor antagonist against *S. rolfsii*. The isolate *T. erinaceaum* being the most effective and a better antagonist compared to other isolates tested {S-1Fig-2(a), 2(b)}.

A Number of species of *Trichoderma e.g.*, *T. harzianum, T.viride* and *T. atroviride* are reported as biocontrol agents against plant pathogens (Rahman et al., 2009; Siameto et al., 2010; Abdollahi et al., 2012; Kumar et al., 2012). To the best of our knowledge, *T. erinaceum* from tree bark has been used for the first time here both as biocontrol agents and biofertilizers. Earlier, Herath et al. (2015), reported a strain of *T. erinaceum* from Sri Lanka as a biocontrol agent. They concluded that control of sheath blight was mediated by the production of chitinase and glucanase enzymes by the *T. erinaceum*.

3.3. Germination and early vigour

Significant differences in mean germination time and vigour indices were observed among different treatments. Mean germination time, Vigour index-I and Vigour index-II of different *Trichoderma* treated seeds ranged from 0.33 to 2.2 days, 2210 to 2740 and 9.67 to 15.00, respectively in variety Karuna (Fig. 2). CRRIT-2 exhibited higher vigour indices. Similarly, Vigour index-I and Vigour index-II significantly differed among the treatments. In Sahabhagidhan highest vigour indices was observed with CRRIT-2 treatment followed by CRRIT-5 (Table 3). Earlier Doni et al. (2014) reported that seven isolates of *Trichoderma* spp. enhanced rice germination rate and seedling vigour. Different phenolics or secondary metabolites along with phytohormones namely auxin, gibberellins released by these isolates may be the responsible for better seedling vigour. Early vigour with better root growth in *Trichoderma* treatments helped the plant to uptake more nutrients to stay green for longer period, thus boosting yield.

3.4. Estimation of chlorophyll content

Significant difference was observed in chlorophyll a and chlorophyll *b* content, total chlorophyll content, and chlorophyll a/b ratio among the treatments in both the varieties. Total chlorophyll content in the Karuna variety ranged from 4.73 mg/g to 17.18 mg/g. CRRIT13 treated plants of variety Karuna measured highest chlorophyll content. Total chlorophyll content in variety Sahabhagidhan ranged from 1.39 mg/g to 25.96 mg/g. CRRIT13 treated plants of Sahabhagidhan variety measured highest total chlorophyll content. Seed treatment with T. atroviride (CRRIT13 and CRRIT5) induced comparatively larger effects on chlorophyll content as compared to other isolates used in the present investigation (Table 4).

Chlorophyll plays a critical role in photosynthetic activity. Higher photosynthetic activity and ageing of any plants depends on chlorophyll content and optimum chlorophyll a/b ratio.

3.5. Growth promotion as indicated by agronomical parameters

Most of the *Trichoderma* treatments influenced the plant growth and yield parameters. Root and shoot length, fresh and dry weight significantly improved in both the varieties Number of tillers/hill varied significantly among the treatments. Yield of Karuna ranged from 20.89 to 39.31 g/hill, control being the lowest and CRRIT-2 the highest. Similarly significant difference was observed in yield of Sahabhagidhan and CRRIT-2 treatment, which recorded highest yield (45.74 g/hill) which comparable to CRRIT-3 and CRRIT-5 (Table 5). Generally isolate CRRIT-2 performed better than other isolates {S-1Fig-3(a), 3(b)}.

Trichoderma spp. produces auxins that are responsible for plant

Table 7
Trichoderma treatment increased the total uptake of NPK by plants in rice varieties.

Treatment Name	Percentage of Potassium in Root		Percentage of Potassium in Shoot		Percentage of Nitrogen in Root		Percentage of Nitrogen in Shoot		Percentage of Phosphorus in Root		Percentage of Phosphorus in Shoot	
	Karuna	Sahabhagidhan	Karuna	Sahabhagidhan	Karuna	Sahabhagidhan	Karuna	Sahabhagidhan	Karuna	Sahabhagidhan	Karuna	Sahabhagidhan
Control	3.37^{E}	3.95^{D}	6.43^{D}	6.93^{E}	0.15^{E}	0.18^{E}	1.14^{E}	0.85^{E}	0.15^{F}	0.15^{F}	0.25^{d}	0.25^{D}
T1	4.28^{D}	4.83^{C}	7.91^{C}	8.00^{D}	0.22^{D}	0.21^{D}	1.45^{D}	1.44^{D}	0.20^{E}	0.20^{E}	0.28^{c}	0.27^{CD}
T2	**7.59^{A}**	**7.07^{A}**	**12.54^{A}**	**12.55^{A}**	**0.49^{A}**	**0.41^{A}**	**1.98^{A}**	**2.09^{A}**	**0.31^{A}**	**0.28^{A}**	**0.35^{A}**	**0.36^{A}**
T3	6.55^{B}	6.83^{AB}	11.78^{A}	11.91^{AB}	0.43^{B}	0.39^{AB}	1.83^{B}	1.88^{B}	0.27^{B}	0.26^{B}	0.34^{A}	0.34^{AB}
T5	6.56^{B}	6.40^{B}	11.86^{A}	11.47^{B}	0.44^{B}	0.38^{B}	1.83^{B}	1.87^{B}	0.25^{C}	0.25^{BC}	0.33^{a}	0.33^{B}
T9	5.47^{C}	5.47^{C}	10.64^{B}	10.40^{C}	0.29^{C}	0.31^{C}	1.70^{C}	1.66^{C}	0.23^{D}	0.23^{CD}	0.30^{b}	0.29^{C}
T13	5.41^{C}	5.43^{C}	10.34^{B}	10.13^{C}	0.29^{C}	0.29^{C}	1.65^{C}	1.64^{C}	0.22^{De}	0.23^{D}	0.30^{b}	0.29^{C}
CV (%)	5.37	3.93	3.54	2.82	4.39	1.64	2.22	3.81	2.95	2.46	2.38	3.50
Tukey's HSD at 5%	0.86	0.6413	1.0324	0.8223	0.0416	0.0145	0.1048	0.1777	0.0198	0.0161	0.0209	0.0305

Means with same letter are not significantly different at $p \leq 0.05$. Bold values indicate the highest values.

growth and root development. Auxins are key hormones effecting plant growth and development that can be produced by fungi in both symbiotic and pathogenic interactions with plants (Gravel et al., 2007; Losane and Kumar, 1992; Shayakhmetov, 2001). IAA produced by *T. virens* and *T. atroviridae* were found to stimulate the growth of Arabidopsis plants *invitro* (Contreras-Cornejo et al., 2009). We have also made similar observation in rice treated with different *Trichoderma* isolates in terms of germination, seed vigour *etc*. They are positive inducers for not only root and shoot length but also for dry and fresh weight of the rice crops. Role of *Trichoderma* as plant symbionts for enhanced nutrient uptake, increased root and shoot growth, improved plant vigour and biotic/abiotic stress tolerance have been extensively reported (Inbar et al., 1994; Yedidia et al., 2001; Harman et al., 2008; Harman, 2011). Seed treatment with *T. erinaceaum* (CRRIT-2) induced comparatively better effects as compared to other isolates. In the present study we report the growth promotion of two direct seeded rice varieties under field condition using *Trichoderma* spp. isolated from above ground sources (S-1Fig-4 and S-1Fig-5).

3.6. Expression of stress related enzymes

Significantly higher expression of the stress related enzymes was observed in *Trichoderma* treated plants as compared to untreated plants over both the years. Maximum SOD activity in shoot and root samples of the rice variety Karuna was observed in CRRIT-2 and CRRIT-3 treated samples. Similarly, CRRIT-2 and CRRIT-3 treated root and shoots of rice variety Sahabhagidhan had significantly higher SOD activity compared to other treatments. Whereas, Catalase activity was significantly higher in CRRIT-2 treatment in both root and shoot of both the varieties. Similar trends were observed in peroxidase expression in root and shoot samples of both the varieties. CRRIT-2 treatment had significantly higher activity of peroxidase compared to other treatments (Table 6).

Both biotic and abiotic stresses lead to the over production of reactive oxygen intermediates, including H_2O_2, causing extensive damage (Drew, 1997). However, the plants that have well defined systems to protect against the superoxide radical (O_2^-) undergo less damage. Enzymes like SOD, CAT and PER are important in protecting from oxidative damage. In the present investigation, stress related enzymes in *Trichoderma* treatments have higher expression than control plants. Similar to our findings, Mohapatra and Mittra (2017) reported that *Trichoderma* spp. activated production of antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase in wheat seedlings which enhanced resistance against diseases. *Trichoderma* isolates are reported to stimulat systemic defense responses through different stress enzymes against *R. solani* in tomato plants (Małolepsza et al., 2017).

3.7. NPK analysis

Higher concentration of N, P, and K was observed in *Trichoderma* treated plants as compared to untreated plants both in shoot and root. In the rice variety Karuna concentration of all the three essential macro elements (*i.e.* N, P, and K) were high in the shoots and roots of the treated plants. CRRIT-2 treatment induced higher uptake of plant nutrients by rice plant followed by the CRRIT-3 and CRRIT-5 (Table 7). In the rice variety Sahabhagidhan the concentrations of three essential macro nutrients (*i.e.* N, P, and K) were more in the shoots and roots of the CRRIT-2 treated plants as compared to others. The K concentration in the roots of CRRIT-2 was at par with CRRIT-3 and CRRIT-5 treatments (Table 7).

The benefit of *Trichoderma* spp. in improving rice plant growth could be explained through enhanced solubilization and uptake of inorganic soil nutrients such as N, P, K, Zn and enhancement of root hair development (Lorito et al., 2010). Doni et al. (2014) found better plant height in rice in *Trichoderma* spp. treated plants as compared to NPK treatment alone. We also found higher N, P, K uptake both in roots and

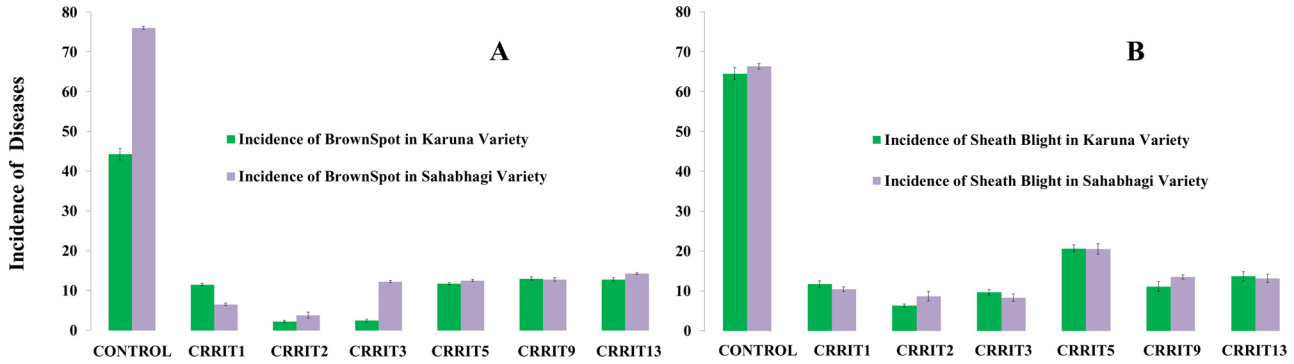

Fig. 3. Average% of Brown Spot and Sheath Blight disease incidence at 60 days and 75 days old plants respectively in rice varieties after seed treated with Trichoderma and control condition.

shoots in *Trichoderma* treated plants as compared to control which also is similar to the observation made by Saba et al. (2012). Better uptake of N, P, and K consequently enhances physiological processes leading to good crop growth. *Trichoderma* spp. are known to enhance growth and nutrient uptake by production of phytohormones, environmental buffering (against pH, drought, water logging), P solubilization and siderophore production (Newman, 2006). Soil application of *Trichoderma* spp. was earlier reported to increase P and Zn uptake by rice in farmers' field in Philippines, by releasing soil-fixed P and secreting beneficial hormones (Cuevas, 2006)

3.8. Reaction to different diseases under field condition

Observations were recorded for their reaction to brown spot disease (60 DAS) and sheath blight disease (75 DAS) both in Karuna and Sahabhagidhan. It was observed that both the varieties showed lower degrees of disease reaction in *Trichoderma* treated plots (Fig. 3). This indicates that all the *Trichoderma* spp. are able to give protection against both the diseases even under field condition in direct seeded rice. However the degrees of disease reaction varied in different *Trichoderma* treated lines and interestingly the isolate CRRI-T2 is the best one followed by CRRI-T3. These data suggest that isolates CRRI-T2 and CRRI-T3 are effective in inhibiting infestation and growth of rice plant pathogens, and are also effective in inhibiting sheath blight and brown spot diseases. The above mentioned formulation using CRRIT2 and CRRIT3 have been filed for Indian patent (File no. 1240/KOL/2015).

4. Conclusion

Trichoderma spp. are commercially being used as biocontrol agents against large number of pathogens across several crops. However, there appears to be a limited efforts to develop effective commercial formulation of *Trichoderma* spp. capable of controlling rice diseases as well as for plant growth promotion. We tried to fill this gap by isolating *Trichoderma* strains from novel habitats (tree bark and wild mushrooms in Odisha state of India) and identified novel strains that are capable of both biocontrol and growth promotion. In the present study, we have demonstrated the bioefficacy of formulated *Trichoderma* strains not only in greenhouse but also in field for over two years, as a step towards l development of *Trichoderma*-based biofungicides for use in commercial rice cultivation. Among more than 200 *Trichoderma* spp. that are defined in literature, only about 10% come from soil/rhizosphere, which have been exploited for development of biocontrol agents, neglecting the major source of *Trichoderma* in nature (wild mushrooms and tree bark). Our present study establishes the importance of "looking up" for identification of superior plant beneficial *Trichoderma* strains for crop health managment.

Acknowledgement

Authors duly acknowledge the technical and financial support provided by the Director, ICAR-National Rice Research Institute, Cuttack and the Department of Science and Technology, Government of India, New Delhi for providing INSPIRE Fellowship to HS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.micres.2018.05.015.

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