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Effect of native rice specific isolates of *Trichoderma* and ecological fitness against aggregated sheath spot of rice caused by *Ceratorhiza oryzae-sativae*

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ABSTRACT

***Trichoderma* is a free-living fungus that interacts heavily with its surroundings found in the root, soil, and foliar regions of plants as well. It is an important biocontrol agent due to its abilities, such as mycoparasitism, production of antibiotic, hydrolytic enzymes, competition for nutrients, as well as induced plant resistance; production of numerous secondary metabolites inhibiting the growth of several plant pathogens. Antagonistic potential of fourteen (n=14) native rice specific *Trichoderma* isolates was evaluated against aggregated sheath spot of rice caused by *Ceratorhiza oryzae-sativae*. It revealed that all native *Trichoderma* isolates significantly inhibited the mycelial growth of the pathogen of *C. oryzae-sativae* with ranges from 71.50% to 97.50% with the highest per cent inhibition by *T. harzianum* (MH257323), and the least percent inhibition by *T. koningiopsis* (MN080228). Bell's scale studied showed that class III category by *T. koningiopsis* (MN080228) and class II showed by *T. harzianum* (MH257323) against *C. oryzae-sativae*. Among isolates of native rice specific *T. harzianum*, MH257323 is found to be the most effective in reducing the rapid growth of pathogen and having high potential ecological fitness.**

Keywords: Aggregated sheath spot, Biocontrol agent, Ecological fitness, Mycoparasitism, Rice, and *Trichoderma*

INTRODUCTION

Rice (*Oryza sativa* L.) is an important cereal crop worldwide. It is the major food crop and staple food for most of the people of India. In Asian countries, where 60% of the world's population resides, more than 90% of the world's rice are produced and consumed (Mahajan *et al.,* 2017). Rice being the staple food of Manipur is widely cultivated occupying nearly 1.80 lakh ha of the total cropped area of Manipur (Goud *et al.,* 2018). Many fungal and bacterial diseases are known to cause heavy grain yield losses. Its productivity is affected by several pathogens that often place major constraints on production. Sheath diseases in rice is responsible for yield loss up to 45% (Margani and Widadi, 2018). Aggregated sheath spot of rice is caused by *Ceratorhiza oryzae-sativae* found to be prevalent in intensively cultivated rice fields. The pathogen is a soil-dwelling saprotroph and facultative parasite which causes lesions on the sheath affecting grain filling and yield in rice (Wu *et al.,* 2012). Agrochemicals are heavily utilized to combat disease-related issues. Modern farmers typically believe that using chemical pesticides is the only way to solve problems, which has led to the indiscriminate use of agrochemicals, which has a number of unfavorable side effects. This bad practice has caused more harm than it has helped to solve. The accumulation of pesticide residues in the environment, which affects the food web and the food chain and causes ecological imbalances as well as the contamination of soil and water resources, is another pressing task that emerges in the wider context. Therefore, a search for alternatives to agrochemicals has shown the crucial role of using bio-control agents, keeping in mind the ever-increasing demand for food safety and security without compromising the environment. One such bio-control agent which has been explored since years is *Trichoderma*. The genus *Trichoderma* houses a variety of free-living fungi that are common in soil and root ecosystems. It is an opportunistic secondary invasive microorganism that grows quickly and produces enormous quantities of spores, antimicrobial compounds, and enzymes like chitinases, glucanases, and proteases that can degrade the fungal cell wall. They are found to be very promising against phytopathogenic fungi. Many *Trichoderma* species are also well known as biocontrol agents (BCA) of important phytopathogenic fungi. Antibiosis, competition for nutrients with the pathogen (Harman and Kubicek, 1998) and mycoparasitism (Papavizas, 1985) are the main strategies of bio-control employed by *Trichoderma* in direct confrontation with pathogenic fungus. The purpose of this research was to determine how native rice-specific isolates of *Trichoderma* affected *C. oryzae-sativae*.

MATERIALS AND METHODS

Isolation and identification of *Trichoderma* from rhizospheric soil of rice

Soil dilution plate technique (Dhingra and Sinclair, 1995) using *Trichoderma* specific medium (TSM) (Elad and Chet, 1983) were followed for the isolation of native rice specific *Trichoderma* spp. Different dilutions were used ranging from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of the soil samples collected from different rice fields of Manipur. The isolates were identified using molecular techniques by amplification of the internal transcribed spacer (ITS) region of fungal isolates in polymerase chain reaction (PCR) amplification. Genomic DNA was extracted from the fungal isolates using a HiPurATM fungal DNA isolation Kit (Himedia, India) following the manufacturer's instructions. The genomic DNA was then used as the template for PCR amplification of the target nucleotide sequences for the fungal isolates. Universal primers for the ITS region viz., ITS1 5'- TCCGTAGG

TGAACCTGCG G - 3' & ITS4 5'- TCCTCCGCTTATTGA TATGC 3' (Xie *et al.,* 2008) were used for the amplification of the target nucleotide and were subjected to sequencing at Xcelris Genomics, Ahmedabad, India. Sequences were submitted to NCBI GenBank and accession numbers were obtained accordingly.

Isolation of pathogen, *Ceratorhiza oryzae-sativae*

The infected rice plant exhibiting the typical symptoms of aggregated sheath spot was collected and studied under a microscope at the laboratory of the Department of Plant Pathology at the Central Agricultural University in Imphal. Later, to remove soil and other debris, the obtained samples were cut into small pieces (< 1.0 cm) and rinsed twice under running water. The cut pieces were surface sterilized by dipping them in 1% sodium hypochlorite (NaOCl) solution and running them successively through three rounds of sterile distilled water for one minute each. With the aid of sterile forceps, the treated sample pieces were blot dried before being placed in groups of four on each plate of sterilized potato dextrose agar medium in petri plates. In order to preserve pure cultures of the fungal isolates, all plates were incubated at 25 ± 2°C for 34 days. The fungal isolates were subjected to PCR amplification using ITS and sequenced to confirm the identification at molecular level.

***In-vitro* antagonistic activity**

In vitro antagonistic activity of the native rice specific isolates of *Trichoderma* against *Ceratorhiza oryzae-sativae* was studied in dual culture technique by following the method by Kucuk and Kivanc (2003). Antagonistic potentials of the rice specific native isolates of *Trichoderma* against *Ceratorhiza oryzae-sativae* were evaluated from the dual culture technique using formula given by Bell (1982). The petri dishes containing sterile PDA were inoculated with 5mm diameter plug of 4-day old pure culture of antagonistic fungi and the pathogen. One mycelial disc of each of the fungus was placed on the opposite poles of PDA plates using sterile cork borer and sterile needle and incubated at 25°C in BOD incubator and radial growths of the pathogen were recorded at 24 hrs interval. A Petri dish without the antagonist served as the control. Each treatment was replicated thrice. The per cent inhibition of the mycelial growth of *Ceratorhiza oryzae-sativae* over the control were calculated using the formula suggested by Dennis and Webster (1971).

Per cent Inhibition of Radial Growth (% IRG) = 100 [(C-T) / C],

where C - linear growth of the fungus in control, T- Linear growth of the fungus in treatment.

Bell's scale with slight modification, class I: The antagonist completely overgrew the test pathogen (100 % overgrowth); class II: The antagonist overgrew at least 2/3rd of the test pathogen surface (75% over growth); class III: The antagonist colonized on half of the growth of the test pathogen surface (50% over growth); class IV: The test pathogen and the antagonist locked at the point of contact; class V: The test pathogen overgrew the antagonist; class VI: The test pathogen and antagonist form inhibition zone (Bell *et al.,* 1982).

Rhizosphere colonization of the potent *Trichoderma* isolate

Further the best isolate which was found potent in vitro was subjected to study of ecological fitness through rhizosphere colonization with the soil collected from the rice rhizosphere. The rhizosphere competence of the potent isolate of *Trichoderma* sp. were conducted inside glasshouse with three different soils viz., unsterilized, sundried, and sterilized soils and observations were recorded at different days after sowing of rice.

RESULTS

Fourteen (14) isolates of native rice specific *Trichoderma* spp. has been identified and accordingly acquired the NCBI Genbank accession as shown in **table 1**. *Ceratorhiza oryzae-sativae* was identified as the isolated phytopathogenic fungus (MH255604) causing aggregated sheath spot of rice. The study further demonstrated the differential bio-control ability of the fourteen (14) isolates of native rice specific *Trichoderma* spp. by dual culture technique against *Ceratorhiza oryzae-sativae* causing aggregated sheath spot of rice which were recorded and percent inhibition tabulated as given in **table 2**, and **fig. 1**. Among the fourteen (14) native rice specific *Trichoderma* spp. used *T. harzianum* (MH257323), resulted in best mycelial growth inhibition by (97.50%) and the least percent inhibition of 71.50% was shown by *T. koningiopsis* (Mn080228).

Table 1: Isolates of native rice specific *Trichoderma* spp. with NCBI Genbank accession number

***Trichoderma* isolates**	**NCBI GenBank Accession**
T. koningii	MH257321
T. brevicompactum	MH257322
T. harzianum	MH257323
T. harzianum	MH257324
T. harzianum	MH257325
T. harzianum	MH257326
T. asperellum	MH257327
T. viride	MH257328
T. asperellum	MH257329
T. harzianum	MH257330
T. harzianum	MH257331
T. harzianum	MH257332
T. harzianum	MH257333
T. koningiopsis	MN080228

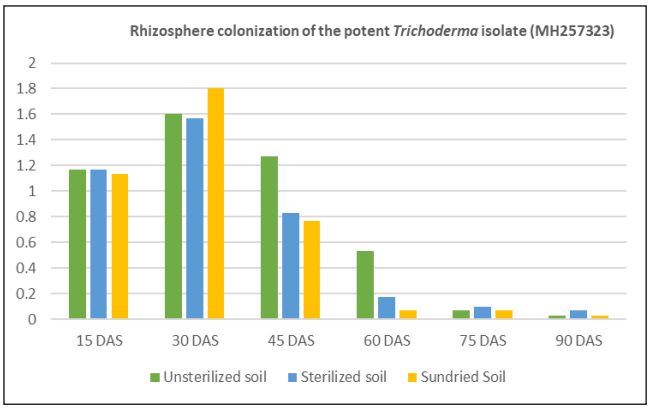

Fig. 1: Rhizosphere colonization of the potent *Trichoderma* isolate at different days after sowing

However, all the species showed considerable mycelial growth inhibition ranging from 71.50% to 97.50% (**Table 2**). Mycelial growth inhibition by *T. koningii* (MH257321) 82.50%, *T. brevicompactum* (MH257322) 77.50%, *T. harzianum* (MH257324) 72.50%, *T. harzianum* (MH257325) 87.50%, *T. harzianum* (MH257326) 90.00%, *T. asperellum* (MH257327) 95%, *T. viride* (MH257328) 72.50%, *T. asperellum* (MH257329) 80%, *T. harzianum* (MH257330) 87.50%, *T. harzianum* (MH257331) 92.50%, *T. harzianum* (MH257332) 85%, *T. harzianum* (MH257333) 95.00% and *T. koningiopsis* (Mn080228) 71.50%.

Table 2: *In-vitro* antagonistic activity of *Trichoderma* isolates against *Ceratorhiza oryzae-sativae*

***Trichoderma* isolates**	**Accession Nos.**	**Bell's Scale**	**Inhibition %***
T. koningii,	MH257321	Class II	82.50
T. brevicompactum	MH257322	Class II	77.50
T. harzianum	MH257323	Class II	97.50
T. harzianum	MH257324	Class III	72.50
T. harzianum	MH257325	Class II	87.50
T. harzianum	MH257326	Class II	90.00
T. asperellum	MH257327	Class II	95.00
T. viride	MH257328	Class III	72.50
T. asperellum	MH257329	Class II	80.00
T. harzianum	MH257330	Class II	87.50
T. harzianum	MH257331	Class II	92.50
T. harzianum	MH257332	Class II	85.00
T. harzianum	MH257333	Class II	95.00
T. koningiopsis	MN080228	Class III	71.50

The Bell's scale classified the antagonistic nature of *T. harzianum* (MH257323), *T. koningii* (MH257321), *T. harzianum* (MH257325), *T. asperellum* (MH257327), *T. viride* (MH257328), *T. harzianum* (Mh257332), *T. harzianum* (MH257330) to class II where the antagonist over grew at least two thirds of the pathogen surface and the rest other antagonists, *T. brevicompactum* (MH257322), *T. harzianum* (MH257324), *T. harzianum* (MH257326), *T. asperellum* (MH257329), *T. harzianum* (Mh257331), *T. harzianum* (MH257333), *T. koningiopsis* (MN080228) to class III where the antagonist which colonized only half of the growth of the pathogen (**Table 2**).

Further the *in-vitro* tested potent/best isolate, *T. harzianum* (MH257323) subjected to ecological fitness evaluation through rhizosphere colonization shows that in all the types of soil, colonization of the potential isolate of *Trichoderma* was found to be increased up to 30 days of sowing then decreased (**Fig. 1**). The rhizosphere colonization of the potent isolate of *Trichoderma* was found to have significant differences up to 45 days of sowing and did not show any significant differences at 60, 75, and 90 days of sowing (**Table 3**).

Table 3: Rhizosphere colonization of the potent *Trichoderma* isolate

Treatments	The population of the potent *Trichoderma* isolate (MH257323) at different days after sowing (DAS) ($x^{8}10$ cfu/g soil)					
	15DAS	30DAS	45DAS	60DAS	75DAS	90DAS
Unsterilized soil	1.17	1.60	1.27	0.53	0.07	0.03
Sterilized soil	1.17	1.57	0.83	0.17	0.10	0.07
Sundried soil	1.13	1.80	0.77	0.07	0.07	0.03
SE(D)±	0.01	0.01	0.01	0.00	0.00	0.00
CD 5%	0.02	0.03	0.02	NS	NS	NS

DISCUSSION

Among the fourteen (14) native rice specific *Trichoderma* spp. used *T. harzianum* (MH257323), resulted in best mycelial growth inhibition of *Ceratorhiza oryzae-sativae. T. harzianum* giving the best inhibition were also reported in findings of (Seema and Devaki, 2012). *Trichoderma* spp. produces substantial and diversified secondary metabolites like pyrones, koninginins, viridins, nitrogen heterocyclic compounds, azaphilones, butenolides and hydroxy-lactones, isocyano metabolites, diketopiperazines, peptaibols, etc., (Francesco *et al.,* 2014). These heterogenic secondary metabolites yielded by *Trichoderma* triggers the activities like mycoparasitism, competition for nutrition (carbon, nitrogen and also free space) and rapid colonization. Baker and Cook (1979) have reported that enzymes may be produced by *Trichoderma* that digest the mycelial walls and septal walls or antibiotics may be formed that inhibit growth or cause endolysis. Dennis and Webster (1971) have reported that *Trichoderma* spp. are known to produce a number of antibiotics such as trichodermin, trichodermol, harzianum and harzianolide as well as some cell wall degrading enzymes such as chitinases, glucanases that break down polysaccharide, chitins and β-glucans, thereby destroying cell wall integrity (Elad, 2000: Devaki *et al.,* 1992).

CONCLUSION

Ceratorhiza oryzae-sativae is an important rice fungal pathogen of rice causing aggregated sheath spot and the prevalent in Manipur state, India. The native *Trichoderma* species isolated from rhizospheric soil of rice of Manipur found very effective against the *C. oryzae-sativae*. The rhizosphere colonization of *Trichoderma* elucidates their ecologically fit nature. These may also play a major role in mycoparasitism because of changes in cell wall integrity and its multiplication and rhizospheric availability further gives edge to the bioactivity of this fungi. All these distinguished features of *Trichoderma* accomplish it as a bio control agent against *C. oryzae-sativae*.

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