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Molecular identification of seed borne non–sporulating endophytic mycobiota of rice landraces and its impact on soil borne rice pathogens

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ABSTRACT

Endophytes were isolated from the seeds of two rice landraces viz. Malabati and Champa collected from Odisha, India. Both were non sporulating cultures. The ITS region of ribosomal DNA was amplified and both isolates were identified by molecular techniques as two species of Dendryphiella. *The GenBank accession numbers fot these endophytes were KT796364 & KT796365.The endophyte from rice variety Malabati was different than the other* Dendryphiella *specie reported earlier. Hence its impact on three soil borne rice pathogens was studied. Sclerotia production by* Rhizoctonia solani *(c.o. of sheath blight of rice) was drastically reduced and causal organisms of Aggregate sheath spot (c.o* R.oryzae sative *) and seedling blight (c.o.* Sclerotium) *could not produce the sclerotia . This endophyte thus has the ability to manage the soil borne sclerotia- producing rice pathogens.*

Key words: *Endophytes, Dendryphiella, Sclerotia, sheath blight of rice, aggregate sheath spot*

Rice is staple food for millions of people. High yielding rice varieties are developed for meeting the requirements of people but rice diseases specially those which are caused by soil borne sclerotia producing pathogens are becoming major constraints for rice cultivation in most of the Asian countries. Cent percent control of these diseases could not be achieved by the application of synthetic fungicides. Host resistance could not be achieved due to the lack of suitable donors against sheath blight disease. Therefore, other options for management of this pathogen were explored. Rodriguez and Redman, (2008) suggested existence of abiotic and biotic stress tolerance through fungal symbiosis in many plant species .Many landraces could survive due to their mutualistic life style. Hence traditional rice landraces from this part of India were collected during 2010 and seed micro-flora was studied to identify the associated endophytes. Seed borne endophytes might play important role for plant adaption to various stresses, hence the seed microflora was studied. Non spore producing endophytes were found to be associated with two traditional rice varieties viz. Malabati (PPVFRA Red/2011/178 dated 14.03.2011) and Champa (PPVFRA Red/2011/166 dated 25.02.2011). Malabati was from Jagatsinghpur district and Champa was from Puri district of Odisha. Both of these long duration varieties are cultivated in low lying area where sheath blight is a serious problem. It was necessary to identify and study the interaction of these fungal cultures with pathogenic micro-flora of soil to have full account of biodiversity and also to know their impact on soil borne rice pathogens.

MATERIALS AND METHODS

The endophyte culture (s) was maintained on MS broth (Murashige & Skoog, 1962) and DNA was extracted by procedure followed by Dhua *et al.* (2011).The PCR amplification of Internal transcribed spacer region of ribosomal DNA was done by the procedure described by White *et al.* (1990). Following

ITS primers (Operon) were used for it: ITS-1(TCCGTAGGTGAACCTGCGG);ITS-4 (TCCTCCGCTTATTGATATGC).

Sequencing was outsourced to Xcelris Labs Ltd. Ahmedabad India. Sequence alignment was done for identifying the microbes. The sequences were analyzed with the BLAST algorithms (developed by Zhang *et al.*, 2000) available in NCBI for analysis of nucleotide sequences. Sequences producing significant alignment with the 'sequence to be identified' were listed , their FASTA files were retrieved from NCBI and were used for further analysis.

Those sequences were at first analyzed in Phylogeny fr platform and cured FASTA files were downloaded which were used while analyzing the evolutionary relationships in MEGA4.The evolutionary history was worked out by the UPGMA method described by Sneath and Sokal (1973). The boot strap test (1000 replicates) suggested by Felsenstein (1985) was conducted and percentage of replicate tree where the taexa grouped together in boot strap test were given adjacent to the branches. The branch length of tree corresponded to the evolutionary distances which were used to conclude the phylogenetic tree. The FASTA files of both isolates were submitted to Gen-Bank to obtain the accession numbers.

The cultural filtrate of twenty day-old *Dendryphiella* sp.crri.33 (NCBI.GenBank accession number KT796364) was collected and centrifuged at 10000 rpm .The clear liquid layer obtained after centrifugation of filtrate was passed via bacteria proof 'Whatman- Cellulose Nitrate' membrane-filters (0.2µm; ø47mm) in a Borosil filtration unit. The refined filtrate was mixed with sterilized Potato Dextrose Agar media (40 ml filtrate in 60 ml PDA) before pouring the media in to the 90 mm diameter Petri-plates. The culture(s) of test organism i.e. soil borne rice pathogens (NCBI Gen Bank accession numbers KC832506, KC832505, KT582015) were grown on PDA and small bits of test culture(s) containing single sclerotia were inoculated on above mentioned media to study the efficacy of Cell free Cultural Filtrate of *Dendryphiella* sp.crri.33 against soil borne rice pathogens.

RESULTS AND DISCUSSION

Nine sequences (7 *Dendryphiella* species and 2 *Alternaria padwickii* isolates) of NCBI database producing significant alignment with isolate crri.33 were included. *Dendryphiella* species had 894-942 maximum score,93% to 96% query coverage and 96%-99% identity with crri.33 (Table 1). The phylogeny was concluded by the Unweighted Pair Group (UPGMA) method. The branch length of tree was 137. Both the Alternaria *padwickii* isolates were together in a major group whereas all *Dendryphiella* species were in another major group which was further divided in to sub groups. *Dendryphiella sp*. crri.33 was in one subgroup and other six *Dendryphiella* species clubbed in another sub group (Fig.1). These findings were further corroborated by the findings of pair-wise distance calculation done for 10 taexas. The NCBI-GenBank accession number for isolate crri.33 was KT796364.

Eleven sequences of NCBI data base (nine *Dendryphiella* species and two of *Alternaria*

Table1. Sequences producing significant alignment with isolate CRRI 33

Gene Bank Accession	Description	Maximum Score	Total Score	Query coverage (%)	E value	Identity (%)	Geographic location of organism
KC871034	*Dendryphiella* sp.DX-FOF2	942	942	96	0	99	China
HM572292	*Dendryphiella* sp. EN12	941	941	95	0	99	India
KC832510	*Dendryphiella* sp.Swarna-1	933	933	96	0	99	India
JQ585678	*Alternaria padwickii* . A12-2	917	917	94	0	99	Iran
FJ971840	*Dendryphiella* sp BR354	915	915	94	0	99	Thailand
KT582010	*Dendryphiella* sp. crri.1	896	896	93	0	96	India
KT582011	*Dendryphiella* sp. crri.2	893	893	93	0	96	India
KT582012	*Dendryphiella* sp. crri.4	894	894	93	0	96	India
JQ907484	*Alternaria padwickii* isolate321-IQ	821	1071	89	0	97	India

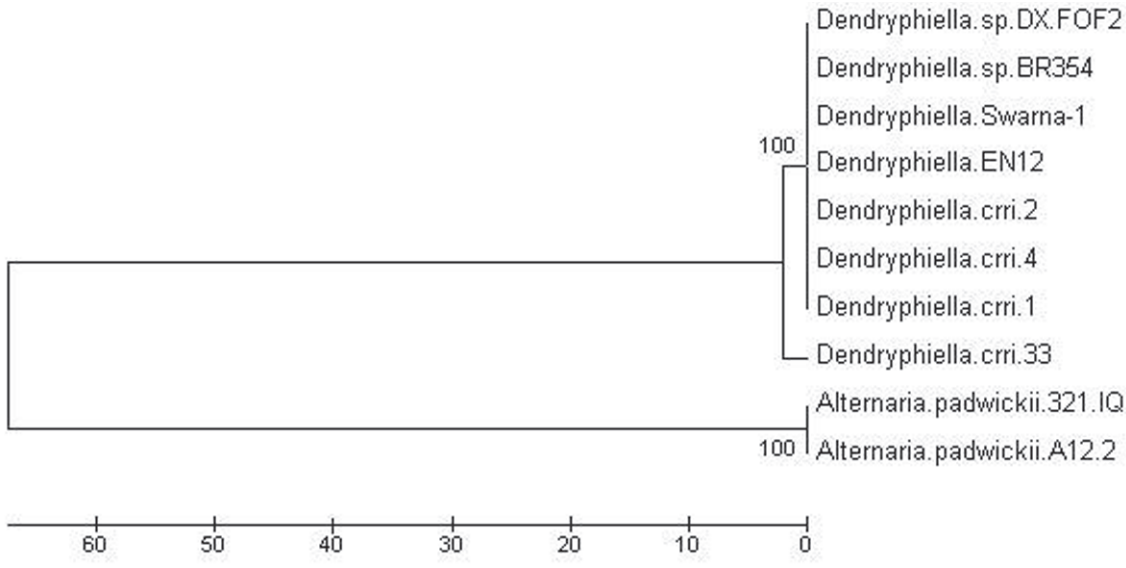

Fig.1. Evolutionary relationships of CRRI.33 and other nine

padwickii isolates) showing significant alignment with isolate crri.34 were included for the identification of this endophyte (Table 2). The *Dendryphiella* species included here clustered together and *Alternaria padwickii* isolates were in separate group indicating that crri.33 was also in genus *Dendryphiella*..The phylogeny tree produced by UPGMA method had 22.25 branch length (Fig.2). *Dendryphiella* sp 39-II , *Dendryphiella* sp Karuna-3, *Dendryphiella* sp.FV 16, *Dendryphiella* sp. FV9, *Dendryphiella* sp.Savitri-4, *Dendryphiella* sp.FV22 grouped with crri.34. Another test i.e. 'Pairwise distance calculation' was also done for 14 texas including *Dendryphiella* sp. crri.34 to estimate the evolutionary divergence between sequences. The findings of this analysis also supported earlier conclusion as the evolutionary divergence between above mentioned six *Dendryphiella* sp. and crri.34 was zero. The NCBI-GenBank accession number for isolate crri.34 was KT796365. These finding indicated that the isolate crri.33 was unique i.e. it was different than the earlier reported species of *Dendryphiella* hence impact of *Dendryphiella* sp.crri.33 on soil-borne sclerotia producing rice pathogens was studied.

The sclerotia of *Sclerotium* sp. (c.o. seedling blight of rice) did not germinate for 10 days in treated plates whereas plenty of sclerotia were produced in untreated control. After ten days it germinated in treated plates but growth was very slow. The colony diameter in treated plate was about 45mm in next four four days but the sclerotia in control plates matured by that time (Fig. 3).

Plenty of mature sclerotia were observed in about 14 days old culture of *Rhizoctonia oryzae sativae*. (c.o. Aggregate sheath spot of rice) in untreated control whereas in treated plates there were no sclerotia but slow mycelial growth i.e. 15 mm colony diameter in 14 days. Effect of CCF of endophyte *Dendryphiella sp.*crri.33 on inoculum producing ability of sheath blight slerotia was studied (Fig.4). The mycelial growth of *R. solani* (c.o. sheath blight of rice) was slow as compared to control but sclerotia production was drastically reduced in treated plates. The endophytic *Dendryphiella* studied here was found to control the growth and sclerotia production of soil borne pathogenic *Rhizoctonia* and *Sclerotium* species (Fig.5).

The *Dendryphiella* species constituted the seed microflora of several rice cultivars specially the land races but exact role of this fungus in rice ecosystem is not well understood. *Dendryphiella* spp. DX-FOF2 was endophytic fungi in seeds of *O. rufipogon*. The endophytic *Dendryphiella* exclusively colonized the seeds (Wang *et al.*, 2015).Rice seeds may likely offer more entry points and place for colonization of endophytic fungi . This mutualism may

Table2. Sequences producing significant alignment with isolate CRRI 33

Gene Bank Accession	Description	Maximum Score	Total Score	Query coverage (%)	E value	Identity (%)	Geographic location of organism
KT582012	*Dendryphiella* sp. crri.4	972	972	98	0	99	India
KJ563122	*Dendryphiella* sp 39-II	972	972	97	0	99	India
KT582011	*Dendryphiella* sp. crri.2	968	968	98	0	99	India
KJ563118	*Dendryphiella* sp Karuna-3	968	968	97	0	99	India
FJ971840	*Dendryphiella* sp BR354	968	968	96	0	99	Thailand
KT582010	*Dendryphiella* sp. crri.1	963	963	98	0	99	India
KC832508	*Dendryphiella* sp.FV 16	963	963	96	0	99	India
KC832507	*Dendryphiella* sp. FV9	961	961	98	0	99	India
KJ563119	*Dendryphiella* sp.Savitri-4	955	955	97	0	99	India
KC832509	*Dendryphiella* sp.FV22	955	955	96	0	99	India
JQ585678	*Alternaria padwickii* isolate A12-2	889	889	89	0	99	Iran
JQ907484	*Alternaria padwickii* isolate 321.IQ	885	1136	94	0	98	India
KC871034	*Dendryphiella* sp.DX-FOF2	883	883	90	0	99	China

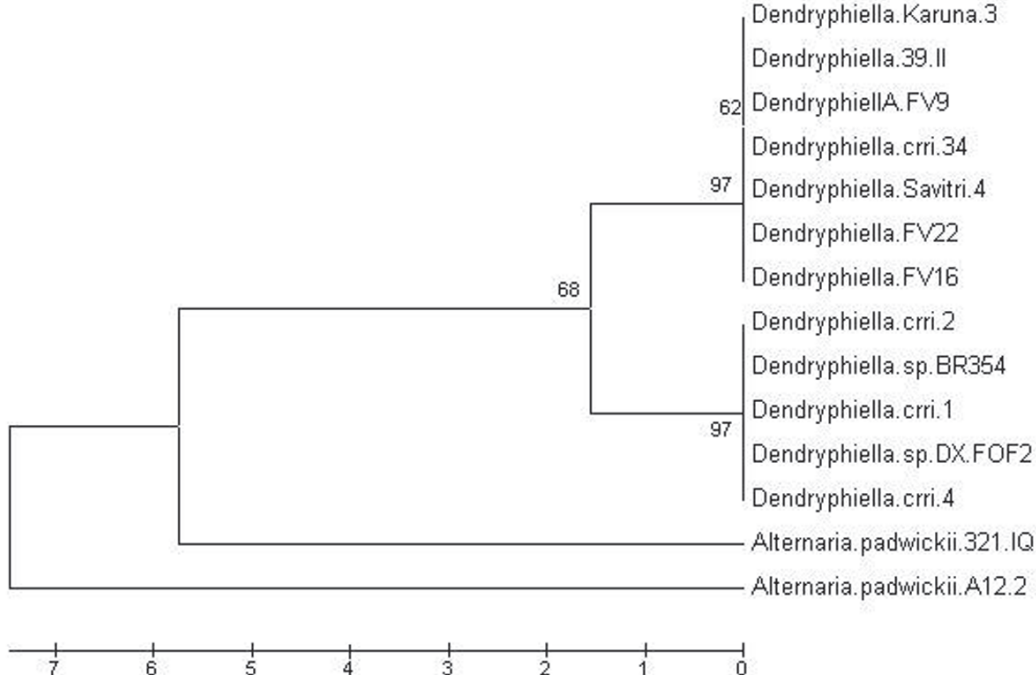

Fig. 2. Evolutionary relationships of CRRI.34 and other thirteen

help both i.e. plant as well as fungi inhabiting in plant for better adaption to various stresses and surviving in those habitats. At the time of senescence this policy helps the endophytic fungi for utilizing most of the plant nutrients(Rodriguez and Redman, 2008). Several fungal endophytes detected in cultivated rice were found to enhance the host growth and reduce/ diminish the effect of biotic stress. (Atugala and Deshappriya, 2015; Zakaria 2010; Naik *et al.*, 2009;Yuan *et al.*, 2007).

Out of the total yield loss due to diseases in rice about, 25% loss is caused by the sheath blight disease of rice, in India . Approximately Rs. 3800 million worth fungicides were used for protecting the rice crop from pathogens during 2010-11. Out of these Rs. 2800million were spent for managing sheath blight and rice blast diseases. The major change in synthetic pesticide use depends up on the variations of pathogens, crop intensity, practices and prices. The changes in pathogen

Fig. 3. Effect of Cell free cultural filtrate of endophyte Dendryphiella sp.CRRI.33 on Sclerotium sp

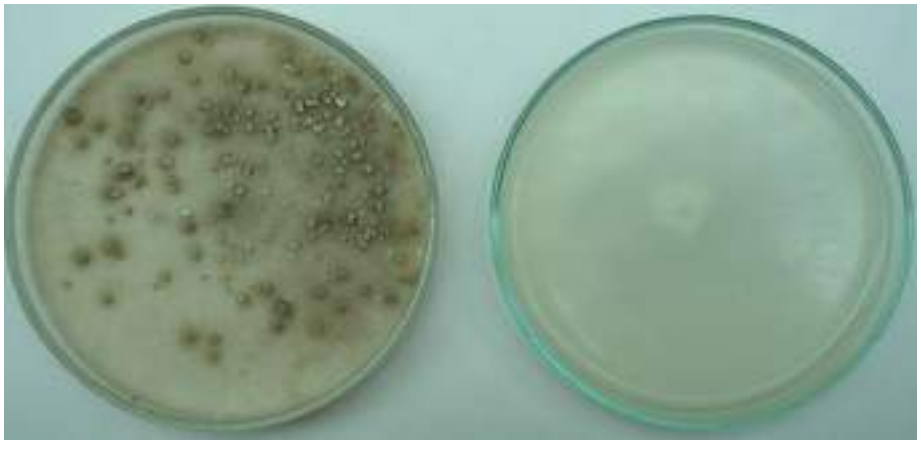

Fig. 4. Effect of Cell free cultural filtrate of endophyte Dendryphiella sp.crri.33 on R. oryzae sativae

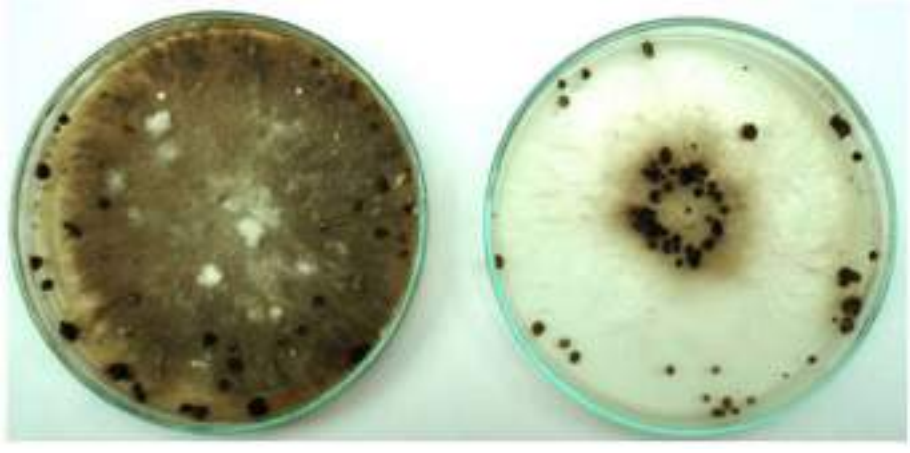

Fig. 5. Effect of Cell free cultural filtrate of endophyte Dendryphiella sp.CRRI.33 on R. solani

can not be predicted. (Kumar *et al.*, 2013). It is essential to develop strategy for eco-friendly management of rice pathogens. The endophytic *Dendryphiella* studied here was found to control the growth and sclerotia production of soil borne pathogenic *Rhizoctonia* and *Sclerotium* species. Extensive use of synthetic pesticides might have disturbed the ecological balance which may be restored by introducing these effective bio-control agents in the ecosystem. Our in vitro findings indicate the possibility of exploiting *Dendryphiella* isolates as a biocontrol of sclerotia-forming rice pathogens. The success will however, depend on the field performance of these endophytes.

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