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Identification of rice endophytes and effect of their cultural filtrates on host cultivars

Urmila Dhua*, Sudhiranjan Dhua, Apurba Chhotaray
Central Rice Research Institute, Cuttack - 753 006 (Odisha)

ABSTRACT

Six endophytes were isolated from seeds of three long duration high yielding rice cultivars namely, Mayurkantha, Utkalprabha and Lunishree suitable for cultivation in rainfed lowland ecosystem of coastal Odisha. The six isolates included in this study were ascomycota fungi, Dendryphiella, Cladosporium, Acremonium, Valsa ambiens and Aspergillus oryzae have NCBI GenBank accession numbers viz. HM572292, HM572293, HM572295, HM572296. Isolate EN 18, isolated from the salt tolerant rice cultivar Lunishree was identified as Dendryphiella sp. which is a marine fungus. The germination percentage was not affected by the treatment with cultural filtrate of these endophytes and their metabolites present in cultural filtrates significantly enhanced length and weight of root and shoot of the rice genotypes.

Key words : *rice, seed, endophytes, cultural filtrates, effect*

Endophytes are fungi or bacteria which live within plant tissues, for entire or part of their life cycle and cause no apparent infection or symptoms. Colonization and propagation of endophytes and their secondary metabolites inside the plants may enhance host-productivity and also may confer host the ability to adapt or resist abiotic and biotic stresses. An "inducible endophyte" was inoculated into rice which established a symbiotic relationship with the rice plant and promoted growth, antioxidant enzyme activity, and photosynthesis (Guo *et al.,* 2004). The endophytes associated with seeds of various rice cultivars were isolated and identified (Dhua *et al*., 2010). Six endophytes were isolated from seeds of three long duration high yielding rice cultivars viz. Mayurkantha, Utkalprabha, and Lunisree. Four fungal cultures (GenBank accession numbers: HM572292, HM572293, HM572295, HM572296) were isolated from the seeds of rice variety Mayurkantha. One fungal endophyte (NCBI-GenBank accession number HM572294) was isolated from rice cv. Utkalprabha.

The endophyte associated with Lunishree was a non-sporulating isolate that belonged to 'mycelia sterilia'. Molecular techniques have been used successfully for phylogenetic placement and segregation of endophytic mycelia sterilia (Promputtha *et al.,* 2002). Internal Transcribed Spacer (ITS) region of Ribosomal DNA is now perhaps the most widely sequenced DNA region in fungi and was found to be useful for the identification of non sporulating endophytes. Hence, the present investigation was carried out to identify the fungus isolate EN18 by these molecular techniques and study the effect of these six fungal endophytes on growth of respective rice cultivars.

MATERIALS AND METHODS

The EN18 culture was maintained on MS broth (Murashige and Skoog, 1962). DNA of this isolate was extracted (Dhua *et al.,* 2008, 2011). Internal Transcribed Spacer (ITS) region of Ribosomal DNA was amplified (White *et al.,* 1990) and sequenced for its identification. Primers used for PCR amplification were ITS-1 (TCCGTAGGTGAACCTGCGG); ITS-4 (TCCTCCGCTTATTGATATGC). Sequencing was outsourced to Chromous Biotech. Pvt. Ltd., Kolkata. Sequence alignment was done for identifying the microbes. The sequences were analyzed with the algorithms afforded by Blast algorithms for nucleotide

or polypeptide homologous sequence analysis in NCBI (Zhang *et al.,* 2000) BLAST. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007)

Six endophytes included in this study were grown in MS broth (Murashige and Skoog, 1962). Seeds of above mentioned rice cultivars (Mayurkantha, Utkalprabha and Lunishree) were soaked for twelve hours in various concentrations (undiluted; 50% dilution; 25% dilution) of cultural-filtrate of fifteen days old culture of respective endophytes.

Seeds of the rice variety Lunishree were treated with EN18; Seeds of Utkalprabha were treated with HM572294; and the seeds of Mayukantha were treated with HM572292, HM572293, HM572295, HM572296.

Treated and untreated seeds were kept at 52^0C in a water bath for 10 minutes followed by shifting to ice cold water. Hot water treated seeds of all the treatments were transferred to Hoagland and Snyder (1934) culture solution.Germination percentage, root length, shoot length of 15 days old seedlings was measured and average length per seedling was calculated. Root and shoot weight of seedlings in all the treatments and replications was recorded.

RESULTS AND DISCUSSION

Database sequences were searched by Mega BLAST. Sequences of *Dendryphiella (*HM572292), *Cladosporium (*HM572295*), Alternaria padwickii* (GU373650), *Alternaria longissima* (FJ971842) *and* uncultured *Ascomycota* (AM901799) had shown pair-wise similarities with fungal isolate EN18. *Dendryphiella* and *Cladosporium* both had 99% similarity as well as E value zero with sequence of EN18 (Table1).

Evolutionary relationships of 5 taxa with isolate EN18 was studied (Fig.1). The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff 1966).Tree #1 out of 4 most parsimonious trees (length = 24) is shown. The consistency index is (0.833333), the retention index is (0.625000), and the composite index is 0.546875 (0.520833) for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein,1985) The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 (Felsenstein,1985; Nei M and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 10 positions in the final dataset, out of which 7 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007).

Pairwise distance and number of base substitutions per site were minimum with *Dendryphiella* (Table 2). All results are based on the pairwise analysis of 6 sequences. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 [(Tamura et al., 2004) and (Tamura et al., 2007)]. Codon positions included were 1^{st} +2^{nd} +3^{rd} + Non coding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 10 *positions* in the final dataset. On the basis of above mentioned analysis the isolate EN 18 was identified as *Dendryphiella sp.*Isolate EN12, EN13, EN15 & EN19 were from local rice cultivar Mayurkantha, EN14 was

Table 1. Database sequences searched by Mega BLAST and showing pair-wise similarities with fungal isolate EN18

Database sequences with max. score showing similarity		Max. Identity	Max. score	Query coverage	E-value
Accession	Description				
HM572292	*Dendryphiella*	99%	931	99%	0
HM572295	*Cladosporium*	99%	926	99%	0
GU373650	*Alternaria padwickii*	98%	850	90%	0
FJ971842	*Alternaria longissima*	92%	643	85%	0
AM901799	uncultured *Ascomycota*	92%	651	85%	0

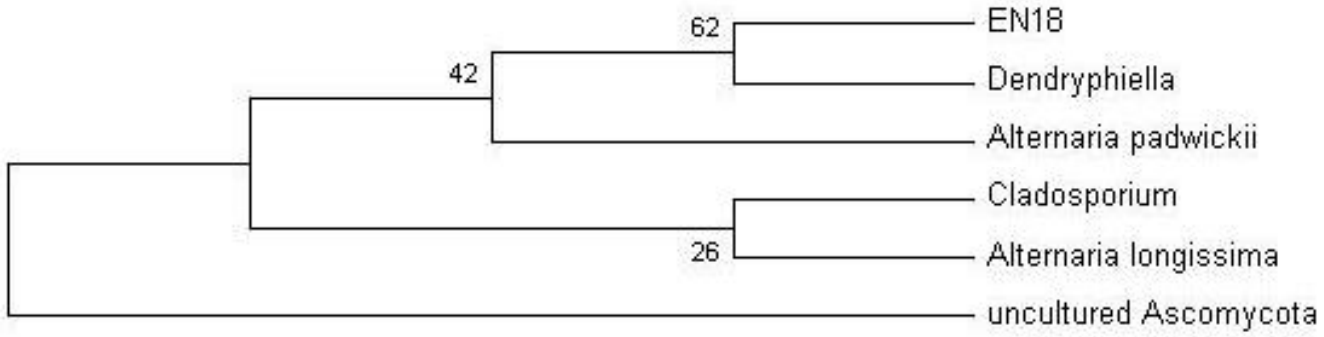

Fig. 1. Evolutionary relationships of 5 taxa with isolate EN18

from high yielding rice variety Utkalprabha. *Dendryphiella* (EN12 and EN18) was associated with salt tolerant rice genotype Lunisree as well as local cultivar of coastal Odisha.

The six isolates included in this study were ascomycota fungi. *Dendryphiella*(EN12, EN18) and *Cladosporium*(EN15*)* both belonged to Pezizomycotina, Dothideomycetes but *Dendryphiella* was from order Pleosporales and *Cladosporium* in Capnodiale(Table 3). *Acremonium* (EN13) and *Valsa ambiens* (EN14) both were in Sordariomtcetes. However *Acremonium* was placed in Hypocreomycetidae, Hypocreales and *Valsa ambiens* was included in order Diaporthales of Sordariomycetidae. *Aspergillus oryzae* (EN19) belonged to Eurotiomycetes, Eurotiomycetidae, Eurotiales (Table 3)

The germination percentage of rice cultivars was not affected by the treatments with cultural filtrate of all the endophytes included in this study. The metabolites of these endophytes present in the cultural filtrates significantly enhanced seedling length and weight of all the cultivars studied. Length and weight of Mayurkantha seedlings treated with endophyte cultural filtrate (EN12, EN13, EN15, EN19 isolated from Mayurkantha) was significantly more than the untreated control (Table 4).

Pure cultural filtrate of Dendryphiella (EN12 and EN18) enhanced seedling growth more than the diluted cultural filtrate. Undiluted cultural filtrate of *Dendryphiella* induced about 1.75 times increase in root length,1.5 times increase in shoot length, 1.4 times increase in shoot weight and approximately 2.5 times

Table 2 . Estimates of Evolutionary Divergence between Sequences

	EN 18
Dendryphiella	9
Cladosporium	14
Alternaria padwickii	153
Alternaria longissima	201
Uncultured Ascomycota	16

increase in root weight of rice cultivars Mayurkantha and Lunisree (Table 4, Fig 2, Fig.3). The cultural filtrates of undiluted *Cladosporium* (EN15) *Acremonium* (EN13*)* and *Aspergillus oryzae* (EN19) promoted shoot growth more than the root growth (Table 4). *Cladosporium* (EN15) treated seedlings had almost double root weight than control though root length was at par with the untreated control. The endophytic *Valsa* enhanced the root length of rice cultivar Utkalprabha about 2.4 times and shoot length was doubled . Diluted cultural filtrate was found to be more effective for enhancement of root length (Table 5), although there was reduction in the root weight.

The six isolates included in this study were endophytic ascomycota fungi associated with rice seeds. Isolate EN 18, isolated from the salt tolerant rice cultivar had similarity with NCBI-GenBank accession number HM572292 and was identified as *Dendryphiella sp*. which is a marine fungi found in various plant and algal substrates from different geographical locations and climatic zones. The culture extract of *Dendryphiella* was reported to be antimicrobial, though production of the biologically active metabolites was strain-specific (Thomas *et al.,*

Table 3. Taxonomy status and passport data of Ascomycota fungal endophytes isolated from rice seeds at CRRI, Cuttack

Isolated from rice genotype	Isolate No.	NCBI GenBank accession no.	Organism	Taxonomic status
Mayurkantha	EN12	HM572292	*Dendryphiella*	Dothideomycetes; Pleosporales,
	EN13	HM572293	*Acremonium*	Sordariomycetes , Hypocreales
	EN15	HM572295	*Cladosporium*	Dothideomycetes Capnodiales
	EN19	HM572296	*Aspergillus oryzae*	Eurotiomycetes; Eurotiales
Utkalprabha	EN14	HM572294	*Valsa ambiens*	Sordariomycetes; Diaporthales
Lunisree	EN18	-	*Dendryphiella*	Dothideomycetes; Pleosporales

2006). This marine fungi, is adapted to survive and complete the life cycle in environments of high salinities (Guo *et al.,* 2004).

The germination percentage was not affected by the treatment with cultural filtrate of these endophytes and their metabolites present in cultural filtrates significantly enhanced length and weight of root and shoot of the genotypes. However, at lower concentration of cultural filtrate enhancement of root length was greater although the root weight was not

Table 4. Effect of 'endophyte-cultural–fitrate(s)' on 15 days old seedlings of rice variety Mayukantha

Treatment	Root Length (mm)	Shoot Length (mm)	Seedling length (mm)	Root wt. (mg)	Shoot wt. (mg)
Control	48.3 ±2.8	25.3±1.4	73.6±3.8	64±2.0	104.0
EN12	78.7±3.3	40.3±2.3	119±4.46	63±6.0	187.6
EN12 (50%)	66.6±3.0	44.8±1.9	111.5±3.49	108.5 ±13.5	158.0
EN13	59.3±3.5	50.6±3.0	109.9±6.0	125 ±9.0	233.6
EN13(50%)	75.8±2.2	53.7±2.5	129.5±4.0	108± 0.0	227.3
EN15	52.0±3.5	55.7±2.0	107.7±4.6	120±4.0	229.6
EN15(50%)	56.5±3.6	54.6±2.0	111.2±4.8	167±20.0	248.3
EN19	59.6±2.7	45±1.65	104.6±4.0	80±1.	132.0
EN19(50%)	55.9±2.3	43.3±2.0	99.2±3.93	94±11.0	141.0

LSD (root/ shoot/ root+shoot length) at 0.01 =9.2 LSD (5 seedling root wt.) at 0.05 = 23.5
LSD (5 seedling shoot wt.) at 0.05 = 14.8

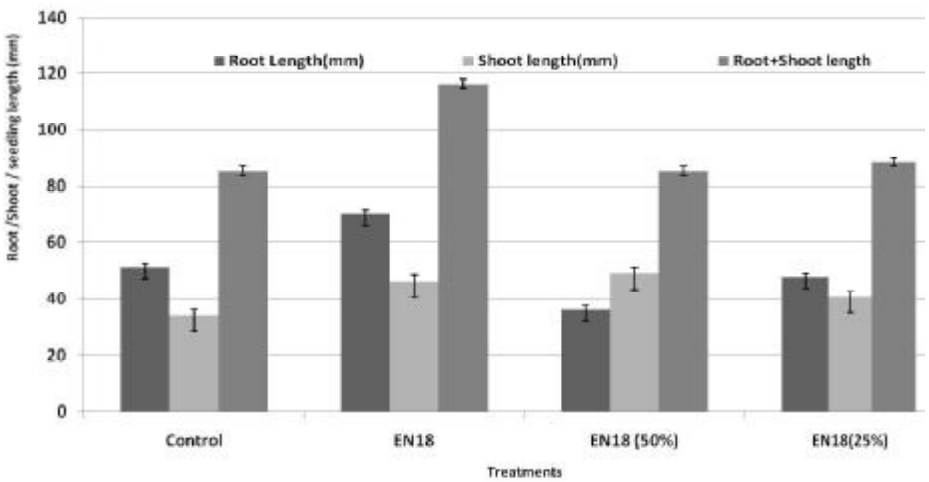

Fig. 2. Effect of EN18 cultural filtrate on root and shoot length of rice cultivar Lunisree

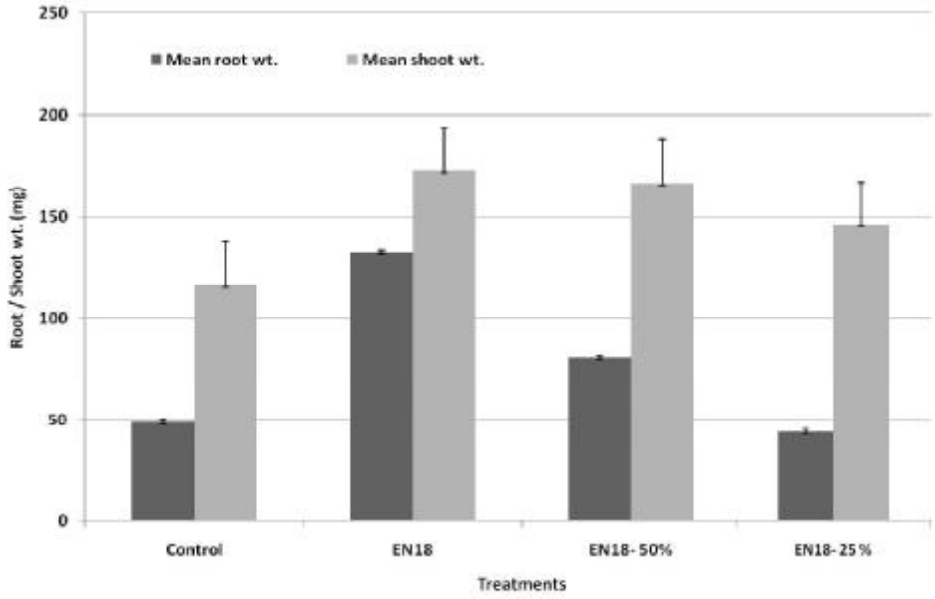

Fig. 3. Effect of EN18 cultural filtrate on root and shoot weight of rice cultivar Lunisree

increased as the increase in length did not increase the total root mass.

Endophytic *Cladosporium* populations were isolated from *Oryza sativa* collected from Bhadra River Project Area, Southern India (Naik *et al*, 2009). Gibberellins production and plant growth-promoting ability of endophytic *Cladosporium* were earlier reported (Muhammad *et al.,* 2009). The plant growth promoting potential of *Aspergillus* is also well known, as it produces substantial amounts of phosphatases (Tarafdar *et al.,* 1988). *Acremonium* the other endophyte which had increased the plant growth significantly during these studies was earlier also found to produce two major groups of substances for plant growth promoter (Lim and Suh, 1998).

Colonization and propagation of these six endophytes inside the plants may enhance productivity of the host and also may help to adapt or resist abiotic stresses.

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Table 5. Effect of 'endophyte-cultural–fitrate(s)' on 15 days old seedlings of rice variety Utkalprabha

Treatment	Root Length in mm	Shoot Length in mm	Root+Shoot Length in mm	Shoot-wt. (mg)	Root wt. (mg)
Control	25.8±2	30± 1.5	55.9± 1.7	93±1.6	49±1.1
EN14	55.8±2	56.9±1.8	112.7±2	173.5±1.8	74.5±1.5
EN14-50%	48.6±1.8	50±1.6	95.8±1.7	168±1.8	78±1.5
EN14-25%	60.5±2.3	62.3±2	122.8±2.1	179±1.9	73±1.4

LSD(root/ shoot/ root+ shoot length) at 0.01 =7.43 LSD (5 seedling shoot wt.) = 27.7; LSD (5 seedling root wt.) = 13.0

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