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Morphological and molecular characterization of *Curvularia* species associated with grain discoloration of rice in Tamil Nadu

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Abstract
Grain discolouration is an emerging disease that reduced the production and productivity of rice. The disease associated number of pathogenic and saprophytic fungi that infect rice grains both in field and storage conditions. A study was carried out to assess the percent disease index and to identify the causal agent of grain discolouration among the popular rice variety growing in various district of Tamilnadu. The result indicated that the maximum PDI was recorded in 37.5% in TKM13 and the least PDI (22.0%) was recorded in CO51 rice variety. The initial identification revealed that *Curvularia* spp. was predominant in rice grain samples expression of the symptoms of grain discolouration collected from the various districts of Tamilnadu. NCBI- BLAST has confirmed the pathogen as *Curvularia lunata*, based on the amplification and sequence analysis of internal transcribed spacer (ITS) region of the rDNA. Pathogenicity test has also proved the cause of grain discolouration by *Curvularia* spp.

Keywords: grain discolouration, pathogenicity, ITS region, *Curvularia lunata*

Introduction
Rice (*Oryza sativa* L.) is one of the most dominantly staple food sources among 90% of produce is consumed in Asia and more than 60 per centage of Indian population (Adam *et al*. 2018) [1]. It provides food security to more than two-third populations and a means of livelihood to millions of rural households (Khamari, 2020) [14]. In world's population it is estimated that will exceed 8 billion people by 2025 and the rice production needs to be increased by 40% more to meet the increasing food demands, by the year 2030 (Yadav *et al*., 2019; Jena *et al*., 2018) [26, 13]. Grain discoloration has been an emerging reducing disease which reduces yield and potent threat to rice crops (Schaad, 2008) [22]. It is otherwise known as "glume discolouration" or "dirty panicle". Baite *et al*., (2019) [5] reported the incidence of grain discoloration ranged from 25 to 92% in different rice varieties.
Many fungal microorganisms *viz*., *Alternaria* spp., *Curvularia* spp., *Fusarium* spp. *Drechslera oryzae*, *Pyricularia oryzae*, *Sarocladium oryzae*, *Sclerotium* spp., *Aspergillus* spp., *Penicillium* spp., *Phoma* spp., *Cercospora* spp. has been reported as causal agents of grain discolouration (Islam and Ahmed, (2017) [12], Persaud *et al*. (2019) [20]. Out of which *Curvularia lunata*, *Helminthosporium oryzae* are commonly found. Currently, *Curvularia* has been classified into family Pleosporaceae under Dothidiomycetes fungi (Ariyawansa *et al*. (2015) [3]. The nomenclature of *Bipolaris* was taken priority over *Cochliobolus* because of the importance as economically phytopathogens (Rossman *et al*. 2013) [21]. *Curvularia* species consist of saprophytes and endophytes pathogens usually it has broad host range (Condon *et al*. 2014) [9]. *Curvularia*, *Bipolaris* and *Fusarium* species were reported to infect plants in Poaceae family including rice crop (Manamgoda *et al*. 2011) [17]. Fungal identification using morphological characteristics is the fundamental step in identifying species. However, species of *Curvularia* has a high degree of similarity based on their morphology and always caused misidentification. Correct fungal species identification is crucial for crop protection and developing disease precautions (McCartney *et al*. 2003) [18]. Therefore, molecular approach was used for species confirmation of *Curvularia* and related species using Internal Transcribed Spacer (ITS) region. ITS region is widely used for species differentiation between *Curvularia* and *Bipolaris* species since it clearly defines interspecific variation (Sharma *et al*. 2014) [23]. Therefore, the objectives of this study are to assess the percent disease index (PDI) and identify the causal agent of grain discolouration of rice.

Materials and Methods

Disease index, sample collection and estimation of loss

Samples were collected from fifteen districts of Tamilnadu to estimate the disease index. Random samples were collected from the fields during summer and rainy season. The samples were brought to the laboratory at Agricultural college and Research Institute, Madurai (9º58'20.0"N 78º12'11.1"E) and used for the analysis. The grains were separated as healthy (no discoloration) and discolored grains based on visual observation. Varieties such as ADT45, ADT43, CO51, ASD16, IR50 and TKM13 growing in different ecologies were selected for the study. The details of the varieties are given in Table 1. All the samples (500 g each) were collected in a polyethylene bags and stored at room temperature until further use for mycological analysis. The disease incidence in the field was calculated by the following formula.

$$PDI = \frac{\text{sum of individual diseased grain rating} \times 100}{\text{Number of grains assesed} \times \text{Maximum disease grade}}$$

Isolation of pathogen associated with grain discolouration

Paddy samples were collected randomly various district of Tamil nadu. Both fresh and discolored seeds were analyzed using the agar plate method as described by Chandramani (2007) and Ora *et al*. (2011) [19]. The standard protocol of International Seed Testing Association (ISTA) (1996) and International Rice Research Institute (IRRI) was followed to detect and identify the signs of fruiting bodies of the fungal microorganisms. The seeds were surface sterilized with 1% sodium hopochlorite for 30 S followed by sterile water. After removing the moisture from seeds using a sterile filter paper, the seeds were placed in potato dextrose agar (PDA) plates amended with streptomycin sulfate. After 5 days of incubation at 26 °C, the plates were observed for mycelial growth. The microscopic slides were prepared from mycelium emerged from the seeds plated in PDA. The spores observed using microscope were carefully separated and plated in PDA. The pure culture obtained using single spore isolation technique was mass multiplied and stored at −4 °C for use in the current study.

Pathogenicity

For the inoculation purposes, eight days old culture was used. By using the hair brush or spatula, the conidia were scrapped and the suspension was collected in the glass beaker. Spore load of conidia was 10^7 ml^{-1} and adjusted using heamocytometer (Waller *et al*., 1998). Ten ml of sterile distilled water containing fungal culture was added to the plate.

Inoculation of pathogen

The pathogenicity of each isolates of *C. lunata* was proved by following Koch postulates. Two kg of sieved garden soil was autoclaved at 1.4 kg cm^{-2} pressure for two hours for two successive days and filled in the earthen pot of 15cm diameter. Rice seedlings were planted @ 3 hills/ pot and replicated three times. The fungal conidial suspension (10^7 spores ml^{-1}) of the pathogens were sprayed on the panicle at flowering and boot leaf stages. The uninoculated seedlings were kept as control. Polythene bags were covered with panicles for two days to maintain humidity. For aeration purposes, the bags were punctured by needle. Each treatment was maintained with three replications. The pots were kept under greenhouse condition. Seedlings were monitored regularly for the disease development. The percent incidence of grain discolouration caused by each isolate was recorded nine days after inoculation. The pathogens were reisolated and maintained.

Morphological identification of *Curvularia* sp.

The morphological characteristics of the isolates were observed based on macro and micromorphology. The colony appearance and pigmentation were recorded after 14 days of incubation on PDA medium for macromorphological characteristics. The growth rate of the isolates was recorded after 72 h of incubation. The micromorphological characteristics such as conidia shape, size and conidiophore were observed on PDA after 7 days of incubation.

Molecular characterization based on internal transcribed spacer (ITS) sequencing

The fungus was cultured on potato dextrose broth at room temperature for two weeks. Then the mycelium was collected, dried and powdered by freezing in liquid nitrogen. The genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Chakraborty *et al*. (2010). The extracted genomic DNA was subjected to PCR with ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primer pair (White *et al*., 1990) [25]. The PCR product was analyzed on 1.2% agarose gel, stained with ethidium bromide and viewed under transilluminator. The amplified fragments were sequenced and confirmed using NCBI database.

Phylogenetic analysis

The purified ITS products were sent to a service provider and were sequenced using an Applied Biosystems 3730xl DNA Analyzer for DNA sequencing by Bioserve scientific company, Hyderabad). Both forward and reverse were sequenced. Both sequences were assembled and aligned using Molecular Evolutionary Genetic Analysis (MEGA 6). The assembled ITS sequences were compared with sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm. gov) to determine the closest matched sequence from the database. All the assembled DNA sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov). All ITS sequences were aligned using CLUSTALW in MEGA6 to construct a phylogenetic tree using Neighbour Joining method with 1000 bootstrap replication value (Tamura *et al*. 2013) [24]. The representative sequences of each species were obtained from GenBank and were included in the tree**.**

Statistical Analysis

Means differences of the treatment were evaluated with ANOVA by using Duncan's Multiple Range Test at 5% significance (Gomez and Gomez, 1984).

Results

Table 1: Survey and assessment of grain discoloration in major rice growing regions of Tamil Nadu

S. No	Place of collection	District	Varieties	Latitude	Longitude	PDI
1.	Kamalaputhur	Thiruvannamalai	Ponni	12.38562	79.152402	35.0^{b} (36.27)
2.	Perumpakkam	Villupuram	Puzhuthi kar nel	11.937804	79.440085	26.2^{g} (30.78)
3.	Muttam	Cuddalore	ADT43	11.140	79.346	30.0^{e}(33.21)
4.	Kathalampet	Vellore	CO51	12.769	79.14	25.5^{g}(30.32)
5.	Chengakovilpatti	Dindugal	CO51	10.017	77.900	23.3^{i}(28.86)
6.	Panpoli	Tenkasi	ASD16	9.026	77.256	27.7^{f}(31.75)
7.	Thirunadriyur	Nagapattinam	IR50	11.103	79.655	33.3^{c}(35.24)
8.	Aranvoyal	Thiruvallur	ADT45	13.102	79.95	24.4^{h}(29.60)
9.	Shoolagiri	Krishnagiri	ADT 45	12.578	78.017	20.2^{k}(26.70)
10.	Devapandalam	Kallakuruchi	ADT 45	11.900	78.931	17.2^{l}(24.50)
11.	Paupparapatti	Dharmapuri	ADT 36	12.221	78.059	28.0^{f}(31.94)
12.	AC & RI	Madurai	TKM 13	9.970	78.204	37.5^{a}(37.76)
13.	Vengalapuram	Thirupathur	CO51	12.477	78.575	24.0^{hi} (29.33)
14.	Aduthurai	Thanjavur	ADT 45	10.998	79.480	32.2^{d}(34.57)
15.	Sevlimedu	Kancheepuram	CO51	12.808	79.685	22.0^{j}(27.97)

The rice grains showing the discolouration symptoms were collected from the major rice growing areas of Tamilnadu and the percent disease index was assessed. The highest disease index (37.5 PDI) was recorded in Agriculture college and Research Institute, Madurai, Tamilnadu and it was on par with the disease index (35 PDI) recorded at Kamalaputhur, Thiruvannamalai district. This was followed by 33.3 of PDI at Thirunadriyur, Nagapattinam district The least incidence was recorded in Devapandalam of Kallakuruchi district (17.7 PDI) (Table.1)

Pathogen associated with grain discolouration

The seed health analysis showed that *Curvularia* spp. was the most predominant fungal pathogen associated with greater than 95% of the sample observed with signs and symptoms of grain discolouration. The presence of *Bipolaris oryzae*, *Sarocladium oryzae*, *Alternaria* spp., *Nigrospora* spp. and *Fusarium* spp. were also observed at low levels below 5% of the samples (Table 2). Rice grains with the discolouration symptoms were selected and sent subsequently to NCBI – BLAST for confrmation of the species. NCBI - BLAST confirmed *C. lunata* as the most dominant fungal pathogen on the grains with the discolouration symptoms. This was based on molecular analysis (sequencing) of the ITS region of the rDNA, for which a 100% match was obtained against published strains of this species *C. lunata*.

Table 2: Pathogen associated with grain discolouration

S. No	Place of collection	*Curvularia* sp.	*Helminthosporium* sp.	*Fusarium* sp.	*Sarocladium oryzae*	*Aspergillus* sp.	*Nigrospora oryzae*
1.	Kamalaputhur	+	+	+	+	+	-
2.	Perumpakkam	+	+	-	-	+	-
3.	Muttam	+	+	+	+	+	-
4.	Kathalampet	+	+	-	+	-	-
5.	Chengakovilpatti	+	+	+	-	+	-
6.	Panpoli	+	+	+	-	-	-
7.	Thirunadriyur	+	+	-	+	-	+
8.	Aranvoyal	+	+	+	-	+	-
9.	Shoolagiri	+	-	-	-	-	-
10.	Devapandalam	+	+	+	-	-	-
11.	Paupparapatti	+	+	-	+	-	-
12.	AC & RI	+	+	-	+	-	-
13.	Vengalapuram	+	+	+	+	-	-
14.	Aduthurai	+	+	+	+	-	+
15.	Sevlimedu	+	-	-	+	-	-

Pathogenecity of the isolates of *C. lunata*

The pathogenicity test has proved *C. lunata* as one of the most predominant and aggressive fungal microorganisms associated with grain discolouration. Isolates of the pathogen were artificially inoculated in rice crop grown under pot culture experiment by spraying the spore suspension. The artificially inoculated pathogens produced similar symptoms with those of naturally infected plants. The result revealed that the highest percent disease index was registered in the isolate of IS (VILL)2 (36.5%) followed by IS(KAG)12 (35.4%) and it was satistically on par with IS (TVM)1 (35%) and the least PDI was recorded in the isolate IS(DHA)11 (17.2%)

Pathogenicity studies of rice grain discolouration isolates

S. No	Isolates	Place of collection	Percent disease index (PDI)
1.	IS (TVM)1	Kamalaputhur	35.0 (36.27)
2.	IS (VILL)2	Perumpakkam	36.5 (37.16)
3.	IS(CUD)3	Muttam	32.2 (34.57)
4.	IS(VLR)4	Kathalampet	18.0 (25.10)
5.	IS(DIN)5	Chengakovilpatti	30.8 (33.70)
6.	IS(TEN)6	Panpoli	33.3 (35.24)
7.	IS(NAG)7	Thirunadriyur	30.0 (33.2)
8.	IS(TLR)8	Aranvoyal	20.0 (26.56)
9.	IS(MDU)9	AC & RI	37.1 (37.52)
10.	IS(DIN)10	Devapandalam	27.0 (31.30)
11.	IS(DHA)11	Paupparapatti	17.2 (24.50)
12.	IS(KAG)12	Shoolagiri	35.4 (36.51)
13.	IS(THR)13	Vengalapuram	25.0 (30.00)
14.	IS(TJR)14	Aduthurai	24.4 (29.60)
15.	IS(KAN)15	Sevlimedu	23.3 (28.86)

Morphological characteristics

The colonies of *Curvularia* sp. grown on PDA medium were grey to brown in colour. The conidia of all the fifteen isolates were boat shaped, swollen at third cell, grey, black to dark brown, third cell from base darkest and largest, with three septa, end cells of the conidia were sub hyaline, germinated at ends and the conidial length and breadth ranged from 22.26 to 17.36 × 11.80 to 7.79 µm. The maximum conidial length was attained from IS(TEN)6 and IS (VILL)2 (22.26 µm) and the maximum width of conidia from IS(DIN)10 (11.80 µm). The minimum length and width of conidia were ranged from IS (KAN)15 (17.36 ×8.90µm) (Table.3).

Table 3: Morphological characteristics of *Curvularia* sp.

Isolate no	Mycelial character	Septation	Size of conidia (µm)	
			Length	Breadth
IS (TVM)1	Grey cottony mycelia with rings on the plate	3	21.5	7.79
IS (VILL)2	Dark brown pigmentation at the centre of plate and light brown towards the edge	3	22.26	9.68
IS(CUD)3	Dark brown pigmentation at the centre of plate and light brown towards the edge	3	17.81	8.90
IS(VLR)4	Dark brown pigmentation at the centre of plate and light brown towards the edge	3	23.71	9.86
IS(DIN)5	Dark brown pigmentation at the centre of plate and light brown towards the edge	3	20.37	9.24
IS(TEN)6	Flattened greyish colonies and black pigmentation on reverse plate	3	22.26	9.86
IS(NAG)7	Grey cottony mycelia with rings on the plate	3	21.48	10.35
IS(TLR)8	Flattened greyish colonies and black pigmentation on reverse plate	3	20.81	8.57
IS(MDU)9	Flattened greyish colonies and black pigmentation on reverse plate	3	20.70	10.35
IS(DIN)10	Dark brown pigmentation at the centre and light brown towards the edge	3	19.26	11.80
IS(DHA)11	Grey cottony mycelia with rings	3	19.37	9.68
IS(KAG)12	Fluffy colonies with grey and white aerial mycelium	3	21.71	8.24
IS(THR)13	Grey cottony mycelia with rings	3	20.04	9.24
IS(TJR)14	Flattened black and grey colonies and black pigmentation on reverse plate	3	19.37	10.46
IS (KAN)15	Flattened greyish colonies and black pigmentation on reverse plate	3	17.36	8.90

Molecular characterization

Virulent isolate of *C. lunata*, *Exserohilum*, and *Fusarium* sp. were successfully amplified and sequenced. The ITS region with expected sizes, approximately 600–650 bp. Based on BLAST search, ITS sequences showed high percentage similarities between 97 and 100% with GenBank database (http://www.ncbi.nlm.gov), virulent isolate *C. lunata* IS (MDU)-9 (MN960393) of ITS sequences were deposited into Genbank (http://www.ncbi.nlm.gov). The phylogenetic tree was constructed and the relationship between all isolates is shown in Fig.1 Based on phylogenetic tree, all isolates were clearly placed into separate clades with their referral control from GenBank. Main clade I and II in this study were referred to Group 1 and 2 respectively. Group 1 represented necrotrophic and highly virulent pathogens, *Curvularia* species while group 2 denoted outer group *Aspergillus nidulans* was chosen as an outgroup control because it is ancestral to the ingroup. The outgroup was distinctly separated from main clade. The phylogenetic tree constructed showed the correlation with morphological characteristics.

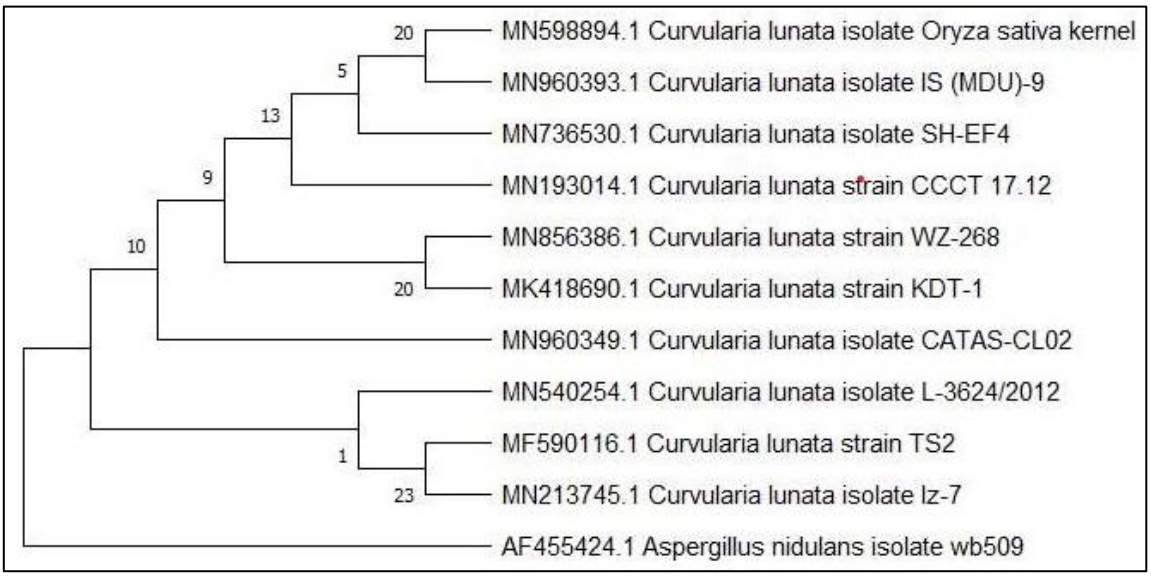

Fig 1: It is ancestral to the in-group.

Discussion

Grain discoloration has been an emerging disease and causes major problem to rice cultivation (Arshad *et al*., 2009) [4]. The disease associated number of microbes including pathogen and saprophytes cause thus, making it a complex problem (Chhabra and Vij, 2020) [8]. Earlier it was considered as a minor problem in the tropical rice-growing areas (Chandramani, 2007) [7]. Recently, it gained importance due to its increase in occurrence and severity across various rice-growing regions of Tamilnadu. Many fungi have found to be involved in grain discolouration and cause discoloured spots on the hulls and kernels (Groth 1991) [11]. The disease causes poor seed and grain quality and associated with many complex fungal microorganisms (Islam and Ahmed 2017) [12]. Ali and Deka (1996) [2] reported that ten fungal species from seven genera (*Curvularia, Drechslera, Nigrospora, Fusarium, Aspergillus and Penicillium*) were found to be associated with grain discolouration of six rice cultivars. In the current study, analysis of discolored grains revealed the predominant association of *Curvularia* spp. with greater than 95% of the sample observed with sign and symptoms of grain discolouration. Along with *Curvularia* spp., *B. oryzae*, *S. oryzae*, *Alternaria* spp., *Aspergillus* spp. and *Fusarium* spp. were also identified to be present on samples at very low levels below 5% of the samples. Similiarly, Gopalakrishnan *et al*. (2010) [10] reported that out of seven fungi, *Curvularia* spp. was predominant followed by *S. oryzae*, *Fusarium* spp., *Alternaria alternata*, *Aspergillus niger* and *H. oryzae*. Subsequently the NCBI - BLAST confirmed *C. lunata*, based on molecular analysis (sequencing) of the ITS region of the rDNA, as the most dominant fungal microorganism on the grains with the discolouration symptoms. This organism identified as a widespread pathogen known to infect rice grains and caused black discolouration on grains. The current research also focused on identified and responsible for causing grain discolouration in rice. Dirty grains were basically a dark, small imperfection that occured on the dorsal surface (the surface opposite the germ) of rice kernels and had a lesion-like appearance due to complex etiology. (Arshad *et al*. 2009) [4]. *Curvularia* spp. were classified as Dothideomycetes fungi which were the most diverse class of fungi (Kirk *et al*. 2008) [15]. From microscopic identification, *Curvularia* spp. conidia were observed with the length of 2.26 to 17.36 and breadth of 11.80 to 7.79 µm. with 3 septation. Based on phylogenetic tree, all the isolates were clearly placed into separate clades with their referral control from GenBank. In this study main clade I and II were referred to Group 1 and 2 respectively. Group 1 as *Curvularia* sp. while group 2 as outer group used as *Aspergillus nidulans.* The phylogenetic tree constructed showed the correlation with morphological characteristics. Kusai *et al*. 2016 [16] constructed tree into two different clades as grouped members of *Curvularia* and *Bipolaris* that indicated the presence of two groups of fungi species *viz*., highly virulent and mild pathogens. Phylogenetic tree constructed by Ariyawansa *et al*. (2015) [3] based on combination genes of ITS, glyceraldehyde 3-phosphate dehydrogenase (GPDH), large subunit (LSU) and translation elongation factor (tef1-a) suggested that *Curvularia* sp. and *Bipolaris* were monophyletic group and could not be combined with each other as showed in this present study. However, the molecular characteristics are more exact and provided the information of genetically pathogenic fungi, accurate identification of pathogenic fungi is vital in disease management of economically important crops especially rice.

Conclusion

Predominant infection by *Curvularia* spp. was recorded in grain discolouration of rice. It was ranged upto 35 percent disease index. Morphological characterization which has been done in this study to *Curvularia* spp. and other related pathogens is the definitive approach of identification which could be aided with molecular characteristics using gene sequences for consistent identification.

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