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MORPHO-MOLECULAR CHARACTERIZATION OF FUNGI ASSOCIATED WITH SEEDS OF HYBRID RICE VARIETIES IN BANGLADESH

SANGIDA AKTER LIZA[1], SHAMIM SHAMSI* AND MD ABDULLAH AL NOMAN

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

Rice, as a dominant crop in Bangladesh, is vulnerable to various fungal pathogens. Many of these pathogens infect the seeds, which can later transmit diseases and serve as an efficient means for spreading seed-borne pathogens over long distances. In this study, seven hybrid rice varieties, specifically BRRI Hybrid Dhan 101 to BRRI Hybrid Dhan 107, were collected from the Bangladesh Rice Research Institute (BRRI) to detect and identify seed-borne fungi using both morphological and molecular techniques. Fourteen fungal species were isolated from the selected rice varieties using "Tissue planting method" and "Blotter method", of which 4 fungal isolates were identified by molecular techniques. Molecular characterization of the isolates was conducted based on the sequence information of internal transcribed spacer (ITS) regions. The sequences obtained using the ITS1 and ITS4 primers were compared with those in the NCBI GenBank using BLAST analysis. Hybrid rice is comparatively more advantageous than inbreed rice because of its high yield, shorter plant life, and higher assimilation translocation. Therefore, it is crucial to identify and manage the pathogenic fungi associated with the seeds to ensure good seed quality, which can contribute to improve the economy of Bangladesh.

Introduction

Rice (*Oryza sativa* L.) is a cereal grain belongs to family Poaceae. Globally, rice is grown in over 100 countries encompassing about 162.06 million hectares, with an annual production of 495.78 million tons of milled rice (FAOSTAT 2020). The average world yield of rice is 3.84 tons/ha (Ahmed *et al*. 2013) and in Bangladesh is only 2.98 tons/ha. Therefore, the development and introduction of hybrid varieties are the topmost priority for the government (Awal *et al*. 2007). In Bangladesh, more than 78 hybrid rice varieties are grown in the field (Bhandari *et al.* 2011). Healthy rice seeds is considered one of the most important constraints in Bangladesh (Nazrul and Fakhrul 2010). Seed-borne fungi associated with rice seeds have been isolated in many countries, including Nigeria, Pakistan, Egypt, Bangladesh, and Cameroon (Nguefack *et al*. 2007, Butt *et al*. 2011, Ora *et al.* 2011, Suleiman and Omafe 2013, Madbouly *et al.* 2014). Rice suffers from more than 60 different diseases, of which 43 are known to occur in Bangladesh. The correct species name of a plant pathogenic fungi is most important for the development of effective disease control management and quarantine purposes (Rossman and Palm-Hernandez 2008). With these considerations in mind, this investigation was conducted to elucidate the morphological and molecular characteristics of fungal isolates associated with selected hybrid rice varieties in Bangladesh. .

Materials and Methods

Seed samples of BRRI Hybrid Dhan 101 - 107 were collected from the Genetic Resources and Seed Division of the Bangladesh Rice Research Institute (BRRI) in Joydebpur, Gazipur. The samples were kept in brown paper bags, labeled, and stored at room temperature (25°C) at Mycology and Plant Pathology Laboratory, University of Dhaka.

*Author for correspondence: <prof.shamsi@gmail.com>. [1]A part of the work of the first author for her MS thesis.

For morphological characterization, isolates were grown on potato dextrose agar (PDA) medium at 25°C. Colony texture, surface appearance, colony margin, colony color, diameter, and conidial size were the parameters considered for morphological characterization. Morphological characters were recorded after 7 days of incubation. Spores were measured at 40X under a compound microscope facilitated with a digital camera (Nikon Optiphot—2 trinocular microscope, Japan) using Image Focus Alpha software. Morphological identification of the isolates was determined following standard literature (Thom and Raper 1945, Raper and Thom 1949, Gilman 1967, Booth 1971, Ellis 1971, 1976, Barnett and Hunter 1972, Ellis and Ellis 1997).

Genomic DNA of four fungal isolates was extracted according to the method used by Noman *et al*. (2021). Before PCR amplification, the DNA quantity was measured by a nanodrop spectrophotometer at 260 nm wavelength and the quality was checked by running on 1% agarose gel. Extracted DNA was stored at -20 °C for further analyses. The ITS regions of the isolates were amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) as forward and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as a reverse primer (White *et al.* 1990). The PCR reactions were carried out in a 25 µl reaction mixture containing 2.0 µL template DNA, 12.5 µl Master mix (Clever Scientific Ltd., Warwickshire, UK), 1.0 µl forward primer, 1.0 µl reverse primer and 8.5 µL nuclease-free water. The reaction mixture was vortexed and centrifuged in a microcentrifuge. The PCR was initiated by an initial denaturation step at 94 °C for 5 min following 30 cycles of 94, 54, and 72 °C each for 30 s, with a final extension step of 5 min at 72°C and ending with 4°C. PCR amplification of ITS regions was confirmed by gel electrophoresis using 1% agarose. The size of the PCR products was estimated by comparing them with a 1 kb DNA ladder (Clever Scientific Ltd., Warwickshire, UK). The purified DNA samples were sequenced through an automated sequencer (Seq Studio Genetic Analyzer, Thermo Fisher Scientific, USA) in the Centre for Advanced Research in Sciences University of Dhaka, Dhaka, 1000.

The obtained sequences were compared with the sequences already available in the National Center for Biotechnology Information (NCBI) using the BLASTn tool (http://www.ncbi. nlm.nih.gov/BLAST), and these sequences were deposited in the GenBank database. Sequences were aligned with CLUSTAL W alignment using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar *et al.* 2016).

Results and Discussion

A total of fourteen fungal species namely *Aspergillus flavus* (Freunde Berlin), *A. niger* (Van Tieghem), *A. fumigatus* (Fresenius Beitragezur), *A. tamarii* (Kita), *A. terreus* (Thom. Amer.), *Curvularia lunata* (Wakker), *Debaryomycetes* sp. (Lodder and Kreger), *Pyricularia grisea* (Sacc.), *Meyerozyma guilliermondii* (Wick.), *Penicillium* sp. 1 (Fr.) Sacc. *Penicillium* sp. 2, *Penicillium oxalicum* (Currie and Thom), *Rhizopus stolonifer* (Bull.), and *Rhizopus* sp. (Ehrenb) were found to be associated with studied seven hybrid varieties of rice seeds.

Based on morphological characteristics, these 14 fungal isolates were provisionally identified (Fig. 1). However, in this investigation, some fungal iolates such as L2, L4, L5, L6 were unable to identify accurately using morphological features alone. Hence, molecular characterization of the fungal isolates were performed for proper identification. Provisional identification of the fungal isolates with their source have been described bellow:

Aspergillus flavus Mag. Ges. Naturf. Freunde Berlin **3** (1): 16 (1809) (**Fig. 1a**)

Colonies effuse, greenish. Mycelia well-developed, septate, profusely branched and hyaline. Cells multinucleated. Conidiophores long. This species produces sclerotium which is well

developed. The sclerotia contribute to an accumulation of inoculum in the soil when they produce conidia at the beginning of the next growing season. Conidia catenulate, dry, usually globose, smooth, green and 5 – 8 µm in diameter.

Based on the features described above SA Liza 02 isolated from BARI hybrid dhan 101 provisionally identified as *Aspergillus flavus.*

Aspergillus fumigatus Fresenius Beitragezur Mykologie **3**:81 (1863) (**Fig. 1b**)

Colonies grayish green. Mycelium well-developed and septate. Cells multinucleated. Conidiophores long. Conidia catenulate, dry, usually globose, smooth, brown in color. Conidiophores produces thousands of minute gray-green conidia (2 – 3 µm).

Based on the features described above SA Liza 03 isolated from BARI hybrid dhan 107 provisionally identified as *Aspergillus fumigatus.*

Aspergillus niger van Tieghem, 1867, Annls sci. Nat. (Bot), Ser. **5**(8): 240 (**Fig. 1c**)

Colonies effuse, black. Mycelium is well-developed, septate, profusely branched and hyaline. Cells are multinucleated. Conidiophores are very long, often with a foot cell, straight or flexuous, swollen at the apex into a spherical vesicle. The surface of the vesicle is covered by closely packed more or less clavate branches. Conidia catenulate, dry, usually globose, echinulate, dark brown in color and 5 – 7 µm in diameter.

Based on the features described above SA Liza 01 isolated from BARI hybrid dhan 104 provisionally identified as *Aspergillus niger* .

Aspergillus tamarii Kita, Centralbl Bakteriol; Abt. **2**: 433 (1913) `(**Fig. 1d**)

Conidial heads are compact and spherical or loosely radiated, 500-600 µm in diameter. Conidiophore stipes are usually 1-2 mm in length, and hyaline is usually roughened. Vesicles spherical, 10-50 mm diameter. Conidiogenus cells are uniseriate and biseriate. Metulae or phialides cover the entire surface of the vesicle. Conidia are echinulate to tuberculate, and sub spherical.

Based on the features described above SA Liza 03 isolated from BARI hybrid dhan 105 provisionally identified as *Aspergillus tamarii* .

Aspergillus terreus Thom. Amer. J. Bot. **5**-(2): 85 (1918) (**Fig. 1e**)

Colonies moderately fast rapidly growing flat, velvety to slightly granular, or powdery, occasionally floccose with thin irregular margins, cinnamon-buff to brown, rarely orange-brown, consisting of a dense felt of conidiophores with reverse yellow to pale brown. Anisolate with deep orange colonies with lemon yellow diffusible pigment has been described. Conidial heads are pale-brown, long, densely columnar, characteristically appearing fan-shaped. Conidiophores are short, 100-250 mm long, flexuous, smooth-walled with dome-shaped vesicles, 10-20 mm diameter. Phialides are biseriate on the upper two-thirds of the vesicle. Conidia hyaline, smooth-walled, spherical to broadly elliptical, 1.5-2.5 mm in diameter.

Based on the features described above SA Liza 04 isolated from BARI hybrid dhan 106 provisionally identified as *Aspergillus terreus* .

Curvularia lunata (Wakker) Boedijn Mycol. Pap. **106**: 2-43 (1966). (**Fig. 1f**)

Colonies effuse grayish black. Stromata very rarely formed in culture, colonies on PDA markedly zonate, Conidiophores solitary, mostly unbranched, straight or slightly undulating, brown, septate up to 37-64 µm long, 9.2-14.4 µm thick. Conidia are mostly three septate, brown, slightly curved,

third cell from the base is broader and darker than others, smooth, 24.4-29.2 × 9.1-12.4 µm in size.

Based on the features described above SA Liza 03 isolated from BARI hybrid dhan 105 provisionally identified as *Curvularia lunata.*

***Debaryomycetes* sp**. Lodder & Kreger-van Rij ex Kreger-van Rij, The Yeasts: a taxonomic study: 130 (1984) (**Fig. 1g**)

Colony greenish, cottony, mycelium profusely branched, septate. Ascospores vary in shape and number usually with one to four per ascus. Pseudo hyphae absent or poorly developed. Cells prominently haploid with sporulation usually preceded by conjugation between independent cells or between a cell and its bud.

Based on the features described above SA Liza 02 isolated from BARI hybrid dhan 109 provisionally identified as *Debaryomycetes* sp..

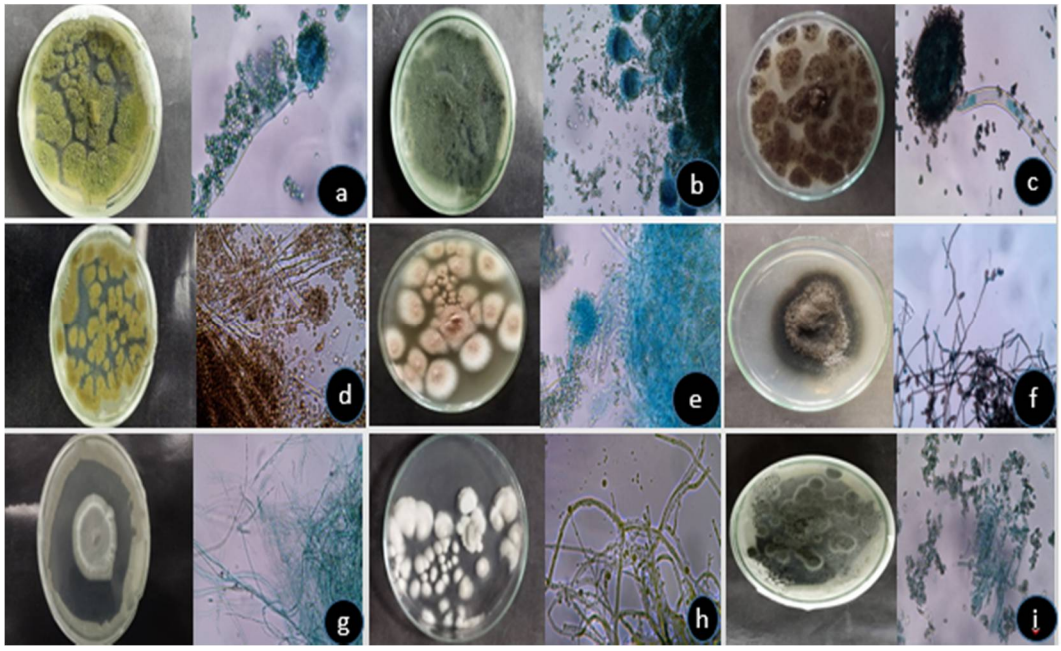

Fig. 1. Colony on PDA medium and conidia under themicroscope (40X) a. *Aspergillus flavus* b. *A. fumigatus* c. *A. niger* d. *A. tamarii* e. *A. terreus* f. *Curvularia lunata* g. *Debaryomyces* sp. h. *Meyerozyma guilliermondii* i. *Penicillium oxalicum.*

Meyerozyma guilliermondii : (Wick.) Kurtzman & M. Suzuki, Mycoscience **51**(1): 7 (2010) (**Fig. 1h**)

Colonies are flat, moist, smooth, and white to cream in color. It produces a cluster of small Blastomeric along the pseudo hyphae and particularly at septal points. Pseudo hyphae are short and few in number. It is a glabrous and yeast-like colony. Spherical to sub-spherical budding yeast-like cells or blast conidia 2.0-4.0 x 3.0-6.5 µm. No capsules are present.

Based on the features described above SA Liza 10 isolated from BARI hybrid dhan 107 provisionally identified as *Meyerozyma guilliermondii.*

Penicillium oxalicum Currie and Thom, Journal of Biological Chemistry **22**: 289 (1915) (**Fig. 1i**)

Colonies growing rapidly 35-50 mm in 7 days, spreading, velvety, sporulation heavy, with conidial masses breaking off in crusts when the culture is jarred cultures have a silky appearance when viewed under the stereo microscope, dull green at the center, with margin shading through pale blue-green to white, exudate and soluble pigment lacking. Conidia elliptical, smooth, very large 5-5.5 x 3-3.5 micrometer.

Based on the features described above SA Liza 07 isolated from BARI hybrid dhan 101 provisionally identified as *Penicillium oxalicum.*

***Penicillium* sp. 1** Link. (Fr.) Sacc. Bur. Anim. Ind., Bul. **118**: 31-33 (1910) (**Fig. 2a**)

The colony on PDA was velvety with areal mycelium and a very fast-growing and ashy color. The reverse color of the plate was yellow to brownish. Conidiophores were smooth, vesiculate, containing phialides and conidia were globose.

Based on the features described above SA Liza 08 isolated from BARI hybrid dhan 105 provisionally identified as *Penicillium* sp. 1.

***Penicillium* sp. 2** Link. (Fr.) Sacc. Bur. Anim. Ind., Bul. **118**: 31-33 (1910) (**Fig. 2b**)

A colony typically exhibits certain striking characteristics. These include color and color change; texture may be varied, floccose, funiculus or fasciculate or corymb form and habit may be restricted or broadly spreading and range from pale to deeply pale to furrowed or wrinkled.

Based on the features described above SA Liza 11 isolated from BARI hybrid dhan 103 provisionally identified as *Penicillium* sp. 2.

Pyricularia grisea Sacc., Michelia **2**(6): 20 (1880) (**Fig. 2c**)

Conidia are solitary, pyriform to obligate, narrowed toward the tip, rounded at the base, 2-septate, hyaline to pale brown, with a distinct basal hilum, sometimes with marginal frill. Colonies effuse, thinly hairy, grey, greyish brown. Conidiophores are single, simple rarely branched. Chlamydospores often produced in culture.

Based on the features described above SA Liza 12 isolated from BARI hybrid dhan 103 provisionally identified as *Pyricularia grisea.*

***Rhizopus* sp.** (Ehrenb.) Vuill., Revue Mycologique Toulouse **24**: 54 (1902) (**Fig. 2d**)

Mycelium coenocyte, well developed, branched and fluffy. Mycelium produces many aerial stolons that develop rhizoids at certain points. Directly above the rhizoids one or more sporangiospores are produced. The top of each sporangiophore becomes swellings the latter reaches maturity and a sporangium is developed. The central portion of sporangium becomes highly vacuolated and it eventually surrounded by a wall that separates it's from the peripheral zone. The central portion is the columellate. Sporangium produces non-motile sporangiospores.

Based on the features described above SA Liza 14 isolated from BARI hybrid dhan 106 provisionally identified as *Rhizopus* sp.

Rhizopus stolonifer Bull. Toney Bot Clup **69**: 592-616 (**Fig. 2e**)

Mycelium coenocytic, well developed, branched and fluffy. Mycelium produces many aerial stolons that develop rhizoids at certain points. Directly above the rhizoids one or more sporangiospores are produced. The top of each sporangiophore becomes swollen as the latter reaches maturity and a sporangium is developed. The central portion of sporangium becomes

highly vacuolated and it is eventually surrounded by a wall that separates it from the peripheral zone. The central portion is the columellate. Sporangium produces nonmotile sporangiospores.

Based on the features described above SA Liza 13 isolated from BARI hybrid dhan 105 provisionally identified as *Rhizopus stolonifer*.

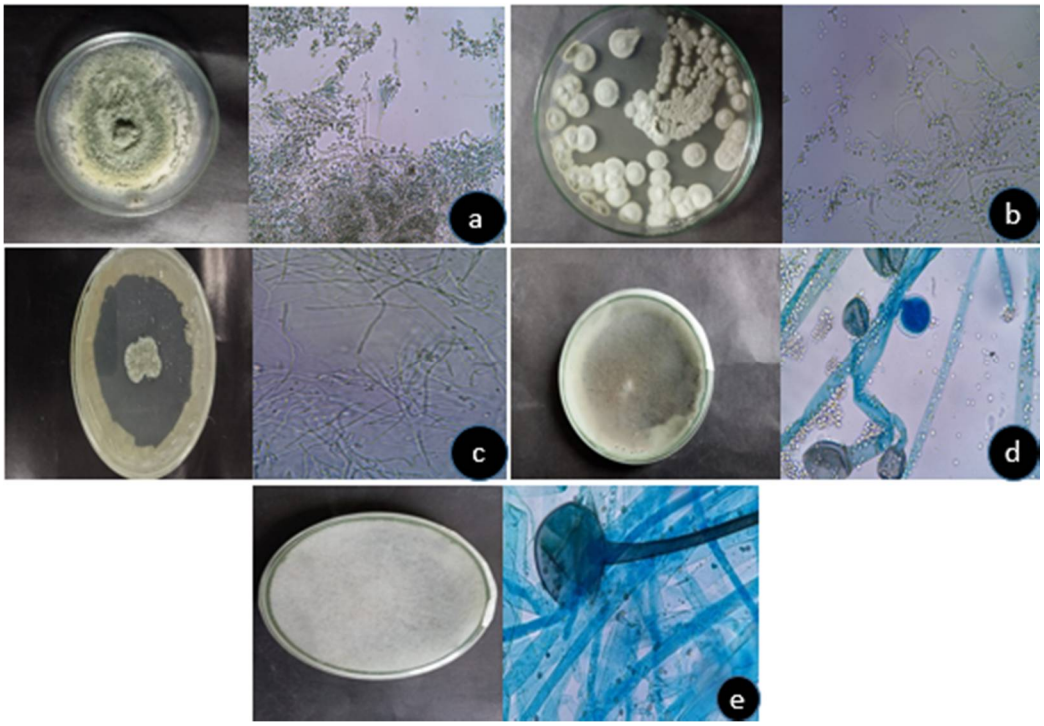

Fig. 2. Colony on PDA medium and conidia under the microscope (40X) a. *Penicillium* sp. 1 b. *Penicillium* sp. 2 c. *Pyricularia grisea* d. *Rhizopus* sp. e. *Rhizopus stolonifer*.

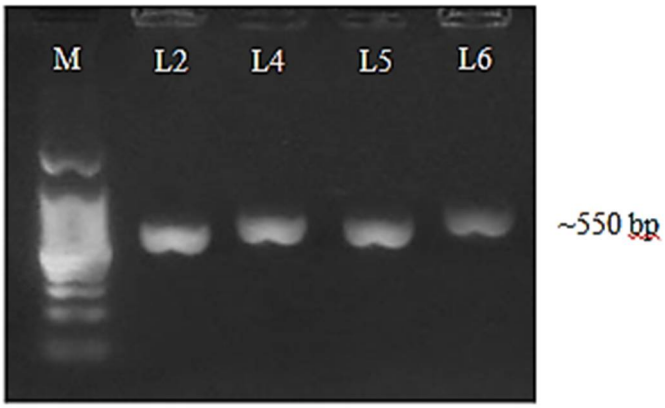

Fig. 3. Gel electrophoresis of the PCR product of five fungal isolates performed by ITS1 (F) and ITS4 (R) primers and showing ~550 bp amplification. (M represents the 1kb DNA ladder).

ITS sequence-based molecular analysis was performed, to identify the unknown fungal isolates. PCR was conducted using ITS1 and ITS4 primers and ~ 550 bp DNA band was amplified (Fig. 3). Sequence analysis of the amplified DNA through BLAST search in GenBank was conducted and found 88.94 to 99.55% similarity and confirmed the identification of the ambiguous fungal isolates (Table 1).

Table 1. BLAST analysis of the amplified sequences from the isolated fungal DNA.

Sample No.	Nameof fungi	Max score	Total score	Query coverage	E value	Per cent identity (%)	NCBI Gene Bank Accession No.
L2	*Meyerozyma gulliermondii*	455	455	81%	3e-123	94.06	MN788421.1
L4	*Pyricularia grisea*	207	207	73%	6e-49	88.44	OM327591.1
L5	*Debaromyces* sp.	148	148	40%	5e-31	88.89	MZ724412.1
L6	*Curvularia lunata*	815	815	99%	0.0	99.55	MT647915.1

Among the isolated fungi *Debaryomyces* sp. is the new record for Bangladesh. Earlier while working with hybrid rice seeds, Ora *et al*. (2011) identified 10 seed-borne pathogens namely, *Xanthomonas* spp. *Rhizopus stolonifer*, *Aspergillus flavus*, *A. niger*, *Fusarium moniliformae*, *Bipolaris oryzae*, *Curvularia lunata*, *Penicillium* sp. *Alternaria tnuissima* and *Nigrospora oryzae.* Tania *et al.* (2020) reported the association of twenty-five fungal species from seeds of 20 inbred rice varieties (BRRI dhan 56 to BRRI dhan 75) from Bangladesh. However, *Aspergillus tamarii*, *Debaryomyces* sp. *Meyerozyma guilliermondii*, *Penicillium oxalicum* and *Pyricularia grisea* found in the present study were not found in the previous work done with inbred rice varieties from Bangladesh.

Present investigation suggests that the molecular technique is a more accurate and rapid means of fungal identification. ITS-based molecular identification methods might be an important complement to conventional mycological detection by culture.

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References

Ahmed M, Hossain M, Hassan K and Dash CK 2013. Efficacy of different plant extract on reducing seed-borne infection and increasing germination of collected rice seed sample. Universal J. Plant Sci. **1**(3): 66-73.

Awal MA, Habib AKMA and Hossain MA 2007. A study on comparative performances of hybrid and conventional rice varieties in aman season. J. Agric. Rural Develop. **5**(1&2): 13-16.

Barnett HL and Hunter BB 1972. Illustrated Genera of Imperfect Fungi. Burgess Pub. Co. U.S.A. pp. 241.

Bhandari HSM and Mossain M 2011. Hybrid Rice in Bangladesh: Current status and future project. In the Proceeding of the 2011 7th ASAE conference, Hanoi, Vietnam. pp. 13-15.

Booth C 1971. The Genus *Fusarium.* The Commonwealth Mycological Institute, England. pp. 267.

Butt AR, Yaseen SI and Javaid A 2011. Seed borne mycoflora of stored rice grains and its chemical control. J Animal Plant Sci. **21**(2): 193-196.

CAB (Commonwealth Agricultural Bureau) 1968. Plant pathologist's pocket book. 1st ed. The Commonwealth Mycological Institute, England. pp. 267.

Ellis MB 1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, England. pp. 608.

Ellis MB 1976. More Dematiaceous Hyphomycetes. The Commonwealth Mycological Institute, England. pp. 507

Ellis MB and Ellis JP 1997. Microfungi on land plants. An identification handbook. The Commonwealth Mycological Institute, England. pp. 868.

Fakir GA, Hossain I, Ahmed MU, Anam MK, Anam MN, Alam MN and Rahman M 2003. Effect of ash, chalk powder and neem leaf on the quality of rice seed stored in gunny bag, motka, plastic drum and tin. Proceeding of review and planning meeting of the rice seed health improvement sub-project held at BRRI, Gazipur, Bangladesh. pp. 1-37.

Food and Agricultural Organisation 2020. FAOSTAT Database. Rome: Food and Agricultural Organization.

Gilman JC 1967. A Manual of Soil Fungi. Oxford and IBH Publishing Co., New Delhi, 2nd edition. Pp. x + 450.

ISTA 1996. International rules for seed testing rules. Seed Sci. Tech. **4**(1): 3-177.

Kumar S, Stecher G and Tamura K 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. **33**(7):1870–1874.

Madbouly AK, Ibrahim MIM and Abdel-Wahhab MA 2014. Efficacy of corn and rice seed-borne mycoflora in controlling aflatoxigenic *Aspergillus flavus*. Comunicata Scientiae **5**(2): 118-130.

Nazrul ASM and Fakhrul ISM 2010. Factor demand in the healthy rice seed use in Boro and T. Aman: A case study of Bangladesh. Bangladesh J. Agric. Res. **35**(2): 297-312.

Nguefack, Nguikwie J, Fotio SK, Dongmo D, Leth B, Nkengfack AE and Amvam ZPH 2007. Fungicidal potential of essential oils and fractions from *Cymbopogon citratus*, *Ocimum gratissimum* and *Thymus vulgaris* to control *Alternaria padwickii* and *Bipolaris oryzae*, two seed-borne fungi of rice (*Oryza sativa* L.). J. Essential Oil Restitution **19**: 581-587.

Noman MAA, Hosen S and Shamsi S 2021. Morphological and molecular characterization of *Pyricularia oryzae* isolates causing wheat blast in Bangladesh. Indian Phytopathol. **74**: 123-131

Ora N, Faruq AN, Islam MT, Akhtar N and Rahman MM 2011. Detection and identification of seed borne pathogens from some cultivated hybrid rice varieties in Bangladesh. Middle-East J. of Scientific Res. **10**(4): 482-488.

Raper KB and Thom C 1949. Manual of the Penicillia, Williams and Wilkins, Baltimore, MD. USA. pp. 875.

Rossman AY and Palm-Hernandez ME 2008. Systematics of plant pathogenic fungi: why it matters. Plant Dis. **92**: 1377-1386.

Santos GR, Castro N, Ignacio M, Furtado GQ, Rancel PHN, Silva LM and Riveiro FF 2009. Resistance of upland rice genotypes to rice disease at the south of Tocantins State. Bio. Sci. J. **25**(6): 96 -105.

Suleiman MN and Omafe OM 2013. Activity of three medicinal plants on fungi isolated from stored maize seeds (*Zea mays* L.). Global J. Med. Plant Res. **1**(1): 77-81.

Tania S, Bashar MA and Shamsi S 2020. Morphological characterization of seed-borne fungi associated with BRRI rice varieties in Bangladesh. Dhaka University J. Biol. Sci. **29**: 75-86.

Thom C and Raper KB 1945. A Manual of the *Aspergilli*. Williams and Wilkins, Baltimore, M.D. USA. pp. 373.

White TJ, Burns T, Lee S and Taylor J 1990. Application and direct sequencing of fungal ribosomal RNA genes for phylogenetics: a guide to methods and amplifications. Academic Press, San Diego, USA.

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