Jahangirnagar University J. Biol. Sci. **10**(1 & 2): 31-38, 2021 (June & December)

Cultural conditions for mycelial growth and molecular identification of *Mucor circinelloides* based on ITS sequence

Nasrin Mahmuda, Sadia Afroj, Durga Dus Bhowmik and Nuhu Alam*
Laboratory of Mycology and Plant Pathlogy, Department of Botany,
Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

Abstract

Mucor circinelloides were isolated from post harvest infected papaya, which was collected from Savar region. *M. circinelloides* was identified by morphology and biology of fungus based on colony features, fungal mycelium, sporangia and sporagiospores as well as molecular approach. Mycelial colonies are floccose, pale greyish-brown. Sporangiophores were transparent, long, erect and shorter branches, 4.5-7 x 3.5-5 µm in size. Sporangia are hyaline, spherical, ranged from 20-80 µm in diameter. Columellae were hyaline, spherical to ellipsoidal up to 50 µm in diameter. The highest mycelial growth was recorded in potato dextrose agar (PDA) medium for the growth and development of *M. circinelloides*. Temperature 30°C was optimum. This fungus grew well in pH 7. So, tested fungi grew well in neutral condition. Complete dark condition was favourable for the vegetative growth of the fungus. The molecular phylogeny in morphologically identified post-harvest pathogenic fungi of papaya such as *M. circinelloides* were studied based on their ITS. The sequences of ITS region of *M. circinelloides* revealed that the total length was 620 bp and sequence analysis suggested that 5.8S of rDNA sequences were identical.

Key words: ITS sequence, *Mucor circinelloides*, Vegetative growth.

INTRODUCTION

Mucor circinelloides is a dimorphic fungus (Lubbenhusen *et al*., 2003) and belongs to the order mucorales. It is worldwide distribution and found mostly in soil, dung and post-harvest fruits and vegetables. *M. circinelloides* causes infection and affected the quality of the fruits, which decreases the market value of fruits. Among the fruits papayas have major post harvest losses due to mucorelean fungi. Isabel *et al*. (2018) reported that the mucorelean fungus causes soft rot in papaya fruits in world wide. Moreover, spores of this fungus found in the orchard soils. It is a source of contamination that affects healthy fruits. *M. circinelloides* is an emerging pathogenic fungus. Some times it has been associated with human disease and causes cutaneous infection in human.

M. circinelloides reproduce both asexually and sexually. Mycelial colonies of *M. circinelloides* are fast growing and go up to 2 cm in height and 60 mm diameter. These colonies appear to be pale grey in colour (Li *et al*., 2011). Sporangiophores of *M. circinelloides* are branched with small sporangia (25 µm). The diameter of the sporangia ranged from 20-80 µm (Li *et al*., 2011). The length and number of tall sporangiophores are decreased with lower temperatures (Watanabe, 2009).

* Corresponding author. E-mail: mnabotju@juniv.edu

DNA barcoding is an important part of mycological research for molecular identification of fungi. Molecular phylogenetic studies suggested that the ITS region of genomic DNA is very useful for the detection of phylogenetic relationships at lower taxonomic levels of fungi (Alam & Rahman, 2020). Considering the above facts, the present research has been under taken to isolation and identification of post harvest papaya fungal pathogen i.e. *Mucor circinelloides* through morphology, biology and ITS sequence analysis.

MATERIALS AND METHODS

Papaya fruit samples were collected for the isolation and identification of selected fungi through classical taxonomy and molecular techniques. Fungal morphology, biology and physico chemical condition was established of the isolated pathogens.

Collection and sterilization of infected samples: Infected samples of papaya were collected from Savar kacha Bazar and Nabinagar Pollibiddut local market, Savar, Dhaka, Bangladesh. After colletion the samples were preserved at the Laboratory of Mycology and Plant Pathology, Department of Botany, Jahangirnagar University, Savar, Dhaka for further investigations.

For surface disinfection, friuts were washed with running tap water and soaked in 5% NaOCl for 3 minutes. Then the samples were washed three to four times with sterilized distilled water and air dried into the laminar airflow cabinet until the surface water was disappeared. Tissue planting method was used to isolate fungal pathogens; subculture was maintained on PDA medium; the pure culture was stored in refrigerator at -4^{0}C. Isolated fungi from the infected tissues of papaya were identified on the basis of colony morphology, morphological characteristic of conidia and conidial measurement using standard manuals.

Effect of culture media, temperature, pH and light: Carrot agar (CA), honey agar (HA), honey peptone agar (HPA), potato dextrose agar (PDA), potato sucrose agar (PSA) and Richard agar (RA) were used as culture media to evaluated the mycelial growth and development of the tested fungus (Sultana *et al*., 2020). To investigate the effect of temperature on fungal mycelial growth, the tested fungus wae inoculated on PDA medium and incubated at 15, 20, 25, 30 and 35°C. The mycelial growth was recorded at 7 days post inoculation (dpi) respectively (Alam *et al*., 2010). The effect of pH on the growth of the fungus was assayed on PDA medium. Five different pH levels *viz*., 5.0, 6.0, 7.0, 8.0 and 9.0 were selected to evaluated the mycelial growth and development of the tested fungus. Before autoclave the PDA medium was adjusted to pH 5, 6, 7, 8 and 9 with the addition of 1 N NaOH or HCl and it will be incubated at 30ºC for 7 days. Radial growth of mycelia on each Petri dish would be measured at 3 directions (Alam & Rahman, 2020). The effect of light on the mycelial growth of the fungus was by exposing the inoculated culture to 24 h light, 24 h dark and alternate cycle of 12 h light and 12 h dark in an environment chamber in which at room temperature (25 ± 2°C) was maintained (Singha *et al*., 2013).

Molecular Characterization: Molecular identification was carried out by using ITS sequence of the fungal genome. The fungal genomic DNA were extracted using the Maxwell Cell Kit (AS1030, Promega, USA). The primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAA AGTCG TAACAAGG-3′) were used for the PCR reaction (Alam *et al*., 2009).

The PCR was performed in a total volume of 25µl reaction mixture by using 5µl DNA template (20ng/µl) and GoTaq® G2 Hot Start Green Master Mix (Promega, USA) as pre-heat at 94°C for 5 min, 35X (94°C for 30 s, 57°C for 30 s, 72°C for 5 min), and 72°C for 10 mins (Sultana *et al*., 2020). The purified PCR products were sequenced bi-directionally in First BASE Laboratories SdnBhd (Malaysia). DNA sequences were analyzed by BioEdit and MEGA6 software. Sequencing data were submitted to the NCBI, under accession number JUF0039. A BLAST search with the ITS sequences were used to reveal the closest matching taxa. Data was converted from fasta to MEGA format with Clustal W. Maximum likelihood, Neighbor-joining, and Maximum parsimony trees were generated (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Characteristics of *Mucor circinelloides*: Mycelial colonies of *M. circinelloides* were floccose, pale greyish-brown. Sporangiophores are transparent with long and shorter branches becoming circinate and 4.5-7 x 3.5-5 µm in size. Sporangia are hyaline, spherical and ranged from 20-80 µm in diameter. Columellae are hyaline, spherical to ellipsoidal and are up to 50 µm in diameter. Chlamydospores, rhizoids and stolons are absent (Fig. 1). Based on these phenotypic traits, the isolated fungus seems to be *Mucor circinelloides* (Schipper & Stalpers, 2003).

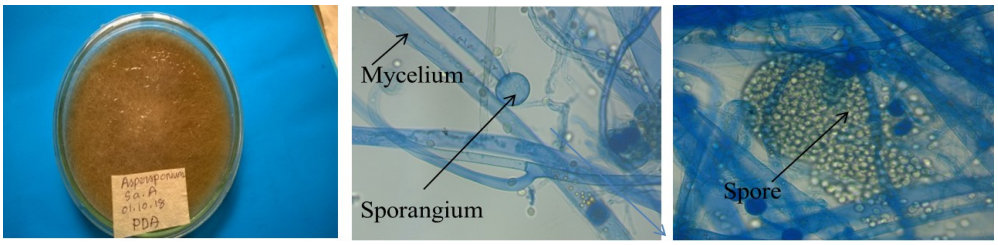

Fig. 1. Morphological view of *Mucor circinelloides*. A: Vegetative growth of *M. circinelloides* on PDA medium, B: Microscopic view of mycelium and sporangium of *M. circinelloides* (10 x 10x), C: Microscopic view of spore of *M. circinelloides* (40 x 10x)

The effect of culture media *viz*., PDA, CA, RA, PSA, HPA and HA on mycelial growth of *Mucor circinelloides* have been presented in Fig. 2. The results showed that the highest mycelial growth (90.13mm) of *M. circinelloides* was recorded on PDA medium which was followed by PSA medium and the lowest growth (60.50 mm) was measured in HPA medium. The current results of *M. circinelloides* is supported by Koley & Mahapatra, (2015) who found that potato dextrose agar is better than other media for growth of

tomato early blight causing fungi-*Alternaria solani*. It was observed that maximum growth of *Alternaria brassicae* was found on PDA. The same observation was also reported by Kumar *et al*. (2008) who working with *Alternaria solani*. The growth pattern on PDA medium was the best under *in vitro* conditions.

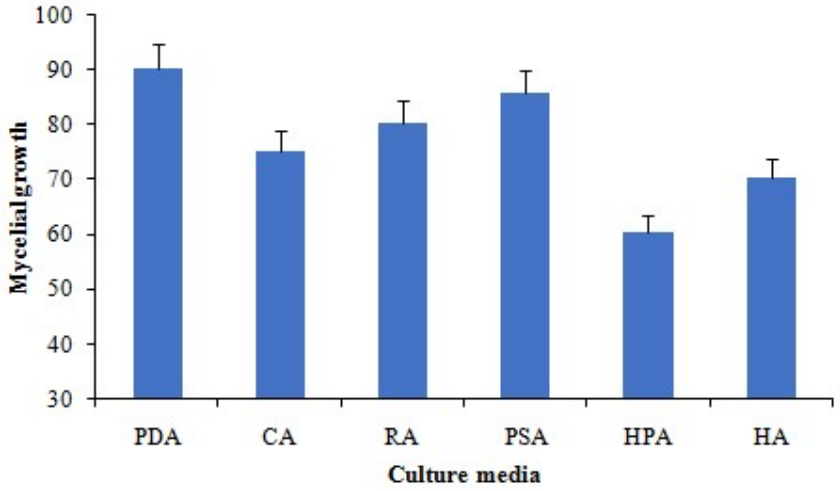

Fig. 2. Effect of different culture media on the mycelial growth of *Mucor circinelloides* at 7 dpi. PDA- potato dextrose agar, CA- carrot agar, PSA- potato sucrose agar, RA- Richard agar, HPA- honey peptone agar, HA- honey agar

Present study suggested that the effect of temperature on radial mycelial growth of *Mucor circinelloides* on PDA media under *in vitro* condition. The experimental plates were incubated at five different temperature viz., 15°C, 20°C, 25°C, 30°C and 35^{0}C and the results have been shown in Fig. 3. The data informed that the highest growth of *M. circinelloides* was recorded at 30ºC, followed by 25ºC. In our experiment, *M. circinelloides* grew the maximum at 30ºC which is consistent with the previous findings of Iwen *et al*. (2007) who cited that the highest mycelia growth and sporulation of *Mucor circinelloides* registered at 30ºC, a sudden fall in mycelial growth and sporulation observed at 30ºC and 35ºC. *Mucor circinelloides* grew with well-developed mycelium at 20ºC to 35ºC. This showed that temperature has no effect on the growth of the mycelial growth of *Mucor circinelloides*. The results also indicated that temperature influenced the growth of *M. circinelloides* and their appropriate combination can be used to inhibit or retard the growth of the mold.

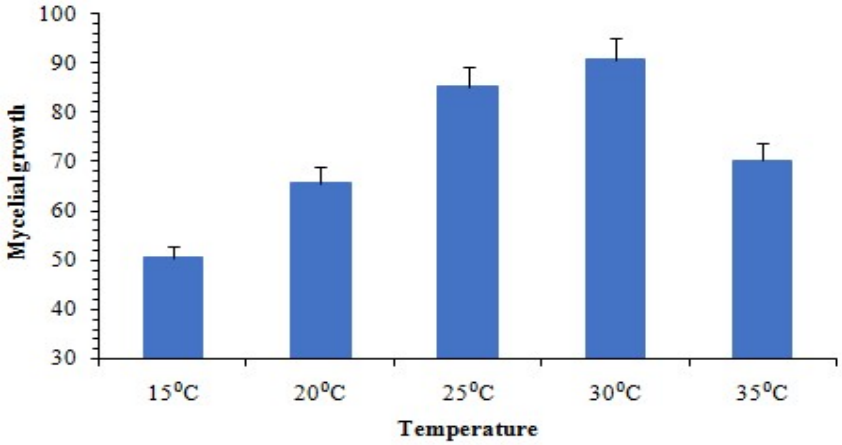

Fig. 3. Effect of different temperature on mycelial growth of *Mucor circinelloides*

pH is an important and special criteria for understanding the ecology of pathogenic fungi. However, in present study, the experimental plates were incubated at five different pH level *viz*., 5, 6, 7, 8 and 9. Maximum mycelia growth (90.13mm) of *Mucor circinelloides* was recorded at pH 7 and followed by pH 8 and pH 6, while minimum mycelial growth was observed at pH9 (Fig. 4). Sonyal *et al*. (2015) reported that the maximum growth of *Ceratocystis fimbriata* at pH 7.5, followed by pH 7.0 and pH 8.0. The mycelial growth of fungus was reduced from pH 5.5 to pH 2.0 and increased from 6.5 to pH 9.0. The highest mycelial growth of *Ceratocystis paradoxa* was recorded at pH 7 (Yadahalli *et al.*, 2007). Result suggested that this fungus grew very well at neutral condition.

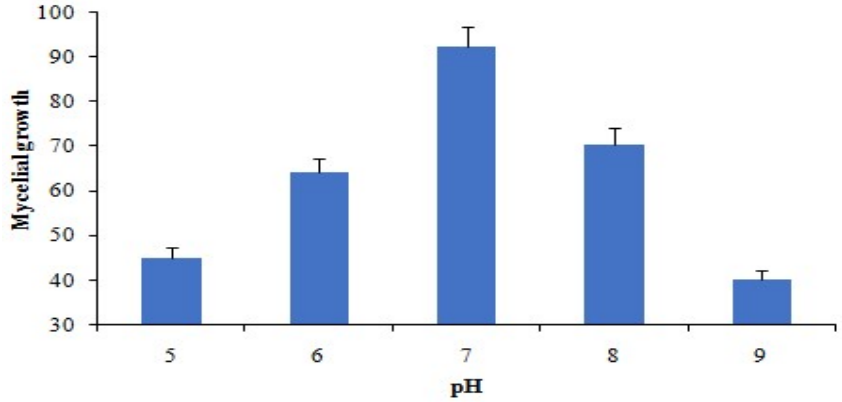

Fig. 4. Effect of different pH on the mycelial growth *Mucor circinelloides*

The consequence of different light duration on mycelial growth and development of *Mucor circinelloides* have presented in Fig. 5. The experimental plates were incubated at three different conditions viz., 24 hours Light, 24 hours Dark and 12/12 hours Light-Dark. *M. circinelloides* showed better mycelial growth under complete dark condition, while least performance observed due to continuous light condition. In the present

findings, complete dark and alternate light/dark gave the highest mycelial growth of *Alternaria* sp. Our results are partially supported by Arunkumar (2015) cited those alternate cycles of 12 h light and 12 h darkness resulted in maximum growth of *A. solani*. The exposure of the fungus to continuous dark and alternate cycles of 12 hour light and 12 hour darkness resulted in the maximum mycelial growth of *M. circinelloides* compared to continuous light.

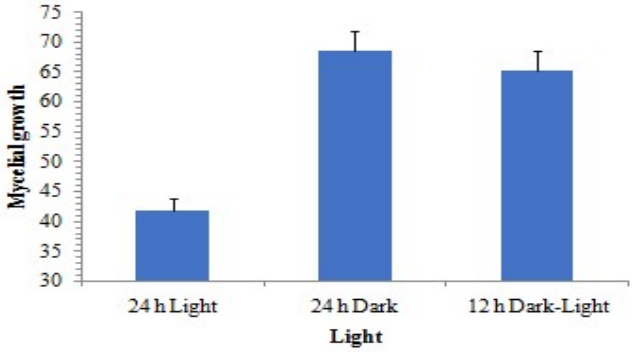

Fig. 5. Effect of light regimes on mycelial growth of *Mucor circinelloides* at7dpi

Molecular Characterization: For molecular identification, the ITS region of 620bp was amplified using ITS4 and ITS5 primers and sequenced (Fig. 6). The ITS of rDNA is considered as a variable region among the species and even among the strains (Alam *et al.*, 2010).

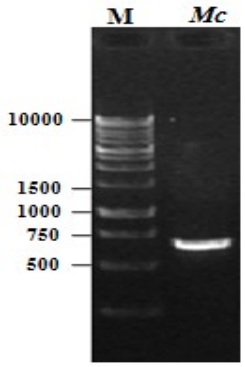

Fig. 6. PCR products of the ITS region of *Mucor circinelloides* (*Mc*). M, molecular size marker (1 kb DNA ladder)

Phylogenetic tree was constructed based on the nucleotide sequences of the ITS regions in 36 fungal taxa were downloaded from the NCBI database for molecular identification of our studied fungus. Percent homology of rDNA sequence of ITS region of our study fungus (JUF0039) was compared with formerly identified fungi *Mucor circinelloides* under accession number KU862913.1, MK260195.1, HQ914900.1, KF435039.1. The maximum parsimony tree, there are eight different clades were found in the phylogenetic tree (Fig. 7). Reciprocal homologies of the ITS region sequences ranged from 98 to

100%. The sequencing data of the selected NCBI GenBank strain (LC194223.1 *Colletotrichum gloeosporiodes*) was used as out group for the comparative studies on phylogenetic relationship with the selected strain of *Mucor circinelloides* (JUF0039). The results indicated that all the individual species of *Mucor circinelloides* belong to single cluster. Alam *et al*. (2010) reported that ITS sequences are genetically constant or show little variation within the species, but vary between species in a genus. Based on molecular evidence, it is clearly indicated that our studied fungus is *Mucor circinelloides* under the family- Mucoraceae.

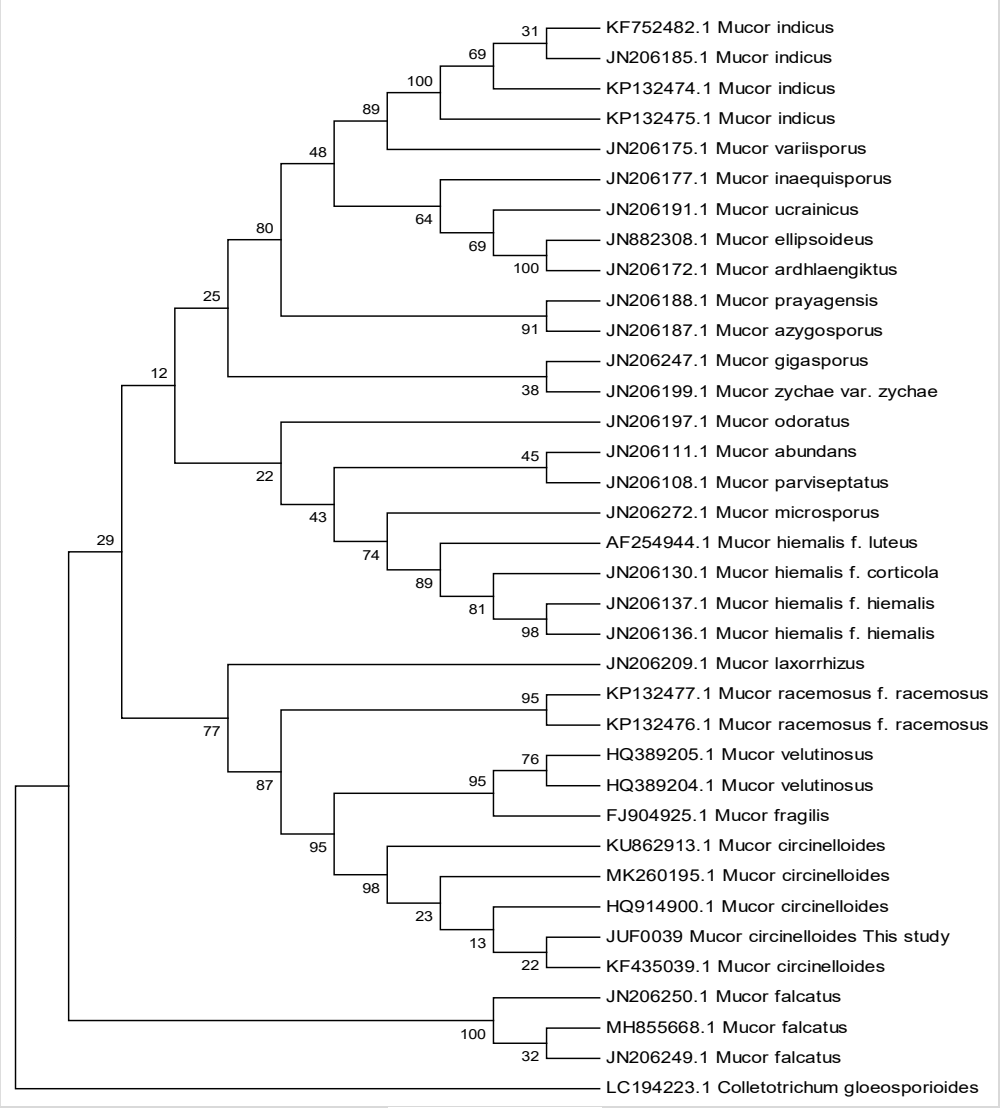

Fig. 7. Maximum likelihood tree of *Mucor circinelloides* with bootstrap value. Our organism marked with JUF0039

Acknowledgements: This research project was funded by Jahangirnagar University research project grand for the fiscal year 2020-2021.

REFERENCES

Alam, N. and Rahman, F. 2020. Vegetative growth and genetic diversity in different strains of *Pleurotus salmoneastramineus* based on pcr polymorphism. *Bangladesh J. Botany*, **49**(1):125-134.

Alam, N., Kim, J. H., Shim, M. J., Lee, U. Y. and Lee, T. S. 2010. Mycelial propagation and molecular phylogenetic relationships of commercially cultivated *Agrocybe cylindracea* based on ITS sequences and RAPD. *Mycobiology*, **38**(2): 89-96.

Alam, N., Shim, M. J., Lee, M. W., Shin, P. G., Yoo, Y. B. and Lee, T. S. 2009. Phylogenetic relationship in different commercial strains of *Pleurotus nebrodensis* based on ITS sequence and RAPD. *Mycobiology*, **37**(3): 183-188.

Arunkumara, K. T., Satyanarayana, C. and Srinivas, N. 2015. Impact of abiotic and nutritional factors on the growth of *Alternaria solani* causing early blight of potato. *Pest Manage. Hort. Ecos.*, 21(2): 190-193.

Isabel, C.L., Isidro, M.Z., Raul, A.M., Josefa, A.S.B., Josefina, L.F., Nancy, L.L., Raymundo, S.G.E. 2018. Diversity of mucoralean fungi in soils of papaya (*Carica papaya* L.) producing regions in Mexico. *Fungal Biol*., **122**(8): 810-816.

Iwen, P. C., Sigler, L., Noel, R. K. and Freifeld, A. G. 2007. *Mucor circinelloides* was identified by molecular methods as a cause of primary cutaneous zygomycosis. *J.Cli. Microbiol.*, **45**(2): 636-640.

Koley, S. and Mahapatra, S. S. 2015. Evaluation of culture media for growth characteristics of *Alternaria solani*, causing early blight of tomato. *J. Plant Patho. Microbiol.*, S1, 005.

Kumar, V., Haldar, S., Pandey, K. K., Singh, R. P., Singh, A. K. and Singh, P. C. 2008. Cultural, morphological, pathogenic and molecularvariability amongst tomato isolates of *Alternaria solani* in India. *World J. Microbiol. Biotech.*, **24**: 1003-1009.

Li, C. H., Cervantes, M., Springer, D. J., Boekhout, T., Ruiz-Vazquez, R. M., Torres-Martinez, S. R., Lee, S. C. 2011. Sporangiospore size dimorphism is linked to virulence of *Mucor circinelloides*. *PLoS pathogens*, **7**(6): 10-17.

Lubbenhusen, T. L, Nielsen, J., McIntyre, M. 2003. Characterization of the *Mucor circinelloides* life cycle by on-line image analysis. *J. Appl. Microbiol.*, **95** (5): 1152-1160.

Schipper, M. A. A. and Stalpers, J. A. 2003. Zygomycetes: The order Mucorales. In: Howard DH (ed) Pathogenic fungi in humans and animals. Marcel Dekker, New York, 67-125.

Singha, S. M., Alam, N., Sarker, N. C. and Shaheen, M. 2013. Influence physicochemical conditions culture medium on *in vitro* mycelial growth in different strains of *Volvariella volvaceae. Bangladesh J. Mush.*, **7**(2): 59-66.

Sonyal, S., Pappachan, A., Palanna, K. B., Mahesha, Manjunath, H. S., Hurakadli, S. and Giri, M. S. 2015. Survival ability of *Ceratocystis fimbriata* causing pomegranate wilt in different temperature and hydrogen ion concentration (pH). *Int. J. Pure Appl. Biosci.*, **3**(4): 49-53.

Sultana, R., Ara, I., Chanda, I.J. and Alam, N. 2020. First report of *Lichtheimia hyalospora* from fresh water dried shrimp in Bangladesh. *Int. J. Fish. Aqua. Stu*., **8**(3): 474-477.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.*, **30**: 2725-2729.

Watanabe, T. 2009. Pictorial atlas of soil and seed fungi: morphologies of cultured fungi and key to species (3rd ed.). Boca Raton, Fla.: CRC. pp. 419.

Yadahalli, K. B., Adiver, S. S. and Kulkarni, S. 2007. Effect of pH, temperature and relative humidity on growth and development of *Ceratocystis paradoxa*- a causal organism of pineapple disease of sugarcane. *Karnataka J. Agri. Sci.*, **20**(1):159-161.